Mice homozygous for a targeted disruption of Hoxd-3 (Hox-4.1) exhibit anterior transformations of the first and second cervical vertebrae, the atlas and the axis

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SUMMARY

Gene targeting in embryo-derived stem (ES) cells was used to generate mice with a disruption in the homeobox-containing gene Hoxd-3 (Hox-4.1). Mice homozygous for this mutation show a radically remodeled craniovertebral joint. The anterior arch of the atlas is transformed to an extension of the basioccipital bone of the skull. The lateral masses of the atlas also assume a morphology more closely resembling the exoccipitals and, to a variable extent, fuse with the exoccipitals. Formation of the second cervical vertebra, the axis, is also affected. The dens and the superior facets are deleted, and the axis shows ‘atlas-like’ characteristics. An unexpected observation is that different parts of the same vertebra are differentially affected by the loss of Hoxd-3 function. Some parts are deleted, others are homeotically transformed to more anterior structures. These observations suggest that one role of Hox genes may be to differentially control the proliferation rates of the mesenchymal condensations that give rise to the vertebral cartilages. Within the mouse Hox complex, paralogous genes not only encode very similar proteins but also often exhibit very similar expression patterns. Therefore, it has been postulated that paralogous Hox genes would perform similar roles. Surprisingly, however, no tissues or structures are affected in common by mutations in the two paralogous genes, Hoxa-3 and Hoxd-3.

Key words: Gene targeting, Hox genes, ES cells, homeotic transformation

INTRODUCTION

A set of genes that may specify the body plan of most, if not all, organisms of the animal kingdom has been identified. These genes have been studied most extensively in Drosophila as members of the Bithorax and Antennapedia complexes and are collectively referred to as the Hom-C genes (Akam, 1987). The eight Hom-C genes encode transcription factors that share a common DNA binding motif, known as the homeobox domain (Kissinger et al., 1990; Otting et al., 1990). Genetic and molecular analyses have shown that these genes act as master switches directing the course of morphogenic development of each parasegment (reviewed in Akam, 1987). Homeobox-containing genes have been isolated from many other species on the basis of cross-hybridization to the Drosophila homeobox (McGinnis et al., 1984a, 1984b; Scott and Weiner, 1984; Scott et al., 1989; Kessel and Gruss, 1990). The corresponding genes in humans and mice are designated as the Hox genes. Humans and mice each contain 38 Hox genes distributed on four linkage groups, designated Hox A, B, C and D, on four separate chromosomes (Scott, 1992). It appears that early in chordate evolution, an ancestral complex common to both invertebrates and vertebrates was quadruplicated (Kappen et al., 1989). Expansion of this gene complex may have played a critical role in the evolutionary progression from invertebrates to vertebrates by supplying the complexity to this network of genes necessary to accommodate the development of a more complex body plan.

A comparison of the chromosomal position of the Drosophila Hom-C genes with the mouse and human Hox genes is provided in Fig. 1. The Drosophila labial (lab) gene is most closely related, with respect to DNA and protein sequence, to the mouse Hoxa-1, Hoxb-1 and Hoxd-1 genes. These mouse genes are members of the same paralogous family, and are referred to as paralogues. Similarly, the DNA sequence of Deformed (Dfd) is most similar to Hoxa-4 and its paralogues. From this Fig. it is evident that the order of the Drosophila Hom-C and the mammalian Hox genes on the chromosome have been largely retained over the 600 million years since the divergence of vertebrates from invertebrates. Further, as first noted by Lewis (1978), the order of Hom-C genes in the Bithorax Complex corresponds with their domains of expression and function along the anteroposterior axis of the developing fly embryo. Similarly, the order of the Hox genes in each linkage group corresponds with their anterior domain of expression in the developing mouse embryo (Duboule and Dolle, 1989;
Graham et al., 1989). These correlations suggest that the mouse Hox genes may also function during development as master switches specifying regional information along the anteroposterior axis of the mammalian embryo. Targeted mutational analysis of Hoxa-1, Hoxa-3, Hoxb-4 and Hoxc-8 has supported this hypothesis (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992; LeMouelic et al., 1992; Ramirez-Solis et al., 1993; Carpenter et al., 1993). In each case, disruption of the gene has resulted in regionally restricted defects along the anteroposterior axis of the mouse.

The expression patterns of paralogous Hox genes are very similar. Thus, for example, paralogous genes of the anterior set (i.e. Hoxa-1 through a-4 and their paralogues) share the same anterior limits of expression in the neural tube, in paraxial mesoderm, and in the branchial arches (Gaunt et al., 1989; Hunt et al., 1991). These observations suggest that members of the same paralogous family might perform very similar if not overlapping or redundant functions (Hunt and Krumlauf, 1992). Previously we described the phenotype of mice homozygous for a targeted disruption of Hoxa-3 (Chisaka and Capecchi, 1991). These mice showed a complex series of regionally restricted defects in tissues derived primarily from neural crest mesenchyme. Here we describe the phenotype of mice homozygous for a targeted disruption of a parologue of Hoxa-3, Hoxd-3. No defects were observed in common between these two mutant mice. Rather Hoxd-3−/Hoxd-3− mice show regionally restricted defects in tissues derived from paraxial and lateral mesoderm.

**MATERIALS AND METHODS**

**Targeting vector construction and electroporation**

A genomic clone that carries the Hoxd-3 gene was isolated by reduced stringency hybridization of a 51-mer oligonucleotide with a genomic λ library prepared from DNA from the ES cell line CC1.2. The sequence of the oligonucleotide is 5′-CTT CGT GTC TTT CTT GTA CTG CCG ACG GTT CTG GAA CCA GAT CTT GAT-3′ which is at the 3′ end of the Hoxb-3 homeobox (Graham et al., 1988). Hybridization of the phage library to this end-labeled oligomer was performed by an overnight incubation in 6× SSPE, 1% SDS at 55°C, followed by washes in 2× SSPE, 0.2% SDS at 55°C for 2 hours and in 2× SSPE, 0.2% SDS at 65°C for 1 hour. DNA sequence analysis showed that the phage clone contained the Hoxd-3 gene (Lonai et al., 1987).

The MC1neo poly A cassette (Thomas and Capecchi, 1987) was inserted into the EarI site at nucleotide 82 of the Hoxd-3 homeobox. A total of 11.7 kb of Hoxd-3 sequence containing the neo cassette was inserted between H1 and H2 thymidine kinase genes (Chisaka and Capecchi, 1991) to create the targeting vector pD3neo2TK. The pD3neo2TK vector was linearized and introduced into CC1.2 ES cells by electroporation. Cells in which one of the two Hoxd-3 alleles had been targeted were enriched by positive-negative selection in medium containing G418 and FIAU (Mansour et al., 1988).

**Genotype of intercross progeny**

Mice heterozygous for the targeted disruption of Hoxd-3 appear outwardly normal and are fertile. Homozygotes were generated by intercrossing heterozygotes. Of 104 progeny generated from such crosses that were analyzed at E19 or at birth, 25 were wild type, 55 were heterozygous and 24 were homozygous for the Hoxd-3 mutation. Genotype analysis of 77 mice that survived to 5 weeks yielded 27 wild type, 46 heterozygotes and 4 Hoxd-3−/Hoxd-3− homozygotes.

**Southern blot analysis**

Genomic DNA was isolated from ES cell clones by lysis in 0.5 ml of 20 mM Tris pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.2 mg/ml proteinase K and incubated at 37°C for 4-12 hours, followed by a phenol extraction, a chloroform extraction, and precipitation by addition of 125 µl 7.5 M ammonium acetate and 700 µl isopropanol. The DNA was dissolved and resuspended in 1× TE. DNA from mouse yolk sacs, newborn skin or liver was isolated using the same protocol as for tail DNA (Mansour et al., 1993).

All probes were labeled with ³²P by random priming (Pharmacia). The Hoxd-3 flanking probe (probe B, Fig. 2) is a 400 bp EcoRI-EcoRV fragment derived from a region immediately 3′ to the targeting vector sequence. The neo probe is a 900 bp coding sequence specific fragment derived from pMC1neo. Genomic DNA from targeted cell lines was digested with XhoI, HindIII, EcoRI and EcoRV and probed with the 3′ flanking probe, the neo probe, and a 3.3 kb SalI-NcoI fragment from the 5′ end of the Hoxd-3 genomic clone. Genomic DNA was digested with EcoRV or HindIII for genotype analysis of the progeny from genetic crosses. Digestions of tail DNA were supplemented with 5 mM spermidine.

**RT-PCR**

Total RNA from E12.5 embryos was extracted by a modification of a previously published method (Cathala et al., 1983). Each embryo was homogenized in 400 µl of guanidine isothiocyanate solution. The homogenate was passed through a 25 ga gauge hypodermic needle 10 times, to shear genomic DNA. RNA was precipitated by adding an equal volume of 6 M LiCl and incubating for 1 hour at 4°C. The pellet was resuspended in 400 µl of 0.1% SDS, extracted with phenol, re-extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in 200 µl of water and reprecipitated with an equal volume of 6 M LiCl, at 4°C for 1 hour. The RNA was resuspended in 200 µl water, reprecipitated with ethanol, washed with 80% ethanol and resuspended in 200 µl water.

Template for the PCR was made by reverse transcription of 10 µg of total RNA, primed with random hexamers. The MMLV enzyme (BRL) was used in a 30 µl reaction volume. After incubation at 37°C for 1 hour, the cDNA was precipitated with ethanol. Each cDNA synthesis was resuspended in 50 µl of water and 10
µl was used in each PCR. Amplification was for 40 cycles with 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C. The PCR primers were chosen to flank the intron between the homeobox exon and the 5′ exon of the Hoxd-3 gene, to eliminate artifacts due to amplification from contaminating genomic DNA. The sequence of the 5′ primer (primer A, Fig. 3B) is 5′-GAACTCCAAGCA-3′ and the sequence of the 3′ primer (primer B, Fig 3B) is 5′-GCAGCTGGCCGGAGTAAGC-3′. The actin primers are the same as those used by Mansour et al. (1993).

**Histology**

Newborn mice were killed by asphyxiation with CO2, fixed in Bouins at 4°C and embedded in paraffin. 10 µm serial sections were collected and regiservely stained with hematoxylin and eosin as described (Chisaka and Capecchi, 1991).

Whole-mount skeletons were stained with alcian blue 8GX and alizarin red S as described (Mansour et al., 1993).

**Whole-mount in situ hybridization**

E8.0-E9.5 mouse embryos were fixed and processed for in situ hybridization as previously described (Carpenter et al., 1993). The digoxigenin RNA probe was transcribed from a subclone of a Hoxd-3 cDNA isolated from an 8.5 day mouse embryo library (Fahrner et al., 1987). This subclone is a 900 bp Pm1k-EcoRI fragment located immediately 3′ of the homeobox.

**RESULTS**

**Disruption of the Hoxd-3 gene**

Fig. 2A shows the structure of the targeting vector used to disrupt the Hoxd-3 locus by homologous recombination.
This vector, pD3Neo2TK, contains 11.7 kb of sequence from a genomic Hoxd-3 clone isolated from an ES cell λ library (Deng and Capecchi, 1992). The pMC1Neo poly A cassette (Thomas and Capecchi, 1987) was inserted into an \textit{Eag}I restriction endonuclease site, located in sequences encoding the second amino acid residue in the second helix of the homeodomain (Lonai et al., 1987). Removal of both helix 2 and 3 from the homebox domain, as well as the

\textbf{Fig. 4.} Homeotic transformation of the atlas and axis vertebrae in \textit{Hoxd-3}\textsuperscript{*} mutant newborns. The skeletons were stained with alizarin red and alcian blue and cleared by treatment with alkali and trypsin. (A) A ventral view of the wild-type (+/+) craniocervical junction. (B) Ventral view of the same skeleton as in A with the anterior arch of the atlas (aaa) removed to reveal the dens (d). The solid arrow indicates the joint between the superior facet of the atlas (at) and the occipital condyle, and an open arrow points to the junction of the inferior atlantal facet and the superior facet of the axis (ax). (C) The craniocervical joint in the \textit{Hoxd-3}\textsuperscript{*} homozygous mutant (−/−). The arrow indicates the portion of the basioccipital bone (bo) resulting from fusion of the atlas anterior arch with the skull. The atlas-like lateral foramen that appears on the mutant axis is indicated (lf). (D) A lateral view of the wild-type (+/+) craniocervical joint. (E) Lateral view of a \textit{Hoxd-3}\textsuperscript{*}/\textit{Hoxd-3}\textsuperscript{*} newborn (−/+), with no fusion of the lateral and dorsal parts of the atlas with the exoccipital bones. The arrow indicates a free-floating piece of ossifying cartilage between the neural arches of the mutant atlas and axis. (F) A \textit{Hoxd-3}\textsuperscript{*} homozygote (−/−) showing nearly complete incorporation of the atlas into the occipital portion of the skull. This individual also clearly has thickened axis neural arches, a morphology similar to the normal atlas. Scale bar, 1 mm; bo, basioccipital bone; ex, exoccipital bone; at, atlas; ax, axis; aaa, anterior arch of the atlas; lm, atlas lateral mass; lf, atlas lateral foramen.
sequences carboxy-terminal to the homeobox domain, should render the \textit{Hoxd-3} gene product non-functional with respect to DNA binding.

The pD3Neo2TK vector was introduced into the CC1.2 ES cell line by electroporation (Thomas and Capecchi, 1987). Cells in which a homologous targeting event had occurred were enriched by using positive-negative selection in medium containing G418 and FIAU (Mansour et al., 1988). Cell lines containing a disrupted \textit{Hoxd-3} allele were identified by Southern blot analysis using a flanking probe derived from sequences outside the targeting vector (probe B, Fig. 2F). Of 94 G418/FIAU resistant cell lines, three had undergone a homologous targeting event. A representative Southern blot for the cell line, 2h6, used to generate the mouse germline chimeras is shown in Fig. 2B. DNA from all three cell lines was further analyzed by Southern blot hybridization using four restriction enzyme digests and the flanking probe (probe B), a \textit{neo} probe (probe A) and a second internal probe from the 5′ region of the targeting vector (Materials and Methods). In all three cell lines the predicted replacement of one of the wild-type \textit{Hoxd-3} genes with the mutant allele occurred with no additional rearrangements or duplications either 5′ or 3′ to the \textit{neo} insertion.

The 2h6 cells were injected into C57Bl/6J blastocysts and

Fig. 5. An intermediate phenotype in \textit{Hoxd-3}− heterozygotes. The panels are ventral views of cleared skeleton preparations of a wild type (A; +/+ ) and a \textit{Hoxd-3}− heterozygote (B; +/− ). The arrow points to the fusion of the anterior arch of the atlas (aaa) with the basioccipital bone (bo) in the heterozygote. Scale bar, 1 mm; bo, basioccipital bone; ex, exoccipital bones; at, atlas; ax, axis; aaa, anterior arch of the atlas.

Fig. 6. Homeotic transformation of the atlas in a \textit{Hoxd-3}− mutant adult. Lateral views of a \textit{Hoxd-3}− heterozygous (A; +/− ) and a \textit{Hoxd-3}− homozygous (B; −/− ) adult skeleton preparation. The arrow in B indicates the ventrally displaced foramen for the first cervical nerve in the homozygote (see Fig. 10). Scale bar, 1 mm; ex, exoccipital bone; at, atlas; ax, axis; lf, atlas lateral foramen.
the resulting male chimeras were test bred to non-agouti C57Bl/6J females. One of these chimeric males transmitted the agouti coat color, characteristic of the mice from which the ES line was derived, to 20% of his progeny. Fig. 2C shows a Southern blot hybridization of the neo probe to tail DNA from one of the agouti progeny that inherited the Hoxd-3 mutation.

The Hoxd-3 mutation is associated with a reduction in postnatal viability

Mice heterozygous for the Hoxd-3−/Hoxd-3− disruption appear outwardly normal and are fertile. Mice homozygous for the Hoxd-3−/Hoxd-3− disruption were obtained from intercrosses between Hoxd-3− heterozygotes. The genotype of the offspring was determined by Southern analysis of tail DNA or yolk sac DNA (Fig. 2D). Intercross progeny were taken at 19 days of gestation (E19) or immediately after birth, or were allowed to be reared to weaning age (4-5 weeks). Some of the litters left to be raised by the mother were monitored daily for the death of any offspring.

The genotypes of intercross progeny collected at E19 or immediately after birth conform to the expected Mendelian 1:2:1 segregation of the Hoxd-3 alleles (Materials and Methods). This suggests that the Hoxd-3 mutation does not cause a loss of viability in utero. Intercross progeny genotyped at 4-5 weeks of age show a significant depression in the number of homozygotes (Materials and Methods), suggesting an effect of the mutation on postnatal viability. Although newborn homozygotes are pink, breathe and nurse as well as their littermates, most died within the first five days after birth. However, four Hoxd-3+/Hoxd-3− mice have survived past weaning age and display normal behavior. The surviving females and males are fertile, each having produced several litters. Homozygotes derived from intercrosses of heterozygous or homozygous parentage are not distinguishable with respect to either phenotype or postnatal viability.

Hoxd-3+/Hoxd-3− embryos do not express normal Hoxd-3 transcripts

To address the question of whether any intact Hoxd-3 transcripts are synthesized in Hoxd-3+/Hoxd-3− embryos, the reverse transcriptase polymerase chain reaction (RT-PCR) was used to assay for the presence of such transcripts in control and mutant embryos. The 5′-RT-PCR-primer was prepared from DNA sequences within exon 1 of Hoxd-3, whereas the 3′-primer was prepared from sequences in the second Hoxd-3 exon, distal to the point of the neo insertion. As can be seen in Fig. 3, the predicted DNA fragment, amplified from the normal Hoxd-3 transcript, was observed in wild-type and heterozygous embryos, but not in Hoxd-3+/Hoxd-3− embryos. In the absence of such transcripts, intact Hoxd-3 protein cannot be synthesized in Hoxd-3− homozygotes. The potential exists for an aminoterminal polypeptide fragment, lacking the homeobox domain, to be synthesized in Hoxd-3− heterozygotes and homozygotes. Such a polypeptide fragment could, in theory, interfere with, or exhibit partial function of, the normal Hoxd-3 gene product. However, it should be kept in mind that such a polypeptide fragment, if synthesized, might be very rapidly degraded relative to the intact, normal protein (Capecchi et al., 1974; Rechsteiner, 1987). Further, as we shall see, the intermediate phenotype of mice heterozygous for the Hoxd-3− mutation is more consistent with the consequences of hemizygosity at the Hoxd-3 locus rather than a gain of function mutation at the Hoxd-3 locus.

Hoxd-3 mutant mice display anterior transformations of the first and second cervical vertebrae

It was anticipated that the role of some of the Hox genes of the anterior set would be to specify cervical vertebrae. To permit examination of the cervical vertebrae, skeleton preparations of normal and mutant mice were stained with alizarin red and alician blue to reveal the bone and cartilage, respectively. Forty-three newborn skeletons were systematically studied, including ten Hoxd-3− homozygotes. The morphology and the number of bones and cartilages of the axial skeleton were scored from the occipital region of the skull to the sacral bone.

These skeleton preparations showed a dramatic restructuring of the craniovertebral joint in Hoxd-3+/Hoxd-3− mice. In normal animals this junction consists of the articulation between the occipital condyles of the skull and the superior facets of the first cervical vertebra (the atlas) as well as the joint between the atlas and the second cervical vertebra (the axis). This complex atlantoaxial junction allows rotation of the head on the vertebral column as well as protection of some of the arteries and nerves traversing the cervical region. The atlantoaxial joint is made up of articulations between the inferior surface of the atlas and the superior surface of the axis. The dens, a bony protuberance attached to the axis, extends cranially and is surrounded ventrally by the anterior arch of the atlas and dorsally by a transverse ligament. The nesting of the dens within the arch of the atlas confers lateral stability to the atlantoaxial joint.

Fig. 4A-C displays ventral views of the craniovertebral regions of a wild-type (+/+) and Hoxd-3+/Hoxd-3− (−/−) newborn. For the skeletal preparation shown in Fig. 4B, the anterior arch of the atlas (aaa) was removed to expose the dens. In the Hoxd-3− homozygote, the anterior arch of the atlas appears to be cleanly assimilated into the caudal part of the basioccipital bone (Fig. 4C). Note that the caudal border of the mutant basioccipital bone in the Hoxd-3+/Hoxd-3− mice has a scalloped shape resembling in outline the anterior arch of the atlas. In addition, the cartilaginous portions of the anterior arch lateral to the ossification center are also incorporated into the basioccipital bone. This phenotype is consistent with an anterior transformation of the anterior arch of the atlas to become part of the basioccipital bone. As a result, the skull now rests on the axis rather than the atlas, and the entire craniovertebral joint is remodeled. This transformation was found in all of the homozygous mutant skeletons examined (Table 1).

The lateral masses and neural arches of the atlas are also altered in all of the Hoxd-3− homozygotes. They are narrower and lack the characteristic shape of the wild-type atlas (Fig. 4E). In addition, the cartilaginous foramina on the lateral masses are missing in the mutant (Fig. 4C). In some cases extra ossified material is found between the neural arches of the atlas and axis (Fig. 4E). In eight out of ten
newborn mutants the atlantal neural arches were incomplete, failing to join dorsally (not shown, Table 1). This feature was also observed in seven of the heterozygotes (26%) but in none of the wild-type skeletons.

The lateral parts of the mutant atlas were fused to varying extents with the exoccipital bones. The cases shown in Fig. 4E and F are representative of the opposite extremes of lateral fusion between the atlas and the skull, seen in Hoxd-3+/Hoxd-3- mice. Alterations in the shape of the lateral masses and the neural arches of the atlas were seen in all of the mutant animals, even in those in which the lateral masses were fused only by a small ossified bridge to the exoccipital bones (Fig. 4E). Fig. 4F shows a mutant skeleton that displayed a complete bilateral fusion of the lateral masses of the atlas to the exoccipital bones. In this individual the transformation of the atlas into an occipital bone was nearly complete, except for the presence of rudimentary and unfused neural arches. Overall, among the ten newborn Hoxd-3+/Hoxd-3- skeleton preparations examined, three showed partial unilateral fusions, one had nearly complete bilateral fusion and the remaining six showed little or no fusion of the lateral masses and neural arches to the exoccipital bone (Table 1).

In the mutant skeletons that do not show fusion of the lateral masses to the exoccipital bones, the articulation between the lateral masses of the atlas and the occipital condyles of the skull is still markedly changed. The lateral views of the skeleton preparations show that the occipital bones and the superior surface of the atlas articulate along a longer portion of their surfaces than in the wild-type skeletons (Fig. 4D,E). Also the occipital condyles are not as prominent in the Hoxd-3+/Hoxd-3- animals as they are in the control animals.

The morphology of the axis in Hoxd-3+/Hoxd-3- mice is also altered. The dens is always missing (Fig. 4C). The laterally oriented superior articular surfaces of the axis are also absent. In five of the mutants, the neural arches of the mutant axis are thicker (compare Fig. 4D,F). In the ventral views it is clear that the cartilaginous foramina present on the lateral parts of the normal atlas are found on the lateral portion of the mutant axis (Fig. 4C). These cartilaginous structures are not as well constructed in the mutants and in a few cases appeared on only one side (3 out of 10; Table 1). These changes were never observed in wild type or heterozygous controls. The thickening of the neural arches and the shift in the position of the lateral foramen can be interpreted as a partial transformation of the axis to more closely resemble the atlas. In no case have we observed a complete transformation of the axis such that it possesses its own ‘atlas-like’ anterior arch.

An intermediate transformation of the atlas has been noted in 15% (4 out of 26) of the animals heterozygous for the Hoxd-3- mutation. This intermediate phenotype consists of a partial fusion of the anterior arch of the atlas with the basioccipital bone producing an ossified bridge between the two bones (Fig. 5B). The fusion does not extend laterally from the ossification center in the anterior arch of the atlas. The atlas remains a largely separate vertebra from the occipital bone. In Hoxd-3- heterozygotes, the axis has a normal morphology and the dens is present.

Some of the Hoxd-3- homozygotes survive to adulthood. To determine if survival correlated with a less extreme transformation of the cranio cervical joint, the skeleton of a 12-week-old Hoxd-3- homozygous mouse was examined. Similar to most extreme transformations seen in the newborn skeletons, this adult displays a complete fusion of the anterior arch and of the lateral masses of the atlas to the occipital bones (Fig. 6B). Again the atlantal neural arches are incomplete, and the axis is remodeled showing ossified lateral foramen that resemble those normally found on the atlas (Fig. 6). The dens is also missing.

**Hoxd-3 expression in mouse embryos**

Homing has shown that mice homozygous for the Hoxd-3 mutation exhibit radical remodeling of the cranio cervical joint, it was important to establish the anterior limit of Hoxd-3 expression in paraxial mesoderm. Though the anterior limit of Hoxd-3 expression in the neural tube and branchial arches of the mouse has been described (Hunt et al., 1991), the expression pattern in somites has not. The Hoxd-3 expression pattern in E8.0-E9.5 embryos was examined by whole-mount in situ hybridization. Fig. 7 shows an E9.5 embryo hybridized with a digoxigenin-labeled Hoxd-3 antisense probe. Hybridization with a control sense probe showed no signal (data not shown). In agreement with the previous report, the anterior limit of Hoxd-3 expression in the neural tube is at the boundary between rhombomeres 4 and 5 with low expression in rhombomere 5 and more intense expression caudal to rhombomere 5. The anterior limit of Hoxd-3 expression in paraxial mesoderm is at the boundary between somite 4 and 5. Somite assignment is the same whether the somites are counted caudally from somite 1 or rostrally from somite 13 at the posterior junction of the forelimb. Interestingly, a recent study using quail-chick chimeras has shown that somites 5 and 6 contribute to the formation of the atlas and the axis (Couly et al., 1993).

### Table 1. Summary of the Hoxd-3+/Hoxd-3- skeletal phenotypes

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<td>Basioccipital-anterior arch fusion</td>
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Paired structures altered bilaterally are indicated (B). Skeletons 37, 42 and 112 appear in Figs 4 and 6. Skeleton 112 is an adult.

*The appearance of lateral foramina on the mutant axis similar to those found on the wild-type atlas.
Though a direct correspondence between the structures derived from somites 1 through 6 of the mouse and chick has not been demonstrated, and the timing in the formation of somites 1 and 2 is different in these two species, nonetheless, based on the migration patterns of cells from these somites, it appears that the contribution of these somites to the formation of the muscles and bones in the cranial cervical regions may be equivalent.
Homeosis of cervical vertebrae in Hoxd-3− mice

Two of the Hoxd-3− homozygotes showed a defective sternum. In the mutant shown in Fig. 8B, the extreme ‘crankshaft’ pattern of the sternum may reflect an alteration in the segmentation of the left half of the sternum. Such a pattern could arise because the sternum is formed from two bilateral bars of cartilage that condense in the lateral mesoderm and later fuse medially (Chen, 1952). The manubrium sterni and the junction of the first costal cartilages with the sternum are normal in this mutant. Starting with the junction of the second costal cartilages, the segments of the sternum are offset between the right and left sides. The left second costal cartilage joins the sternum at a more posterior position relative to the right costal cartilage.

Fig. 9. Changes in the pathway and size of the vertebral arteries in Hoxd-3− homozygous newborns. (A,B) Parasagittal sections of the cervical region of newborn mice stained with hematoxylin and eosin. (A) Normally, as in this Hox-D3− heterozygote (+/−), the vertebral artery enters the spinal canal (arrow) between the atlas neural arch (at) and the exoccipital bone (ex). (B) In the Hoxd-3− homozygote (−/−), the vertebral artery entry point (arrow) is between the axis (ax) and the third cervical vertebra, immediately ventral to the dorsal root ganglion of the third cervical nerve (g3). (C,D) Transverse sections through newborn mice at the level of the sixth cervical vertebra. (C) Normal morphology of the vertebral artery (va, arrow) in a wild-type newborn (+/+). (D) Marked stenosis of the vertebral artery (va, arrow) in a stillborn Hoxd-3/Hoxd-3− mouse. Scale bar, (A,B) 0.5 mm; (C,D) 0.2 mm; bo, basioccipital bone.

Hoxd-3− homozygote with a defective sternum

Two of the Hoxd-3− homozygotes showed a defective sternum. In the mutant shown in Fig. 8B, the extreme ‘crankshaft’ pattern of the sternum may reflect an alteration in the segmentation of the left half of the sternum. Such a pattern could arise because the sternum is formed from two bilateral bars of cartilage that condense in the lateral mesoderm and later fuse medially (Chen, 1952). The manubrium sterni and the junction of the first costal cartilages with the sternum are normal in this mutant. Starting with the junction of the second costal cartilages, the segments of the sternum are offset between the right and left sides. The left second costal cartilage joins the sternum at a more posterior position relative to the right costal cartilage.
mice, newborn mice have normal homozygotes. Normally, the vertebral arteries run mutants mutants, the vertebral arteries enter the spinal canal on the left side, the fourth half-sternebra has failed to form and part of the left third half-sternebra is fused with the smaller right fourth half-sternebra. As a result, the three posterior left costal processes insert at the same point on the sternum instead of the normal insertion of two as is seen on the right side (Fig. 8B). The xiphoideal process in this mutant is normal.

**Histological examination of Hoxd-3/Hoxd-3 mice**

A total of eight Hoxd-3/Hoxd-3- E19 or newborn mice were sectioned for histological analysis, four in the sagittal and four in the transverse plane. Examination of these sections showed that all mutants had the same transformation of the craniocervical joint seen in the cleared skeleton preparations. Deletion of the dens and fusion of the anterior arch of the atlas to the basioccipital bone were clearly seen in all cases (data not shown). In addition, the intervertebral disc that is normally found between the dens and the body of the axis was found to be absent or greatly reduced in the homozygous mutants.

The sections were examined for alterations in other segmental structures in the cervical region. Both sagittal and transverse sections show that the entry point of the vertebral artery into the spinal canal is shifted caudally in the Hoxd-3- homozygotes. Normally, the vertebral arteries run cranially through the transverse foramen of the cervical vertebrae and then course over the lateral masses of the atlas, entering the spinal canal between the neural arches of the atlas and the exoccipital bones (Fig. 9A). In the Hoxd-3/Hoxd-3 mutants, the vertebral arteries enter the spinal canal at the level of the intervertebral foramen between the atlas lateral masses and C2 or between C2 and C3, immediately ventral to the spinal ganglia found in each of these foramina (Fig. 9B). In twelve of the fourteen entry points examined, the artery enters between C2 and C3, in one case it enters between C2 and the mutant atlas, and in the remaining case a small branch enters between C2 and the atlas, while the main part of the artery entered between C2 and C3. An additional anomaly of the vertebral arteries was noted in two of the Hoxd-3- homozygotes that were stillborn. In these two cases the vertebral artery appears to be abnormally narrow along its entire length (Fig. 9D).

The first cervical nerve in the mutant homozygotes is rerouted. Instead of exiting around the lateral masses of the atlas, this nerve runs through a foramen in the lateral parts of the floor of the mutant skull, between the lateral masses of the mutant atlas and the exoccipital bones (Fig. 10B). This additional foramen can be seen clearly in many of the skeleton preparations (arrow in Fig. 6B). In addition, an extra bundle of nerve fibers is seen to extend from the spinal cord to the lateral portion of the neural arches of the mutant atlas (Fig. 10D). This extra nerve collides with the bone and does not appear to exit the spinal canal (Fig. 10D). Caudally, the dorsal root ganglia of the cervical region are present and appear to be normal (Fig. 9B). The cranial nerves and ganglia also appear normal in whole-mount preparations of mutant 10 day embryos stained with a neurofilament monoclonal antibody, and in histological sections of newborns (data not shown).

**Tissues altered or eliminated in the Hoxa-3/Hoxa-3- mutants are normal in Hoxd-3/Hoxd-3- mice**

The mouse Hoxd-3 gene is a member of the paralogous gene family containing Hoxa-3 and Hoxb-3. Members of a paralogous family are related with respect to DNA and protein sequence both within and outside the homeobox domain (Graham et al., 1989) and show extensive overlaps in their RNA expression domains. Thus, for example, Hoxa-3, Hoxb-3 and Hoxd-3 show the same anterior limit of expression in the neural tube at the prospective boundary between rhombomere 4 and 5, in the branchial arches and in paraxial mesoderm (Hunt et al., 1991; and this paper). Such observations have suggested that paralogous Hox genes might perform similar functions. The mutant phenotype resulting from targeted disruption of Hoxa-3 has been described (Chisaka and Capecchi, 1991). Unlike Hoxd-3/Hoxd-3- mice, Hoxa-3/Hoxa-3- mice show a normal craniocervical joint. To extend the comparison of these two mutant mice further, all of the tissues altered in Hoxa-3- homozygous mice were carefully examined in Hoxd-3/Hoxd-3- mice.

Among the structures that are altered in the Hoxa-3/Hoxa-3- mutants are the cartilages and bones of the throat and jaws. The hyoid bone, thyroid and cricoid cartilages are remodelled, the lesser horns of the hyoid bone are missing, and the maxillae and mandibles are shortened and altered in shape in Hoxa-3- homozygotes. All of these cartilages and bones are normal in Hoxd-3/Hoxd-3- mice and indistinguishable from those observed in control littermates.

Hoxa-3/Hoxa-3- mice are also athymic, aparathyroid and have reduced thyroid and submaxillary tissue. In contrast, Hoxd-3/Hoxd-3- newborn mice have normal thyroid, parathyroid, thymus and submaxillary glands (Fig. 11B). Hoxa-3- homozygotes have defects in the musculature of the throat, tongue, epiglottis, esophagus and trachea. These defects cause the newborn Hoxa-3/Hoxa-3- mutants to bleat as a result of air being pumped into the stomach rather than into their lungs. The Hoxd-3/Hoxd-3- newborns never bleat and histological sections show that the epiglottis, esophagus, trachea, and soft palate appear normal in these mice (Fig. 11D).

Hoxa-3/Hoxa-3- mice were also found to have a spectrum of cardiovascular defects including poor septation, poorly formed valves, hypertrophy of both atria, hypertrophy of the major veins, poorly formed aorta, and missing carotid arteries. As a consequence, newborn Hoxa-3/Hoxa-3- mutants died at birth or within a few hours of birth from cardiovascular and pulmonary dysfunction. In contrast Hoxd-3/Hoxd-3- newborns are pink and breathing normally at birth. Their behavior is indistinguishable from their littermates. Most Hoxd-3- homozygotes survive for a few days following birth and a few survive into adulthood. Histological analysis of Hoxd-3/Hoxd-3- mice does not reveal any defects in the heart and major vessels issuing from the heart, the ductus arteriosus is closed, the aorta and pulmonary artery appear normal, hypertrophy of the atria and major veins is not observed and the structure of the
valves and the ventricles appears normal (data not shown). In summary, no defects common to both 589/583 and 589/583 mice were found.

**DISCUSSION**

**Low postnatal viability**

589 homozygous mice rarely survive to weaning age. This poor viability may be a result of instability in the craniocebral joint caused by the radical remodeling of the atlas and axis. The critical features of this complex joint, including the dens and the anterior arch of the atlas, which normally provide lateral stability, are absent from the mutant mice. Loss of these stabilizing structures may contribute to accidental cervical dislocation. The force needed to cause such dislocations could be provided by the mothers as they pick up the pups by the neck to move them in the nest. In humans, congenital abnormalities or injuries at this joint do lead to instability, which can result in accidental cervical dislocations (Georgopoulos et al., 1987). Unfortunately, diagnosis of accidental cervical dislocation in 589 homozygotes has been difficult because the dead pups are rapidly cannibalized. Consistent with the hypothesis of an accidental cause of death among 589/583 pups, we found that up to the time of death, the mutant pups appear normal. Further, the adult 589/583 mouse, whose craniocebral joint was analyzed, showed an extreme anterior homeotic transformation of the atlas and axis (Fig. 6). Survival to adulthood thus appeared to be unrelated to the extent of remodeling of the craniocebral joint in the mutant animals. However, even the mildest of transformations would still be expected to have reduced lateral stability at this joint since the dens of the axis and the anterior arch of the atlas are always missing.

Alternatively or additionally, the early death in 589 homozygous mice could be attributed to the altered pathway of the vertebral arteries. Normally these arteries are protected by foramen of the atlas as they course over this vertebra and into the spinal canal. In the mutant animals, the point of arterial entry is usually between the second and third cervical vertebrae. This abnormal entry point may subject the arteries to excessive compression and stretching during movement of the cervical spine. In the two cases examined of 589/583 pups found dead at birth, the vertebral arteries were abnormally narrow (Fig. 9C,D). This may have contributed to their very early demise.

**Anterior homeotic transformations and deletion of structures**

The primary function of 583 appears to be the appropriate specification of the first and second cervical vertebrae, the atlas and the axis. In the absence of 583, we observe a clear homeotic transformation of the anterior arch of the atlas into an extension of the basioccipital bone of the skull. This aspect of the phenotype is fully penetrant. Apparently, the cells that normally form the anterior arch of the atlas behave more like mesenchyme of the occipital sclerotomes and fuse with this mesenchyme to form an extension of the occipital portion of the skull. The fusion of the lateral parts of the atlas to the exoccipital bones is more variable, ranging from no fusion to complete fusion. However, in all cases, the lateral and dorsal portions of the mutant atlas differ in shape from the wild-type atlas and assume a morphology more closely resembling that of the exoccipitals.

Formation of the second cervical vertebra, the axis, is also affected in 589/583 embryos. The dens and the surfaces of the axis that normally articulate with the atlas are always missing. In addition, the axis takes on some of the characteristics normally associated with the atlas, suggesting that it is partially transformed to the atlas. However, this transformation is not complete. For example, the mutant axis does not acquire a ventral tubercle typical of the atlas. In this context it is interesting that disruption of 583 results in a more complete transformation of the axis to an atlas, but in these mutant animals the axis always retains its dens (Ramirez-Solis et al., 1993). A schematic summary of the effects of the loss of 583 function on the craniocebral joint is shown in Fig. 12. This diagram illustrates the assimilation of the anterior arch of the atlas into the basioccipital bone, the loss of the dens and of the intervertebral disk, the displacement of the lateral foramen of the atlas to the axis, and the unilateral fusion of the lateral masses of the atlas to the exoccipital bone.

From the above discussion it appears that different parts of the same vertebra are altered in different ways by the loss of the 583 gene product. For example, parts of the mutant axis, the dens and the superior facets of the axis, appear to be deleted, as is the intervertebral disc that normally forms between the axis body and the dens, whereas other parts of the atlas and axis are not missing but rather appear to be homeotically transformed to resemble more anterior structures. If the atlas and axis are predominantly of fifth and sixth somite origin (Couly et al., 1993), then the above results imply that the fate of different cells within these sclerotomes is differentially coded by 583. Alternatively, the same vertebra may originate from separate mesenchymal condensations at a later stage in development and these separate condensations are subject to different fates as a consequence of the mutation in the 583 gene. Anatomical interspecies comparisons of the craniocebral joint have suggested that the dens and superior facets of the mammalian axis were derived from the atlas vertebral body of lower vertebrae (DeBeer, 1937; Jenkins, 1969). Further, embryological analysis suggests that different parts of the same vertebra may originate from separate mesenchymal condensations (Theiler, 1988). It has been suggested that the dens and superior facets of the axis are derived from more medial mesenchymal condensations immediately surrounding the notochord. It is these structures that are deleted in the 589/583 mice. On the other hand, the structures that are subject to homeotic transformation like the atlas anterior arch may be derived from condensations that form ventral to the notochord (the hypochondral mesenchyme) and other condensations within the sclerotome (Theiler, 1988).

A model that could account for the observation that disruption of 583 may result in very different qualitative effects on the formation of different parts of the atlas and axis would be that a possible function of 583 is to differentially control the rates of proliferation of the groups of cells that will give rise to these structures. In this model, in
the absence of Hoxd-3 function, the rate of proliferation of the precursor cells that give rise to the dens or the superior facets of the axis would be severely retarded, resulting in these structures not being formed. However, loss of Hoxd-3, perhaps as a result of compensation by another Hox gene, might only slow down the proliferation of the cell precursors that will give rise to the anterior arch of the atlas, thereby placing their progeny into a condensation pattern adjacent to and akin with those that are forming the basioccipital bone. As a consequence, the cells that would normally form the anterior arch of the atlas now participate in the formation of an extension of the occipital portion of the skull. In this model quantitative changes in the rates of proliferation of different groups of cells would be translated into very different qualitative fates in the affected groups of cells. In the context of this model, it is interesting that Hoxb-

**Fig. 10.** Ventral displacement of the first cervical nerve in the Hoxd-3<sup>−/−</sup> mutants. Transverse sections of newborn mice. (A) The normal course of the first cervical nerve (arrow) around the lateral mass of the atlas (lm) in a wild-type newborn (+/+). (B) Ventral displacement of the first cervical nerve (arrow) in a Hoxd-3<sup>−/−</sup> homozygote (−/−), exiting over the lateral mass of the atlas (lm) and adjacent to the enlarged basioccipital bone (bo). (C) Higher magnification view of same section as in (A). (D) Additional nerve bundle (arrow) found in Hoxd-3<sup>−/−</sup> homozygotes (−/−) immediately caudal to the first cervical nerve. Scale bars, 0.5 mm; sc, spinal cord; d, dens; na, atlas neural arch.

**Fig. 11.** The glands and muscles affected by the Hoxa-3<sup>−/−</sup> mutation are normal in the Hoxd-3<sup>−/−</sup> homozygotes. Parasagittal sections of Hoxd-3<sup>−/−</sup> heterozygous (A, C; +/−) and Hoxd-3<sup>−/−</sup> homozygous (B,D; −/−) newborns. Scale bars, (A,B) 1 mm; (C,D) 0.5 mm; thd, thyroid; pth, parathyroid; thy, thymus; sm, submandibular gland; ep, epiglottis; pa, soft palate; to, tongue; tr, trachea; es, esophagus; thd c, thyroid cartilage.
Fig. 11
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structure (Lewis, 1978). In the

Fig. 12. Schematic summary of the Hoxd-3 phenotype, ventral view. The star indicates the portion of the mutant basioccipital bone derived from fusion of the atlas. The lateral mass (lm) of the atlas in the mutant is drawn as a separate bone on one side to reflect the varying degree of fusion between the lateral masses and the exoccipital bones found in the mutant skeletons. bo, basioccipital bone; eo, exoccipital bone; oc, occipital condyle; lf, lateral foramen of the atlas; at, atlas; d, dens; ax, axis; id, intervertebral discs; C3, third cervical vertebra.

8 is transcriptionally activated in mouse myeloid leukemia WEHI-3B cells and that transfection of this gene into NIH-3T3 cells produces fibrosarcomas in nude mice (Aberdam et al., 1991). By this definition, Hox genes can function as oncogenes and thereby are implicated in controlling cell proliferation. Postulating a role for Hox genes in regulating the proliferation rates of precursor cells is conceptually quite different from postulating a role in the specification of cell identity. The former may be less complex and might not require a complex ‘Hox-code.' Of course these postulated roles are not mutually exclusive.

Loss of function mutations in the Drosophila homeotic genes are often associated with anterior transformations of segment identity (for a review see Akam, 1987). For example, loss of function mutations in Ultrabithorax (Ubx) result in conversion of T3 thoracic structures to T2 thoracic structures (Lewis, 1978). In the Hoxd-3/−Hoxd-3/− mice we also observe an anterior homeotic transformation of structures, the conversion of the first cervical vertebra (C1) to part of the occipital bone and a partial transformation of the second cervical vertebra (C2) to the first cervical vertebra (C1).

Similarly, loss of function mutations in the mouse Hoxc-8 (LeMouellic et al., 1992) and Hoxb-4 genes (Ramirez-Solis et al., 1993) are associated with anterior transformations of vertebrae. The similar consequences of loss of function mutations in the Drosophila Hom-C genes and the mouse Hox genes suggest a common mechanism for Hox and Hom-C gene function.

In the Hoxd-3/−Hoxd-3/− mice, the region affected by the mutation represents only a small subdomain of Hoxd-3 expression, corresponding to the anterior limit of Hoxd-3 expression in mesoderm-derived tissue. Significantly, the anterior expression limit of Hoxd-3 in somite 5 coincides with the somites that have recently been shown to contribute to the formation of the atlas and axis in chick (Coulby et al., 1993). Defects were not observed in the central nervous system, in neural crest derived tissue, or in somite derived tissue caudal to the second cervical vertebra. Similarly, with targeted disruptions in Hoxa-1, Hoxa-3 and Hoxb-4, the affected tissues were also restricted to the anterior subdomain of their respective expression domains (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992; Ramirez-Solis et al., 1993).

The shift in the entry point of the vertebral artery into the central nervous system of Hoxd-3− homozygotes may arise as a secondary effect of the anteriorization of the vertebral segments. Vascular endothelial cells are a highly mobile and invasive cell population and obtain their cues for the patterning of blood vessels from surrounding structures (Noden, 1988, 1990). The anterior transformation of the vertebrae in Hoxd-3− homozygotes could misplace endothelial cells and cause the vertebral artery entry point to be shifted caudally.

The first cervical nerve is shifted ventrally in the Hoxd-3/−Hoxd-3/− mice. In many cases a new foramen for this nerve in the mutant skulls could be seen between the exoccipital bone and the lateral masses of the mutant atlas. Superficially, this new foramen resembles the foramen for the hypoglossal nerve, a more anteriorly placed cranial nerve. This suggests that the patterning of the passageway for this nerve out of the skull has been anteriorly shifted to resemble the foramen of the hypoglossal nerve.

Functional relationships between paralogous Hox genes

Expression studies suggested that Hox genes belonging to the same paralogous family would perform very similar functions (Hunt and Krumlauf, 1992). Therefore, it is surprising that the first genetic analysis of two paralogous Hox genes has revealed no common defects. The defects observed in Hoxa-3/−Hoxa-3/− (hox-1.5/−hox-1.5/−) mice are primarily in tissues and structures derived from and/or modeled by mesenchymal neural crest cells (Chisaka and Capecchi, 1991) whereas the Hoxd-3/−Hoxd-3/− abnormality can be attributed to defects in paraxial and lateral mesoderm. The same region along the A-P axis is affected in both mutant mice, but in completely separate tissues.

Finding that Hoxa-3/−Hoxa-3/− and Hoxd-3/−Hoxd-3/− mice have no defects in common does not imply that these two genes have no functions in common. If a common function between these genes were completely redundant, then it would not be revealed in mice homozygous for targeted disruptions in only one of the genes. Such functions would only become evident in mice homozygous for both mutations. Since Hoxa-3 and Hoxd-3 are on different chromosomes, the mice homozygous for both targeted mutations required to examine this issue can be generated by breeding.

In terms of function, Hoxd-3 and Hoxb-4 are more related to each other than are Hoxd-3 and Hoxa-3. Both Hoxd-3 and Hoxb-4 are required for specification of cervical vertebrae, the primary functions of Hoxd-3 and Hoxb-4 being the specification of the atlas (C1) and the axis (C2) respectively (this paper and Ramirez-Solis et al., 1993). Interestingly, both Hoxd-3 and Hoxb-4 have a partial role in the specification of C2 However, the overlap of function in C2 is not redundant. Whereas Hoxd-3 is uniquely required for the specification of the dens, Hoxb-4 is uniquely required for the specification of the remainder of the axis. As we discussed
previously, this division of labor on forming the axis may not be fortuitous. Comparative anatomists have argued that the dens is evolutionarily an extension of the formation of the vertebral body of the atlas (DeBeer, 1937; Jenkins, 1969). It will be of interest to explore the functional relationship between Hoxd-3 and Hoxb-4 both genetically and molecularly.

Variability in penetrance and expressivity
Two of the Hoxd-3- mutants showed a defective sternum. Interestingly, targeted disruption of Hoxb-4 is also accompanied by defects in the formation of the sternum (Ramirez-Solis et al., 1993). The sternum arises from lateral mesoderm in the thoracic region and the remodeling found in the Hoxd-3 mutant suggests an effect on the joining of the second costal cartilages to the sternum. On the left, this rib is inserted at a more posterior point than on the right, leading to an offset in the segmentation of the left and right halves of the sternum. This may be due to a change in the cues for the insertion of this rib causing it to attach at an inappropriately caudal point. In explant cultures of mouse sternal rudiments, Chen (1953) showed that the insertion points of the costal portions of the rib determined the segmental pattern of the sternum. The removal of ribs led to the hypertrophy and ossification of the sternebrae, while insertion of an extra rib inhibited the formation of the sternebrae. Therefore, changing the insertion points of the ribs could lead to a change in the segmental patterns of the sternum.

It is curious that the penetrance of the sternum phenotype is low and the effect on the formation of the sternum is asymmetric. Variability in the penetrance and expressivity of a mutation has been classically attributed either to variability in genetic background or to ‘leakiness’ of the mutation. We have no reason to suspect that this mutation is leaky since it is not revertible nor can we detect any intact Hoxd-3 transcripts in homozygous mutant mice. Variability in the genetic background could account for the variability of penetrance of this phenotype. The Hoxd-3 mutation is maintained on a mixed background of 129Sv and C57Bl/6, the respective sources of the ES cells and the recipient blastocysts used to generate the germline chimeras. However, we have recently observed variability in the expressivity of targeted mutations which could not result from leakiness of the mutation nor variability in the genetic background. For example, disruption of int-2 (fgf-3) results in aberrant development of the inner ear (Mansour et al., 1993). However, in many int-2/int-2- mice one inner ear develops normally whereas the other is completely defective. Variability in genetic background cannot explain this observation since the variability in expressivity is seen in the same animals. Further, the mutation was carefully characterized to ensure that it was a complete loss of function mutation. The only plausible explanation for this variability of expressivity is to suggest the existence of an alternative pathway, containing one or more genes, that is stochastically used to compensate for the int-2 mutation. The asymmetry of the sternum phenotype in Hoxd-3- mutants argues that the variability in the expressivity of this phenotype might also involve stochastic compensatory mechanisms. Another site in which variability in expressivity of the Hoxd-3/Hoxd-3- mutation was observed was in the extent of fusion between the lateral masses of the mutant atlas and the exoccipitals. Interestingly, this variability in expressivity was also often asymmetric, showing unilateral as well as bilateral fusions of the mutant lateral masses with the exoccipital bone.

Heterozygous mice show a phenotype
Mice heterozygous for targeted disruptions in Hox genes usually do not exhibit a phenotype. In contrast, a small fraction (15%) of the mice heterozygous for the Hoxd-3- mutation do exhibit an intermediate phenotype. This could be due to a dosage effect, reflecting a higher sensitivity to gene dosage of Hoxd-3 in some individuals. The presence of a phenotype in heterozygotes, albeit at low penetrance, may be of particular relevance in assessing whether some human congenital malformations may be attributable to mutations in human Hox genes. The probability of observing a human congenital disorder is much greater if humans, heterozygous for the genetic lesion, exhibit a visible phenotype. Clinical literature cites numerous cases of cranio-cervical malformations. Fusions of various cervical vertebrae, incorporation of the atlas into the skull (occipitalization of the atlas) and fusions of the dens, atlas and basioccipital bones have all been reported (McRae and Barnum, 1953; Gunderson et al., 1967; Gunther, 1980). However, only in the case of Klippel-Feil syndrome (Gunderson et al., 1967), which involves fusions of cervical vertebrae, has a familial occurrence been established. In the case of type II Klippel-Feil syndrome, fusion of C2 and C3 vertebrae is frequently associated with occipitalization of the atlas. Families tend to share the same levels of fusion, suggesting that a single gene may influence this aberrant development. Pedigrees of this syndrome suggest that it is an autosomal dominant mutation (i.e. heterozygotes exhibit a visible phenotype). This syndrome has not yet been mapped.

Arabian horses show a familial vertebral malformation that appears very similar, if not identical, to that described herein for Hoxd-3- mice. This congenital syndrome is autosomal recessive and is characterized by the transformation of the anterior arch of the atlas into an extension of the basioccipital bone, unilateral or bilateral fusion of the lateral masses of the atlas to the exoccipital bones, hypoplasia of the dens, as well as partial transformation in the shape of the axis to more closely resemble that of the atlas (Noden and Lahunta, 1985). The extensive similarities between the mouse and horse phenotypes make the Hoxd-3 gene a strong candidate for the locus of this equine congenital malformation.

Conclusion
Targeted disruption of Hoxd-3 in the mouse has shown that the primary function of this gene is to specify the formation of the cranio-cervical joint. Unlike its paralogous gene Hoxa-3, which primarily controls the formation of structures and tissues derived from mesenchymal neural crest cells, Hoxd-3-mediated defects are restricted to structures derived from paraxial and lateral mesoderm. In the absence of Hoxd-3 function the atlas appears to be homeotically transformed to a more anterior structure resembling the occipital bones of the skull. Comparative anatomy has suggested that the occipital region of the skull has developed through the modification of occipital vertebrae into the contiguous occipital
bones (DeBeer, 1937). Lower vertebrates such as the agnathans (lampreys) have features thought to reflect the more ancestral skull, including structures homologous to occipital neural arches. In transgenic mice, expression of Hoxa-7 and Hoxd-4 genes in cells anterior to their normal expression boundaries has resulted in partial segmentation of the occipital bone, which can be interpreted as atavistic posterior transformations (Kessel et al., 1990; Lufkin et al., 1992). These transformations partially reveal the ancestral segmented structures of the occipital skull. The Hoxd-3/ Hoxd-3' phenotype is an example of the opposite transformation, remodeling a vertebra into an occipital bone. It shows that a radical reconstruction of the craniocervical joint and an enlargement of the skull can be accomplished with the loss of a single gene. Such observations lend credence to the hypothesis that changes in the circuitry of genes within the Hox complex could account for some of the dramatic changes in animal body form during evolution.

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