Homeotic transformations and limb defects in *Hox A11* mutant mice

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Hox A11 is one of the expanded set of vertebrate homeo box (*Hox*) genes with similarities to the *Drosophila* homeotic gene, *Abdominal-B* (*Abd-B*). These *Abd-B*-type *Hox* genes have been shown to be expressed in the most caudal regions of the developing vertebrate embryo and in overlapping domains within the developing limbs, suggesting that these genes play important roles in pattern formation in both appendicular and axial regions of the body. In this report whole-mount in situ hybridization in mouse embryos gave a precise description of *Hox A11* gene expression in the developing limbs and in the axial domain of the developing body. In addition, we generated a targeted mutation in *Hox A11* and characterized the resulting phenotype to begin to dissect developmental functions of the *Abd-B* subfamily of *Hox* genes. *Hox A11* mutant mice exhibited double homeotic transformations, with the thirteenth thoracic segment posteriorized to form an additional first lumbar vertebra and with the sacral region anteriorized, generating yet another lumbar segment. Furthermore, skeletal malformations were observed in both forelimbs and hindlimbs. In mutant forelimbs, the ulna and radius were misshapen, the pisiform and triangular carpal bones were fused, and abnormal sesamoid bone development occurred. In mutant hindlimbs the tibia and fibula were joined incorrectly and malformed at their distal ends. Also, an enlarged sesamoid developed ventral to the tibiale bone. Both heterozygous and homozygous mice displayed mutant phenotypes adding an additional level of complexity to the *Hox* code hypothesis.

[Key Words: *Hox A11*; mouse; development; homeotic transformation; limb defects]

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The genetic program of pattern formation during vertebrate development is only beginning to be deciphered. In *Drosophila*, however, it has been clear for some time that transcription factor genes carrying the homeo box sequence are among the master switch regulators of development, capable of initiating genetic cascades that control the developmental destinies of groups of cells (Gehring 1987). Striking similarities in sequence, organization and expression patterns between *Drosophila* and vertebrate homeo box (*Hox*) genes originally suggested important developmental functions for the vertebrate counterparts (Graham et al. 1989). This view was supported further by transgenic fly and transgenic mouse experiments in which mammalian *Hox* genes were introduced into flies and vice versa (McGinnis et al. 1990; Malicki et al. 1990, 1992; and Awgulewitsch and Jacobs 1992). Significant conservation of *cis*-regulatory element and coding sequence functions were observed, despite the rather large evolutionary separation. Moreover, recent studies of mice carrying misexpression or null mutations of *Hox* genes have found structure deletions and homeotic transformations similar to those seen previously in flies, demonstrating further the importance of these genes in mammalian development (Kessel et al.

1990; Chisaka and Cappechi 1991; Lufkin et al. 1991, 1992; Chisaka et al. 1992; Kaur et al. 1992; LeMouellic et al. 1992; McLain et al. 1992; Ramirez-Solis et al. 1993).

The potential roles of *Hox* genes in limb development are of particular interest. Limb formation has served as an excellent model system for pattern generation in vertebrates. The morphogenetic principles of limb formation are well conserved, in that grafted pieces of limb buds from mammals or reptiles are capable of driving appropriate chicken limb development (Fallon and Crosby 1977). At the posterior edge of the limb bud resides the zone of polarizing activity (ZPA), which appears to be the source of a morphogen gradient important in establishing anteroposterior polarity (Tickle et al. 1975). Moreover, the outgrowth of the limb bud is controlled by the apical ectodermal ridge (AER), a specialized ectodermal structure along the rim of the distal tip of the limb bud. The AER apparently signals the group of mesenchymal cells beneath it, the progress zone, where pattern specification of limb cells is determined (Summerbell et al. 1973). In addition, the Turing reaction-diffusion process has been proposed as a mechanism for the generation of standing waves of certain morphogens in the developing limb (Turing 1952).

A number of murine *Hox* genes of the *Abd-B* subfam-

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ily have been shown to be expressed during limb development, suggesting that they play an important role in limb morphogenesis. Interestingly, the 5' ends of the vertebrate Hox clusters have been expanded considerably when compared with *Drosophila*. The most 5' gene in the *Drosophila* cluster is *Abd-B*, whereas the vertebrate clusters have a total of 15 genes that are either direct homologs of *Abd-B* or are located even more 5'. Each of the 15 genes carries sequence characteristics that mark it as a member of the *Abd-B* subfamily. In mammals, therefore, a striking 40% of all Hox genes are of the *Abd-B* group; and for most of these genes, expression in the developing limb has now been shown. The *Hox D9*, *D10*, *D11*, *D12*, and *D13* genes, for example, establish an anterior-to-posterior gradient (Dolle and Duboule 1989; Dolle et al. 1989, 1991; Nohno et al. 1991), *Hox C10* is expressed in an antero-proximal region (Peterson et al. 1992), and there is evidence that the *Hox A10*, *A11*, and *A13* genes are expressed in proximodistal-restricted domains in developing chick and mouse limbs (Yocouchi et al. 1991; Haack and Gruss 1993).

To begin to understand the developmental functions of the *Abd-B* subfamily genes we have examined the expression pattern of the *Hox A11* gene during mouse embryogenesis using whole-mount in situ hybridization. Furthermore, we have generated a targeted mutation in *Hox A11* and characterized the resulting phenotype. Interestingly, *Hox A11* mutant mice develop with skeletal malformations in both forelimbs and hindlimbs. In the trunk we observed a double homeotic transformation, with one vertebral segment posteriorized and others apparently anteriorized. Moreover, mutant effects were observed in both heterozygous and homozygous animals.

Results

Expression of Hox A11

We originally cloned the *Hox A11* gene using a degenerate oligonucleotide probe carrying a combination of sequences capable of encoding the most conserved KIWFQNR amino acid sequence region of the homeo domain (Singh et al. 1991). Sequence analysis and chromosomal mapping allowed this gene to be identified as *Hox-1.9*, which is now *Hox A11* under the new system of nomenclature (Scott 1992).

The *Hox A11* expression pattern during embryogenesis has been examined previously in both mice and chickens using traditional in situ hybridization procedures (Yocouchi et al. 1991; Haack and Gruss 1993). Nevertheless, to generate a more precise three-dimensional picture of the transcription pattern of this gene in developing limbs, and to determine the rostral-caudal boundary of expression in the mesoderm of the body, which has not been described previously, whole-mount in situ hybridizations were performed. A segment of ~300 bp located immediately 3' of the homeo box was made by PCR and subcloned into pBS (KS) for preparation of sense and antisense riboprobes.

At embryonic day 8.5 (E8.5) there was very little *Hox A11* expression, although a faint hybridization signal was reproducibly observed in a caudal-lateral region of the body, as shown in Figure 1A. One day later, however, at E9.5, *Hox A11* was transcribed extensively in the four developing limb buds and in the caudal portion of the body posterior to the hindlimbs. At this time there was a gradient of expression in the limb buds, with the distal regions showing the highest abundance of *Hox A11* tran-

Figure 1. Expression pattern of *Hox A11*. Whole-mount in situ hybridizations were used to determine expression at E8.5 (A), E10.5 (B), E9.5 (C), and E10.75 (D). The arrowhead in A marks the weak but reproducible hybridization signal observed on the most caudal portion of the tail. The arrowhead in C marks the intense hybridization of the developing hindlimb. The brain ventricles were perforated to reduce antibody trapping and resulting false signal.

scripts. Although not apparent in Figure 1C, we also observed a separate region of expression at the base of the limbs, as described previously (Haack and Gruss 1993). Expression in the trunk at E9.5 extended posteriorly to the tip of the tail, with a diffuse anterior boundary. In Figure 1C this anterior boundary coincided roughly with the mid-region of the hindlimb; but in other embryos, presumably representing slightly different times of gestation, there was a faint indication of expression as far anterior as the rostral edge of the hindlimb bud.

By day E10.5 the *Hox A11* expression in the torso was much reduced, and expression in the forelimbs was now restricted to the most distal region (Fig. 1B). At E10.75 the expression in the body was not detectable above background, and the continued outgrowth of the limb buds had produced a ringed pattern of *Hox A11* expression, with the most distal regions devoid of transcripts. As shown in Figure 1D, however, this proximodistal-restricted expression was not symmetric about the circumference. In the hindlimb, for example, the anterior edge of the expression domain was considerably more distal than the posterior edge. Likewise, the levels of expression were not uniform, with the anterior portion of the hindlimb having higher levels of *Hox A11* transcripts. It should also be noted that during this E9.5–E10.75 period of development, *Hox A11* expression was apparent on both the dorsal and ventral surfaces of developing limbs. All other apparent signals in Figure 1, such as the otic vesicles and brain ventricles, were the result of antibody trapping and are also seen with sense strand probes used as controls.

Targeted disruption of *Hox A11*

The replacement construct, *pGKOHox A11-129*, was used for targeted disruption of the *Hox A11* gene; it included 10 kb of homologous 129/Sv genomic DNA and deleted a 2.7-kb region containing the 3' portion of the *Hox A11* gene, including the homeo box (Fig. 2A). *pGKOHox A11-129* was electroporated into D3 embryonic stem (ES) cells (Doetschman et al. 1985), and ~300 G418 and gancyclovir-resistant colonies were analyzed by Southern blot hybridization using a DNA probe from a region flanking the replacement construct (Fig. 2). One clone (20) gave the predicted patterns of hybridization for homologous recombination when ES cellular DNA was digested with *Bst*XI, *Nco*I, or *Spe*I. Hybridization with a *neo* probe further confirmed that clone 20 was targeted (data not shown). Clone 20 ES cells were injected into C57Bl/6J blastocysts, and 10 male germ-line chimeras were generated.

Mice heterozygous for a disruption in *Hox A11* were interbred to generate homozygotes. Figure 2B shows a Southern blot genotyping of the resulting wild-type, heterozygous, and homozygous mutant mice. The progeny of *Hox A11* heterozygous matings, were genotyped, identifying 43 wild-type, 84 heterozygous, and 45 homozygous mice, which closely approximates the predicted Mendelian ratio. Homozygous mice were viable, and careful dissections detected no alterations in soft tissues.

External morphology and behavior were indistinguishable between the targeted mice and their wild-type littermates.

Skeletal analysis of *Hox A11* mutant mice

Interestingly, skeletal staining revealed malformations of both axial and appendicular skeletal elements in both heterozygous and homozygous mutant mice. Figure 3 illustrates the axial skeletons of one wild-type, two heterozygous, and two homozygous mutant mice. In both homozygotes the thirteenth thoracic vertebra (T13) was converted to the first lumbar vertebra (L1) with no attached ribs. In most homozygotes, this transformation was complete, resulting in the total absence of the thirteenth pair of ribs (Fig. 3I), but in some cases distal cartilaginous remnants of ribs were observed (Fig. 3J). Somewhat surprisingly, this transformation was also seen in heterozygotes, although generally in an incomplete form, with significant portions of the ribs remaining. The proximal and distal elements of the ribs were most resistant to the mutation effects, as these components often remained in heterozygotes, with the central rib regions absent (Fig. 3G). The observed T13-to-L1 transformation appeared to be near the anterior boundary of the observed expression domain of the *Hox A11* gene. It is important to note that although the thirteenth thoracic segment was converted to a first lumbar segment, the twelfth thoracic vertebra retained its normal identity, as determined by rib morphology. There was no apparent posteriorization of any other thoracic vertebrae.

The skeletons of the *Hox A11* mutants also displayed a second homeotic transformation at the lumbosacral transition. This was evidenced by the presence of two extra lumbar vertebrae in many of the *Hox A11* mutant mice. As shown in Figure 3 and summarized in Table 1, six lumbar segments were generally observed in wild-type mice, although occasionally we saw five (a normal wild-type variant). In mutants, seven lumbar segments were often counted, the result of conversion of a thoracic to a lumbar; but about half of the mutants actually have yet another additional lumbar segment, or eight total. One explanation is an anteriorization of sacral segments generating an additional lumbar segment. We cannot exclude, however, an alternate model, stating that the mutation simply resulted in the formation of an extra lumbar vertebra, with no homeotic transformation of sacral segments. The net results of these two models are very similar and experimentally indistinguishable, as the tails of wild-type mice have variable numbers of vertebrae. It is therefore impossible to make meaningful comparisons of total numbers of vertebrae.

In addition to the vertebral transformations, two homozygous mutants displayed malformations in which one of the the second ribs was fused inappropriately at the sternum. In one mutant, the second rib was fused to the first rib near the sternum (Fig. 4B), and in the other mutant, the second rib was fused caudal to its normal position causing misalignment of the remaining ribs (r3–r7) and abnormal sternal segmentation along the length

Figure 2. The *Hox A11* gene targeting vector, wild-type allele, targeted allele, and mouse genotype analysis. (A) Structure of the replacement targeting vector, *pGKOHox A11-129*, used to disrupt the *Hox A11* gene in ES cells. *Bam*HI (B), *Not*I (Nt), and *Xba*I (X) sites were eliminated (in parentheses) when 7.5-kb *Bam*HI–*Not*I and 2.5-kb *Bam*HI–*Xba*I fragments were subcloned, 5' and 3', respectively, of the *pGKNeo* cassette. Construction of *pGKOHox A11-129* deleted a 2.7-kb region that included the *Hox A11* homeo box. (A) Diagram of the wild-type and targeted *Spe*I (S), *Nco*I (N), and *Bst*XI (Bx) fragments that hybridized to probe A, a 350-bp *Xba*I–*Nco*I-flanking DNA fragment, not present in the targeting vector. (B) Genotyping of 12 littermates from a *Hox A11* +/– intercross. DNA was digested with *Spe*I, with a 12-kb band of hybridization indicating a wild-type allele and a 5.4-kb band indicating a targeted allele. Littermates that are wild type (lanes 3,6,10,12), heterozygous (lanes 1,4,5,8,9), and homozygous for a disruption in *Hox A11* (lanes 2,6,11) are shown. (HSV–TK) The herpes simplex *thymidine kinase* gene cassette [Mansour et al. 1988]; (*pGKNeo*) the neomycin resistance gene cassette [Soriano et al. 1991]. (■) The *Hox A11* homeo box.

of the sternum (data not shown). These malformations were unexpected because they appeared anterior to the observed expression domain of *Hox A11*.

The targeted ablation of the *Hox A11* gene described in this report directly alters the Hox code expression pattern during limb development; and, as a result, malformations were observed in both forelimbs and hindlimbs of *Hox A11* mutant mice. Initial analysis of newborn skeletons showed a malformation of two carpal bones in mutant forelimbs. The pisiform and the triangular were fused together in mutants to form a single bone. As shown in Figure 5A, there was usually complete fusion of these two bones along their entire contiguous lengths. This formation of one bone from what would normally be two also results in a subtle spacing difference of the other carpal bones in the mutants. As summarized in Table 1, there was some variability in this phenotype, with 19 of 23 homozygous mutant forelimbs showing complete fusion but 2 forelimbs showing only partial fusion and 2 forelimbs appearing normal. While all of the wild-type and 34 of the heterozygote forelimbs were normal, 7 of the heterozygote forelimbs showed partial fusion and 10 had complete fusion. There was no apparent correlation in individuals between the severities of the axial and forelimb malformations.

Analysis of adult mutant skeletons revealed additional forelimb malformations not evident in newborn skeletons. In the adult, the pisiform is not only fused to the triangular but is distinctly altered in shape, as shown in Figure 5, B and C. The distal end of the ulna, the ulnar epiphysis, was more rounded in shape in mutants than in wild types (Fig. 5B), and mutant radius and ulna bones

appeared wider along their entire lengths as compared with wild type (Fig 5C). In addition, in all homozygotes examined, abnormal sesamoid bone development occurred near the distal–medial side of the radius (Fig. 5C). This sesamoid bone was only observed in 1 of 10 wild-type forelimbs analyzed and was much smaller in size. In heterozygous mutants this sesamoid was present and intermediate in size.

Adult *Hox A11* mutant skeletons also revealed several hindlimb defects. As shown in Figure 6A, the fibula of homozygous mutant mice joined with the tibia at a position distal to that observed in wild-type hindlimbs. In most homozygous mutants analyzed, the fibula failed to fuse completely with the tibia, as evidenced by a small gap between the two bones. In heterozygotes these bones attached at an intermediate position. Also in mutant hindlimbs, the distal ends of the tibia and fibula, the medial malleolus and the lateral malleolus, were severely malformed, lacking the contours normally seen in wild-type bones; and similar to what was observed in the radius and ulna of mutant forelimbs, both the mutant tibia and fibula appeared wider as compared with wild type (Fig. 6A–C). In addition, the sesamoid located ventral to the tibial bone appeared larger in size in both heterozygotes and homozygotes (Fig. 6B,C).

Discussion

Expression pattern

In this report we describe the *Hox A11* expression pattern and mutant phenotype. The use of whole-mount in

Figure 3. Comparison of skeletons from *Hox A11* $+/+$ (A,F), *Hox A11* $+/-$ (B,C,G,H), and *Hox A11* $-/-$ (D,E,I,J) mice. (A) Dorsal view of ribs and vertebrae of a *Hox A11* $+/+$ skeleton. (r1) The first rib pair that is attached to the first thoracic vertebra; (r12 and r13) the twelfth and thirteenth pair of ribs attached to the twelfth and thirteenth thoracic vertebrae, respectively. (F) Close-up view of the same skeleton; (T13) the thirteenth thoracic vertebra; (L1 and L6) the first and sixth lumbar vertebrae, respectively. (B) Partial development of the thirteenth pair of ribs in a *Hox A11* $+/-$ skeleton. (G) Close-up view of B. (C) *Hox A11* $+/-$ skeleton with rudimentary thirteenth ribs indicated by arrowheads. (H) Close-up view, showing the transformation of T13 to L1. For reference, the vertebra that would normally be T13 is labeled as such even though it is transformed into L1. (D) Complete absence of the thirteenth pair of ribs in a *Hox A11* $-/-$ skeleton. (I) Close-up view of D showing transformation of T13 to L1. (E) A single rudimentary rib located near T13, indicating the transformation of T13 to L1. (J) The development of an additional lumbar vertebra, L7. This $-/-$ skeleton has 12 pairs of ribs and a total of 8 lumbar vertebrae.

situ hybridizations allowed a precise description of *Hox A11* expression in developing limbs and defined the axial domain of expression in the developing body. Early-forming limb buds, at E9.5, showed widespread expression. But later, as paddle structures were generated, there was a gradient of expression with proximal regions of the limbs apparently devoid of *Hox A11* transcripts and with the distal tips showing the greatest hybridization signals. By E10.75, however, continued limb outgrowth had produced a proximodistal-restricted band of expression. This ring of expression included both dorsal and ventral sides of the developing limbs but was uneven about the circumference in terms of both proximo-distal location and level of expression.

In the embryo body, *Hox A11* expression was observed in only the most caudal region, as might be predicted

based on its 5' location in the *Hox A* cluster of genes. Expression was transient, with peak levels seen at E9.5–E10.0. During this period there was some fluctuation in the diffuse anterior boundary of expression, with a distinct hybridization signal detected as far rostral as the anterior edge of the hindlimb bud, which corresponds to prevertebrae 19 (T12) and 20 (T13) (Holland and Hogan 1988).

Mutant phenotype

Mice heterozygous and homozygous for a mutation in the *Hox A11* gene showed homeotic transformations of the thirteenth thoracic vertebra to the first lumbar vertebra. In heterozygotes, this transformation was often incomplete, and occurred in ~50% of the mice. In ho-

Table 1. Newborn skeleton morphology according to Hox A11 genotype

Morphology	+	+	+/-	-/-
Rib and vertebrae transformations				
13 ribs (normal)	25	22	0	
6 lumbar vertebrae ^a				
12 ribs with partial thirteenth rib(s)	0	15	0	
6 lumbar vertebrae				
12 ribs with rudimentary thirteenth rib(s)	0	12	2	
7 lumbar vertebrae (T13–L1)				
12 ribs with thirteenth rib(s) completely absent	0	5	12	
7 lumbar vertebrae (T13–L1)				
12 ribs with rudimentary thirteenth rib(s)	0	0	6	
8 lumbar vertebrae (T13–L1; +L8)				
12 ribs with thirteenth rib(s) completely absent	0	0	6	
8 lumbar vertebrae (T13–L1; +L8)				
Total	25	54	26	
Forelimb malformations				
normal forelimb (carpal bones not fused) ^b	20	34	2	
partial fusion of carpal bones ^b	0	7	2	
complete fusion of carpal bones ^b	0	10	19	
Total	20	51	23	

^aOne +/+ skeleton had five lumbar vertebrae.^bPisiform fused to triangular.

mozygous mutants, the T13-to-L1 transformation was complete in nearly all mice, and many of these mutants also showed a second homeotic transformation in the lumbosacral region, with the addition of an eighth lumbar vertebra. Moreover, two homozygous mutants had inappropriate costosternal attachments. Limb defects were also found in *Hox A11* mutants, with the pisiform and triangular carpal bones fused, the ulnar epiphysis, ulna and radius misshapen, and abnormal sesamoid development in the forelimb, as well as malformations of the tibia and fibula, and an enlarged sesamoid in the hindlimbs. The homeotic transformations seen in *Hox A11* mutant mice support a role for *Hox A11* in specifying segment identity during mouse development, and malformations found in the forelimbs and the hindlimbs of mutant mice show directly for the first time that at least one of the expanded set of *Abd-B* type *Hox* genes plays a role in limb development.

The observed mutant phenotype in heterozygotes is of particular interest for several reasons. First, heterozygotes for targeted mutations in the four *Hox* genes examined previously appeared normal (Chisaka and Cappechi 1991; Lufkin et al. 1991; Chisaka et al. 1992; Le-Mouellic et al. 1992; Ramirez-Solis et al. 1993). This contrasted with the heterozygous mutant phenotypes found for *Pax* genes (Epstein et al. 1991; Hill et al. 1991), suggesting an inherent functional distinction between

the clustered and dispersed *Hox* genes. The effects in heterozygotes described in this report cast doubt on this conclusion. Second, the observed *Hox A11* heterozygote phenotype argues against a simple Hox code hypothesis in which the combinations of *Hox* genes in the expressed mode directly determine segment identity. The heterozygous effects are likely attributable to reduced levels of *Hox A11* transcripts, the result of having only one functional *Hox A11* gene. This indicates that Hox expression levels play an important role in specifying segment identity and that the Hox code is assigned by the combination of active *Hox* genes, and, at least in some cases, by the level of *Hox* gene expression. Finally, the mutant phenotype observed in *Hox A11* heterozygotes is particularly interesting because *Drosophila Abd-B* heterozygous mutants also show weak homeotic transformations of abdominal segments, reflecting a gene dosage effect (Duncan 1987). This striking parallel adds to the accumulating body of evidence showing surprising functional conservation between the *Drosophila* and vertebrate *Hox* genes.

The rule of posterior prevalence states that a *Hox* gene has its major influence in the domain limited by its anterior boundary and that of the next most 5' gene in the cluster (Duboule 1991). That is, *Hox* genes expressed in

Figure 4. Comparison of ribs from *Hox A11*+/+ and *Hox A11*-/- mice. The rib cage was dissected away from the thoracic vertebrae. (A) The seven pairs of ribs attached to the sternum in a wild-type mouse. In B, the second rib on one side is inappropriately fused to the first rib near the sternum in a mutant mouse (arrowhead).

Figure 5. Comparison of forelimbs from *Hox A11* +/+ and *Hox A11* -/- mice. (A) Dorsal view of left forelimbs with the radius and ulna removed shows space between the pisiform and triangular carpal bones in a newborn wild-type mouse (*left*). These bones are fused in the mutant mouse (*right*) as indicated by the arrowhead. A side view of the right forelimbs in B, with the small arrowheads indicating space between the pisiform and triangular bones in an adult wild-type mouse (*left*) and fusion of these bones in the mutant (*right*). The large arrowhead in B points to the misshapen ulnar epiphysis in the mutant. (C) Ventral view of adult left forelimbs from wild-type (*left*) and mutant (*right*) mouse shows the large arrowhead pointing to the abnormal sesamoid in the mutant forelimb. This view also shows the misshapen mutant pisiform bone (small arrowhead), and the increased width of the radius and ulna bones.

more posterior domains tend to have dominant effects over *Hox* genes expressed more anteriorly. As mentioned, the anterior boundary of the *Hox A11* axial domain of expression corresponds to prevertebrae 19 (T12) and 20 (T13), (Holland and Hogan 1988). Therefore, the homeotic transformation of T13 to L1 seen in *Hox A11* mutant mice corresponds to this gene's rostral domain of expression and, in this regard, supports the concept of posterior prevalence and resembles phenotypes of other

Hox gene mutants (Chisaka and Cappechi 1991; Lufkin et al. 1991; Chisaka et al. 1992; Ramirez-Solis et al. 1993).

Although the T13-to-L1 transformation occurred where a phenotypic effect of the mutation might be predicted, the observed posteriorization was unexpected. Because *Hox* genes are expressed with varying rostral boundaries that overlap caudally, ablation of one gene will convert the expressed *Hox* code for one segment to

Figure 6. Comparison of hindlimbs from *Hox A11*^{+/+}, *Hox A11*^{+/-}, and *Hox A11*^{-/-} mice. (A) Dissected tibia and fibula bones from adult wild-type (left), heterozygous (middle), and homozygous (right) mutant mice. In both the heterozygous and homozygous hindlimbs, the fibula and tibia were joined incorrectly at a position distal to that seen in wild-type hindlimbs. In the homozygous hindlimb, the fibula fails to fuse to the tibia, as evidenced by the small gap between the two bones (small arrowhead). In addition, the tibia and fibula appeared wider in mutant hindlimbs, and the distal ends of these bones, the lateral malleolus, and the medial malleolus are malformed (large arrowhead). (B) Side view of wild-type (top) and homozygous mutant (bottom) hindlimbs with the sesamoid bones indicated by arrowheads. The sesamoid is larger in the mutant hindlimb.

the same code normally found one segment anterior (Kessel and Gruss 1991). Hence, one predicts and normally observes anteriorization for *Hox* gene inactivation and posteriorization for *Hox* gene misexpression, although exceptions to these generalizations have been described in *Drosophila* (Kuziora and McGinnis 1988; Gibson et al. 1990).

The observed posteriorization could be the result of misexpression of other *Hox* genes. It has been shown that ectopic expression of *Hox* genes in transgenic mice can sometimes cause posteriorization of vertebral segments (Kessel et al. 1990; Lufkin et al. 1992). The disruption of the *Hox A11* gene could alter the genetic regulatory network, causing other *Hox* genes to be expressed inappropriately, indirectly resulting in posteriorization. It is also possible that the insertion of the *neo* gene has caused misexpression of flanking *Hox* genes, although in this region of the *Hox A* cluster, the genes are set apart by ~10 kb (Haack and Gruss 1993).

The presence of an eighth lumbar vertebra indicates additional homeotic transformations in *Hox A11* homozygous mutants. The simplest explanation is an anteriorization of the sacral region by one segment, producing an extra lumbar vertebra. Whereas the transformation at the thoracolumbar boundary altered only one segment, the generation of an additional lumbar segment at the lumbosacral boundary affected an entire region. At the thoracolumbar border, T12 remained unchanged and T13 was absent in mutants. At the lumbosacral border, an additional lumbar segment was generated without deleting any sacral segment, presumably because each segment in the entire region was anteriorized. Here, as in *Hox C8* homozygous mutants described previously, transformations occur within an extended region (Le-Mouellic et al. 1992). In *Hox C8* mutants, homeosis occurred in vertebrae T7–L1, with each vertebra transformed to the identity of the vertebra one segment anterior. Both *Hox A11* and *Hox C8* transformations show the anteriorization that is predicted for *Hox* gene null mutants; however, the regions altered do not correspond to the most anterior domains of expression for either gene. Instead, the extended regions are located within the axial domains of expression. Results of this sort demonstrate that posterior prevalence is a tendency rather than a rule.

Incorrect positional information in rib somitic mesoderm appears to have caused misalignment of ribs near the sternum in two homozygous mutant mice. The ribs and sternum originate independently from the somitic region and the lateral mesoderm, respectively (Chen 1952). During normal development, the sternum becomes segmented as a result of inhibition of chondroblastic hypertrophy and subsequent ossification in regions fused to the rib tips (Chen 1953). In mutants, however, incorrect sternal segmentation results when ribs are positioned improperly, causing inappropriate inhibition of ossification on one side of the sternum. This defect is intriguing because it appears outside the observed *Hox A11* domain of expression. It is interesting to note that *Hox B4* null mutants developed with a split ster-

num caused by the failure of the sternal rudiments to fuse (Ramirez-Solis et al. 1993). This defect also occurred in a region where *Hox B4* transcripts had not been observed. Transient or low levels of *Hox A11* and/or *Hox B4* expression may not have been detected or, alternatively, the absence of normal inductive interactions may play a role in these mutant malformations.

Hox A11, as well as other *Abd-B*-type *Hox* genes, is expressed in spatially and temporally restricted domains within the developing limbs. These overlapping domains of gene expression suggest that these genes play important roles during limb morphogenesis. The *Hox* code hypothesis states that the various combinations of *Hox* genes active in a particular region of the developing limb will play a role in cell type specification. This model was tested by Morgan et al. (1992), when they used a retroviral vector to misexpress the *Hox D11* gene during chick limb development. Interestingly, homeotic transformations of digit identity were observed. Our data indicate that targeted disruption of *Hox A11* directly alters the *Hox* code expressed within the developing limbs. As a result, *Hox A11* mutant mice developed abnormal forelimbs and hindlimbs. Mutant forelimbs developed with the fusion of two carpal bones and an abnormal sesamoid. In addition, the radius, the ulna, and the ulnar epiphysis were malformed. Hindlimb development was also effected with improper fusion of the fibula to the tibia and with malformations of the fibula, tibia, lateral malleolus, and medial malleolus. Abnormal sesamoid development was also observed. At E13.5 within the developing limbs, undifferentiated mesenchyme forms precartilaginous condensations, and these condensations later chondrify and ultimately undergo bone replacement. Studies in the chick have shown that individual carpal bones are derived from unique precartilaginous elements (Hinchcliffe 1977). Therefore, improper positional information in *Hox A11* mutant mice limbs may have effected development of these precartilaginous elements. Future experiments that examine altered *Hox* gene expression patterns in mutants and the phenotypic effects of multiple paralogous mutations will further clarify the genetic regulatory network of pattern formation in mammals.

Materials and methods

Whole-mount *in situ* hybridization

The whole-mount hybridization procedure was as described by Rosen and Beddington (1993), except that proteinase K digestion (10 μ g/ml) was substituted for the detergent treatment. A segment of ~300 bp immediately 3' of the homeo box was created by PCR, subcloned into pBS (KS, Stratagene), and used to make both sense and antisense riboprobe. Sense probe (not shown) gave occasional weak signals in the otic vesicle and brain ventricles because of antibody trapping.

Construction of the *Hox A11* replacement vector

A single-copy 0.7-kb *HindIII* DNA fragment isolated from the original DBA *Hox A11* genomic clone (Singh et al. 1991) was used to screen a 129/Sv genomic library. Two positive clones,

14-10 and 15-8, were used to construct the *Hox A11* homologous recombination construct, *pGKOHox A11-129*. First a general gene knockout vector, *pGKOV*, was constructed. A 2-kb *KpnI* herpes simplex virus thymidine kinase (HSV-TK) gene cassette (Mansour et al. 1988) was subcloned into pBS II (SK, Stratagene), and a 2.9-kb *XhoI* *pGKNeo* cassette (Soriano et al. 1991) was then blunt end ligated into the *BamHI* site. In the process, unique restriction enzyme sites, that is, *XhoI* and *NotI*, were available to subclone regions of target homology 5' and 3' of the *neo* gene. To make *pGKOHox A11*, a 2.5-kb *BamHI*-*XbaI* fragment was subcloned into the *NotI* site of *pGKOV* and, a 7.5-kb *BamHI*-*NotI* fragment was subcloned into the unique *XhoI* site. The final construct contained 10 kb of target homology and deleted a 2.5-kb region at the targeted locus, which included the *Hox A11* homeo box.

Cell culture and transfections

D3 ES cells (Doetschman et al. 1985) were maintained on primary G418-resistant mouse embryo fibroblasts in Dulbecco's modified Eagle medium (DMEM) containing 15% heat-inactivated fetal calf serum, 0.1 mM β -mercaptoethanol, 1 mM sodium pyruvate, 10 mM penicillin/streptomycin, and 1000 U/ml of LIF (GIBCO) at 37°C, 5% CO₂. For electroporation, two confluent 100-mm plates of ES cells were trypsinized and resuspended in 3 ml of PBS containing 20 μ g/ml of linearized *pGKOHox A11-129*. Three 1-ml aliquots were electroporated at 900 V and 14 μ F in a 0.4-cm-wide cuvette (IBI Gene Zapper). Cells were distributed equally on twelve 100-mm plates. G418 at 150 μ g/ml was added to the media 24 hr later, and gancyclovir was added 48 hr after electroporation at a final concentration of 2 μ M. Selection was continued for 7 more days. Approximately 300 individual surviving colonies were picked, trypsinized, and passaged to 24-well plates. After 2 or 3 days in culture, one-half of the cells from each well were frozen, and the other half were passaged to gelatin-coated six-well plates. DNA was then prepared for each clone.

DNA analysis

ES cells were lysed in 0.5 ml of buffer containing 0.2 M NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.2% SDS, and 20 μ g/ml of proteinase K at 65°C for 10 min. Potassium acetate (200 μ l) was then added, and samples were incubated on ice for at least 1 hr. DNA samples were then centrifuged for 20 min at 12,000 rpm. Ice-cold 100% ethanol was added to the supernatant, and genomic DNA was spooled out of solution. Mouse tail DNA preps were performed in a similar manner, except the lysis buffer contained 750 μ g/ml of proteinase K, and samples were incubated overnight at 65°C.

Southern blot analysis was performed using GeneScreen Plus as recommended by the membrane manufacturer. DNA probes used for Southern analysis were subcloned, digested, and purified away from vector sequences. To facilitate identification of +/+, +/-, and -/- mice, PCR analysis was used. Three primers, Neo-5' (TCTGGATTCATCGACTGTGG), H1.9-5' (AATGGCGTACTCTCTGAAGG), and H1.9-3' (TTCCACGT-CAGCTTACGT), were used. The resulting PCR products were ~500 and 300 bp for the heterozygous allele and homozygous alleles, respectively.

Embryo injection and mouse breeding

C57Bl/6J blastocysts (3.5 days postcoitum) were flushed from the uteri of superovulated 4.5-week-old females with M2 media (Hogan et al. 1986). Approximately 10–15 ES cells were micro-

injected into the blastocoel of each blastocyst. Injected blastocysts were cultured in a drop of M2 under a layer of paraffin oil at 37°C for 1–3 hr and were reimplanted into 2.5-day pseudo-pregnant CD-1 female mice. Chimeric males were bred to CF-1 females, and germ-line transmission was determined by the presence of agouti offspring in the litter.

Skeletal analysis

Newborn mice were sacrificed, and their skeletons were stained with alizarin red for bone and alcian blue for cartilage, according to Kuczuk and Scott (1984). Adult limbs were stained with alizarin red only and were heated at 55°C for 2–5 hr to facilitate clearing.

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