Compound mutants for the paralogous *hoxa-4*, *hoxb-4*, and *hoxd-4* genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed

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The *Hox* gene products are transcription factors involved in specifying regional identity along the anteroposterior body axis. In the mouse, several single mutants for *Hox* genes show variably penetrant, partial homeotic transformations of vertebrae at their anterior limits of expression, suggesting that compound *Hox* mutants might show more complete transformations with greater penetrance than the single *Hox* mutants. Compound mutants for the paralogous group 3 genes, *hoxa-3* and *hoxd-3*, show deletion of a cervical vertebrae, which is not readily interpretable in terms of an alteration in regional identity. Here, we report the skeletal phenotypes of compound mutants in the group 4 *Hox* genes, *hoxa-4*, *hoxb-4*, and *hoxd-4*. Mice mutant for each of these genes were intercrossed to generate the three possible double mutant combinations and the triple mutant. In contrast to the *hoxa-3*, *hoxd-3* double mutants, group 4 *Hox* compound mutants displayed clear alterations in regional identity, including a nearly complete transformation of the second cervical vertebrae toward the morphology of the first cervical vertebra in one double mutant combination. In comparing the types of homeotic transformations observed, different double mutant combinations showed different degrees of synergism. These results suggest a certain degree of functional redundancy among paralogous genes in specifying regional identity. Furthermore, there was a remarkable dose-dependent increase in the number of vertebrae transformed to a first cervical vertebra identity, including the second through the fifth cervical vertebrae in the triple mutant. Thus, these genes are required in a larger anteroposterior domain than is revealed by the single mutant phenotypes alone, such that multiple mutations in these genes result in transformations of vertebrae that are not at their anterior limit of expression.

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The specification of regional identity along the anteroposterior body axis of many organisms culminates in the generation of a diversity of structures along this axis. The proper specification of regional structures along this axis is dependent on the action of an evolutionarily conserved set of genes, which were first identified in *Drosophila melanogaster* for their ability to effect homeotic transformations, reidentification of a body segment

(Lewis 1978). These homeotic genes encode putative transcription factors that share a common DNA-binding motif known as the homeo domain (Slack et al. 1993). In insects and lower chordates, these homeotic genes comprise a single cluster (which is split in *Drosophila*; Lewis 1978; Kaufman et al. 1980; Garcia-Fernández and Holland 1994). Presumably, duplications of a single ancestral cluster (the *HOM-C* complex) during evolution resulted in the mouse and human genomes having four independent clusters, lettered A through D (Fig. 1A) (Scott 1993), which contain at least 38 so-called *Hox* genes. Thus, for a given ancestral gene in the *HOM-C* complex, the mouse may have up to four genes, called

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Figure 1. The *Hox* clusters and the group 4 *Hox* genes, with their anterior limits of expression in the axial skeleton. (A) The *Hox* clusters in mouse. Note that group 4 is one of only two groups with four members. (■) The three most anteriorly expressed members of this group that were used for combination mutant analysis. (B) Anterior limits of expression of group 4 *Hox* genes in E12.5 cervical prevertebrae (Gaunt et al. 1989; Geada et al. 1992), with lighter shading representing weaker expression in the anterior-most vertebrae. It should be noted that *hoxb-4* expression is very weak in C1 and some groups have placed its anterior limit at C2 (Ramírez-Solis et al. 1993).

paralogs, which are closely related to each other and to the ancestral gene, particularly in the homeo domain.

In mouse, as in *Drosophila*, *Hox* genes are expressed along the anteroposterior axis of the embryo with discrete anterior limits that generally correlate with their relative position within a given cluster, such that the most 3' genes are expressed in the most anterior structures (Duboule and Dollé 1989; Graham et al. 1989). Because paralogs share analogous positions within clusters, they sometimes share similar anterior limits of expression as well. In the mouse, these discrete anterior limits are most evident in the central nervous system and in the somites and prevertebrae, which will give rise to the axial skeleton.

In *Drosophila*, the generation of a large number of mutant alleles of the *HOM-C* genes has been invaluable in elucidating the singular and combined functions of these genes (Lawrence and Morata 1994). A similar effort has been undertaken in the mouse, utilizing transgenic techniques to create both gain-of-function and loss-of-function alleles. In general, loss-of-function mutations lead to anterior transformations near the anterior limit of expression of the gene, similar to what is seen in *Drosophila* (Duboule and Morata 1994). Although these genes are expressed in a number of tissues in the mouse, these transformations have been primarily seen in the vertebrae of the axial skeleton. In many instances, only partial transformations of vertebral identity occur, involving the modification of some aspects of vertebral morphology whereas other aspects remain unchanged (Krumlauf 1994). In addition, these phenotypes are often incompletely penetrant, suggesting that other genes may

partially compensate for their loss. The most obvious candidate genes for compensation are the paralogous genes.

There are four members of the group 4 paralogous *Hox* genes, *hoxa-4*, *hoxb-4*, *hoxc-4*, and *hoxd-4* (Rubin et al. 1986; Wolgemuth et al. 1986; Featherstone et al. 1988; Graham et al. 1988; Geada et al. 1992). They share extensive amino acid identity in the homeo domain as well as in other domains in the protein. They have very similar expression patterns, with similar but distinct anterior limits of expression in the prevertebrae and central nervous system (Gaunt et al. 1989; Geada et al. 1992). Mice mutant for three of the group 4 genes (*hoxa-4*, *hoxb-4*, and *hoxd-4*) have been generated via homologous recombination in embryonic stem cells. *hoxa-4* mutants show a partial transformation of the third cervical vertebra (C3) to the second cervical vertebra (C2) (Horan et al. 1994; Kostic and Capecchi 1994) as well as a cervical rib at C7 (Horan et al. 1994). Neonatal mice homozygous for a mutation in the first exon of *hoxb-4* display partial homeotic transformations of C2 to C1, defects in the closure of the sternal rudiments, and variably penetrant neonatal lethality (Ramírez-Solis et al. 1993). Heterozygous and homozygous *hoxd-4* mutants have more complete homeotic transformations of C2 to C1 than those seen in *hoxb-4* mutants, as well as malformations of the neural arches of C1 and ectopic ossification at the basioccipital bone (Horan et al. 1995). In each of these mutants, the anterior transformations were at the respective anterior limits of expression of these genes (Fig. 1B), suggesting that they were important for establishing the regional identity of the second and third cervical vertebrae. However, each of these phenotypes

were incompletely expressive and variably penetrant, even on an inbred genetic background (Ramírez-Solis et al. 1993; Horan et al. 1994, 1995).

To address the question of whether the incomplete penetrance and expressivity in these mutant phenotypes was the result of partial functional redundancy among the paralogs, we intercrossed these three mutants to produce the three double mutant combinations and the triple mutant. We found that in the *hoxb-4*, *hoxd-4* double mutants, the C2 to C1 transformations became completely penetrant, with greater expressivity. Surprisingly, there was a dose-dependent increase in the number of vertebrae transformed, such that vertebrae that were not at the anterior limit of expression of these genes were also transformed. In the triple mutant *hoxa-4^{-/-}*, *hoxb-4^{-/-}*, *hoxd-4^{-/-}* there was a remarkably long-range transformation of identity, such that even C5 partially adopts the morphology of C1. Thus, these *Hox* genes are collectively required in a larger anteroposterior domain than is defined by the single mutant phenotypes. We discuss these results as they apply to current models of how *Hox* genes specify regional identity in the axial skeleton.

Results

Differential viability in different double mutant combinations

Both the *hoxa-4^{-/-}* and *hoxd-4^{-/-}* mice are viable (Horan et al. 1994, 1995), whereas a 50% lethality is seen in *hoxb-4^{-/-}* mice (only *hoxb-4^r* mutants were used in these studies; Ramírez-Solis et al. 1993). The viability of the three different double mutant combinations are summarized in Table 1. *hoxa-4^{-/-}*, *hoxd-4^{-/-}* mice were viable, whereas *hoxa-4^{-/-}*, *hoxb-4^{-/-}* mice always died within the first week after birth. Although nearly all *hoxb-4^{-/-}*, *hoxd-4^{-/-}* mice died within the first week after birth, two mice have survived for more than 6 months. The exact cause of death in the *hoxa-4^{-/-}*, *hoxb-4^{-/-}* mutants is still being studied, but because the skeletons of some *hoxa-4^{-/-}*, *hoxb-4^{-/-}* mice showed no major morphological abnormalities, it is likely that this lethality was because of abnormalities in tissues other than the skeleton.

hoxa-4, *hoxb-4* double mutants show novel phenotypes in the cervical vertebrae

Wild-type morphology of the cervical vertebrae Compound group 4 *Hox* mutants on a C57BL/6×129 hybrid genetic background were examined for abnormalities in the axial skeleton at birth and as adults, except for *hoxa-4^{-/-}*, *hoxb-4^{-/-}* mice that do not survive to adulthood. Abnormalities were limited to the cervical vertebrae. Wild-type mice have seven cervical vertebrae, designated C1 to C7 (anterior to posterior) (Fig. 2A). In general, vertebrae are composed of a vertebral body located ventral

Table 1. Genotypes of group 4 *Hox* mutant combinations

A. Genotypes of offspring from double heterozygote matings	a4, b4	b4, d4	a4, d4
	observed (expected)		
-/-, -/-	0 (8)	1 (2)	15 (17)
-/-, +/-	29 (32)	7 (4)	35 (33)
+/-, -/-	6 (16)	40 (34)	25 (33)
+/-, +/-	77 (63)	63 (68)	54 (66)
-/-, +/+	14 (16)	2 (2)	22 (17)
+/-, -/-	8 (8)	14 (17)	14 (17)
+/-, +/+	35 (32)	32 (34)	30 (33)
+/+, +/-	23 (32)	39 (34)	42 (33)
+/+, +/+	11 (16)	17 (17)	27 (17)
Total	203	215	264

B. Genotypes of offspring from <i>hoxb-4</i> heterozygote matings on a <i>hoxa-4</i> or <i>hoxd-4</i> homozygous mutant background	a4, b4	d4, b4
	observed (expected)	
-/-, -/-	0 (12)	1 (7)
-/-, +/-	77 (70)	38 (36)
-/-, +/+	40 (35)	22 (18)
Total	117	61

All of the analysis presented here involves mice on a C57BL/6 × 129 hybrid genetic background, generation F3 or greater. +/+, +/-, and -/- are wild-type, heterozygous, and homozygous mutants, respectively. (A) Observed numbers are the mice for each possible genotype surviving beyond 3 weeks of age. Expected numbers represent the predicted mendelian ratios, except for genotype combinations involving *hoxb-4* homozygous mutants, which result in variably penetrant lethality. For these combinations, the number of mice homozygous for the *hoxb-4* mutation and wild type for the other *Hox* mutations was used as an internal control to calculate the expected numbers of the other mutant combinations involving *hoxb-4^{-/-}*. For this reason, the total of the expected numbers does not equal the total of the observed numbers in these combinations. (B) Because there is no such internal control in these crosses, predicted values were calculated based on the 35.7% survival of *hoxb-4^{-/-}* mice compared with wild type in the crosses of A.

to the neural tube and neural arches that surround the neural tube laterally and dorsally. C1 and C2, also known as the atlas and the axis, have a number of unique morphological characteristics that distinguish them from the other cervical vertebrae. First, C1 lacks a vertebral body (which is incorporated into the dens of C2) but possesses a ventral arch, known as the anterior arch of the atlas (aaa; Fig. 2A). In addition, C1 has the widest neural arches of the cervical vertebrae and a U-shaped, rostral notch in the lateral portions of the neural arches. Its foramina, through which the vertebral arteries pass, are located on the lateral surface of the vertebra and can best be seen in views of separated vertebrae (Fig. 5B, below). In contrast, C2, like the rest of the cervical vertebrae, has a vertebral body and ventral foramina but is distinct in having an anterior projection of the vertebral body known as the dens (Fig. 5B, below). The neural arches of C2 are slightly wider than those of C3 to C7.

Figure 2. Skeletal abnormalities in *hoxa-4*, *hoxb-4* and *hoxa-4*, *hoxd-4* double mutants. In the cervical vertebrae (numbered 1 through 7) of wild-type (wt) neonates (A), only C1 (the atlas) has an anterior arch of the atlas (aaa). C6 is morphologically distinguishable by its anterior tuberculi (at). There are articular processes on the lateral neural arches of C2 through C7. The most anterior articular process is on the caudal portion of C2 in the wild-type skeleton (*) and is marked by an asterisk (*) in each of the mutants (B–F). In *hoxa-4* mutants (B), cervical ribs form on C7 (arrow) with low penetrance. In ~20% of *hoxb-4* mutants (C), an aaa forms on C2 (arrow) and there is widening of the neural arches of C2. In *hoxa-4*^{-/-}, *hoxb-4*^{-/-} double mutants (D), there is extensive fusion of the neural arches of C2 and C3 (short arrow). The articular processes between C2 and C3 are lost in all mice, and in 15%, the rostral articular process of C4 is also lost (white curved arrow). In addition, the anterior tuberculi are on C7 (black curved arrow) instead of C6. The *hoxd-4* phenotype (E) is similar to *hoxb-4* (aaa on C2, arrow) but involves transformation of more features of the C2 vertebrae and malformation of the neural arches of C1. In *hoxa-4*^{-/-}, *hoxd-4*^{-/-} double mutants (F), there is a low penetrance of fusion of the cartilaginous portions of the dorsal neural arches of C2 and C3 (arrow). (ex) Exoccipital bone.

C3, C4, and C5 are morphologically similar, whereas C6 is distinct in having bilateral ventral projections known as anterior tuberculi (at; Fig. 2A). C7 is morphologically similar to C3 through C5.

Novel skeletal abnormalities in *hoxa-4*, *hoxb-4* double mutants In neonatal skeletons, *hoxa-4*^{-/-} mice have a variably penetrant cervical rib on C7 (Fig. 2B) (Horan et al. 1994). *hoxb-4*^{-/-} mice have an anterior arch of the atlas at C2 (Fig. 2C), indicative of a partial C2 to C1 transformation (Ramírez-Solis et al. 1993). Although these phenotypes shared no common elements, the *hoxa-4*^{-/-}, *hoxb-4*^{-/-} double mutants showed phenotypes not seen in either single mutant. In 78% of these mice, the dorsal cartilages and/or the lateral ossified portions of the neural arches of C2 and C3 were fused (Fig. 2D; and Table 2A) and the lateral articular processes between C2 and C3 were lost. In some cases, the rostral articular process on C4 was missing as well (Fig. 2D; Table 2A).

C6 to C5 and C7 to C6 transformations in *hoxa-4*, *hoxb-4* double mutants The formation of a cervical rib at C7, a phenotype seen in some *hoxa-4*^{-/-} mice, was suppressed in *hoxa-4*^{-/-}, *hoxb-4*^{-/-} mice. There was also a variably penetrant shift of the anterior tuberculi from C6 to C7 in these mice and in *hoxa-4*^{+/-}, *hoxb-4*^{+/-}, and *hoxa-4*^{-/-}, *hoxb-4*^{+/-} mice (Fig. 2D; Table 2A), interpreted as transformations of C7 to C6 and C6 to C5.

hoxa-4, *hoxd-4* double mutants show a phenotype similar to *hoxa-4*, *hoxb-4* double mutants but with lower penetrance and expressivity

Just as the *hoxd-4* (Fig. 2E) mutants are similar to the *hoxb-4* mutants in having an anterior arch of the atlas at C2 (Horan et al. 1995), so were *hoxa-4*^{-/-}, *hoxd-4*^{-/-} mice similar to *hoxa-4*^{-/-}, *hoxb-4*^{-/-} mice in having fusions of the dorsal cartilages of the neural arches of C2 and C3 (Fig. 2F). There are some C2–C3 fusions in *hoxd-4* single mutants (Horan et al. 1995); however, those fusions resulted from the neural arches forming at abnormal angles to each other. In contrast, the fusions in the *hoxa-4*^{-/-}, *hoxd-4*^{-/-} mice involved a piece of cartilage that extended anteroposteriorly to connect the dorsal cartilages of the neural arches that formed parallel to each other. Thus, the C2–C3 fusions in *hoxa-4*^{-/-}, *hoxd-4*^{-/-} mice were more similar to those of *hoxa-4*^{-/-}, *hoxb-4*^{-/-} mice than to those seen in *hoxd-4* single mutants. However, the penetrance (17%) of the C2–C3 neural arch fusion in *hoxa-4*^{-/-}, *hoxd-4*^{-/-} was significantly lower, and the lateral ossified portions of the neural arches were never fused (Fig. 2F; Table 2B) as they were in many of the *hoxa-4*^{-/-}, *hoxb-4*^{-/-} mice. There was also no loss of the rostral articular process of C4 (Fig. 2F; Table 2B). Furthermore, the cervical rib phenotype was not suppressed, and the C7 to C6 and C6 to C5 transformations were not seen. In most of the *hoxa-4*^{-/-}, *hoxd-4*^{-/-} skeletons, the phenotype was simply an addition of the two single mutant phenotypes. Taken together, these results seem to indicate that there was

Table 2. Percent penetrance of neonatal skeletal abnormalities in group 4 *Hox* double mutants

Type of transformation	Description	a4 ^{+/-} b4 ^{+/-}	a4 ^{+/-} b4 ^{-/-}	a4 ^{-/-} b4 ^{+/-}	a4 ^{-/-} b4 ^{-/-}
C6 to C5 } C7 to C6 }	A. <i>hoxa-4, hoxb-4</i>				
	fusion of neural arches: C2/C3	0	0	5	78
	loss of articular processes between C2 and C3	0	(1/3)	0	100
	loss of rostral articular process on C4:	0	0	0	15
	anterior tuberculi: shifted to C7	0	(2/3)	20	85
	total number analyzed	8	3	20	13
C6 to C5 } C7 to C6 }	B. <i>hoxa-4, hoxd-4</i>	a4 ^{+/-} d4 ^{+/-}	a4 ^{+/-} d4 ^{-/-}	a4 ^{-/-} d4 ^{+/-}	a4 ^{-/-} d4 ^{-/-}
	fusion of neural arches: C2/C3	0	6	0	17
	loss of articular processes between C2 and C3:	0	33	0	56
	anterior tuberculi: shifted to C7	0	0	0	0
	total number analyzed	10	18	13	18
		b4 ^{+/-} d4 ^{+/-}	b4 ^{-/-} d4 ^{+/-}	b4 ^{+/-} d4 ^{-/-}	b4 ^{-/-} d4 ^{-/-}
C2 to C1	C. <i>hoxb-4, hoxd-4</i>				
C3 to C1	anterior arch of the atlas: on C2	71	100	100	100
C2 to C1	on C3	0	20	0	100
	lateral foramen on C2: extensive	0	0	32	100
	complete	0	0	8	90
C3 to C2	{ dens: intermediate of C2 and C3	0	30	40	0
	on C3	0	20	20	100
	ectopic ossification at } extensive	7	20	48	90
	basioccipital bone } fused to aaa	0	0	24	90
	incomplete neural arches: C1	7	50	76	100
	C2	0	20	84	90
	C3	14	50	52	80
	fusion of neural arches: C1/C2	0	20	20	100
	loss of articular processes between C2 and C3:	0	100	96	100
C6 to C5 } C7 to C6 }	anterior tuberculi: shifted to C7	0	50 10	0 25	70 10
	total number analyzed	14	10	25	10

All abnormalities listed are either novel to the double mutants, or the penetrance of the abnormalities is significantly changed in the double mutants when compared with the single mutants. (A) Abnormalities in *hoxa-4*^{+/-}, *hoxb-4*^{-/-} mice are expressed as a fraction rather than as a percentage penetrance because of the low number of animals examined.

less synergism in the *hoxa-4, hoxd-4* combination than in the *hoxa-4, hoxb-4* combination.

In adult skeletons, the C3 to C2 transformation shows hoxa-4 dosage dependence on a hoxb-4^{-/-} *or hoxd-4*^{-/-} *background*

Because *hoxa-4*^{-/-} mice exhibit a partial C3 to C2 transformation that is only evident in adult skeletons (Horan et al. 1994; Kostic and Capecchi 1994), mutant skeletons were also analyzed at adulthood. Wild-type and *hoxa-4*^{+/-} mice have large processus spinosi on the dorsal surface of C2 but never on C3 (Fig. 3A). *hoxa-*

4^{-/-} mice (Fig. 3B) have large processus spinosi on both C2 and C3. *hoxa-4*^{+/-}, *hoxb-4*^{-/-} mice also had processus spinosi on both C2 and C3 (Fig. 3C). Similarly, this C3 to C2 transformation was seen in *hoxa-4*^{+/-}, *hoxd-4*^{-/-} mice (Fig. 3D). Thus, this phenotype revealed a dose-dependent requirement for *hoxa-4* on a *hoxb-4*^{-/-} or a *hoxd-4*^{-/-} background.

More complete and penetrant homeotic transformations in hoxb-4, hoxd-4 double mutants are also dose-dependent

C2 to C1 transformations in hoxb-4, hoxd-4 mutants In single mutants for both *hoxb-4* and *hoxd-4*,

Figure 3. Dose-dependent requirement for *hoxa-4* on a *hoxb-4* or *hoxd-4* mutant background. In adult skeletons, there is a large processus spinosus on C2 but never on C3 in wild-type and *hoxa-4*^{+/−} mice (A). In *hoxa-4*^{−/−} mice (B), a processus spinosus forms on C3 (arrow), a partial transformation of C3 to C2. Processus spinosi are not present on C3 of *hoxb-4*^{−/−} mice but are present (arrow) in *hoxa-4*^{+/−}, *hoxb-4*^{−/−} mice (C). A processus spinosus also forms on C3 (arrow) of *hoxa-4*^{+/−}, *hoxd-4*^{−/−} mice (D). The orientation of the cervical vertebrae in D is slightly different from A–C to more easily see the processus spinosus on C3.

there are partial transformations of C2 to C1. In neonatal skeletons, ~20% and 33% of *hoxb-4* and *hoxd-4* single mutant mice, respectively, have an anterior arch of the atlas and widened neural arches on C2 (Ramírez-Solis et al. 1993; Horan et al. 1995). In all of the *hoxb-4*, *hoxd-4* double mutant mice (Fig. 4C; Table 2C), C2 was almost

completely transformed to a C1 identity, as defined by five distinct morphological criteria. The C2 of double mutants had an anterior arch of the atlas, widened neural arches, complete lateral foramina, loss of the vertebral body, and loss of the caudal articular process (Fig. 5B; Table 2C). In some *hoxb-4*^{−/−}, *hoxd-4*^{−/−} skeletons, the neural arches of C2 were malformed. However, in other skeletons, distinctive U-shaped rostral notches, normally seen only on C1, were seen in the neural arches of C2, such that the only element missing from a complete transformation was the lack of fusion at the dorsal cartilages of the neural arches. In addition, the neural arches of C1 and C2 were often fused, similar to the C2–C3 fusions in *hoxa-4*^{−/−}, *hoxb-4*^{−/−} mice.

C3 to C1 and C3 to C2 transformations in *hoxb-4*, *hoxd-4* mutants Unexpectedly, an anterior arch of the atlas also formed on C3, indicative of a C3 to C1 transformation. The vertebral body of C2 was incorporated into an extended dens on C3 (Fig. 5B; Table 2C), which was interpreted as a C3 to C2 transformation.

Dose-dependent intermediates in *hoxb-4*, *hoxd-4* mutants The *hoxb-4*^{+/−}, *hoxd-4*^{−/−} (Fig. 4A) and *hoxb-4*^{−/−}, *hoxd-4*^{+/−} mice (Fig. 4B) represented an intermediate state between the single and double mutants, in terms of both the penetrance and expressivity of these transformations (Table 2C). The anterior arch of the atlas at C2 phenotype was completely penetrant in these mice, and other aspects of the C2 to C1 transformation were more penetrant (Table 2C). Thus, as the number of wild-type alleles of *hoxb-4* and *hoxd-4* was incrementally decreased, there was a corresponding incremental shift in the position at which C1- and C2-specific structures are formed. For instance, the dens always originates from C2 in wild-type mice (Fig. 5B). It sometimes

Figure 4. Stepwise anterior transformations in *hoxb-4*, *hoxd-4* double mutants and *hoxa-4*, *hoxb-4*, *hoxd-4* triple mutants. An anterior arch of the atlas (aaa), a C1-type structure, forms on progressively more posterior vertebrae (straight arrows) as more group 4 *Hox* mutations are combined, going from *hoxb-4*^{+/−}, *hoxd-4*^{−/−} (A) to *hoxb-4*^{−/−}, *hoxd-4*^{+/−} (B) to *hoxb-4*^{−/−}, *hoxd-4*^{−/−} (C) to *hoxa-4*^{+/−}, *hoxb-4*^{−/−}, *hoxd-4*^{−/−} (D) to *hoxa-4*^{−/−}, *hoxb-4*^{−/−}, *hoxd-4*^{−/−} (E). Similarly, the position of the most anterior articular process (*) is shifted to more posterior vertebrae in (A–E). In addition, in *hoxb-4*^{−/−}, *hoxd-4*^{−/−} double mutant mice (C), the neural arches of C1 are always incomplete and often severely shortened (open arrow). There can also be fusion of the neural arches of C1 and C2 and shifting of the anterior tuberculi to C7 (curved arrow). (ex) Exoccipital bone.

Figure 5. C1- and C2-specific structures form on progressively more posterior vertebrae in *hoxb-4*, *hoxd-4* double mutants and *hoxa-4*, *hoxb-4*, *hoxd-4* triple mutants. In a rostral view of partially separated vertebrae (A), the vertebral body (vb) of a wild-type (wt) C2 can clearly be seen. The shape of both C1 and C2 (fused) of *hoxb-4*^{-/-}, *hoxd-4*^{-/-} double mutant mice is similar to a wild-type C1, including absence of a vertebral body. In *hoxa-4*^{-/-}, *hoxb-4*^{-/-}, *hoxd-4*^{-/-} triple mutant mice, C1, C2, and C3 (fused) all lack vertebral bodies. In a ventral view (B), the dens, a structure that projects anteriorly from the vertebral body of C2, is shifted to C3 in *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice and to C4 in *hoxa-4*^{-/-}, *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice (some mechanical breakage of the dens occurred during separation of vertebrae). The dens at C3 or C4 is more extensive than a dens at C2, presumably because the vertebral bodies of C2 and C3 have been incorporated into this structure. Lateral foramina (lf) form on C1 in wild-type mice, on C2 (arrow) in *hoxb-4*^{-/-}, *hoxd-4*^{-/-} double mutant mice, and on C2, C3 and C4 of *hoxa-4*^{-/-}, *hoxb-4*^{-/-}, *hoxd-4*^{-/-} triple mutant mice (not visible in this view).

originated from C2 on one side and from C3 on the other in *hoxb-4*^{+/-}, *hoxd-4*^{-/-} and *hoxb-4*^{-/-}, *hoxd-4*^{+/-} mice, and it always originated from C3 in *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice (Fig. 5B; Table 2C).

C6 to C5 and C7 to C6 transformations in *hoxb-4*, *hoxd-4* mutants In addition to transformations toward a C1 identity, *hoxb-4*, *hoxd-4* mice also showed transformations of C6 to C5 and C7 to C6, as seen in *hoxa4*, *hoxb-4* mice. This was seen as a shift in the position of the anterior tuberculi from C6 to C7 (Fig. 4C; Table 2C).

Other skeletal abnormalities in *hoxb-4*, *hoxd-4* mutants Another abnormality that is specific to *hoxd-4*

mutant mice in the single mutants was exacerbated in the *hoxb-4*, *hoxd-4* double mutants. Ectopic ossification at the basioccipital bone, which was minimal in *hoxd-4*^{-/-} mice, was more extensive in the *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice, such that a bony bridge often fuses the basioccipital bone to the anterior arch of the atlas (Table 2C).

As was mentioned previously, the C2 neural arches of *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice often failed to fuse at the dorsal cartilages. These incomplete neural arches were also seen on C1 and C3 (Table 2C). This phenotype was most severe in the C1 neural arches of *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice, which were often severely reduced not just dorsally but laterally as well (Fig. 4C; Table 2C).

hoxa-4, *hoxb-4*, *hoxd-4* triple mutants show a greatly expanded domain of anterior transformations toward a C1 identity

The number of vertebrae transformed to a C1 identity increased remarkably in the triple mutants. Specifically, in three *hoxa-4*^{-/-}, *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice (Fig. 4E), there were anterior arches of the atlas on C1 through C5 and lateral foramina on C2, C3, and C4. The vertebral bodies of both C2 and C3 were incorporated into an extended dens on C4 (Fig. 5A,B). Therefore, in the absence of wild-type alleles of three of the group 4 *Hox* genes, the five most anterior cervical vertebrae assumed a nearly complete or partial C1 identity. In addition, small anterior arches of the atlas were present on C4 in 4 of 6 *hoxa-4*^{+/-}, *hoxb-4*^{-/-}, *hoxd-4*^{-/-} (Fig. 4D) mice. Hence, even though the *hoxa-4* mutation never produced transformations to a C1-type vertebral element (anterior arch of the atlas or lateral foramen) in either of the double mutant combinations, these transformations were seen for the first time at C4 and C5 when the *hoxa-4* mutation was combined with the *hoxb-4* and *hoxd-4* mutations.

C6 to C5 and C7 to C6 transformations in *hoxa-4*, *hoxb-4*, *hoxd-4* mutants In *hoxa-4*^{-/-}, *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice, the anterior tuberculi were always absent from C6, interpreted as a C6 to C5 transformation. In two of three mice, the anterior tuberculi were very small on one side of C7, indicative of a transformation of C7 to C6, whereas in the third mouse, they were completely absent from C7. This absence of the anterior tuberculi from C7 may represent either a lack of transformation at C7 or a transformation of C7 to C5.

***hoxb-5* expression is unchanged in *hoxa-4*, *hoxb-4* and *hoxb-4*, *hoxd-4* double mutants**

The C6 to C5 and C7 to C6 transformations in *hoxa-4*^{-/-}, *hoxb-4*^{-/-} and *hoxb-4*^{-/-}, *hoxd-4*^{-/-} mice are also found in *hoxb-5*^{-/-} mice (Rancourt et al. 1995). Thus, these phenotypes could be the result of altered *hoxb-5* expression in these group 4 double mutants. Therefore, we immunostained *hoxa-4*^{-/-}, *hoxb-4*^{-/-} and *hoxb-*

$4^{-/-}$, $hoxd-4^{-/-}$ embryos at embryonic day 12.5 (E12.5) with a *hoxb-5* antibody (Wall et al. 1992) to examine the distribution of *hoxb-5* protein. In all of the embryos examined, the spatial distribution of *hoxb-5* protein in the double mutants was similar to that observed in the control embryos (Fig. 6; data not shown) and appeared to be unchanged in the affected prevertebrae, pv 6 and pv 7 (Wall et al. 1992; Rancourt et al. 1995).

Discussion

Dosage-dependent increases in the expressivity and penetrance of homeotic transformations in compound mutants suggest functional redundancy among paralogs in specifying regional identity

In the other compound *Hox* mutants reported, the link between mutant phenotypes and changes in regional identity is less clear. In *hoxa-3*, *hoxd-3* double mutants, the deletion of C1 structures seen in *hoxd-3^{-/-}* mutants is exacerbated such that there is a nearly complete deletion of C1 in the double mutant; however, there is no clear homeotic transformation (Condie and Capecchi 1994). In *hoxb-5/hoxb-6* transheterozygotes, there is ho-

meotic transformation similar to that seen in *hoxb-5^{-/-}* and *hoxb-6^{-/-}* mutants (Rancourt et al. 1995). Neither case, though, revealed a requirement for the genes in regions not affected in either of the single mutants, nor did they result in more complete homeotic transformation. In contrast, the homeotic transformations seen in compound mutants of the group 4 paralogous *Hox* genes can be readily interpreted as more complete transformations of vertebral morphology, consistent with more complete changes in regional identity. Furthermore, the homeotic transformations seen in each double mutant combination and in the triple mutant were clearly dosage dependent. Given that these genes encode similar proteins with similar expression patterns, this dosage dependence probably reflects a certain degree of functional redundancy in terms of transcriptional regulation of the same downstream target genes. However, the possibility that these genes act through parallel pathways cannot be excluded.

Compound mutant phenotypes reveal a larger functional domain than is seen in the single mutant phenotypes

In addition to an increase in the expressivity and penetrance of homeotic transformations, the number of vertebrae transformed was also increased in combination mutants. This suggests that the domain of activity for the group 4 genes extends beyond that which is defined by the single mutant phenotypes. This was most dramatically visualized by the formation of an anterior arch of the atlas, a C1-specific structure, in the more posterior cervical vertebrae (Fig. 7). This C1-type transformation occurred at more posterior vertebrae in a dose-dependent fashion as the *hoxb-4* and *hoxd-4* mutations were combined and as the *hoxa-4* mutation was added to the *hoxb-4*, *hoxd-4* mutant background, such that only C2 was transformed in the single mutant, both C2 and C3 were transformed in the double mutant, and C2, C3, C4, and C5 were transformed in the triple mutant.

This expansion in the affected domain was also evident in the most posterior cervical vertebrae, C6 and C7. As mentioned previously, both the *hoxb-4*, *hoxd-4* and the *hoxa-4*, *hoxb-4* double mutants showed transformations at C6 and C7 similar to those seen in *hoxb-5* single mutants (Rancourt et al. 1995). In addition, *hoxa-5* mutants showed the C6 to C5 transformations but not the C7 to C6 transformations (Jeanotte et al. 1993), and *hoxb-6* mutants showed a low penetrance of both types of transformations, but never bilaterally (Rancourt et al. 1995). Thus, whereas the formation of anterior arches of the atlas at more posterior vertebrae was unique to group 4 *Hox* mutants, certain double mutant combinations produced phenotypes in the posterior region of their functional domain that were similar to the phenotypes of single mutants for more posterior *Hox* genes.

A similar interpretation applies to the shortening of the neural arches of C1, C2, and C3 in the *hoxb-4*, *hoxd-4* double mutants. The sometimes severely shortened neural arches of C1 resembled the most severely

Figure 6. Expression of *hoxb-5* in E12.5 control and *hoxa-4*, *hoxb-4* double mutant embryos. Lateral views of hemisected *hoxa-4* single mutant (A) and *hoxa-4*, *hoxb-4* double mutant (B) embryos immunostained for *hoxb-5* protein and counterstained with alcian blue to visualize the cartilaginous skeleton. Although the strength of the antibody staining among different embryos of all genotypes was variable, no differences in the spatial distribution of *hoxb-5* protein were observed in the prevertebrae of the double mutants. Staining was weak in prevertebra 2 (pv 2) stronger in pv 3 (curved arrow) through pv 7, and weak in pv 8. The prevertebrae that show a similar phenotype in *hoxb-5* mutants and in these double mutants are pv 6 and pv 7 (white arrows). However, no differences in *hoxb-5* antibody staining were detected in these prevertebrae. Similarly, no differences were found in *hoxb-4*, *hoxd-4* double mutant embryos (data not shown). The *hoxb-5* protein localization in these embryos was identical to that observed in wild-type embryos (Wall et al. 1992; Rancourt et al. 1995). The anterior limits of *hoxb-5* expression in the hindbrain of controls and the double mutants were identical (large black arrow).

Figure 7. Schematic representation of the progressive anterior transformations of cervical vertebrae in group 4 *Hox* mutants. The anterior limits of group 4 *Hox* gene expression in E12.5 prevertebrae (pv) is depicted at *left*, with lighter shading representing weaker expression in the anteriormost vertebrae. Moving from *left* to *right*, the number of group 4 wild-type alleles decreases, whereas the extent of anteriorization of the cervical vertebrae increases. The transformation to a C1 identity is nearly complete in those prevertebrae for which group 4 *Hox* expression is completely lost. For example, in *hoxa-4*, *hoxb-4*, *hoxd-4* triple mutants, there are no wild-type group 4 *Hox* genes expressed in C2 and C3, and these vertebrae are completely transformed to a C1 identity. *hoxc-4* and the group 5 *Hox* genes are expressed in C4 and C5, which show partial transformations to a C1 identity. The group 5 genes are more strongly expressed in C6 and C7, which are never transformed to a C1 identity; however, their morphological changes are consistent with anterior transformations.

affected *hoxd-3* single mutants (Condie and Capecchi 1993). Thus, *hoxb-4*, *hoxd-4* double mutants showed a phenotype in the most anterior region of their functional domain that was similar to the phenotype of the single mutant for a more anterior *Hox* gene.

A final example of overlapping phenotypes was the fusion of the neural arches of C1 and C2 in *hoxb-4*, *hoxd-4* and of C2 and C3 in *hoxa-4*, *hoxb-4* mutants. These fusions were similar to the fusion of C1 and the exoccipital bone in *hoxd-3* mutants (Condie and Capecchi 1993). These mutant combinations therefore produced similar phenotypes in different anteroposterior regions that correspond with the different anterior limits of expression of the genes.

One possible explanation for these overlapping phenotypes is that the expression of these other *Hox* genes is altered in the double mutants. This could be because group 4 *Hox* genes are directly required for proper regulation of the expression of these other *Hox* genes, or it could be the result of more indirect mechanisms. The *hoxa-4*, *hoxb-4* double mutants, the *hoxb-4*, *hoxd-4* double mutants, and the *hoxb-5* single mutants (Rancourt et al. 1995) showed the most extensive similarities of any of these overlapping phenotypes, and thus, *hoxb-5* represented the best candidate for having altered expression in the double mutants. However, whole-mount immunostaining of E12.5 embryos revealed no changes in the spatial distribution of *hoxb-5* protein in both double mutant combinations, notably in the affected prevertebrae, pv 6 and pv 7. Thus, these transformations are not the result of a complete loss of *hoxb-5* expression throughout development. We cannot, however, rule out the possibility that subtle quantitative changes in the expression of *hoxb-5* or other *Hox* genes at different stages of

development play a role in effecting these types of transformations.

Different double mutant combinations show different degrees of synergism

Although the different group 4 *Hox* combination mutants show extensive similarities with each other and with neighboring nonparalogous *Hox* genes, this study also permits us to analyze the differences between the three different mutant combinations. For instance, although *hoxb-4* and *hoxd-4* have almost identical anterior limits of expression and overlapping single mutant phenotypes, their double mutant combinations with *hoxa-4* showed distinct phenotypes. Specifically, the *hoxa-4*, *hoxb-4* skeletal phenotype seemed to show greater synergism than did the *hoxa-4*, *hoxd-4* skeletal phenotype. These differences could be the result of small differences between *hoxb-4* and *hoxd-4* in their timing, levels, and anterior limits of expression or functional differences in the proteins. It is interesting to note, however, that in comparing the single mutant phenotypes of these three genes, *hoxd-4* affects the most anterior structures, whereas *hoxa-4* affects the most posterior structures. Thus, the least synergism was seen in the double mutant combination for which the affected domains of the single mutants are most separated.

Paralogous Hox genes and axial patterning

These novel observations suggest certain refinements to the current hypotheses of how *Hox* genes cooperatively pattern the axial skeleton. For example, based on the loss of the dens at C2 in *hoxd-3* mutants and the formation of

the anterior arch of the atlas in *hoxb-4* mutants, it was suggested that *Hox* genes might cooperatively pattern vertebrae by separately specifying different morphologic features of the same vertebrae (Krumlauf 1994). Although this may still be one way in which these genes cooperatively specify identity, the *hoxb-4*, *hoxd-4* mutants show more complete transformations of all aspects of vertebral morphology in double mutants, even for structures that are not affected in the *hoxb-4* mutant (e.g., the lateral foramina) and for structures not affected in either single mutant (e.g., the incorporation of the vertebral body of C2 into a dens on C3). This suggests that there is not such a fine division of labor in specifying individual structures but rather a more extensive cooperativity in which several *Hox* genes with similar anterior limits of expression may each play a role in the proper specification of many aspects of vertebral morphology, whether directly or indirectly. Likewise, the similarities between the group 4 compound mutant phenotypes and the mutant phenotypes of neighboring nonparalogous genes suggest that this cooperativity exists among nonparalogous genes as well.

The posterior prevalence model has been useful in describing the action of *Hox* genes in vertebrates (Duboule 1991). In this model, genes with more posterior limits of expression are dominant in specifying identity over more anterior genes expressed in the same region. Thus, it suggests a functional hierarchy among vertebrate *Hox* genes similar to that seen in *Drosophila*. This model predicts that loss-of-function mutations should produce transformations only at their anterior limit of expression and not in more posterior vertebrae where more posterior *Hox* genes are expressed. The complete anterior transformations of C2 and C3, which are at the anterior limit of *hoxa-4*, *hoxb-4*, and *hoxd-4* expression, and the lack of transformations in the thoracic vertebrae of group 4 compound mutants are in agreement with this model. However, the transformations of C5, C6, and C7, where more posterior *Hox* genes are strongly expressed (Gaunt et al. 1988, 1990; Graham et al. 1989), are apparently inconsistent with this model. It has been proposed, though, that there is a quantitative aspect to this model, in which relative amounts of *Hox* gene products may be important in determining this functional hierarchy, perhaps by direct competition among *Hox* genes (Duboule and Morata 1994). If we interpret our mutant phenotypes using this more quantitative approach, then we would expect that combining mutations in *Hox* genes with very similar anterior limits of expression should produce a stronger anteriorizing effect, owing to decreased competition with more anterior genes. This effect would then be moderated in progressively more posterior vertebrae where increasingly greater quantities of posterior genes are expressed. For example in the group 4 triple mutant, the degree of competition against the more anterior genes would be low in C5, where only *hoxc-4* (the fourth group 4 paralog) is strongly expressed and *hoxa-5* and *hoxb-5* are weakly expressed; greater in C6 and C7, where all of the group 5 genes are strongly expressed; and greatest in the thoracic vertebrae, where both group 5

and group 6 *Hox* genes are expressed. Correspondingly, C5 is strongly transformed, to a C1 identity; C6 and C7 are more weakly transformed, to a C5 and C6 identity; and the thoracic vertebrae are not transformed at all. Thus, the degree of anteriorization of these vertebrae inversely correlates with the quantity of more posterior genes expressed, which is consistent with a quantitative posterior prevalence model. In *Drosophila*, the phenotypic suppression model (González-Reyes et al. 1990) is the equivalent to the posterior prevalence model in mouse. Similarly, in this model, there is not an absolute dominance of the more posterior genes and some abnormalities can occur in regions more posterior to the anterior limit of expression of the *Drosophila* HOM-C genes (Manak and Scott 1994).

Alternatively, the transformations in these more posterior vertebrae could be explained by more indirect effects. For instance, there could be secondary interactions that maintain anteroposterior order in the cervical vertebrae, so that the nearly complete transformations of identity at C2 and C3 lead to a secondary anteriorization of the remaining cervical vertebrae. Finally, the observation that the group 4 *Hox* paralogs function cooperatively in specifying regional identity of the axial skeleton suggests that similar mechanisms might also be operating in other group 4 *Hox*-expressing tissues.

Materials and methods

Skeleton preparations

Neonates were sacrificed, skinned, eviscerated, and fixed in 95% ethanol. Their skeletons were subsequently prepared by alkaline digestion and stained with alizarin red S for ossified bone and alcian blue 8GX for cartilage (Kochhar 1973).

Southern analysis

Tails were biopsied for DNA genotyping by Southern blot analysis (Ramírez-Solis et al. 1993; Horan et al. 1994, 1995).

hoxb-5 immunohistochemistry

E12.5 embryos were processed for *hoxb-5* whole-mount immunohistochemistry as described (Wall et al. 1992; Rancourt et al. 1995).

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