Axial skeletal patterning in mice lacking all paralogous group 8 Hox genes

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Accepted 26 February; published on WWW 19 April 2001

SUMMARY

We present a detailed study of the genetic basis of mesodermal axial patterning by paralogous group 8 Hox genes in the mouse. The phenotype of Hoxd8 loss-of-function mutants is presented, and compared with that of Hoxb8- and Hoxc8-null mice. Our analysis of single mutants reveals common features for the Hoxc8 and Hoxd8 genes in patterning lower thoracic and lumbar vertebrae. In the Hoxb8 mutant, more anterior axial regions are affected. The three paralogous Hox genes are expressed up to similar rostral boundaries in the mesoderm, but at levels that strongly vary with the axial position. We find that the axial region affected in each of the single mutants mostly corresponds to the area with the highest level of gene expression. However, analysis of double and triple mutants reveals that lower expression of the other two paralogous genes also plays a patterning role when the mainly expressed gene is defective. We therefore conclude that paralogous group 8 Hox genes are involved in patterning quite an extensive anteroposterior (AP) axial region. Phenotypes of double and triple mutants reveal that Hoxb8, Hoxc8 and Hoxd8 have redundant functions at upper thoracic and sacral levels, including positioning of the hindlimbs. Interestingly, loss of functional Hoxb8 alleles partially rescues the phenotype of Hoxc8- and Hoxd8/Hoxc8/null mutants at lower thoracic and lumbar levels. This suggests that Hoxb8 affects patterning at these axial positions differently from the other paralogous gene products. We conclude that paralogous Hox genes can have a unique role in patterning specific axial regions in addition to their redundant function at other AP levels.

Key words: Paralogous Hox genes, Vertebral column, Axial skeleton, Vertebrae, Ribs, Mouse, Functional redundancy, Hoxb8, Hoxc8, Hoxd8, Transformation

INTRODUCTION

The paraxial mesoderm-derived axial skeleton of vertebrates comprises part of the skull and the vertebral column, and associated thoracic ribcage. The vertebral column consists of a series of anatomical elements, the vertebrae, which, on the basis of their relative position along the anteroposterior (AP) axis and distinct anatomical features, are grouped in five distinct types: cervical, thoracic, lumbar, sacral and caudal. The relative number and arrangement of these vertebral types is highly variable and is responsible for much of the morphological variation among vertebrate species. For example, the chick has 14 cervical vertebrae while mice and humans have seven. Using quail-chick chimaeras it has been shown that the entire vertebral column as well as the ribs (which can be regarded as vertebral processes) are generated from the somite-derived sclerotomes (Christ et al., 1974; Huang et al., 2000). Heterotopic grafting experiments in chick suggest that positional identity in the paraxial mesoderm is already determined before compaction of the somites; when unsegmented paraxial mesoderm from the future thoracic region is transplanted to the cervical region, the ability to form ribs is retained in the graft (Kieny et al., 1972). Factors certainly involved in this early specification process are the Hox gene products. These homeodomain-containing transcription factors are encoded by the vertebrate homologues of the clustered Drosophila homeotic genes (reviewed by McGinnis and Krumlauf, 1992). In mammals, four linkage groups (a, b, c and d) exist (Scott, 1992) which are thought to have arisen during evolution by two successive duplications of a single ancestral gene cluster (Kappen et al., 1989; Bailey et al., 1997). Both the Hox and homeotic genes are expressed along the AP axis in the same order as the way they are organised on the chromosome (Lewis, 1978; Duboule and Döll, 1989; Graham et al., 1989). In the developing mouse embryo, the Hox genes are expressed in partially overlapping domains in the mesoderm, neurectoderm and some endodermal structures, from the neck region to the tail. Expression of the most 3’ Hox genes is initiated in the most posterior part of the embryo at the late primitive streak stage during gastrulation, and expression of more 5’ genes progressively later. The expression domains of these genes then spread forward in the nascent mesoderm and the ectoderm of the primitive streak and further rostrally, each gene eventually reaching its specific anterior boundaries (reviewed by Deschamps et al., 1999). In Drosophila, the homeotic genes act as master switches...
governing the genetic program leading to the correct identity of each parasegment; mutations in these genes result in the partial or complete transformation of the identity of a parasegment into that of a neighbour (homeosis). The type of transformation induced by most, but not all, mutations in *Drosophila* seems to obey the posterior dominance rule: of all genes expressed in a parasegment at a given position, the most posteriorly restricted gene is the gene governing the developmental program (reviewed by McGinnis and Krumlauf, 1992; Duboule and Morata, 1994). In accordance with this rule, null mutations in homeotic genes generally lead to anterior homeotic transformation of the most rostral segment within the expression domain, and gain-of-function mutations or ectopic overexpression to posterior transformation of the segments that rostrally flank the expression domain; the most crucial functions of the homeotic genes therefore appear to be exerted within their expression domain near their anterior boundary of expression.

In the mouse, gain- and loss-of-function studies of individual Hox genes have demonstrated that changes in Hox expression can lead to changes in the regional identity of mesodermal, neuroectodermal and endodermal derivatives (reviewed by Krumlauf, 1994; for a description of most Hox gene loss-of-function mutants, see Mak, 1998 and references therein). The direction of the transformations is, however, generally less predictable than in *Drosophila* (Krumlauf, 1994; Crawford, 1995). For example, there are Hox null mutant mice displaying defects indicative of posterior transformation of particular structures. In addition, the AP levels at which the transformations take place are not always near the rostral expression boundary of the gene; in some null mutants, defects have been observed in structures located far more posterior than their anterior boundary of expression. Strikingly, transiently delayed or precocious expression of 5′ Hox genes either owing to deletion of an enhancer (Zákány et al., 1997), or to deletion of a ‘cluster encompassing’ repressor element (Kondo and Duboule, 1999) were shown to lead to, respectively, anterior or posterior skeletal transformations. This points to the importance of a well-timed early activation phase of Hox gene expression for axial patterning (Kondo and Duboule, 1999).

Paralogous Hox genes occupy the same relative position within different Hox clusters and they bear highly homologous sequences both in and outside their homeodomain (Graham et al., 1989; Duboule and Dollé, 1989). Mice which are double or triple mutant for paralogous Hox genes display more severe and more penetrant phenotypes than single mutant mice for any of the two or three genes (Condie and Capecchi, 1994; Davis et al., 1995, Horan et al., 1995b; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Chen and Cappechi, 1997; Manley and Capecchi, 1997; Manley and Capecchi, 1998; Chen et al., 1998; Gavalas et al., 1998; Studer et al., 1998). The transformations in the double or triple mutant mice were often more complete and/or found in a larger domain (extending farther from the anterior boundary of expression) than those in the single mutant mice. These results point to a large functional redundancy among Hox genes. Besides evidence for functional redundancy between paralogous genes, evidence for functional redundancy between neighbouring genes (Rancourt et al., 1995; de la Cruz et al., 1999; Davis and Capecchi, 1996; Zákány and Duboule, 1996) and even non-paralogous genes in separate clusters (Favier et al., 1996) has also been found.

We have studied the axial skeleton of newborn mice that are single, double and triple mutant for members of the Hox paralogous group 8 (Hoxb8, Hoxc8 and Hoxd8). The three genes have been reported to have different expression boundaries in the mesoderm. At embryonic day 12.5, the most rostral pre-vertebra (pv) expressing *Hoxb8* is pv 7 (Deschamps and Wijgerde, 1993). At this stage, the anterior limits of *Hoxc8* and *Hoxd8* expression in the mesoderm have been reported to be at the level of pv 8 (Tiret et al., 1993) and pv 18 (Izpisúa-Belmonte et al., 1990), respectively.

*Hoxd8* was inactivated by gene targeting, and the mutant phenotype was investigated. These *Hoxd8* loss-of-function mutants, *Hoxb8* (van den Akker et al., 1999) and *Hoxc8* (LeMouelic et al., 1992; Tiret et al., 1993) null mice show phenotypical differences. By crossing these mice, and performing bone and cartilage staining on newborn double and triple mutants, we found evidence for interactions between all three genes along a large A-P domain of the vertebral column. Although most skeletal defects in double and triple mutant mice suggest synergistic interactions between these genes, the attenuation of some defects suggests a different contribution of these genes at specific AP levels.

**MATERIALS AND METHODS**

**Generation of single mutant mice and newborns**

*Hoxd8* mutant mice were generated using a construct depicted in Fig.1. Briefly, a 4.3 kb *XhoI*-*HindIII* 129/Sv genomic fragment containing the *Hoxd8* gene was subcloned in pSL1190 where the *EcoRI* site is missing. A 1 kb *PMC1Neo* cassette (Thomas and Capecchi, 1987) was inserted in the same transcriptional orientation as *Hoxd8* into the *EcoRI* site of the homeobox to give pSLXHNeo. This insertion interrupts the *Hoxd8* protein sequence at the level of amino acid 18 of the homeodomain. A 3 kb *HindIII*-*XhoI* genomic fragment corresponding to *Hoxd8* 5′ sequences was cloned in blueprints SK+ (Stratagene) and excised by *EcoR-V-XhoI* before subcloning between *Snabl* and *XhoI* in pSLXHNeo to give pSLHXHNeo. A PGK-TK cassette purified from plasmid pD352 (Rijli et al., 1994) was inserted in the *KpnI* site at the 5′ extremity of the genomic fragment giving the plasmid pTKd8pmcneo. After elimination of the 5′ *HindIII* site the vector was linearised by *HindIII* before electroporation of D3 embryonic stem cells (Gossler et al., 1986). The generation of *Hoxb8* and *Hoxc8* mutant mice has been described previously (van den Akker et al., 1999 and Le Mouellic et al., 1992, respectively). In both of these mutant lines, a *HoxLacZ* fusion protein is expressed instead of the normal Hox protein. Of the three *Hox8* mutant lines described (van den Akker et al., 1999), we used the *Hoxb8lacZneo*− line in which the neo selection cassette has been removed in vivo using the *Cre*loxp system.

*Hoxb8*−/− mice are viable, but display a number of visible defects; they tend to be smaller than control mice, they develop skin wounds, and they show an aberrant clasping reflex and signs of abnormal locomotion (van den Akker et al., 1999). As *Hoxb8*−/− males are fertile but appear to have breeding difficulties (which may be linked to the neurological defects observed) and *Hoxb8*−/− females often have problems in feeding their offspring (E. A. and J. D., unpublished) this line was maintained mainly using heterozygotes, which are healthy and fertile. The *Hox8* mutant line was maintained in a similar manner, as most *Hoxc8*−/− mice die shortly after birth from a still unknown cause. Viable *Hoxc8*−/− mice could, however, be used for further breeding (see also Le Mouellic et al., 1992). The *Hoxc8* and...
Hoxd8 mutations were studied in an almost pure FVB background. The Hoxb8neo mutation was originally in a mixed genetic background (FVB/129Ola/B6SJL). As subsequent crosses (see Results) were with FVB all double and triple mutants were in a mixed but mostly FVB background.

Generation of double and triple mutant mice
Hoxb8+/− and Hoxc8+/− mice were crossed to obtain transheterozygous animals. As Hoxb8+/−/Hoxc8+/− mice appeared normal and healthy, and were fertile, these mice were intercrossed. Mice of all expected genotypes were present at birth at normal Mendelian ratios (not shown). Hoxb8+/−/Hoxc8+/− mice were viable and appeared to have similar defects to those observed in the Hoxb8−/− mice. Like Hoxc8-null animals, Hoxb8+/−/Hoxc8+/− and Hoxb8−/−/Hoxc8+/− mice died shortly after birth. Hoxb8−/− mice were crossed with Hoxb8+/− and Hoxc8+/− mice to produce Hoxb8+/−/Hoxc8+/− and Hoxc8+/−/Hoxb8−/− mice, respectively. These mice appeared normal. By crossing these transheterozygotes with Hoxd8+/− mice, we obtained Hoxb8+/−/Hoxd8+/− and Hoxb8−/−/Hoxd8+/− mice, which appeared healthy and fertile. Hoxb8−/−/Hoxd8−/− compound mutant mice. Hoxb8+/−/Hoxd8−/− double null mice are viable and show neurological defects, which appear similar to those observed in the Hoxb8−/− mice (not shown). Intercrosses of Hoxc8−/−/Hoxb8+/− mice produced Hoxc8−/−/Hoxd8−/− newborns at normal ratios at birth; as expected, all newborns with this genotype died within a few hours. By crossing Hoxb8−/−/Hoxd8−/− and Hoxc8−/−/Hoxd8−/− mice we obtained Hoxb8−/−/Hoxd8−/−/Hoxc8−/− mice. These mutants appeared healthy and fertile. Intercrosses produced mice of all possible genotypic combinations at birth, at approximately expected Mendelian ratios (not shown). At weaning, of the possible triple mutant genotypic combinations, only Hoxb8−/−/Hoxc8+/−/Hoxd8−/− and Hoxb8−/−/Hoxc8−/−/Hoxd8−/− mice were found to be present. The latter mice all die within a few months. As predicted from the lethality of the Hoxb8 homozygous mutation, all triple mutants that lack both copies of the Hoxc8 gene died perinatally.

Genotype analysis
DNA was isolated from tail or ear biopsy samples from adult mice, organs from newborn mice and yolk sacs from embryos using standard techniques. Hoxd8 genotypes were determined by Southern analysis using an EcoRI digest and a HindIII-Xhol probe (Fig. 1). Hoxb8 and Hoxc8 genotypes were determined by PCR (Tiret et al., 1998). For Hoxb8 genotyping, a common forward primer 5′-CTGGGCCCCAATTAGTGACTGC (primer 1) could be used in one PCR reaction with reverse primers 5′-GAGGTTGGGAGG-GTTATGGATT (primer 2) and 5′-CGGGGCTCGAAGCTTTAGT-ACG (primer 3). The combinations of primer 1.2 and primer 1.3 make it possible to identify the wild type (628 bp product) and targeted (374 bp product) Hoxb8 loci, respectively. Amplification conditions were: denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 30 seconds for 35 cycles.

Skeletal staining
Skeletons of newborn mice were stained according to the following procedure. Newborns were skinned and eviscerated, fixed in 96% ethanol overnight, stained for cartilage in 80% ethanol/20% acetic acid and 0.5 mg/ml Alcian Blue (Sigma) overnight, and rinsed twice for 1 hour in 96% ethanol. After digestion of soft tissues in 1.5% KOH for 5 hours, bone was stained overnight in 0.5% KOH and 0.15 mg/ml Alcian Red S (Sigma). Embryos and newborns were then destained in 0.5% KOH/20% glycerol for 3 days or longer, and stored in 20% ethanol / 20% glycerol.

Whole mount in situ hybridisation
Whole mount in situ hybridisation of embryos with Hoxb8 (350 bp SacI-KpnI), Hoxc8 (180 bp Aval-PstI) and Hoxd8 (220 bp EcoRI-Aval) probes was carried out (Wilkinson, 1992).

RESULTS

Comparison between the mesodermal expression patterns of the three paralogous group 8 Hox genes
Hoxb8 expression has been reported to be detected from the level of somite 10/11 to more posterior somites (Charité et al., 1994) around day 9.5. Hoxc8 is expressed weakly in somite 15 and more strongly between somites 16 and 24 (pv21, L1) (Le Mouellec et al., 1992). Hoxd8 is expressed caudally to pv18 (T11, in part originating from somite 21) at day 12.5 (Izpisúa-Belmonte et al., 1990). With a view to anticipate and later interpret the phenotypes of single and compound mutants, we directly compared the expression of the three genes in day 9.5 embryos (between 22 and 26 somites) within the same experiment. Whole-mount in situ hybridisation confirmed that Hoxb8 is expressed weakly in somite 10, and quite strongly in the paraxial mesoderm between somite 11 and 22. (Fig. 1A). Hoxc8 is expressed the most strongly between somites 15 and 23 (Fig. 1B) but very low levels of expression can be detected up to s11 (data not shown). Hoxd8 is expressed at very low levels in the paraxial mesoderm posterior to the forelimb (s11)

Table 1. Penetration (%) of vertebral column defects in Hox paralogous group 8 single mutants

<table>
<thead>
<tr>
<th>Type of transformation</th>
<th>C7 to C6</th>
<th>T1 to C7</th>
<th>T2 to T1</th>
<th>T7 to T6</th>
<th>T8 to T7</th>
<th>T12 to T10</th>
<th>T11 to T10</th>
<th>L1 to T13*</th>
<th>S1 to L6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>AT on C7</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Extra</td>
<td>Eighth rib</td>
<td>TV=T11 of T12</td>
<td>14th rib on</td>
<td>S1 loose</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T1 rib</td>
<td>T2 rib</td>
<td>sternbra</td>
<td>T6 and T7 ribs</td>
<td>T12</td>
<td>L1</td>
<td>from sacrum</td>
<td></td>
</tr>
<tr>
<td>Wild type (n=21)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 (0)</td>
<td>0</td>
<td>5 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Hoxb8+/− (n=61)</td>
<td>3 (2)</td>
<td>2 (0)</td>
<td>2 (2)</td>
<td>0</td>
<td>2 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hoxb8+/− (n=30)</td>
<td>17 (0)</td>
<td>23 (7)</td>
<td>27 (10)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hoxb8−/− (n=24)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>25 (17)</td>
<td>5‡</td>
<td>4 (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hoxb8−/− (n=18)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>67</td>
<td>56 (33)</td>
<td>100</td>
<td>89 (33)</td>
<td>28 (11)</td>
<td></td>
</tr>
<tr>
<td>Hoxb8+/− (n=17)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>18 (0)</td>
<td>0</td>
<td>6 (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hoxb8−/− (n=22)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The percentage of animals with bilateral presence of phenotypes is indicated between brackets.

*For the L1 to T13 transformation the numbers between brackets represent the percentage of animals with two fully developed ribs on L1.

‡n=22.
Fig. 1. (A-C) Comparison of the expression pattern of Hoxb8 (A), Hoxc8 (B), and Hoxd8 (C) in day 9.5 embryos upon whole-mount in situ hybridisation. The staining reactions to reveal the hybridised transcripts were allowed to continue for 13 hours, until signal became visible in the somitic mesoderm posterior to the forelimb buds in embryos hybridised with the Hoxd8 probe. (A) 24-somite embryo hybridised with the Hoxb8 probe. Transcripts are detected weakly in somite 10 (s10, contributing to future pv7, T7), and more strongly in s11 (pv8, T1) and more posteriorly. (B) 22-somite embryo hybridised with a Hoxc8 probe. The gene is expressed weakly in somite 14 (pv11, T4) and stronger between somites 15 (pv12, T5) and 23 (pv 20, T13). (C) 26-somite embryo hybridised with a Hoxd8 probe. Expression is strong in the posterior paraxial mesoderm (still mostly unsegmented, from where lumbar, sacral and caudal vertebrae will arise) up to somite 22 (contribute to pv19, T12), and weak in the region between s23 and s18 (corresponding to pv15, T8); very low levels of transcripts are detected between s17 and s11 (pv8, T1). Scale bar: 0.1 mm.

to the level of somite 17, at slightly higher levels between somites 18 and 23, and more strongly posterior to somite 23 (Fig. 1C, and data not shown).

Generation and phenotype of Hoxd8-null mice

The Hoxd8 gene was disrupted using a strategy depicted in Fig. 2 (for a detailed description, see Materials and Methods). Southern analysis (not shown) confirmed correct disruption of the Hoxd8 gene in four out of 87 analysed embryonic stem cell clones. Two lines were used to generate chimaeric mice that were able to transmit the Hoxd8+/− alleles. Two lines were used to generate chimaeric mice that were able to transmit the Hoxd8+/− alleles. Two lines were used to generate chimaeric mice that were able to transmit the Hoxd8+/− alleles. Two lines were used to generate chimaeric mice that were able to transmit the Hoxd8+/− alleles.

Le Mouellic et al., 1992; Tiet et al., 1993; Tiet et al., 1998. We extended and repeated these studies, respectively, performing skeletal staining on newborns (Table 1).

In total we studied the skeletons of 61 Hoxb8+/− and 30 Hoxb8−/− newborns of the Hoxb8lacZneo− (see Table 1). The Hoxb8−/− mice show variable defects in the upper thoracic rib cage (Fig. 3B,C,H-I) with a penetrance of 27%. In two Hoxb8−/− newborns, the T1 and T2 ribs fused before attaching to the top of the sternum on one side, while the T1 and T2 ribs had the same attachment point at the top of the sternum on the other side, resulting in an abnormal sternum with a reduced number of segments (Fig. 3C,I,J). In one Hoxb8−/− newborn, the T1 and T2 ribs had the same attachment point at the top of the sternum on both sides, also resulting in a shorter sternum (not shown). Two other Hoxb8−/− newborns showed unilateral shifts of the attachment point of the T2 thoracic and lower ribs (not shown). In three Hoxb8−/− newborns the T1 and T2 ribs fused and split again before attaching to the sternum, resulting in a normal sternum (Fig. 3B,H). The T2 rib defects could be considered as anterior transformation of the T2 vertebra towards the identity of T1, as either the proximal part of the rib or the whole rib (including the attachment point to the sternum) is always shifted anteriorly. Consistent with this, we observed that the spinous process normally present on T2 was shorter in some Hoxb8−/− animals (Fig. 3H) compared with

Different skeletal phenotypes in newborn Hoxb8, Hoxc8 and Hoxd8 loss of function mice

The skeletal phenotypes of Hoxb8, Hoxc8 and Hoxd8 mutant mice have been described previously (van den Akker et al., 1999; E. van den Akker and others, 1999; Le Mouellic et al., 1992; Tiet et al., 1993; Tiet et al., 1998).

Table 1. Hoxd8−/− newborns show variable defects in the upper thoracic rib cage (Fig. 3B,C,H-I) with a penetrance of 27%. In two Hoxb8−/− newborns, the T1 and T2 ribs fused before attaching to the top of the sternum on one side, while the T1 and T2 ribs had the same attachment point at the top of the sternum on the other side, resulting in an abnormal sternum with a reduced number of segments (Fig. 3C,I,J). In one Hoxb8−/− newborn, the T1 and T2 ribs had the same attachment point at the top of the sternum on both sides, also resulting in a shorter sternum (not shown). Two other Hoxb8−/− newborns showed unilateral shifts of the attachment point of the T2 thoracic and lower ribs (down to T7) resulting in an irregularly patterned ‘crankshaft’ sternum, and fusion of the T1 and T2 ribs (not shown). In two Hoxb8−/− newborns, the T1 and T2 ribs fused and split again before attaching to the sternum, resulting in a normal sternum (Fig. 3B,H). The T2 rib defects could be considered as anterior transformation of the T2 vertebra towards the identity of T1, as either the proximal part of the rib or the whole rib (including the attachment point to the sternum) is always shifted anteriorly. Consistent with this, we observed that the spinous process normally present on T2 was shorter in some Hoxb8−/− animals (Fig. 3H) compared with

Fig. 2. Gene targeting of Hoxd8. Restriction maps describing the structure of the targeting vector, the wild-type genomic locus and the recombinant locus for Hoxd8. The position of the 3' and 5' external probes for Southern blot analysis is indicated under the wild-type locus. B, BamHI; E, EcoRI; H, HindIII; X, XbaI; Xh, Xhol. Grey letters represent the restriction sites destroyed by the cloning protocol.
Paralogous Hox genes and axial patterning

the long spinous process found on T2 in wild-type animals (Fig. 3G). In one of the Hoxb8−/− animals with a strong phenotype, the long spinous process was clearly shifted to T3 (Fig. 3I, J), suggesting transformation of the T3 vertebra towards a T2 identity in this animal. The T1 rib defects appear to be more ambiguous and only clear shortening of the T1 rib can be easily explained in terms of transformation of the T1 vertebra towards the identity of C7. With low penetrance, we observed an abnormality of the cervical vertebrae in the Hoxb8−/− mice. The anterior tuberculum (AT), a ventral process that is normally found exclusively on the 6th cervical vertebra (C6; Fig. 3G) was found to be present either on the 7th cervical vertebra (C7; Fig. 3J) or on C6 and C7 in 17% of the Hoxb8−/− mice. We interpret presence of an anterior tuberculum on C7 as anterior transformation of the C7 vertebra towards the identity of C6 (Table 1). These results suggest a role for Hoxb8 in patterning the upper thoracic vertebral column.
fully developed) ribs were present on vertebra L1 (Fig. 3D,E). Six mice out of the 18 examined had a fully developed pair of ribs on L1 (Fig. 3D). These abnormalities of the Hoxc8 mutants have been described previously and suggest a role for Hoxc8 in specifying the identity of elements forming the ventral part of the thoracic vertebral column at least from the sixth thoracic to the first lumbar vertebra (Le Mouellic et al., 1992; Tiret et al., 1993). We also observed defects that have not been described before, possibly owing to differences in genetic background between this and previous studies. Thoracic vertebrae normally carry a dorsal process that points posteriorly from T3 to T9, and anteriorly from T11 and more caudally. T10 is therefore called the transitional vertebra. Although in wild-type mice T10 was always the transitional vertebra (Fig. 3K), T11 was in one of the Hoxc8−/− mice, and T12 was in all 18 Hoxc8−/− mice (Fig. 3L). Furthermore, a partial or complete transformation of the 1st sacral vertebra towards the identity of the 6th lumbar vertebra was observed in Hoxc8−/− mice (Fig. 3M-P). This transformation results in a unilateral or bilateral posterior shift in the position of the hindlimb, which is always associated with the position of the sacrum. This strongly suggests that Hoxc8 is involved in positioning the hindlimbs. In addition, we observed a subtle defect in the appendicular skeleton of Hoxc8 mutants: in all studied Hoxc8−/− newborn mice, the deltoid crest of the humerus appeared slightly malformed (not shown). These results suggest that Hoxc8 has a minor role in forelimb morphogenesis.

Fig. 4. Typical examples of double and triple null mutant skeletons. (A-C) Hoxb8−/−/Hoxc8−/− newborn with bilateral fusion of the first and second ribs (in this case resulting in fusion of the first two sternbral segments). As in most Hoxc8−/− newborns, an extra sternbra is visible between the sixth and seventh ribs (arrow), but only seven pairs of ribs are attached to the sternum (as in wild types) and only one rudimentary rib is present on L1 (L1’). The S1 vertebra however is completely transformed to a lumbar identity (S1’). As in the Hoxc8−/− newborns, the transitional vertebra (see text) is T12. (D-F) Hoxb8−/−/Hoxd8−/− newborn with unilateral fusion of the first and second rib. No abnormalities are present in more posterior regions. (G-I) Hoxc8−/−/Hoxd8−/− newborn with extra sternbra between the sixth and seventh pairs of ribs, bilateral attachment of an eighth rib to the sternum, bilateral presence of a 14th rib on L1 (L1’) and full transformation of S1 towards a lumbar identity (S1’). The position of the transitional vertebra (asterisks) is as in the Hoxc8−/− newborns shifted to T12 (now designated T12’). (J-L) Triple mutant newborn with bilateral fusion of the first and second thoracic ribs before the sternum. The number of sternbrae is normal due to the presence of an extra sternbra between the sixth and seventh pairs of ribs. Seven pairs of ribs attach to the sternum and two rudimentary ribs are present on L1. S1 is transformed to a lumbar identity (L1’) and the transitional vertebra (asterisks) is T12’.

Enhanced penetrance of Hoxb8 and Hoxc8 specific mutant phenotypes in double mutants
Double mutant mice were generated as described in Materials and Methods. Defects in the upper thoracic rib cage, which are specific for the Hoxb8 mutation, were observed much more
with a much higher frequency upon additional loss of Hoxb8 (hallmarks of the extra (pair of) rib(s) attached to the first lumbar vertebra of animals with an 8th rib attached to the sternum or with an increase in the penetrance of upper thorax defects compared with the Hoxb8-null mutant). The skeletal phenotypes as they are observed in Hox8 double null mutants show an increase in the penetrance and expressivity of transformations between thoracic and sacral levels compared to the Hoxc8-null mutant. In Hoxb8/c8 double null mutants, an increase in the penetrance of upper thorax defects compared with the Hoxb8-null mutant accompanies a small decrease in penetrance and expressivity of lower thoracic/lumbar defects compared with the Hoxc8-null mutant. In the triple null mutants, the penetrance and expressivity of upper thorax defects is further increased (indicated by shortening of the T1 rib resulting in fusion with the T2 rib before attaching to the sternum) and the anterior tuberculum is often present on C7 instead of C6. Compared with the Hoxc8/d8 double null mutant decreased penetrance and expressivity of transformations between lower thoracic and sacral levels was observed in the triple null mutant. An indication of the relative expression level of the Hoxb8, Hoxc8 and Hoxd8 genes in the prevertebrae at different A-P levels is given on the right (based on data obtained in the present study in day 9.5 (Fig. 1) and 11.5 (not shown) embryos, and expression data from Deschamps and Wijgerde, 1993; Charié et al., 1994; van den Akker et al., 1999 (Hoxb8); Le Mouellec et al., 1992; Tiret et al., 1993 (Hoxc8); Izpisúa-Belmonte et al., 1990; N. van der Lugt and J.D., unpublished (Hoxd8) for day 12.5 embryos. Lighter colour represents weaker expression. For more details see text, Figs 3 and 4, and Tables 1 and 2.

Frequently in Hoxb8/Hoxc8 double mutants than in Hoxb8 single mutants (Table 2A). We also observed an increase in the percentage of Hoxc8 null mice with an extra lumbar vertebra causing a shift in the position of the hindlimbs upon additional loss of one or two functional Hoxb8 alleles (compare Table 2A with Table 1). This S1 to L6 transformation was partial in some Hoxc8+/− mice missing one or two Hoxb8 alleles (Table 2A), in spite of the fact that it was never observed in single Hoxc8+/− mice (Table 1).

**Hoxc8 mutant phenotypes at lower thoracic levels are attenuated by additional loss of Hoxb8**

Interestingly, we did not observe an increase in the percentage of animals with an 8th rib attached to the sternum or an extra (pair of) rib(s) attached to the first lumbar vertebra (hallmarks of the Hoxc8 loss-of-function mutation) in Hoxb8/Hoxc8 double mutants. A decrease in the frequency and an even stronger decrease in the severity of the defects at these levels were found in Hoxb8+/−/Hoxc8+/− double mutants compared with the Hoxc8+/− mice (compare Table 2A with Table 1). Thus, the Hoxb8 mutation appears to attenuate the effects of the Hoxc8 mutation at this level of the vertebral column. A representative Hoxb8+/−/Hoxc8+/− double mutant skeleton is shown in Fig. 4A-C.

**Enhancement of Hoxb8 mutant skeletal phenotypes by additional loss of Hoxd8**

Hoxb8 loss-of-function mutants exhibited upper thorax defects with a much higher frequency upon additional loss of functional Hoxd8 alleles (compare Table 2B with Table 1). This was unexpected given the fact that Hoxd8 is expressed at considerably low levels in the somites and pre-vertebrae at that AP level. We tested the possibility that the lower penetrance of upper rib cage defects in Hoxb8−/− single mutant compared with Hoxb8+/−/Hoxd8−/− double mutant mice could be due to an increased expression of Hoxd8 in the Hoxb8 null mutant, resulting in a partial rescue of the Hoxb8 null mutant phenotype. No significant difference in Hoxd8 mRNA distribution was however apparent between Hoxb8−/− and wild-type embryos at day 9.5 and 11.5 (not shown). Hoxc8 expression was also normal in the Hoxb8 null mutants (not shown). We conclude that the low expression of both Hoxd8 alleles is important for correct patterning at upper thoracic levels when Hoxb8 is fully inactivated.

No defects were found in the appendicular skeleton of Hoxb8/Hoxd8 double mutants. An example of a Hoxb8+/−/Hoxd8−/− double mutant skeleton is shown in Fig. 4D-F.

**Enhancement of Hoxc8 mutant skeletal defects by additional loss of Hoxd8**

Hoxc8 mutant mice carrying one or two mutant alleles of Hoxd8 (Table 2C) showed an increased penetrance and severity of the T8 to T7 transformation, compared to Hoxc8 single mutants (compare Table 2C with Table 1). In addition, we observed twice as many mice with a fully developed pair of ribs on L1 when Hoxc8-null mice lacked both functional Hoxd8 alleles (compare Table 2C with Table 1). Moreover,
Table 2. Penetrance (%) of vertebral column defects in Hox paralogous group 8 double and triple mutants

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<th>Type of transformation</th>
<th>C7 to C6</th>
<th>T1 to C7</th>
<th>T2 to T1</th>
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<th>T8 to T7</th>
<th>T12 to T10</th>
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<td>L1</td>
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<td></td>
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<td>T2 rib</td>
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<td>on L1</td>
<td>from sacrum</td>
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The percentage of animals with bilateral presence of phenotypes is indicated between brackets.

*For the L1 to T13 transformation the numbers between brackets represent the percentage of animals with 2 fully developed ribs on L1.

‡‡n=4.

§§n=21.

**Abnormalities in the Hoxc8+/−/Hoxd8+/− mutants are expressed in a fraction because of the low number of animals examined.

††n=1.

§§n=10.

anterior transformation of vertebra S1 into L6 and the associated caudal shift in the position of the hindlimb, another feature of the Hoxc8 null mutation, increased in frequency in genetic combinations comprising one or two Hoxd8 mutant alleles (compare Table 2C with Table 1). This phenotype became fully penetrant in Hoxc8/Hoxd8 double null mice. This points to a contribution of Hoxd8 in vertebral morphogenesis at axial levels where Hoxc8 is the main patterning gene, in spite of the normal phenotype at this level in single Hoxc8 mutants.

The malformation of the humerus, present in Hoxc8 single null mutants, was not more severe in any of the Hoxc8/Hoxd8 double mutants. A summary of defects in Hoxc8/Hoxd8 double mutants is given in Table 2C and a representative Hoxc8+/−/Hoxd8+/− double mutant is shown in Fig. 4G-I.

Skeletal phenotypes in triple mutant mice

An increase in the penetrance of upper ribcage abnormalities (and associated malformations of the sternum, a hallmark of the Hoxb8 mutation) was observed in the triple mutant mice compared with the double and single mutants (compare Table 2D with Tables 1 and 2A-C). These upper thorax defects are fully penetrant in mice that totally lack or only possess one functional paralogous group 8 Hox allele (Table 2D). The most severe upper thorax defects were observed in the triple null mutants. In 45% of the triple null mutants the anterior tuberculum was positioned abnormally, suggesting transformation of the C7 vertebra towards C6. This is a clear increase compared with all other genotypes (see Tables 1 and 2).

Interestingly, the T8 to T7 transformation was observed with a decreased frequency in triple mutants compared with Hoxc8/Hoxd8 double null mice (Table 2C,D). The severity of the phenotypes (percentage of bilateral transformations) was even lower. A threefold decrease in the percentage of animals with bilateral attachment of an extra rib to the sternum was observed in triple null mutants compared to Hoxc8/Hoxd8 double null mutants (Table 2C,D). Loss of functional Hoxb8 alleles therefore seems to partially rescue the phenotype at this level. In addition, a decrease in the percentage of mice with an extra (pair of) rib(s) on L1, and an even more striking decrease in severity of this phenotype was observed when Hoxc8/Hoxd8 double null newborns lost one or both functional Hoxb8 alleles (Table 2C,D). Thus, the Hoxb8 mutation clearly appears to attenuate some of the aspects of the Hoxc8/Hoxd8 double mutant phenotype.

As in all double mutant combinations for the paralogous group 8 Hox genes, we did not observe additional defects in fore- and hindlimb bones in any of the newborn triple mutant combinations compared with the single mutants; triple mutants missing both functional alleles of Hoxc8 showed the same malformation of the humerus as single Hoxc8-null mutants. The defects in the triple mutants are summarised in Table 2D.
and a representative example of a triple null mutant skeleton is shown in Fig. 4J-L. A schematic representation of the defects observed in the different single, double and triple mutants is shown in Fig. 5.

DISCUSSION

The present study contributes new data concerning the genetic basis of axial patterning by paralogous mouse Hox genes. We describe the phenotype of Hoxd8 loss-of-function mice, and present novel features of Hoxc8-null mutants, suggesting involvement of the gene in positioning the hindlimbs, and in morphogenesis of the forelimbs. In addition, we provide evidence for genetic interactions between the three paralogous group 8 Hox genes in vertebral patterning along an extensive AP domain of the axis.

Each of the Hox paralogous group 8 single mutant mice exhibits distinct phenotypical abnormalities at specific AP levels

The Hoxd8 loss-of-function mutation leads, at low penetrance, to the attachment of an extra rib to the sternum (T8 to T7 transformation) and to development of a rudimentary rib at the first lumbar vertebra (L1 to T13 transformation). These abnormalities resemble a subset of defects that have already been found with much higher penetrance in Hoxc8 mutant mice, suggesting an overlap in function between the two genes. In the Hoxc8-null mutants, in addition to the phenotypical abnormalities at lower thoracic (T7 to T6 and T8 to T7 transformations) and lumbar levels (L1 to T13 transformations) (Le Mouellic et al., 1992; Tiret et al., 1993; this paper), we observed a fully penetrant shift in the position of the transitional vertebra (T12 instead of T10). In addition, we occasionally observed the partial or complete transformation of the first sacral into a lumbar vertebra (S1 to L6). These results show that Hoxc8 has a patterning function in a broader domain than suspected before. They also reveal that this gene plays a role in positioning the sacrum and associated hindlimbs. A very similar phenotype has been described in mice carrying a site-directed mutation in an enhancer element of Hoxd10 and Hoxd11 (Zákány et al., 1997), suggesting the contribution of several non-paralogous gene products in patterning the same axial structures. In addition we observed a mild malformation of a humeral process, the deltoid crest, in all the examined Hoxc8-null mutants, suggesting an involvement of this gene in morphogenesis of the forelimb. This is reminiscent of the fact that overexpressed Hoxb8 interferes with proper AP patterning of the forelimb (Charité et al., 1994). Nevertheless, neither Hoxb8 loss-of-function mice, nor double and triple null mutants for paralogous group 8 Hox genes exhibit forelimb AP patterning defects, suggesting involvement of other Hox genes for this function. Hoxb8 null animals exhibit irregular attachment of upper thoracic ribs (T1 and T2) and associated defects of the sternum (van den Akker et al., 1999; this paper).

It is unlikely that the phenotype in any of the Hox paralogous group 8 mutant lines is due to an effect of the neo resistance cassette on the expression of neighbouring genes. In the Hoxb8 mutant line the neo cassette has been deleted (van den Akker et al., 1999). The expression of neighbouring genes was found to be unchanged in the Hoxc8 mutants (Tiret et al., 1993), and the very mild phenotypic abnormality that we found in the Hoxd8 mutants occurs at an axial level that is different from the defects in Hoxd4- and Hoxd9-null mutants (Horan et al., 1995a; Fromental-Ramain et al., 1996a).

In conclusion, mice mutant in the three Hox paralogous group 8 genes show different phenotypes in the axial skeleton, like they do in the nervous system (LeMouellic et al., 1992; Tiret et al., 1998; van den Akker et al., 1999; E. v.d.A., W. d.G. and J. D., unpublished). Recent data concerning the Hoxa3 and Hoxd3 genes show that these paralogous genes can totally substitute for each other’s functions, suggesting that the differences in phenotype in the single mutants are based only on quantitative differences in expression, and not on qualitative differences between the protein products of these genes (Greer et al., 2000). Overexpression of Hoxb8, Hoxc8 and Hoxd8 in transgenic mouse embryos using the RARβ2 promoter resulted in similar phenotypes in the vertebral column and peripheral nervous system (A. Schouwstra, I. Valarché and J. D., unpublished). It is therefore tempting to suggest that differences in the relative distribution of Hoxb8, Hoxc8 and Hoxd8 mRNA (see Fig. 1) and protein, rather than qualitative differences between the three gene products, underlie the differences in phenotype found between the mutants for each gene. These differences would derive from gene-specific variations in transcriptional regulation. Hoxb8 is the most abundantly expressed of the three proteins at the level of the upper thoracic rib cage, possibly explaining why we only find a phenotype in this region in the Hoxb8 single mutant. Hoxc8 expression is particularly high in the lower thoracic (pre)vertebrae compared with the levels of Hoxb8 and Hoxd8, and it is in this domain that the most penetrant phenotypes are found in the Hoxc8-null mutant. The highest level of Hoxd8 expression in embryos is found at the level of sacral vertebrae (see above), that are very likely to be specified by more additional (5’) Hox genes than vertebrae at more anterior levels. The poorly penetrant Hoxd8 mutant phenotype therefore correlates with relatively low levels of gene expression in the paraxial mesoderm, possibly explaining why absence of Hoxd8 protein in this region will in general not lead to a phenotype in Hoxd8 mutants.

Positive and negative interactions between Hoxb8, Hoxc8 and Hoxd8 in patterning the axial skeleton

In spite of the distinct range of action of each of the three paralogous group 8 Hox genes revealed by analysis of the single mutants, phenotypes of double and triple mutant mice suggest that functional interactions between these three genes occur along an extensive domain of the vertebral column, from the level of the cervicothoracic transition to the level of the sacrum. Most of the interactions appear to be synergistic. We observed, for example, a strong increase in the percentage of Hoxb8/Hoxc8 and Hoxb8/Hoxd8 double null mutants with upper ribcage defects compared with Hoxb8 single null mutants. In the triple null mutants, the T1/T2 patterning defects became fully penetrant. Although Hoxc8 had been reported to be expressed weakly up to the level of pv 8 (T1), and T2 defects had been found in an isolated Hoxc8-null mutant (Tiret et al., 1993), the contribution of Hoxd8 to patterning of the upper thoracic vertebrae was rather surprising, as the anterior expression boundary of Hoxd8 had been reported to be far
more posterior in the paraxial mesoderm (Izpisúa-Belmonte et al., 1990) (even though our whole-mount in situ analysis of gene expression revealed that Hoxd8 is expressed in the paraxial mesoderm up to s11 at very low levels). Hoxb8-null mice additionally lacking one or more functional alleles of weakly expressed Hoxc8 and/or Hoxd8 clearly suffered from upper thorax defects with increased penetrance and severity. An alternative explanation might have been that Hoxb8 normally negatively regulates the expression of Hoxc8 and/or Hoxd8 in the upper thoracic region and that these genes are upregulated in the Hoxb8 mutant mice, making it possible for their products to compensate for the loss of Hoxb8 function. We could rule out the latter hypothesis, as no differences were observed in Hoxc8 and Hoxd8 mRNA distribution between wild-type and Hoxb8-null embryos. Synergistic actions were also found for the Hoxc8 and Hoxd8 genes. A clear increase in the penetrance and severity of Hoxc8-specific defects was observed upon additional loss of functional Hoxd8 alleles.

Interestingly, we observed apparently antagonistic interactions between Hoxb8 on the one hand, and Hoxc8 and Hoxd8 on the other hand. Loss of functional Hoxd8 alleles in a Hoxc8 null or Hoxc8/Hoxd8 double null mutant background led to a decrease in the penetrance and severity of anterior transformations at the lower thoracic levels (T8 to T7 and L1 to T13 transformations) while at the same time it led to an increase in the penetrance and severity of the upper thorax defects (see above). The mechanism by which two mutations in paralogous Hox genes partially complement each other is not clear. Apparently mutant prevertebral anlagen missing two functional genes can interpret the remaining positional information as less aberrant than when they miss only one gene. This suggests that different interactions have been disrupted by each mutation, leading to rescue of mutual effects. The balance of target events at the given axial levels in the double and triple mutant situation would correspond to a situation closer to wild types than to single mutants.

**Different roles of paralogous Hox products point to qualitative differences between the modes of action of Hox proteins**

A model assuming that the sum of the expression levels of equivalent gene products would specify the identity of the prevertebrae can explain some but not all data described in this work. The partial compensation of single and double Hoxc8/Hoxd8 mutant defects by additional mutations in Hoxb8 alleles at lower thoracic levels could be better explained by the existence of qualitative differences between paralogous group 8 Hox proteins, for example in subdomains of the gene products important for protein-protein interactions. Depending on the axial level, the Hox proteins could interact similarly or differently with local protein partners, resulting in similar or different effects on the regulation of target genes. The existence of qualitative differences between the Hoxb8 and Hoxc8 genes has already been suggested by Pollock et al., who showed that ectopic expression of the Hoxb8 and Hoxc8 genes driven by a transcriptional control region of Hoxa4 resulted in an array of common transformations as well as morphological changes unique to each gene (Pollock et al., 1995). The only definitive way to test whether there are qualitative differences between these Hox proteins would be to exchange the gene coding sequences as has been done for the Hoxa3 and Hoxd3 genes (Greer et al., 2000).

We thank Frits Meijlink and Rolf Zeller for comments on the manuscript. D. Duboule for the Hoxb8 probe, P. Gruss for the Hoxc8 probe, M. Capecci for the PMC1Neo cassette, and D. Lohnes for the PGK-TK cassette.

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