Targeted Mutations in Hoxa-9 and Hoxb-9 Reveal Synergistic Interactions

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Mice were generated with a targeted disruption of the homeobox-containing gene hoxb-9. Mice homozygous for this mutation show defects in the development of the first and second ribs. In most cases the first and second ribs are fused near the point at which the first and second pairs of ribs normally attach to the sternum. Abnormalities of the sternum accompany the rib fusions. These include abnormal attachment of the ribs to the sternum, a reduction in the number of intercostal segments of the sternum, and abnormal growth of the intercostal segments. Over half of the homozygous mutants, as well as some heterozygotes, also have an eighth rib attached to the sternum. These results show that hoxb-9 plays a significant role in the specification of thoracic skeletal elements. To reveal potential interactions between the paralogous Hox genes hoxa-9 and hoxb-9, mice heterozygous for both mutations were intercrossed. Mice homozygous for both mutations show more severe phenotypes than predicted by the addition of the individual mutant phenotypes. Both the penetrance and the expressivity of the rib and sternal defects are increased, suggesting synergistic interactions between these genes. In particular, the sternum defects are greatly exacerbated. Interestingly, the defects in hoxb-9 and hoxa-9/hoxb-9 mutant mice are concentrated along the axial column at points of transition between vertebral types.

INTRODUCTION

Hox genes encode transcription factors belonging to the Antennapedia homeodomain class. The mammalian Hox complex contains 39 genes distributed on 4 linkage groups designated as HoxA, B, C, and D. This organization is believed to have arisen early in vertebrate phylogeny by quadruplication of an ancestral complex common to vertebrates and invertebrates (Pendleton et al., 1993; Holland and Garcia-Fernandez, 1996). Based on DNA sequence and the position of the genes on their respective chromosomes, individual members of the 4 linkage groups have been classified into 13 paralogous families. Members of a paralogous family often share similar gene expression patterns.

In Drosophila the homologous genes (Hom C genes) are used to pattern the developing embryo along its rostrocaudal axis (Akam, 1987; Gehring, 1987). Mutations in some of these genes change the identity of one parasegment into that of a neighboring parasegment (Lewis, 1978). Mutations in some of these genes change the identity of one parasegment into that of a neighboring parasegment (Lewis, 1978). Mutations in some of these genes change the identity of one parasegment into that of a neighboring parasegment (Lewis, 1978). Mutations in some of these genes change the identity of one parasegment into that of a neighboring parasegment (Lewis, 1978).
Targeted Mutations in Hoxa-9 and Hoxb-9

Although all 39 Hox genes are likely to be involved in the formation of the axial skeleton, the specific malformations caused by mutations in individual Hox genes are difficult to predict. Some of this difficulty can be attributed to overlap of function between genes of the same paralogous group or between genes of different paralogous groups. The rostral-caudal direction of apparent homeotic transformations of vertebrae seen in Hox mutants is also unpredictable. In fact, the two polarities can appear in the same mutant (Jeannotte et al., 1993; Small and Potter, 1993). Often the defects are observed at the anterior limit of the Hox gene expression pattern, a phenomenon that has led to the proposal of the

FIG. 1. Disruption of the hoxb-9 gene and analysis of the mutant genotype. (A–C) Diagrams of the wild-type hoxb-9 locus, the targeting vector, and the targeted allele, respectively. The black box indicates the homeobox of hoxb-9. Hoxb-9 is transcribed from left to right. The bars indicate restriction fragments produced by EcoRI digest. RI, EcoRI; RV, EcoRV; E, Eco47III; S, SalI; X, XhoI; B, BamHI. (D, E) Southern transfer analyses of the targeted cell line and of the intercross genotypes, respectively. Genomic DNA was digested with EcoRI and probed with the 3' flanking probe. The 12- and 4.3-kb bands were from the wild-type and mutant alleles, respectively. (F) PCR analysis of the intercross genotypes. The wild-type band is 188 bp and the mutant band is 333 bp.
FIG. 2. Defects in the thoracic skeleton in hoxb-9−/− mice. (A–E) Ventral views of the thoracic skeletons with the vertebral columns removed. (F–J) Lateral views of the upper thoracic regions of the same embryos showing different types of first and second rib fusions. The black arrow points to a ventral rib element which connects only to the sternum.

posterior prevalence model (Duboule, 1991). However, even this correlation has many exceptions. A model that, in its broad sense, is likely to be correct is that the combination of Hox genes expressed in a given region determines the identity of structures in that region. However, such combinatorial models should not be viewed as a code involving simple addition of elements, because these gene products, as already pointed out, are likely to interact positively, negatively, and in parallel with each other. Simple combinatorial models based on overlapping Hox gene expression patterns have in fact been woefully inaccurate in predicting vertebral identities. Instead, the interactions among Hox genes are sufficiently complex that their role in specifying the vertebral column must be functionally determined on a case by case basis through analyses of both individual and appropriate combinations of Hox gene mutations.

Herein we describe the phenotypic consequences of disrupting hoxb-9 in mice, as well as the effects of combining hoxb-9 and hoxa-9 mutations. These mice show defects in the formation of the thoracic skeleton.

### TABLE 1

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>+/+ (n = 11)</th>
<th>+/- (n = 27)</th>
<th>−/− (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First and second rib fusion</td>
<td>0</td>
<td>0</td>
<td>20 (83%)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Unilateral</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Articulating eighth rib</td>
<td>0</td>
<td>2 (7.4%)</td>
<td>13 (54%)</td>
</tr>
<tr>
<td>Both sides</td>
<td>0</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>One side</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

### MATERIALS AND METHODS

#### Targeting Vector

A 9.9-kb genomic clone containing the hoxb-9 gene was isolated from a λDNA library prepared from mouse CC1.2 embryo-derived stem (ES) cells and used to construct a replacement-type targeting vector...
vector. In order to disrupt the hoxb-9 coding sequence, the MC1neo poly(A) cassette (Thomas and Capecchi, 1987) was inserted into the Eco47III site within the homeodomain. Completion of the targeting vector involved flanking the hoxb-9 genomic sequences with the HSV1 and HSV2 thymidine kinase genes (Fig. 1).

**Electroporation and the Generation of hoxb-9 Mutant Mice**

The targeting vector was linearized by digestion with XhoI and electroporated into R1 ES cells (Deng and Capecchi, 1992; Nagy et al., 1993). ES cells containing a disruption of the hoxb-9 gene were enriched by positive-negative selection (Mansour et al., 1988). DNA samples isolated from colonies of ES cells were digested with EcoRI and probed with 5' and 3' flanking probes (Fig. 1). Two percent of the ES cell lines contained the desired hoxb-9 mutation. The targeted ES cell line (1g6) was used to generate chimeric mice that transmitted the hoxb-9 mutant allele to their progeny (Capecchi, 1989, 1994).

**The Generation of Mice Deficient for Both hoxa-9 and hoxb-9**

The hoxa-9 mutant mice used in this study were generated by Peterson, Chisaka, and Capecchi (unpublished data). Since both hoxa-9<sup>−/−</sup> mice and hoxb-9<sup>−/−</sup> mice are fertile (Fromental-Ramain et al., 1996), compound heterozygotes for hoxa-9 and hoxb-9 mutations (hoxa-9<sup>+</sup><sup>−/−</sup> and hoxb-9<sup>+</sup><sup>−/−</sup>) were obtained from crosses between these homozygous mutant mice.

**Genotype Analysis**

DNA was prepared from tail biopsies of adult and newborn mice and from yolk sacs of embryos. Hoxb-9 genotypes were determined either by Southern transfer analysis with the 3' flanking probe or by amplification of DNA fragments using the polymerase chain reaction (PCR). Hoxa-9 genotypes were determined by PCR. The sequences of the PCR primers used for this analysis were: hoxb-9 forward primer, 5'TCTCCAATGCCAGGGGAGTAG3'; hoxb-9 reverse primer, 5'CTTCTCTAGCTCCAGCGTCTGG3'; MC1neo reverse primer for hoxb-9, 5'GTGTTCGAATTCGCCAATGAC-AAG3'; hoxa-9 forward primer, 5'CCTCCTAGGCTCCAGCTTGCTGG3'; MC1neo reverse primer for hoxa-9, 5'CGCTGGAACGTGGGAGG-AGTCTCTG3'; hoxa-9 reverse primer, ATCCTCGCTTCTGCGGAC-AGCAATG3'; MC1neo reverse primer for hoxa-9, 5'TCTATCGCCTTCTGAGCAGTT3'. An example of the hoxb-9 genotyping results is given in Fig. 1.

**Histology**

Newborn mice were euthanized by asphyxiatiion with CO<sub>2</sub>, fixed in 4% formaldehyde in phosphate-buffered saline (PBS) overnight.

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**FIG. 3.** Sagittal sections of hoxb-9<sup>+</sup><sup>−/−</sup> and −<sup>−/−</sup> newborn mice. Sections (A) and (B), (C) and (D), (E) and (F), (G) and (H) were from similar positions of the mice. Sections from top to bottom were taken progressively further away from the midlines of the mice. The space in the upper thoracic region and the size of the thymus are reduced in the hoxb-9<sup>−/−</sup> mutants with rib fusions.
at room temperature (Manley and Capecchi, 1995), and embedded in paraffin according to standard protocols. Ten-micrometer serial sagittal sections were collected and progressively stained with hematoxylin and eosin (Chisaka and Capecchi, 1991). Newborn and adult whole mount skeletons were prepared as described by Mansour et al. (1993).

Whole Mount in Situ Hybridization

Whole mount in situ hybridization on E9.0–E12.5 embryos was performed as described (Carpenter et al., 1993; Manley and Capecchi, 1995). The concentration of digoxigenin–UTP-labeled RNA probes in the hybridization mixture was approximately 0.4 µg/ml. Alkaline phosphatase-conjugated anti-digoxigenin Fab fragment was used at a 1:4000 dilution. The templates for hoxb-9, hoxb-8, and hoxb-7 RNA in situ probes were as follows: a 517-bp fragment in the 3' untranslated region (UTR) of hoxb-9, a 356-bp SacI–KpnI fragment from the 3' UTR of hoxb-8, and a 524-bp fragment from the 3' UTR of hoxb-7, respectively.

RESULTS

Generation of hoxb-9 Mutant Mice

The structure of the targeting vector used to disrupt the hoxb-9 gene in R1 ES cells is shown in Fig. 1. The insertion of the neo cassette into the hoxb-9 homeobox terminates the protein prior to all three helices of the homeodomain and therefore should render the gene product nonfunctional with respect to DNA binding. Southern transfer analyses, using 5' and 3' probes flanking the targeting vector as well as an internal probe, were used to ensure that no rearrangements of the hoxb-9 locus had occurred other than the desired neo insertion into the homeodomain. A representative targeted cell line was used to produce chimeric males that passed the hoxb-9 mutation through the germ line.

Hoxb-9 Mutants Are Viable and Fertile

Mice heterozygous for the hoxb-9 mutation were intercrossed to produce homozygotes. Adult hoxb-9+/− mice were obtained at a frequency predicted from a Mendelian distribution of mutant and wild-type alleles, indicating no loss of mutant alleles as a consequence of embryonic or postnatal lethality. Hoxb-9+/− mice appear outwardly normal and animals of both sexes are fertile.

Hoxb-9 Mutant Mice Display Skeletal Defects in the Thoracic Region

The skeletons of hoxb-9 mutant mice showed malformations in the thoracic region (Table 1, Fig. 2). Of 24 hoxb-9−/− homozygotes examined, 20 had fusions of the first and second ribs. Ninety percent of the fusions were bilateral.

The sternum of a normal mouse consists of six ossified segments, the manubrium, four sternebrae, and the xiphoid process. In animals with first and second rib fusions on both sides of the body, the normal cartilage centers associated with the attachment of the second pair of ribs are absent (Fig. 2). As a result, the upper sternum has one less ossified segment. The absence of that ossified segment causes the upper rib cage to be shortened by approximately 35% in the distance from the manubrium to the point where the third rib articulates with the sternum. This shortening is in part due to the loss of two growth plates normally present at the ends of each ossified segment. Segmentation of the sternum results from the attachment of the ribs to the sternum which locally inhibits the hypertrophy of the cartilaginous cells of the sternum near the attachment site (Chen, 1953). Thus, the observed abnormal segmentation patterns of the sternum can be understood in terms of the abnormal patterns of first and second rib attachment to the sternum.

The majority of the first and second rib fusions occurred at the point of attachment with the sternum (Fig. 2G). About 25% of the fusions occurred by alternative pathways. For example, in some animals the first and second ribs fused prior to attachment to the sternum, formed a common ventral rib, and then attached to the sternum (Fig. 2H). In other mutants, the first rib branched after fusion. In some of these cases both branches were articulated with the sternum; in others only one branch was attached. Finally, in two heterozygotes the first rib attached to the sternum but lost connection with either the second rib or the dorsal portion of the first rib, implicating remodeling after first rib attachment (Fig. 2J).

Thirteen of the 24 hoxb-9−/− mice examined had an eighth rib attached to the sternum on one or both sides (Figs. 2B, 2D, and 2E). A similar phenotype has been described in hoxc-8 and hoxc-9 mutant mice (LeMouelic et al., 1993; Suemori et al., 1995). Such eighth rib attachment appears to occur independently of the first and second rib fusions, since some animals had eighth rib articulations with the sternum but did not show first and second rib fusions (Fig. 2E). Approximately 7% of the hoxb-9−/− heterozygotes also showed eighth rib attachment to the sternum. This phenotype was never observed in wild-type control animals. The thoracic vertebral bodies of hoxb-9−/− mice appear normal. Thus, the defects appear to be restricted to the patterning of the ribs and sternum (i.e., the ventral aspects of the thoracic axial column). Formation of the appendicular axis appears normal in hoxb-9−/− mutant homozygotes.

Histological Examination of hoxb-9−/− Mice

Sagittal and parasagittal sections of newborn hoxb-9 homozygous mutant mice reveal a significant reduction in the size and an alteration in the shape of the thymus relative to wild-type and hoxb-9 heterozygous littermates (Fig. 3). The cortex and medulla of the thymus in hoxb-9−/− mice, however, appear normal. We presume that the altered shape of the thymus is an indirect effect of the reduced space in the upper thoracic region in hoxb-9 mutant homozygotes. Consistent with this hypothesis, hoxb-9 expression is not observed in tissues contributing to the formation of thymus.

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TABLE 2

| Genotype       | With rib fusion | Thymus weight \( T \) (g) | Body weight \( B \) (g) | \( T/B \)  
|----------------|-----------------|---------------------------|------------------------|----------
| \(-/-\)         | Yes             | 0.022                     | 3.9                    | 0.0056   
| \(-/-\)         | No              | 0.024                     | 3.6                    | 0.0067   
| \(-/-\)         | No              | 0.027                     | 4.2                    | 0.0064   
| \(-/-\)         | Yes \( ^c \)    | 0.026                     | 4.2                    | 0.0060   
| \(-/-\)         | Yes             | 0.022                     | 4.5                    | 0.0049   
| \(-/-\)         | No              | 0.026                     | 4.5                    | 0.0058   
| \(-/-\)         | Yes             | 0.021                     | 4.2                    | 0.0050   

Note. Thymus weight was independently normalized to heart weight and the results were indistinguishable from those shown above.

\( ^{a} \) These are 8-day-old littermates from a cross between two hoxb-9 homozygous mutants.

\( ^{b} \) If we use \( T/B_{A} \) for the average value of \( T/B \) for mutants with the rib fusions (Nos. 1, 4, 5, 6, and 7), we get \( T/B_{A} \approx 0.0055 \); if we use \( T/B_{A} \) for the average value of \( T/B \) for mutants without the rib fusions (Nos. 2 and 3), we get \( T/B_{A} \approx 0.0066 \). The difference between these two values is 20%.

\( ^{c} \) No. 4 had rib fusions that were not as severe as the other rib fusions observed in this litter.

Also, mutant animals do not appear to be compromised with respect to their immune function. For example, fluorescence–cytometric analysis of blood from mutant and control mice showed a normal distribution of T and B lymphocytes (data not shown). Finally, as previously mentioned, the rib fusions which are responsible for the reduction in the size of the upper thoracic cavity are not present in all hoxb-9 mutant homozygotes. When the weight of excised thymic tissues was compared between hoxb-9 mutant homozygous mice with and without the first and second rib fusions, the average ratio of thymus to body mass in mutants with the rib fusions was 20% lower than in mutants with normal ribs (Table 2).

E13.5 embryos were immunostained with the 2H3 antibody directed against a subunit of the neurofilament protein (Dodd et al., 1988) to reveal possible changes in the pattern of neurons in the peripheral nervous system. No neuronal defects were apparent in hoxb-9 mutant homozygotes either in the body wall or in the limbs (data not shown).

Hoxb-9 Is Expressed at the Axial Level From Which Affected Skeletal Elements Arise

The defects in the upper thoracic region of hoxb-9 homozygotes are found at significantly more rostral levels than the reported anterior limits of expression for hoxb-9 or its paralogues at E12.5 (Bogarad et al., 1989; Burke et al., 1995). To resolve this discrepancy, we reexamined the expression of hoxb-9 at E9.5, E10.5, E11.5, and E12.5 using whole mount RNA in situ hybridization (Fig. 4). In E9.5 embryos, the expression of hoxb-9 was strong in the neural tube with an anterior limit at the level of somites 7–8 (pV3). At this time, a high level of expression in the somites was detected in the posterior portion of the embryo but lower levels were seen in the rostral portion of the embryo. Expression in the somites could, however, be readily detected up to the level of the seventh to eighth somite. At E10.5, the anterior limit of expression in the neural tube shifted rostrally by one to two somites. Expression in the spinal ganglia was also apparent. Paraxial mesoderm expression appeared to be enhanced in ventral portions of the somites. At E11.5 and E12.5, changes in neural tube expression were not apparent. However, the intensity of the RNA hybridization signal decreased progressively in the anterior region of the embryos in paraxial mesoderm-derived structures which included the vertebral bodies and rib primordia. Hoxb-9 expression was evident in the kidneys from E12.5 onward.

From these studies, it is evident that at E9.5, the anterior limit of hoxb-9 expression is sufficiently anterior to cover the region of thoracic defects observed in hoxb-9 mutant mice. The anterior limit of expression in paraxial mesoderm-derived structures then shifts caudally. However, hoxb-9 expression is apparent in the regions of the rib and sternum primordia (i.e., the tissues affected by the hoxb-9 mutation).

The hoxb-9 Mutation Does Not Affect the Expression of Neighboring Hox Genes

It has been reported that in some cases a mutation in one Hox gene can affect the expression of a neighboring Hox gene (Suemori et al., 1995; Barrow and Capelchi, 1995; Boulet and Capelchi, 1996). To determine if the hoxb-9 mutation affected hoxb-8 or hoxb-7 expression, we conducted whole mount RNA in situ hybridization experiments on E10.5 and E12.5 embryos of all three hoxb-9 genotypes (i.e., +/+, +/−, −/−). Neither the anterior limits of hoxb-8 expression in the neural tube or in paraxial mesoderm-derived structures nor the overall level of hoxb-8 expression was distinguishable in embryos of these three hoxb-9 genotypes (Fig. 5). Similarly, the expression pattern of hoxb-7 was not affected in hoxb-9 heterozygous and mutant homozygous embryos (data not shown). Thus, neither the presence of the neo insertion in hoxb-9 nor the absence of functional hoxb-9 protein appears to affect the expression of the neighboring Hox genes.

As a further test for relationships between hoxb-9 and neighboring Hox genes, hoxb-9/hoxb-8 and hoxb-9/hoxb-7 transheterozygotes were constructed by crossing hoxb-9 mutants with hoxb-8 (Greer and Capelchi, data not shown) or hoxb-7 (Chen and Capelchi, unpublished data) mutants. At low frequencies (20%), hoxb-9/hoxb-7 transheterozygotes (i.e., hoxb-9/+; hoxb-7/−/+ mice) and (14%) of hoxb-9/hoxb-8 transheterozygotes showed first and second rib fusions very similar to those observed in hoxb-9 homozygotes. Such fused ribs have never been observed in mice heterozygous for hoxb-9, hoxb-8, or hoxb-7 alone. Such non-

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allelic noncomplementation suggests that hoxb-7, hoxb-8, and hoxb-9 gene products directly or indirectly interact to specify the upper thoracic region of the mouse (Rancourt et al., 1995).

**Hoxa-9/hoxb-9 Double Mutants**

Hoxa-9 mutant mice have been described by Fromental-Ramain et al. (1996). Since both hoxa-9−/− and hoxb-9−/− mice are fertile, compound heterozygotes for both hoxa-9 and hoxb-9 could be obtained from crosses between hoxa-9 and hoxb-9 mutant homozygotes. These compound heterozygotes appeared outwardly normal and were fertile. Mice of all nine possible genotypes were obtained from crosses between such compound heterozygotes, and the nine genotypes were obtained at the expected Mendelian ratios, indicating that all genotypes, including double mutant homozygotes, were viable.

**Skeletal Defects in hoxa-9/hoxb-9 Double Mutants**

Hoxa-9 mutant homozygotes have an extra pair of fully grown ribs on the 21st vertebra (i.e., show an anterior homeosis of the 1st lumbar vertebra to a thoracic vertebra; Fromental-Ramain et al., 1996). Hoxa-9 heterozygotes occasionally have small rib anlage on the 1st lumbar vertebra (Peterson, Chisaka and Capecchi, unpublished results). Hoxa-9 heterozygotes carrying one or two mutant alleles of hoxb-9 had a pair of full-size 14th ribs (Table 3, Fig. 6). Thirty-eight percent of hoxa-9+/−; hoxb-9+/− mice had pairs of fully grown ribs. The penetrance increased to 53% in hoxa-9+/−; hoxb-9−/− mice, indicating a quantitative effect of the hoxb-9 mutant alleles on mediation of this homeosis in mice heterozygous for the hoxa-9 mutation. An increase in the penetrance of the 1st and 2nd rib fusion phenotype was also observed when hoxb-9 homozygous mutant mice received one or two copies of the hoxa-9 mutant allele (Table 3 and Fig. 6). Approximately 83% of hoxb-9 homozygous mice show 1st and 2nd rib fusions (Table 1), while 93% of the hoxa-9+/−; hoxb-9−/− mice and 100% of the hoxa-9−/−; hoxb-9−/− mice show rib fusions. Moreover, the rib fusions in hoxa-9/hoxb-9 double mutants are more severe. Some double mutants even had the 3rd rib fused to the 1st and 2nd rib fusion (Fig. 6). Exacerbation of sternum defects is also evident in the hoxa-9/hoxb-9 double mutants (Fig. 6H). In such animals the length of all of the intercostal segments is greatly reduced. As is evident from Figs. 6F, 6G, and 6H, the expressivity of the sternum defects varies in the hoxa-9/hoxb-9 double mutants, suggesting that other members of this paralogous family may play important roles in patterning the thoracic vertebrae. The more severe abnormalities evident in hoxa-9−/−; hoxb-9−/− mice compared to defects observed in hoxa-9 and hoxb-9 single mutant homozygotes support the hypothesis that these two genes function synergistically to pattern the thoracic vertebrae.

**FIG. 4.** The expression of hoxb-9 in wild-type embryos. The anterior limit of neural tube expression (white arrow) and the anterior limit of paraxial mesoderm expression (black arrow) are at approximately P3 at E9.5 (A). The anterior limit of neural tube expression shifts rostrally by one to two somites at E10.5 (B) and remains unchanged at E11.5 (C) and E12.5 (D). The anterior limit of paraxial mesoderm expression shifts caudally in the upper thoracic region between E9.5 and E10.5. The intensity of the signal from mesoderm expression decreases progressively and becomes restricted to structures that appear to be primordia of the vertebral bodies (black triangle) and ribs (white triangle).

**DISCUSSION**

Eighty-three percent of the hoxb-9 homozygous mutants examined had first and second rib fusions while 54% of homozygotes and a small number of heterozygotes showed abnormal attachment of the eighth rib to the sternum. Both of these defects can be interpreted in terms of anterior homeotic transformations. In many hoxb-9 mutant homozygotes, the articulation angle and position of sternal attachment of the second rib resembles those of the first rib. Normally, the first rib has two attachment points with the
FIG. 5. The expression pattern of hoxb-8 does not change in hoxb-9 mutants. (A) hoxb-9+/+, (B) hoxb-9+/−, (C) hoxb-9−/−. White arrows indicate the anterior limits of neural tube expression. Black arrows indicate the anterior limits of mesoderm expression.
The ribcage (Fig. 2). This indicates that the ribs with intervening ribs appearing normal. However, the more extensive sternum defects observed in hoxa-9/hoxb-9 double mutants support the second hypothesis. In most of the hoxa-9/hoxb-9 double mutants, the entire sternum is hypoplastic and malformed. Such extensive sternum malformations are not readily interpreted as resulting solely from the defects in the articulations of the first, second, or eighth ribs, but instead suggest an additional role for hoxa-9 and hoxb-9 in sternum formation. This is an important distinction since ribs and sternum originate from separate mesodermal lineages.

It is curious that in hoxb-9 mutant homozygotes, defects are observed in the formation of the first, second, and eighth ribs with intervening ribs appearing normal. However, closer examination indicates that the angle of articulation of the ribs with the sternum is abnormal along the entire ribcage (Fig. 2). This indicates that the hoxb-9 mutation contributes to mispatterning of the ribs, and possibly the sternum, from T1 through T8, with the defects only being more apparent at the ends of this block of vertebrae.

A consequence of the rib fusions seen in some hoxb-9 mutant mice is a reduction in the size of the upper thoracic cavity. Interestingly, in those hoxb-9 mutant homozygotes that have first and second rib fusions, the size of the thymus is also reduced. The reduction in the size of the thymus appears to result as a secondary response to the reduction in the size of the upper thoracic cavity since in hoxb-9 mutant mice lacking rib fusions the thymus is normal in size. These observations suggest that the size of the thymus is regulated with respect to the size of the thoracic cavity.

**Hoxa-9/hoxb-9 Double Mutants**

Mice homozygous for mutations in either hoxa-9 and hoxb-9 do not show overlapping phenotypes. Yet double mutants show exacerbation of all of the defects observed in either hoxa-9- or hoxb-9- homozygotes. In addition, both the penetrance and expressivity of the defects correlate with the number of mutant alleles present in the mouse. For example, mice heterozygous for either the hoxa-9 or the hoxb-9 mutation never show full-size 14th ribs. However, such ribs are apparent in some hoxa-9, hoxb-9 compound heterozygous mice and the frequency is increased in hoxa-9+/−, hoxb-9+/− mice. Conversely, hoxa-9−/− mice have never been observed to have 1st and 2nd rib fusions. However, the frequency of such fusions in hoxb-9−/− mice progressively increases with the addition of one and then two hoxa-9 mutant alleles. Similar observations have been made in mice containing combinations of hoxa-3 and hoxd-3 mutant alleles (Condie and Capecchi, 1994). These observations again emphasize the extensive quantitative interactions among Hox genes required to specify the vertebrate body plan. Each Hox gene appears to have individual unique functions as well as more extensive roles in combination.
with other Hox genes. The concentration of Hox gene products in different cells must be very tightly regulated since the reduction of Hox protein concentration in heterozygotes can result in marked phenotypic consequences.

Genetic interactions between hox-a-9 and hox-a-11 have been reported (Fromental-Ramain et al., 1996). In this case, the interactions are observed in more caudal aspects of the vertebral column compared to those seen in hox-a-9/hox-a-11 double mutants. Specifically, Fromental-Ramain et al. (1996) observed in mice mutant for both hox-a-9 and hox-a-11 an exacerbation of defects in the lumbar sacral axial skeleton. Thus, the domain of influence of hox-a-9 extends from the first thoracic vertebra, when interacting with hox-b-9, to sacral vertebrae, when interacting with hox-d-9.

As the functions of increasing numbers of Hox genes are unraveled, patterns are emerging. With respect to the formation of the axial column, there appear to be hot spots for the accumulation of defects. Such defects are particularly evident at the boundaries between changes in vertebral type, such as the base of the skull and the first cervical vertebra, C7 and T1. T7 and T8, T13 and L1, L6 and S1, and S4 and C1. For example, mutations in hox-b-2, hox-b-4, hox-d-1, hox-d-3, and hox-d-4 show defects in the formation of the first cervical vertebra, the atlas (Barrow and Capecchi, 1996; Ramirez-Solis et al., 1993; Condie and Capecchi, 1993; Horan et al., 1995a, b). Mutations in hox-a-4, hox-a-5, hox-a-6, hox-b-5, hox-b-6, hox-b-7, hox-b-8, and hox-a-9 have defects in C7, T1, or both and so on. Why is this the case? Part of the answer is operational. It is very apparent when C7 acquires ectopic ribs or T1 loses ribs. On the other hand, transformations of C5 to C4 are more difficult to score. Second, we can anticipate that the generation of the major morphological differences associated with the different vertebral classes will require the concerted activity of more Hox genes than the generation of the smaller differences within a vertebral class. Finally, the vertebral classes may be formed as units using prescribed developmental programs. Consistent with this hypothesis, the expression patterns of Hox genes in the prevertebrae of the chick and mouse maintain register with the type of vertebral column rather than the position of the vertebra along the axial column (Burke et al., 1995). In mice in which the continuity of the normal program has been disrupted by a Hox mutation, the discontinuities in the specification of cells are likely to be most apparent at the transitions between vertebral types, making such boundaries particularly vulnerable to dysmorphology.

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