The mouse *Hox-1.3* gene is functionally equivalent to the *Drosophila Sex combs reduced* gene

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To test whether the mouse *Hox-1.3* gene is a cognate of the *Drosophila Sex combs reduced* (*Scr*) gene, we inserted a *hsp70-Hox-1.3* fusion gene into the *Drosophila* genome. Transgenic flies displayed *Scr*-like homeotic transformations after ectopic expression of *Hox-1.3* induced by heat shock. In larvae, the thoracic segments T2 and T3 are transformed toward T1. In adults, head structures are dramatically disrupted, including transformation of antenna towards leg. Transformations are not the result of ectopic activation of the endogenous *Scr* gene. Rather, *Hox-1.3* appears to directly regulate *Scr* target genes, as demonstrated by the ectopic activation of *fork head* by *Hox-1.3*. The results suggest that mouse *Hox-1.3* cannot only substitute functionally for *Drosophila Scr* in the determination of external structures but also can participate in the regulatory hierarchy of insect organogenesis.

[Key Words: *Hox-1.3*; *Sex combs reduced*; *fork head*; homeotic transformation; evolution]

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The conserved homeo box sequences first found in *Drosophila* regulatory genes encode a 60-amino-acid residue domain, known as the homeo domain (McGinnis et al. 1984a,b; Scott and Weiner 1984). Substantial genetic evidence demonstrates that mutations in homeo box-containing genes can dramatically affect the development of *Drosophila*. As homeotic mutations have not been found naturally in mice, the mouse homeo box-containing (*Hox*) genes were isolated solely on the basis of homology to *Drosophila* homeo box sequences. Most *Hox* genes are tightly linked in four gene clusters (*Hox-1*, *Hox-2*, *Hox-3*, and *Hox-4* clusters) on chromosomes 6, 11, 15, and 2. Based on homeo box sequence similarity, two groups independently observed that the members of the four mouse *Hox* clusters can be aligned with the *Drosophila Antennapedia* complex (ANT-C) and *Bithorax* complex (BX-C) (collectively known as HOM-C) and classified into cognate subfamilies (Duboule and Dolle 1989; Graham et al. 1989; for review, see McGinnis and Krumlauf 1992). Interestingly, the physical order of homeotic genes in *Drosophila* HOM-C corresponds to the order along the anterior-posterior axis of the segments affected by the genes (for review, see Akam 1987). The correlation between regions of gene expression along the body axis and physical order along the chromosome is also conserved in mouse (Duboule and Dolle 1989; Graham et al. 1989).

In vitro and in vivo studies of *Drosophila* and mammalian homeo domain proteins indicate that they are transcription factors and that the homeo domain is required for nuclear targeting and sequence-specific DNA-

binding activity (Desplan et al. 1985, 1988; Fainsod et al. 1987; Hall and Johnson 1987; Hoey et al. 1988; Müller et al. 1988; Kuziora and McGinnis 1989; Odenwald et al. 1989; Treisman et al. 1989; Kissinger et al. 1990). These findings, along with the observation that mammalian *Hox* genes are expressed in a tissue-specific fashion during embryonic development, suggest that *Hox* genes may play important roles in controlling embryogenesis in vertebrates as do *Drosophila* homeo box-containing genes. This idea is strongly supported by recent results using homologous recombination to disrupt endogenous *Hox* genes in mice (Chisaka and Capecchi 1991; Lufkin et al. 1991; Le Mouellie et al. 1992).

The *Hox-1.3* gene was identified initially by screening a mouse genomic library with an *Antennapedia* (*Antp*) homeo box probe under low stringency (Colberg-Poley et al. 1985). The gene was then characterized by Odenwald et al. (1987) and Fibi et al. (1988). It consists of two exons and one intron. The 270-amino-acid residue *Hox-1.3* open reading frame (ORF) is rich in proline, serine, and glycine and is phosphorylated at multiple sites (Odenwald et al. 1989). The homeo domain is located between residues 195 and 254. The protein also contains an octapeptide (MSSYFVNS) and a pentapeptide (IYPWM) that are conserved among many vertebrate and *Drosophila* homeo box genes (Odenwald et al. 1987). The distribution of *Hox-1.3* is progressively restricted during early mouse embryogenesis. In very early embryos, it is expressed in nearly all cells (Tani et al. 1989). In mid-gestation embryos, *Hox-1.3* mRNA is expressed at high levels in the mesoderm and neuroectoderm with an anterior

boundary near the junction of the hindbrain and spinal cord (Dony and Gruss 1987; Fibi et al. 1988). In adults, the mRNA and protein have also been detected in some tissues, including liver, lung, kidney, and central nervous system (Odenwald et al. 1987; Garbern et al. 1989). DNA-binding assays showed that Hox-1.3 high-affinity binding sites contain an ATTA core motif (Odenwald et al. 1989), which is also the core of *Drosophila Antp* class high-affinity binding sites (for review, see Scott et al. 1989). Nevertheless, the biological function of Hox-1.3 is unknown.

If homeo box-containing genes play a basic role in embryonic development that is conserved throughout the animal kingdom, they would be under selective pressure to maintain certain functional specificities during evolution. One way to test this notion is to assay the ability of a given homeo box-containing gene from one organism to functionally substitute for a putative cognate gene in another organism. Two previous studies have shown that the mouse *Hox-2.2* and human *Hox-4.2* genes can carry out some of the functions of their *Drosophila* cognates when introduced into flies (Malicki et al. 1990; McGinnis et al. 1990).

We have chosen to use the mouse *Hox-1.3/Drosophila Sex combs reduced (Scr)* putative cognate pair as a model to examine the functional conservation of *Hox* genes at both morphological and regulatory levels. We have inserted the mouse *Hox-1.3* ORF into the *Drosophila* genome under the control of a *Drosophila* heat shock promoter (*hsp70*) that gives rise to ubiquitous expression after heat treatment. Abnormalities induced by the ectopic expression of Hox-1.3 protein during both embryonic and larval phases of development were *Scr*-like. These results strongly suggest that mouse *Hox-1.3* is the bona fide cognate of *Scr* but not the closely related and neighboring *Antp* gene. We show further that the mouse *Hox-1.3* gene can participate in *Drosophila* regulatory networks by activating the *fork head (fkh)* gene, a downstream target of *Scr*. Thus, the functional properties of this cognate pair appear to have been conserved >600 million years since the divergence of vertebrates and arthropods.

Results

Expression of the mouse Hox-1.3 gene in transgenic flies

The mouse Hox-1.3 and three *Drosophila* homeo domain proteins (*Scr*, *Antp*, and *Dfd*) are highly homologous in the homeo domain (Fig. 1A). In addition, two other short regions (Odenwald et al. 1987), one at the amino terminus of the proteins (the octapeptide) and the other just adjacent to the homeo domain (the pentapeptide), are also highly conserved among these proteins. However, the overall similarity of the 270-amino-acid Hox-1.3 protein to the three is low: 28% (75 amino acids) to *Scr*; 26% (70 amino acids) to *Antp*; and 24% (66 amino acids) to *Deformed (Dfd)*. Because of the slightly higher degree of sequence similarity and the position of *Hox-1.3* in the

A

<i>Hox-1.3</i>	MSSY*FVNS		PQIYPWMRKLHI		GPE	GKRARTAYTRYQTLELE	
<i>Scr</i>	---Q---	-----	---KRV-L		-*	T-Q-S	-----
<i>Antp</i>	-T--*-T-		-*L-----		N*	R-G-QT-	-----
<i>Dfd</i>	---F*****		RI-----	K-I-V		-MD	P-Q-----H-I---

<i>Hox-1.3</i>	KEFHFNRYLTRRRRIEIAHALCLSERQIKIWFQNRMRKWKKN	KLKSM	
<i>Scr</i>	-----T-----	EH	-MA--
<i>Antp</i>	-----T-----	E-	-T-GE
<i>Dfd</i>	---Y-----	T-V	--PNT

B

Figure 1. *Hsp70-Hox-1.3* transgene constructs and amino acid sequences comparison. (A) Alignment of mouse Hox-1.3 amino acid sequences with three *Drosophila* homeotic gene products *Scr*, *Antp*, and *Dfd* [*Scr* (LeMotte et al. 1989), *Antp* (Schneuwly et al. 1986), *Dfd* (Regulski et al. 1987)]. Within the conserved domains, identical residues are marked with a broken line. Homeo domain residues are in boldface type. The asterisks (*) indicate missing positions in terms of alignment. The dots represent large nonconserved regions (not to scale). (B) Schematic representation of pCaSpeR-*hs-Hox-1.3* transgenic plasmids. The vector contains the mini *white* (*w*⁺) gene flanked by the ends of the P transposon (Pirota 1988). The multiple cloning site separates the *hsp70* promoter (−245 to +207) from the *hsp70* trailer sequences (a 525-bp fragment-containing polyadenylation site). The full-length *Hox-1.3* cDNA was inserted into the polylinker sites (see Materials and methods). The orientations of *Hox-1.3* and *white* ORFs are indicated by arrows. Besides the homeo domain (HD), two other short conserved regions are indicated by O (the octapeptide) and P (the pentapeptide).

Hox-1 cluster, the mouse *Hox-1.3* gene was proposed to be a cognate of the *Scr* gene (Duboule and Dolle 1989; Graham et al. 1989).

We examined the potential *Scr*-like function of Hox-1.3 protein in transgenic flies by placing the *Hox-1.3* gene under the control of a heat-inducible-promoter-*hsp70* (Fig. 1B). The full-length *Hox-1.3* ORF (Odenwald et al. 1987) was inserted between the *Drosophila hsp70* promoter and trailer sequences in the P-element vector CaSpeR-hs (Pirota 1988) to create the fusion gene *hs-Hox-1.3*. The construct was inserted into the *Drosophila* genome by P-element-mediated transformation (Rubin and Spradling 1982; Spradling and Rubin 1982). Ten *hs-Hox-1.3* independent transformant lines were generated, and all described phenotypes were observed in at least three independent lines.

Under normal growth conditions (25°C), transformant embryos carrying *hs-Hox-1.3* do not express detectable levels of Hox-1.3 proteins (Fig. 2A). However, after a 1-hr heat shock, Hox-1.3 protein products are distributed in most nuclei of the embryo (Fig. 2B). The expressed Hox-1.3 protein products persist in the embryo and are still abundant even after 3 hr of recovery at 25°C (Fig. 2C). To

Figure 2. Induction of mouse *Hox-1.3* protein in transgenic embryos. (A) No *Hox-1.3* protein is detected in the non-heat-shocked transgenic embryos at the germ-band extension stage. (B) Heat-induced expression of *Hox-1.3* in *hs-Hox-1.3*-bearing embryos. *Hox-1.3* protein was detected by immunostaining with anti-*Hox-1.3* antibodies after 1-hr recovery at 25°C following a 1-hr heat shock at 6 hr + 30 min AEL. (Note that the signal is located in the nuclei of all embryonic cells.) (C) Embryos were heat treated as in B, but immunostaining was carried out after 3 hr of recovery at 25°C.

rule out general effects of heat shock and protein ectopic expression, we also inserted a *Hox-1.3* gene fragment lacking the coding sequence for the homeo domain and part of the carboxyl terminus into the *Drosophila* genome. The protein products of the truncated form are detected in the cytoplasm of the cell (data not shown).

Homeotic transformations induced by ectopic expression of Drosophila Scr

To compare the morphological consequences induced by ectopic expression of the mouse *Hox-1.3* and *Drosophila* *Scr* genes in transgenic flies, we have re-examined the defects produced in *hs-Scr* flies. Here, we expand the description of the induced phenotypes.

Larval transformations were induced optimally by a 1-hr heat shock (37°C) of *hs-Scr*-bearing embryos at 6 hr + 30 min after egg laying (AEL). The cuticular phenotypes were essentially the same as reported previously (Gibson et al. 1990). Briefly, >80% of heat-shocked *hs-Scr* cuticles display thoracic transformation, although to

varying degrees. Compared with a wild-type larva (Fig. 3A), the transformation is marked by the unique T1 ventral beards appearing ectopically in the T2 and T3 segments (Fig. 3B). After a 1-hr heat shock, most resulting *hs-Scr* larvae have the T2 toward T1 transformation, and 70% of them had T1 epidermal identity in both T2 and T3. Shorter heat shocks (e.g., 20 min) stimulate ectopic beards only in T2 (data not shown). Additional effects induced by ectopic expression of *Scr* are found in the head segments of resulting larvae. Head involution is disrupted, and the cephalopharyngeal skeleton is dramatically reduced. The extent of malformation in various head structures is variable and depends on the time and length of heat shock. Even though there is a strong transformation of T3, no transformation of abdominal segments was observed in these *hs-Scr* cuticles.

In the adult, morphological changes were induced by treating *hs-Scr*-bearing larvae with multiple heat shocks starting at late second-instar or early third-instar larval stage, as described in Materials and methods. The result is largely consistent with a previous report (Gibson et al. 1990). Compared with a normal head (Fig. 3C), the aberrant expression of *Scr* leads to a dramatic reduction of the compound eyes (observed in 42 of 45 flies), induces arista transformation (or thickened arista, observed in 40 of 45 flies; Fig. 3D), and causes disfigured maxillary palps, labial palps (observed in 24 of 45 flies; Fig. 3E), and swollen occiputs with many thickened bristles in the hindhead (observed in 30 of 45 flies; Fig. 3F). The occiput transformation is similar to a phenotype induced by ectopic expression of *Antp* (Schneuwly et al. 1987; Gibson and Gehring 1988) and a natural dominant mutation *Cephalothorax* (*Ctx*) in the *Antp* complex (Duncan and Lewis 1982). The hindhead seems to be a sensitive and plastic region that can be modified to thoracic identity by both *Antp* and *Scr* genes.

Homeotic transformations induced by the ectopic expression of mouse Hox-1.3

To compare the phenotypic consequences of ectopically expressed mouse *Hox-1.3* to those of *Scr*, ~5.5 to 6.5-hr-old *hs-Hox-1.3*-bearing embryos were subjected to heat treatment at 37°C for 1 hr, as was done for *hs-Scr*-bearing embryos. As shown in Figure 4B, the larvae display *Scr*-like transformations (cf. Fig. 3B). Characteristically, mouse *Hox-1.3* induces the transformation of thoracic segments T2 and T3 toward T1. The ventral beard, a unique feature of T1, clearly appears in the T2 and T3 thoracic segments. The thoracic segment transformation induced in *hs-Hox-1.3* animals is *Scr*-like but it is not *Antp*-like, because the ectopic expression of *Antp* causes the loss of the T1 ventral beard, an antagonistic transformation to that induced by *Scr* (Gibson and Gehring 1988; Gibson et al. 1990). Other obvious abnormalities caused by misexpression of *Hox-1.3* are the failure of head involution and the absence or misplacement of many cuticular head structures (e.g., epistomal sclerite, ectostomal and hypostomal sclerites, the pigmented H-piece, the mouth hooks, and sensory organs). Trans-

Figure 3. Transformations resulting from ectopic expression of *Drosophila Scr*. (A) Wild-type first-instar larva cuticular structures. The arrow indicates the ventral beard that marks the first thoracic segment (T1). (B) Morphological consequences induced by ectopic expression of *Scr* in *hs-Scr*-bearing embryos. Embryos were subjected to a 1-hr heat shock at 6 hr + 30 min AEL and allowed to develop to larvae. Authentic and ectopic T1 beards of a first-instar larva are indicated by the arrows. (C) Wild-type adult head. (D) An example of phenotypes induced in the adult head by misexpression of *Scr* during larval development (see text for details). (E) The malformed mouth parts of a heat-shocked *hs-Scr*-bearing animal. Note that the maxillary palps are severely disfigured (arrows). (F) The dorsal head of a heat-shocked *hs-Scr*-bearing animal. The arrows indicate the *Ctx*-like transformation.

formation of cuticular pattern is not detectable in abdominal segments. Embryos carrying the *Hox-1.3* fragment lacking homeo domain-coding sequence are still able to hatch after heat shock, and the larval cuticles are indistinguishable from that of wild-type larvae (data not shown), as are those of non-heat-shocked *hs-Hox-1.3*-bearing embryos (Fig. 4A).

To test whether the ectopic expression of *Hox-1.3* could induce *Scr*-like homeotic transformations in adult animals, *hs-Hox-1.3*-bearing larvae were subjected to one or more heat treatments at varying stages of development. Ectopic expression of *Hox-1.3* reproduced all *Scr*-like transformations in the external head structures of transgenic flies (Fig. 5A, cf. Fig. 3D). Although some

flies eclosed after heat treatment, many of the phenotypes were observed in animals that were dissected from pupal cases. The abnormalities include reduced and irregular compound eyes (observed in 157 of 203 flies), malformation of mouth parts (observed in 91 of 203 flies), and thickening of the arista (observed in 102 of 203 flies). We have often observed a *Ctx*-like transformation in the hindhead (155 of 203 flies). Intriguingly, the antenna is transformed toward a relatively well-formed leg (observed in 42 of 203 flies; Fig. 5B). For *hs-Hox-1.3* animals, this antenna toward leg transformation could be induced by even a single 1-hr heat shock between 63 and 93 hr AEL (observed in 10 of 65 flies). Occasionally, structures strongly resembling sex comb teeth were seen

Figure 4. Ectopic expression of mouse *Hox-1.3* induces segmental transformations in *Drosophila* larva. (A) Cuticular pattern of a non-heat-shocked *hs-Hox-1.3* bearing first-instar larva. There is no transformation detected (cf. the wild-type larva in Fig. 3A). The T1 beard is indicated by an arrow. (B) Embryos carrying the *hs-Hox-1.3* fusion gene were subjected to a 1-hr heat shock at 6 hr + 30 min AEL and 37°C and allowed to develop to larvae. A typical first-instar larva is shown. Authentic and ectopic T1 beards are indicated by arrows.

on the well-transformed antennal legs (Fig. 5C), suggesting that the ectopic legs have T1 leg identity. In contrast, ectopic expression of *Antp* (*hs-Antp*) induces transformation of antenna toward T2 leg, as indicated by the presence of an apical bristle on the ectopic leg (Schneuwly et al. 1987; Gibson and Gehring 1988). We have never observed an apical bristle on the *hs-Hox-1.3*-in-

duced ectopic legs. In some *hs-Hox-1.3* adult heads, the maxillary palps were severely affected and split into two segments (Fig. 5D). This morphological consequence is similar to the transformation described previously for *hs-Dfd* and *hs-Hox4.2* transgenic flies (McGinnis et al. 1990). As for the embryonic phenotype, no transformation was observed in embryos carrying the *Hox-1.3* fragment lacking the homeo domain (data not shown). Thus, these transformations are not the result of nonspecific effects of heat shock. In addition, the homeo domain is required for all transformations observed.

Hox-1.3 action is not mediated by *Drosophila* homeotic genes

Because *Hox-1.3* can cause *Scr*-like homeotic transformations of posterior thoracic segments toward T1 in embryos and *Scr*- and *Dfd*-like defects in the adult head, we investigated whether the induced defects might result from activation of the endogenous *Scr* and/or *Dfd* genes. For these purposes, we compared the localizations of *Scr* and *Dfd* proteins in treated *hs-Hox-1.3* embryos with wild-type embryos by immunostaining. In wild-type embryos, *Scr* protein is present in the anterior compartment of T1, in the labial segment, and in a small group of cells in the posterior region of the maxillary segment at the germ-band-extension stage (Riley et al. 1987; LeMotte et al. 1989). *Dfd* protein is localized to the maxillary and mandibular segments (Jack et al. 1988).

In heat-shocked *hs-Hox-1.3*-bearing embryos, *Scr* and *Dfd* expression appears normal. No expansion of expression of *Scr* or *Dfd* in either posterior or anterior direction has been observed (Fig. 6A, B), although there are abundant *Hox-1.3* protein products in the whole embryo (see

Figure 5. Adult phenotypes induced by ectopic expression of mouse *Hox-1.3*. (A) Adult head phenotypes induced by misexpression of *hs-Hox-1.3*. Larva carrying the *hs-Hox-1.3* fusion gene were subjected to multiple heat shocks (see Materials and methods). Typical transformations shown include the thickening of the arista, small compound eyes, irregular shape of maxillary palps, and *Ctx*-like transformation on the dorsal head. (B) One example of transformation of antenna toward leg in a *hs-Hox-1.3* fly. Arrows indicate the ectopic legs. (C) High-power magnification showing a sex comb tooth (arrow) on an ectopic leg. (D) Malformations of mouth parts induced by improper expression of *Hox-1.3*. [Note the maxillary palps are split into two segments (arrows)].

Figure 6. The expression patterns of *Drosophila Scr* and *Dfd* are not affected by *Hox-1.3*. Embryos carrying the *hs-Hox-1.3* fusion gene were subjected to a 1-hr heat shock at 6 hr + 30 min AEL and 37°C and allowed to recover at 25°C for 3 hr (for *Scr* immunostaining) and 2 hr (for *Dfd* immunostaining). Expression patterns of *Scr* (A) and *Dfd* (B) proteins were detected by immunostaining. No difference from wild-type patterns was detected. For C and D, larvae were subjected to two 1-hr heat shocks at the third-instar larval stage (see Materials and methods). No ectopic expression of *Scr* was detected in the eye-antennal imaginal disc (C), whereas expression in T1 leg discs was normal (D). Thus, ectopic expression of *Hox-1.3* did not induce the endogenous *Scr* gene.

Fig. 2C). We conclude that the endogenous *Scr* and *Dfd* genes are not activated by *Hox-1.3* in heat-treated embryos. Furthermore, *Antp* gene expression was normal in heat-shocked *hs-Hox-1.3*-bearing embryos (data not shown). Similar experiments were carried out to test the role of endogenous *Scr* in inducing the observed adult phenotypes. Larvae were subjected to two heat shocks, as described previously. Although strong transformations were observed in adults in these experiments, no ectopic expression of *Scr* was observed in eye-antennal discs (Fig. 6C). In addition, we have observed *Hox-1.3*-induced embryonic transformations in *Scr* mutant animals (data not shown). These results are consistent with the lack of genetic evidence that the endogenous *Scr* gene is autoregulated and strongly suggest that *Hox-1.3* is acting directly to regulate the downstream targets of *Scr*.

Hox-1.3 activates *fkh*, a target gene of *Scr*

Drosophila Scr is expressed in the primordia of the salivary gland and is required for its development (Panzer et al. 1992). One of the *Scr* target genes involved in this process is *fkh*, a transcription factor participating in the development of the salivary gland and gut (Weigel et al. 1990). In *Scr* null mutant embryos, no *fkh* expression and no salivary placode are detected, whereas misexpression of *Scr* induces ectopic salivary placodes in the procephalon, in parasegments 1 and 3, and in A8 (Panzer et al. 1992). A *fkh-lacZ* fusion gene containing a *fkh* salivary gland-specific enhancer element directs *lacZ* expression specifically in salivary placodes of early embryos (strain P[ry⁺, HNK01]; Panzer et al. 1992; Fig. 7A). This fusion

gene responds to *Scr* overexpression as does the endogenous *fkh* gene (see Fig. 7B; Panzer et al. 1992). To test the ability of mouse *Hox-1.3* to participate in the salivary gland regulatory pathway of *Drosophila*, we crossed the *hs-Hox-1.3* strain with P[ry⁺, HNK01]. After a 30-min heat shock of 1- to 4-hr-old hybrid embryos and recovery at 25°C for 6 hr, ectopic expression of the *fkh-lacZ* fusion gene is observed. β -Galactosidase is detected ectopically in the procephalon and in small patches in ventrolateral parts of almost all other parasegments (Fig. 7C). This expression pattern is very similar to that induced by ectopic *Scr* expression (cf. Fig. 7B). Thus, the mouse *Hox-1.3* gene mimics the effects of *Scr* in regulating the expression of the *fkh* gene.

Discussion

The ectopic expression of mouse Hox-1.3 mimics Scr homeotic transformations

In this paper we show that the mouse *Hox-1.3* can induce specific homeotic transformations when ubiquitously expressed in *Drosophila*. These transformations are similar to those morphological consequences induced by ectopic *Scr* gene expression.

After a 60-min heat shock to germ-band-extended embryos, first-instar larvae bearing either *hs-Scr* or *hs-Hox-1.3* display a prominent abnormality in the thoracic region. The two posterior segments (T2 and T3) gain T1 epidermal identity evidenced by the presence of ectopic ventral beards. This transformation must result from the presence of *Scr/Hox-1.3* proteins in the whole thoracic

Figure 7. Ectopic expression of a *fkh-lacZ* fusion gene induced by misexpression of mouse *Hox1.3*. (A) The expression of the *fkh-lacZ* fusion gene in HNK01 transformant embryos was detected with anti- β -galactosidase antibodies. (Note the prominent band of expression in the salivary gland primordia.) (B, C) Hybrid embryos carrying either *hs-Scr* and *fkh-lacZ* (B) or *hs-Hox-1.3* and *fkh-lacZ* (C) were subjected to a 30-min heat shock at 1–4 hr AEL and 37°C and allowed to develop at 25°C for another 6 hr. *fkh-lacZ* expression is detected in patches that extend to the posterior segments of the embryo in each case. Embryos at the germ-band extension stage are shown (lateral view).

region, which reprograms the fate of ventral beard precursor cells and, possibly, additional thoracic precursors by overriding the effects of *Antp* and *Ubx* activities in this region. These results suggest that there must be strict selective pressure to maintain the *Scr* expression pattern within sharp boundaries. Other morphological abnormalities are displayed mainly in head segments, including the failure of head involution and the absence or misplacement of head structures. However, these alterations may not reflect the functional specificity of *Scr* and *Hox-1.3*, as ectopic expressions of other *Drosophila* homeotic selector genes and a mouse cognate of *Antp* (*Hox-2.2*) also generated similar abnormalities (Gibson and Gehring 1988; Kuziora and McGinnis 1988; Malicki et al. 1990; Mann and Hogness 1990).

More dramatic homeotic transformations are generated in adult animals after heat treatment of the larvae, the most striking being a transformation of antenna toward leg. These legs appear to correspond to T1 legs be-

cause sex comb teeth were displayed in a few *hs-Hox-1.3* male pupae. This result strongly indicates that the transformation is *Scr*-specific because one of the functions of *Scr* is to produce sex combs on T1 legs of male flies (Pattatucci et al. 1991). The transformation of antenna toward leg by *Hox-1.3* is stronger than that induced by *Scr* and is detected with a higher frequency (observed in 42 of 203 flies examined). This rather surprising observation may be explained by the increased viability of *hs-Hox-1.3* flies as compared with *hs-Scr* flies after heat shock. Under equivalent conditions, we found that more *hs-Hox-1.3* flies develop to adult than do *hs-Scr* flies. This most likely results from the mouse *Hox-1.3* having lost functions present in the *Drosophila* *Scr* that prevent development during larval and pupal stages. Thus, the potential for stronger transformation of antenna toward leg is uncovered in animals that can develop to adult.

In addition to antennal transformations, both *Scr* and *Hox-1.3* induce a *Ctx*-like transformation in adult flies (swollen occiputs with many dark ectopic bristles in the dorsal aspect of hindhead; Duncan and Lewis 1982; Schneuwly et al. 1987; Gibson and Gehring 1988). At present, we are unable to determine the segmental identity of this transformation and, thus, cannot distinguish between *Antp*- and *Scr*-induced effects on these structures. Unlike dominant *Scr* mutant males that have ectopic sex comb teeth on both T2 and T3 legs (Pattatucci et al. 1991), *hs-Scr* and *hs-Hox-1.3* adult animals do not exhibit a posterior leg toward foreleg transformation. Perhaps the timing or persistence of heat-induced expression differs from that of the *Scr* protein produced in dominant gain-of-function mutants. It is possible that in these mutants, a rearranged *cis*-acting regulatory element leads to ectopic expression of *Scr* exclusively in the primordial cells of the T2 and T3 leg imaginal discs that have the potential to generate sex combs. In this case, adults with ectopic T2 and T3 sex combs might not be observed because animals subjected to heat treatment sufficient to induce high levels of *Scr* expression in these specific cells die as a result of poisoning effects of *Scr* in other parts of the larva.

The anteriorized transformation in heat-shocked larvae containing Scr/Hox-1.3

As shown in Figure 3, the misexpression of *Scr* causes transformation of T2 and T3 toward T1. The anteriorization is probably the result of the ectopic presence of *Scr* protein products in T2 and T3, because it is similar to the phenotype of *Antp* recessive mutants where *Scr* proteins can be found in T2 and T3 (Riley et al. 1987). This observation is consistent with the model that posteriorly acting homeotic genes normally down-regulate anteriorly acting genes in the thoracic region. For instance, *Antp* can repress *Scr* (Riley et al. 1987) and *Ultrabithorax* (*Ubx*) can repress *Antp* (Hafen et al. 1984; Harding et al. 1985). Thus, the improper expression of *Scr* can override *Antp* and *Ubx* in two other thoracic segments (T2 and T3) and cause them to gain the epidermal identity of a T1 segment that is normally determined by *Scr*. Ec-

topic expression of *Antp* leads to a transformation of T1 to T2 identity (Gibson and Gehring 1988). T3 is not transformed, presumably because its identity normally is determined by both *Antp* and *Ubx*. A transient excess of *Antp* protein cannot override *Ubx* activity and alter the developmental program of T3. Ectopic expression of *Ubx*, which normally determines T3 (together with *Antp*) and A1 (alone) segment identities, causes a transformation of all thoracic segments toward A1 (González-Reyes and Morata 1990; Mann and Hogness 1990).

Why does the anteriorized transformation not occur in all abdominal segments of embryos in which anteriorly acting genes are ectopically expressed? One explanation is that BX-C gene products present in abdominal segments are functionally dominant to ANT-C gene products such that ectopic expression of ANT-C genes cannot override their effects in abdominal segments even when expressed at high levels, a phenomenon termed phenotypic suppression (González-Reyes and Morata 1990; González-Reyes et al. 1990; Mann and Hogness 1990). Thus far, almost all reported homeotic transformations induced by misexpression of HOM-C genes fit within this model. Nevertheless, our results and a previous paper (Panzer et al. 1992) demonstrate that this model may not be suitable for explaining other sorts of transformations. *fkh*, a downstream gene of *Scr*, can be activated ectopically in the ventrolateral epithelial cells of most posterior segments by overexpression of either *Scr* or *Hox-1.3* (see Fig. 7). Apparently, the functional dominance of BX-C genes is not applicable to this subset of cells in posterior segments. Consistent with this idea, ectopic expression of ANT-C genes also altered the developmental program of the peripheral nervous system in the abdominal segments (Heuer and Kaufman 1992). Thus, when markers for individual cell identity are utilized, transformation can be observed at a cellular level along the entire anterior–posterior body axis. These transformations may not be manifested at the level of morphological change in cuticular pattern, because of the large number of cells and/or sets of genes in each individual cell that must be simultaneously reprogrammed to produce such a dramatic phenotypic change as segmental transformation.

A primordial gene for Hox-1.3 and Scr

Several investigators proposed that a common ancestor for vertebrates and arthropods contained five homeotic genes in a primordial cluster (Duboule and Dolle 1989; Graham et al. 1989; McGinnis and Krumlauf 1992). These genes were arrayed in an order corresponding to *lab*, *pb*, *Dfd*, one of *Scr*/*Antp*, and *Abd-B* of the HOM-C of *Drosophila*. Akam et al. (1988) suggested that *Scr*, *Ubx*, and *Abd-A* emerged after the divergence of vertebrates and arthropods by duplication of a single *Antp*-like primordial gene. As shown in Figure 1A, *Hox-1.3* shares 55 of 61 amino acids within the homeo domain of both *Antp* and *Scr*, leaving the assignment of primordial gene identity ambiguous. Our observations demonstrate that despite this sequence similarity, *Hox-1.3* is func-

tionally equivalent to *Scr* and not to *Antp*. In addition, *Hox-1.3* shares more homology to *Scr* than to *Antp* because of the conserved octapeptide and pentapeptide regions (see Fig. 1A). This homology suggests that *Hox-1.3* and *Scr* arose from a single primordial gene, although we cannot rule out convergent evolution of *Scr* and *Hox-1.3*.

The results presented here reflect the strongest conservation of functional specificity among the three cognate pairs of *Hox* genes that have been examined. In the *Dfd*/*Hox-4.2* case, the resulting transformation is probably the consequence of activation of endogenous *Dfd* by ectopic expression of a human cognate *Hox-4.2* (McGinnis et al. 1990). In embryos carrying the mouse *Hox-2.2* transgene, an *Antp*-like transformation was not dependent on the activation of the endogenous *Antp* gene (Malicki et al. 1990). However, there is a difference between the cuticular phenotypes induced by *Antp* and *Hox-2.2*. *Hox-2.2* has little effect on the morphology of T1, whereas the ectopic expression of *Antp* resulted in T1 to T2 transformation. This may be attributed to a partial loss of functional specificity during evolution or, alternatively, to a convergent evolution of *Antp* and its vertebrate cognates. In this study we have shown that phenotypes induced by ectopic expression of *Hox-1.3* are virtually identical to (if not stronger than) those of *hs-Scr*. Thus, we suggest that an independent primordial gene for *Scr* and *Hox-1.3* existed in a primordial cluster of a common ancestor. Possibly, this gene was retained under more stringent selective pressure because it plays a more important role than an *Antp*-like gene for pattern formation in vertebrate development.

How does a cognate maintain its regulatory specificity?

The present study supports the previous finding that mammalian *Hox* genes can functionally substitute for cognate *Drosophila* homeotic selector genes in fruit flies (Malicki et al. 1990; McGinnis et al. 1990). For each one of the three examined cognate pairs [*Antp*/mouse *Hox-2.2* (Malicki et al. 1990), *Dfd*/human *Hox-4.2* (McGinnis et al. 1990), and *Scr*/mouse *Hox-1.3* (this paper)], comparison of primary amino acid sequence reveals only small regions of conservation, the strongest homology being in the homeo domain (57/61, 54/60, and 56/61, respectively). In addition, similarities in the octapeptide and pentapeptide regions between *Scr* and *Hox-1.3* suggest that these regions may contribute to functional specificity as well. However, these regions of similarity are extremely short.

Thus, the remarkable specificities of these proteins seem to be based only on a few individual amino acids in conserved regions, relatively independent of the rest of the protein sequence. At the same time, however, functional specificity of different homeo box-containing genes is accomplished despite the striking similarities of their homeo domains (*Antp* vs. *Scr*, 56/61; *Antp* vs. *Dfd*, 49/60; and *Scr* vs. *Dfd*, 50/60). These analyses suggest that specificities of these proteins may be accomplished by secondary or tertiary protein structures whose exis-

tence is not revealed by primary amino acid sequence analysis and whose function is to interact with auxiliary factors (Hayashi and Scott 1990). Furthermore, the combined results of the mouse/fly cognate experiments suggest that position within a *Hox* cluster may be more predictive of function than is analysis of primary amino acid sequence.

Although the different members of mouse *Hox* complexes are expressed in patterns having unique anterior boundaries, there are large regions of the body in which several members of each complex are expressed. For example, the expression domains of *Hox-1.2*, *Hox-1.3*, and *Hox-1.4* partially overlap (Gaunt et al. 1988, 1989, 1990; Dressler and Gruss 1989). In flies, the *fkh* gene is activated by ectopic expression of *Scr* but not *Antp* (Panzer et al. 1992). The observation that the *fkh* gene is activated by *Hox-1.3* in flies suggests that it might have the ability to regulate specific genes that are not regulated by other members of the *Hox-1* cluster, for example, *Hox-1.2*, the *Antp* cognate. A further speculation is that each *Hox* gene may thus regulate different sets of target genes. The overlapping expression of different combinations of *Hox* genes in the mouse embryo would thereby provide a combinatorial code that would give each cell in the developing embryo a unique identity.

Hox-1.3 activates a downstream gene of *Scr*

The ectopic activation of *fkh* after the induction of mouse *Hox-1.3* in fly embryos provides the first molecular evidence to support the notion that mammalian *Hox* gene products can activate a target gene of an insect homeotic selector gene and participate in the genetic circuitry of *Drosophila*. The transformation and the activation of the *fkh-lacZ* fusion gene observed in *hs-Hox-1.3*-bearing animals do not appear to be attributable to the activation of the endogenous cognate *Scr* gene, as ectopic expression of *Scr* is not observed after induction. Panzer et al. (1992) pointed out that the action of *Scr* on *fkh* expression is restricted to the early phase of embryogenesis (within 4 hr AEL). After that, *Scr* is unable to induce *fkh* ectopically. The response time phase of *fkh* to *Scr/Hox-1.3* induction further supports the hypothesis that *Hox-1.3* directly activates *fkh* transcription. In *hs-Hox-1.3/fkh-lacZ*-bearing hybrid embryos, the ectopic presence of β -galactosidase is indistinguishable from that of *hs-Scr/fkh-lacZ* embryos (see Fig. 7). It is unlikely to be mediated by activation of the endogenous *Scr* gene after heat-induced expression of *Hox-1.3*, because the *Scr* gene has an ~25-kb-long transcription unit (LeMotte et al. 1989). Thus, enough *Scr* protein products would only accumulate after the sensitive period for *fkh* induction in *hs-Hox-1.3* embryos.

It is possible that the extent of conservation in developmental programs between vertebrates and invertebrates is far more than expected. Sequence comparison reveals that the mammalian HNF-3 gene family, whose products were originally purified from rat liver nuclear extracts, is the counterpart to *Drosophila fkh* (Lai et al. 1991). Similar to *fkh*, which is specifically expressed in

gut and salivary gland tissues, the HNF-3 family is also expressed at different levels in the organs or tissues (such as liver, lung, and intestine etc.) derived from the embryonic gut tube. Lai et al. (1991) suggested that the HNF-3 family may maintain certain *fkh* functions in forming gut-related terminal embryonic structures during evolution (Lai et al. 1991). Interestingly, *Hox-1.3* transcripts also have been found in liver and lung (Odenwald et al. 1987; Garbern et al. 1989). The colocalization of *Hox-1.3* and HNF-3 gene products in the same tissues may not be a coincidence. Rather, the mammalian *Hox-1.3* may participate in organogenesis by activating HNF-3 in a particular group of cells, as *Scr* and *fkh* dictate the ontogenesis of the *Drosophila* salivary gland. The HNF-3 products, a new class of transcription factors, may then act on downstream targets to direct the formation of terminal organ structures.

Materials and methods

DNA construction

The P-element vector CaSpeR-hs (Pirotta 1988) was cleaved within the polylinker sites with restriction enzymes *HpaI* and *XbaI*. A full-length *Hox-1.3*-coding sequence from plasmid pCDM81.35+ (obtained from W. Odenwald, National Institutes of Health, Bethesda, MD) was cleaved with *XhoI*. Protruding ends were filled in with Klenow enzyme, and the linerized DNA was digested with *XbaI*. The fragment produced by this manipulation contains the *Hox-1.3* ORF flanked by the 5' end TCGAGCAACAAGGAA linker and the 3' end GCATCTGAGCGGCCAAA GTACTGTGACTCTAGA untranslated sequence. The fragment and the vector DNA were ligated together to generate *hs-Hox-1.3*. Standard methods were used for all procedures (Sambrook et al. 1989).

Transgenic flies

Flies were grown at 25°C on standard medium containing cornmeal, sugar, yeast, and agar. P-element transformation of *Df(1)w* embryos was carried out using standard techniques with the helper plasmid p π 25.1wc (Rubin and Spradling 1982; Karess and Rubin 1984). Stocks were maintained as homozygous or with the balancer chromosomes *yw/CyO* or *yw/TM6b*. All experiments were repeated with three or more of the 10 independent transformant lines established to rule out positional effects of insertion.

Three independent *hs-Scr* lines were kindly provided by D. Andrew, W.-L. Zeng, and M. Scott (Stanford University, CA). P[ry⁺, HNK01] carrying a *fkh* enhancer-*lacZ* fusion gene was a gift from B. Zhou and S. Beckendorf (University of California, Berkeley, CA).

Heat treatment and phenotypic analysis

Heat shock treatments were performed as described by Gibson and Gehring (1988; G. Gibson, pers. comm.) except for some modification. For examining the cuticular consequences of heat shock, embryos are precollected on yeasted apple juice plates for 1 hr and collected on fresh yeasted apple juice plates for 30 min. They are allowed to mature at 25°C for ~5.5–6 hr until heat shock treatment. Embryos are immersed in still water of a 37°C water bath for 60 min. After heat shock treatment, the embryos are allowed to mature further for another 20 hr on plain apple juice plates. They are dechorionated in 3% sodium hypochlorite

(supplied by VWR Scientific) for 2 min, and vitelline membranes are removed by shaking vigorously in heptane/methanol (1 : 1) solution. After washing twice with methanol, embryos are incubated in acetic acid/glycerol (4 : 1) at 45°C for 2 days and mounted in Hoyer's medium/lactic acid (1 : 1) on a glass slide (Van der Meer 1977). They are placed at 45°C for 1 week. The cuticular structures are analyzed and photographed using dark-field and phase-contrast microscopy. By delivering a 20-min heat shock at different times to *hs-Hox-1.3* embryos, we found that the most sensitive stage to ubiquitous *Hox-1.3* expression was at 5–6 hr AEL; 70% of the treated embryos were not able to hatch to first-instar larvae.

For larval heat shock, embryos are collected for 1 hr, washed, and allowed to age on plain apple juice plates at 25°C for 24 hr. To test in which period *hs-Hox-1.3* larvae would be affected most severely by heat shock, hatched larvae were picked and inoculated into several yeasted cornmeal vials, in batches of 20. Animals in each vial grew at 25°C to a predetermined stage and were heat-shocked at 36.8°C for 45 min (heat shock is given to individual vials at 3-hr intervals starting at 48 hr and ending at 120 hr AEL). To obtain stronger transformations, heat shock is given multiple times, usually from 68 to 92 hr AEL at 36.8°C for 45 min with minimal 4-hr intervals. Heat is delivered to animals by simply placing the vials in a 36.8°C water bath. It was found that the temperature of the water bath is crucial, and a 0.3°C higher temperature could kill all larvae before they reach the pupal stage. After heat shock, usually, 70–80% of the larvae die before eclosion (for *hs-Scr* flies, >90% die). Adult flies and unhatched pupae (that were first dissected out of sacs) are treated as described by McGinnis et al. (1990).

Immunohistochemical staining

Embryos are allowed to age 6 hr and are heat-shocked for the indicated time. For *Hox-1.3* antibody staining, the heat treatment is for 1 hr and embryos are recovered at 25°C for 1 hr. For *Scr* and *Dfd* antibody staining, embryos are heat-shocked for 1 hr and allowed to recover at 25°C for 3 hr and 2 hr, respectively. Embryo collection and heat treatment for the *fkh* enhancer-*lacZ* construct is according to the previous description (Panzer et al. 1992). Immunostaining is carried out by modifications of standard methods (T. Gutjahr and M. Noll, pers. comm.) using a preformed ABC (avidin and biotinylated horseradish peroxidase macromolecular complex) reagent (Vectastain Elite ABC Kit). The *Hox-1.3* epitope is located in a nonconserved region from amino acid residue 53 to 72, and the preparation of *Hox-1.3* antiserum was described previously (Odenwald et al. 1987). The *Scr* and *Dfd* monoclonal antisera are gifts from D. Brower and W. McGinnis. The anti- β -galactosidase antibody is from Capell. Imaginal disc staining was carried out as described by Patatucci and Kaufman (1991).

Photography

The immunostaining embryos were photographed with Nomarski optics, and the larval cuticles were photographed in dark field on a Zeiss Axiophot microscope. The adults were photographed on an Olympus BH-2 microscope. We used Kodak TMAX100 film for black and white photography and Ektachrome ET160 film for color photography. Films were processed with Adobe Photoshop Macintosh Version 2 (Adobe Systems Inc.) and printed on a Kodak XL7700 printer.

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Note

The new nomenclature for *Hox-1.3* is *HoxA5* (Scott 1992).

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The mouse Hox-1.3 gene is functionally equivalent to the Drosophila Sex combs reduced gene.

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