Homeotic transformation of cervical vertebrae in Hoxa-4 mutant mice
(pattern formation/skeleton)

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ABSTRACT Hoxa-4 (previously known as Hox-1.4) is a mouse homeobox-containing gene that is expressed in the presumptive hindbrain and spinal cord, prevertebrae, and other tissues during embryogenesis. To understand the role of Hoxa-4 during development, we generated Hoxa-4 mutant mice. Homozygous mutants were viable and fertile. Analysis of neuronal skeletons revealed that the development of ribs on the seventh cervical vertebra at variable penetrance and expressivity. A low frequency of alterations in sternal morphogenesis was also observed. In addition, we analyzed the skeletons of transgenic mice that overexpress Hoxa-4 and found that the formation of the small rib anlagen that often develop on the seventh cervical vertebra was suppressed. Analysis of adult homozygous mutant skeletons revealed that the dorsal process normally associated with the second cervical vertebra was also found on the third cervical vertebra. These results demonstrate that Hoxa-4 plays a role in conferring positional information along the anteroposterior axis to specify the identity of the third and the seventh cervical vertebrae.

The cells of a developing embryo must have positional information to form secondary structures at the proper locations along the embryonic axes. A group of genes involved in this process was originally identified in Drosophila, because mutations at these loci resulted in homeotic transformations (respecification of segments) along the anteroposterior axis (1). These homeotic genes map to two clusters in the Drosophila genome, the Antennapedia complex and the bithorax complex. The relative positions of genes within these clusters correlate with their functional domains along the axis: the 5′-most gene is required for the most posterior structures, and genes located toward the 3′ end of the cluster affect progressively more anterior domains (2, 3). In addition, these genes share a DNA sequence known as the homeobox that encodes the DNA-binding homeodomain, which is structurally similar to the helix–turn–helix motif of transcriptional repressors (4). Because the homeotic genes encode transcription factors, it is thought that they regulate specific sets of downstream genes, in a cascade leading to the morphogenesis of a given segment (1).

Cross-hybridization with Drosophila homeobox sequences facilitated the cloning and identification of similar gene clusters in the mouse. There are 38 murine Hox genes localized in four clusters on four different chromosomes (5). Many of these genes can be divided into paralogous subfamilies, each containing up to four members encoding homeodomains that are more similar to each other and to those encoded by their Drosophila homologues than to the homeodomains encoded by the other mouse Hox genes. The Hox genes are usually expressed in the mesoderm of late-gastrulation-stage embryos and, subsequently, in the central nervous system. A discrete anterior limit of expression can be seen in the central nervous system and in the prevertebrae (6). In situ hybridization studies show that Hoxa-4 has an anterior limit of expression in the hindbrain at the rhombomere 6/7 boundary (7). In prevertebrae at 12.5 days of gestation (E12.5), the anterior limit of Hoxa-4 transcription is at the second cervical vertebra (C2), although transcripts are detected weakly in C2 and more strongly in C3 through C7. Expression is also detected in the mesodermal components of other organs, including lung, kidney, and portions of the gut. In the adult, Hoxa-4 expression is restricted to meiotic and postmeiotic male germ cells (8, 9).

Loss-of-function studies using homologous recombination in embryonic stem (ES) cells have begun to reveal the specific functions of individual Hox genes in vivo, including neural crest specification, limb development, and patterning of the axial skeleton (10). Therefore, to understand the function of Hoxa-4 during development, we mutated the Hoxa-4 locus in ES cells and used the mutated ES cells to generate Hoxa-4 mutant mice. Hoxa-4 homozygous mutant mice are viable and fertile but display homeotic transformations of cervical vertebrae; C3 is partially transformed to have characteristics of the C2 and C7 adopts the identity of a thoracic vertebra (T). In light of this finding, we examined the skeletons of mice overexpressing Hoxa-4 and found that the formation of rib anlagen at C7 was suppressed. Our results suggest that Hoxa-4 functions to specify the identity of C3 and C7, and its action in determining the identity of C7 is dependent on the level of Hoxa-4 expression. We also compare these results with the mutant phenotypes of one of its paralogs, Hoxb-4.

MATERIALS AND METHODS

Disruption of the Hoxa-4 Locus in Mouse ES Cells. A gene-targeting vector was generated from a λ phage DNA clone containing the Hoxa-4 gene previously isolated from a C57BL/6J (B6) mouse genomic library (Fig. 1). The vector contains 6.0 kb of sequence homology with genomic DNA and extends from a 5′ Kpn I site to an EcoRI site that lies 3′ of the homeodomain. A PGKNeoBP1 cassette was inserted in reverse orientation relative to the direction of Hoxa-4 transcription into the EcoRI site located in helix 1 of the homeodomain (11). An MC1tkPa cassette was also added to enrich for homologous recombinants by using negative selection with 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) (12). Ten micrograms of linearized vector was electroporated into 10⁵ AB-1 ES cells (originally derived from a Black Agouti 129 mouse embryo) and cultured on a monolayer of SNL 76/7 STO cells in the presence of G418 and FIAU (11, 13). A total of 140 G418/FIAU-resistant ES clones

Abbreviations: B6, C57BL/6J; Cn, cervical vertebra n; En, gestational day n; ES, embryonic stem; Th, thoracic vertebra n.
were screened by Southern analysis by hybridizing EcoRI digested genomic DNA with a unique 5' probe that lies external to the vector homology. Correctly targeted cell clones were then expanded and reanalyzed by hybridizing EcoRV digested DNA with a 3' probe external to the vector homology.

**Germ-Line Transmission of the Hoxa-4 Mutant Allele.** The Hoxa-4 mutant ES clones were microinjected into B6 blastocysts to generate chimeras that were subsequently bred with B6 mates (14). Tail DNA samples from the agouti pups that resulted from those matings were analyzed by Southern blotting with the 5' or 3' probes to identify individuals that were heterozygous for the Hoxa-4 mutation. The chimeras were also bred with 129/SvEv mates to produce an inbred mouse strain carrying the Hoxa-4 mutation.

**Northern Blot Analysis.** Total RNA was isolated by lithium chloride precipitation (15). Total RNA (25 µg per sample) was analyzed by Northern blotting as described (16). The integrity of the RNA on the blots was confirmed by the appearance of the 18S and 28S rRNA bands after ethidium bromide staining. Two Hoxa-4 probes were used for the analysis. One probe is located 5' of the neo insertion and contains ~300 bp of Hoxa-4 cDNA sequence between the Nru I and EcoRI sites. The other probe is an ~680-bp Xba I–HindIII fragment that is located 3' of the neo insertion and that contains 3' untranslated sequence (17).

**Skeleton Preparations.** Skeletons were prepared by alkaline digestion and stained with alizarin red S for ossified bone and alcan blue 8GX for cartilage (18).

**Hoxa-4 Overexpressing Transgenic Mice.** The transgenic mouse strain Tg(Hoxa-4)69Bri carries multiple copies of a mouse Hoxa-4 transgene (17). This mouse line was initially generated in a B6 × SJL hybrid genetic background. It has subsequently been maintained by crosses with outbred Swiss mice for four generations and for subsequent generations with B6 × DBA/2 F1, hybrids. The genotype of mice carrying the transgene was determined with a simian virus 40 probe that is specific for the transgene.

**RESULTS**

**Hoxa-4 Mutant Mice Are Viable and Fertile.** To disrupt the Hoxa-4 gene in mouse ES cells, we generated a vector that contains a neomycin resistance (neo) expression cassette inserted into the EcoRI site in the first helix of the Hoxa-4 homeodomain (Fig. 1). Correctly targeted clones can be detected by the presence of an additional 7.8-kb band on a Southern blot of genomic DNA digested with EcoRI and hybridized with a 5' EcoRI–Kpn I fragment (1.1 kb) or by the presence of a 4.8-kb band when genomic DNA is digested with EcoRV and hybridized with a 3' EcoRI fragment (0.5 kb). Correct targeting of the Hoxa-4 locus results in the disruption of the homeobox by the neo cassette. Thus, there should be no Hoxa-4 transcripts with a continuous coding region, and, at most, a truncated protein lacking DNA-binding activity could be produced. Three correctly targeted ES clones were identified, and two of these clones, 1D3 and 2D4, were found to successfully contribute to the germ line of chimeric mice (Fig. 2).

Male and female Hoxa-4 heterozygotes appeared normal and were fertile. These heterozygotes were subsequently interbred to generate mice homozygous for the Hoxa-4 mutation. The genotypes of the offspring from these heterozygous crosses (Fig. 2) followed predicted Mendelian frequencies, and the homozygous mutants were externally indistinguishable from their wild-type littermates. All male and female homozygous mutants were fertile.

**Northern Analysis of Hoxa-4 Mutant Embryos.** To determine whether the mutation in the Hoxa-4 gene disrupted the transcription of wild-type Hoxa-4, RNA isolated from 12.5 wild-type, heterozygous, and homozygous mutant embryos was analyzed by Northern blotting. The blots were hybridized either with a probe containing a portion of the 3'-untranslated region of the Hoxa-4 gene located 3' of the neo insertion or with a cDNA probe containing portions of exon 1 and exon 2 that are 5' of the neo insertion (Fig. 3). Both of the Hoxa-4 probes detected the 1.7-kb embryonic transcript in the wild-type and heterozygous embryos but not in the homozygous mutant embryos. The intensity of the hybridization signal in the heterozygotes was approximately one-half that of the wild-type embryos. Although there were faint bands at ~3.1 and 4.0 kb detected in all samples, it is clear that the major embryonic wild-type transcript has been abolished by the targeted mutation.

**Homoeotic Transformations of Cervical Vertebrae in Hoxa-4 Mutant Mice.** Histological analysis of various organs including liver, lung, kidney, small intestine, colon, brain, testis, epididymis, stomach, ovary, thymus, and spleen from adult homozygous mutants revealed no obvious differences in comparison to wild-type controls. Since targeted mutations in several Hox genes resulted in homeotic transformation of skeletal segments (10), we looked for similar changes in the Hoxa-4 mutants from matings between F1 heterozygotes. A significant number of skeletons from newborns had an extensive ossified rib on C7 (Fig. 4). Since small ossified rib anlagen at C7 are common in some strains of mice and extensive ribs have been reported in 1 of 48 wild-type mice of a hybrid strain (19), we quantified the cervical-rib effect by scoring each skeleton for the presence or absence of a rib anlage and for the presence of an extensive rib (one with a
length approximately 2 or more times its width). These scores were then compared with genotype. An extensive rib was observed in 22 of 46 (48%) homozygous mutants and in 1 of 41 (2.4%) wild-type pups of the 2D4 line. Nine (20%) of the extensive ribs in the homozygous mutants were long enough to fuse to the costal cartilage of the first thoracic (T1) rib (Fig. 4B), but none fused directly to the sternum. Eleven (24%) of the extensive ribs were bilateral. Interestingly, 20 of 109 heterozygotes (19%) also had extensive ribs, including two that were fused to the ribs at T1. Extensive cervical ribs that fused to the ribs at T1 were never observed in wild-type mice.

To determine if the penetrance of the cervical rib phenotype is higher in an inbred background, we analyzed 47 neonatal skeletons from heterozygote crosses of inbred 129 mice. While some of the mutants had rib anlagen slightly longer than their widths, there were no extensive ribs in these skeletons. Thus, the expressivity of the Hoxa-4 cervical rib phenotype decreased in strain 129 mice.

It was surprising that the more anterior cervical vertebrae of neonatal Hoxa-4 mutants were not affected, since the anterior limit of Hoxa-4 expression is at the level of C2. Therefore, we also examined the cervical region of skeletons from adults (animals greater than 8 months of age). In wild-type skeletons, a skeletal element known as the processus spinosus is found on C2 but not on C3 (20). This dorsal process is a fin-shaped axial ossification that projects dorsocaudally. In five of seven (71%) Hoxa-4 homozygous mutants, a dorsal process was also found on C3 (Fig. 5). The six heterozygotes that were examined did not possess the ectopic dorsal process on C3.

**Sternal Malformations in Hoxa-4 Mutant Mice.** In neonatal skeletons from the products of matings of F2 homozygous mutants to either F1 heterozygotes or F2 homozygous mutants, 7 of 21 (33%) Hoxa-4 homozygous mutants had sternal abnormalities, most commonly one or two fused sternbrae. In two mutant heterozygotes (10%), the malformation was more severe: there was asymmetric ossification of the sternbrae (Fig. 6), similar to that seen in Hoxa-5 and Hoxd-3 mutants (21, 22). Presumably, in the mutants, the offset in attachment points of the costal cartilages on opposite sides of the sternal bars results in the direct apposition of ossified and nonossified segments. In one of these mutants (Fig. 6), the costal cartilage of one of the T1 ribs is bifurcated. On the left side, the two processes from the bifurcation attach to the sternum at different sites, one at the normal attachment site for T1 and one at the normal attachment site for T2.

**Suppression of Cervical Rib Anlagen in Transgenic Mice That Overexpress Hoxa-4.** We previously generated lines of transgenic mice that overexpress Hoxa-4 in the tissues in which Hoxa-4 is normally expressed (17). Although expression of the transgene is highest in the developing gut, it is also expressed at levels comparable to the endogenous levels of Hoxa-4 in the prevertebrae. The combined expression from both the transgene and the endogenous loci results in overexpression of Hoxa-4 within its normal domain. Since the loss of Hoxa-4 transcripts in Hoxa-4 mutant mice correlates with more extensive cervical ribs, we asked whether over
expression of Hoxa-4 transcripts could suppress cervical rib formation. We analyzed the skeletons of neonates from matings between Hoxa-4 transgenic males and Swiss females. In this genetic background, 22 of 31 (71%) wild-type mice had small rib anlagen at C7, while only 6 of 28 (21%) transgenic mice had them. These results suggest that overexpression of Hoxa-4 suppresses the formation of ribs on C7 \( (P < 0.001, \chi^2 \text{ analysis}) \). This is consistent with the interpretation that the intermediate phenotype seen in mice heterozygous for the Hoxa-4 mutant allele may be due to loss of expression of the wild-type gene from one allele rather than to a dominant action of the mutant allele, particularly since Northern analysis shows the intensity of the wild-type transcript from heterozygotes is decreased relative to wild types (Fig. 3).

**DISCUSSION**

We generated Hoxa-4 mutant mice to investigate the role of Hoxa-4 during development. There is no decrease in viability associated with this Hoxa-4 mutation. While the embryonic tissues in which Hoxa-4 is expressed are quite diverse, in the adult, expression is restricted to meiotic and postmeiotic male germ cells (9). The highly restricted expression pattern in the male gonad suggested that this homeoprotein has a function during spermatogenesis. Surprisingly, our Hoxa-4 mutant mice were fertile. In addition, mice carrying a partial deletion allele of Hoxa-4 were also found to be fertile (23). These results suggest that Hoxa-4 is not essential for spermatogenesis. Other Hoxa-4 paralogs may provide redundant or compensatory functions during male germ cell development. Hoxd-4 is expressed in the somatic cells of the testis but not in germ cells and is thus an unlikely candidate for redundant function. However, Hoxb-4 is transcribed in spermatogenic cells, though at much lower levels than Hoxa-4 (24). It should be noted that Hoxb-4 mutant male mice are also fertile (25). If Hoxa-4/Hoxb-4 double-mutant males are viable, perhaps they will have defects in spermatogenesis.

The formation of the C2-type processus spinosus on the C3 of homozygous mutants is indicative of a transformation of C3 toward a C2 identity. This is a partial anterior transformation, since C3 does not assume other characteristics of C2, specifically the dens and widened neural arches. In Hoxa-4 mutants, the presence of extensive ribs at C7 is characteristic of a posterior transformation: a cervical vertebra assuming the fate of a thoracic vertebra. Intriguingly, mice with mutations of the Hox-5 gene (21) and the Hox-6 gene (23), which are located just 5' of Hoxa-4, have a similar phenotype. These mice have a more complete C7 to thoracic transformation that is also more penetrant, although different genetic backgrounds were analyzed. Nonetheless, this is a clear example of overlapping phenotypes among three different Hox mutants (Hoxa-4, Hoxa-5, and Hoxa-6), suggesting the possibility that some phenotypes may be shared between Hox mutants for genes whose anterior limits of expression are close. Interestingly, another Hoxa-4 mutation that is different from the one reported here causes a similar phenotype at C3, but posterior transformations of C7 were not mentioned (23). This mutation differs from the one reported here because it deletes the coding region of the recognition helix, 3' to the EcoRI site in which the disruption reported here was made. While it is possible that the differences could be due to different partially functional gene products in the two mutants, it is more likely that the cervical rib phenotype and sternal defects were not reported in the other mutation because the low expressivity and penetrance of these phenotypes, combined with the influence of genetic background, obscured their detection.

The posterior prevalence model states that the function of a given Hox gene manifests in the axial region in which it is the gene with the most posterior limit of expression (26). Thus, a loss-of-function mutation should result in deficiencies or transformations of axial regions near the anterior limit of expression. Although Hoxa-4 is expressed in C2, it is expressed only weakly, and is more strongly expressed in C3 through C5. Thus, it is perhaps not surprising for the Hoxa-4 mutants to show a transformation at the level of C3. The incompleteness of the transformation suggests that different Hox genes may be responsible for specifying different aspects of the same vertebra. Alternatively, in the absence of Hoxa-4 other Hox genes may substitute for Hoxa-4 function.

The cervical rib phenotype does not strictly conform to the posterior prevalence model. Perhaps this is because the specification of the identity of C7 is more susceptible to perturbations in the expression of Hox genes and to other events that are involved in rib formation. Indeed, the formation of extensive ribs in three different Hox mutants, in retinoic acid-treated embryos (19), and in a small percentage of wild-type animals suggests that this is a process which is easily perturbable. In the B6 × 129 F2 genetic background, extensive cervical ribs were significantly more frequent in homozygous mutants than in heterozygotes, and, in turn, extensive ribs were more frequent in heterozygotes than in wild-type littermates. Thus, the frequency of extensive cervical ribs increases as the number of wild-type Hoxa-4 alleles decreases, probably due to a concomitant decrease in wild-type transcripts. In an independent experiment, we compared the frequency of small cervical rib anlagen in transgenic mice overexpressing Hoxa-4 with that of their wild-type littermates. We showed that the frequency of formation of a small cervical rib anlage in the Hoxa-4 transgenics was significantly reduced compared with controls. Thus, overexpression of Hoxa-4 can suppress cervical rib formation, while loss of wild-type Hoxa-4 transcripts correlates with formation of extensive cervical ribs. It is still possible that these effects could be due to altered expression of other Hox genes in these mutants; however, there is still no report of significant changes in the expression of other Hox genes in any Hox mutant. While it is likely that Hoxa-4 is only one of many genes involved in specifying the identity of C7, these results indicate that levels of Hoxa-4 expression can influence the expressivity and penetrance of the cervical rib phenotype.

A significant number of the Hoxa-4 homozygous mutant mice from matings of F2 homozygotes to either F1 heterozygotes or F2 homozygotes had sternospondylosis, a phenotype in a transgenic mouse of both C5 and Hoxd-3 mutants (21, 22). It was suggested that the low penetrance and variability in expressivity of this phenotype could be due to the existence of an alternate pathway (controlled by one or more genes) that is stochastically used to compensate for the mutation. Indeed, the identification of more and more Hox mutants with sternal abnormalities suggests that a number of genes could be involved. The expression of Hoxa-4 in prevertebra T1 through T7 could be responsible for these minor defects in sternum morphogenesis.

Finally, the Hoxa-4 mutation had no obvious effects in any of the other tissues, not even in the central nervous system, where Hoxa-4 is most strongly expressed. The high percentage of amino acid identity among the homeodomains encoded by different Hox genes suggests that different Hox proteins may bind to the same or similar sites in downstream target genes. It has therefore been proposed that other Hox genes can compensate for at least some of the functions of the mutated gene. The most obvious candidate genes for functional compensation are the Hox paralogs. Like the Hoxa-3 and Hoxd-3 mutants (22, 27), which share no common phenotypes, the Hoxa-4 and Hoxb-4 mutants also have no phenotype in common. Hoxb-4 mutants exhibit partial anterior transformations of C2, specifically the development of an anterior arch of the atlas and widening of the neural arches.
(25), while Hoxa-4 mutants show partial anterior transformations of C3 and posterior transformations of C7 to a thoracic identity. However, the Hoxa-4, Hoxa-5, and Hoxa-6 mutants do share a common phenotype. This apparent overlap in function suggests that there may be shared functions among the Hox genes, particularly among genes whose anterior limits of expression are very close, as is the case with Hoxa-4 and Hoxa-5. Now that mutants for the paralogous genes Hoxa-4 and Hoxb-4 have been generated, it will be possible through matings to generate double mutants to determine the degree of functional redundancy among paralogs. Likewise, creation of mice that are mutant for both Hoxa-4 and Hoxa-5 or Hoxa-6 should better define the degree of functional redundancy between nonparalogous Hox genes.

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