Specification of axial identity in the mouse: role of the *Hoxa-5* (*Hox1.3*) gene

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Numerous lines of study have suggested that the *Hox* genes, encoding putative transcription factors, are key genes in the establishment of the body plan of the mammalian embryo. To examine the role of *Hoxa-5* (*Hox1.3*) gene during development, we have used targeted mutagenesis in embryonic stem cells to produce a strain of mice carrying a disrupted *Hoxa-5* allele. The viability of homozygous mutant mice is markedly reduced, with 50% of the mutant animals dying at birth or shortly thereafter. Analysis of the skeleton of *Hoxa-5* mutants reveals a number of homeotic transformations restricted to the cervical and thoracic regions. Of these, one of the most frequent morphological abnormalities is the posterior transformation of the seventh cervical vertebra into the likeness of a thoracic vertebra complete with a pair of ribs. These results demonstrate that the *Hoxa-5* gene has an important role in the establishment of the skeleton during development and contributes to the process whereby the axial structures are determined.

[Key Words: Gene disruption; *Hox* gene; axial identity; homeotic transformation]

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Embryonic development can be viewed as being controlled by the expression of a hierarchy of regulatory genes. In *Drosophila*, a critical position in the developmental hierarchy is occupied by the homeotic genes (Akam 1987). These genes are thought to control the formation of body segment-specific structures by regulating the expression of specific sets of downstream effectors that, in turn, direct the morphogenetic events leading to the formation of complex body forms. Consistent with this notion, mutations in homeotic genes result in alterations to segmental identities (Lewis 1978).

Hybridization analysis has revealed the presence of numerous homeo box-containing genes in the genomes of vertebrates. In human and mouse, the 38 homeo box genes of the *Antennapedia* (*Antp*) class identified so far are arranged in four gene clusters located on different chromosomes (McGinnis and Krumlauf 1992; Scott 1992). Comparative studies of mammalian homeo box gene clusters with the *Drosophila* homeotic complex (*HOM-C*) have shown numerous similarities in protein sequences, gene organization, and patterns of expression suggesting that the organization of these complexes predates the evolutionary divergence of insects and verte-

brates (Schughart et al. 1989). This evolutionary conservation also raises the issue as to whether the functions of these proteins have been similarly conserved.

Possible developmental functions for the mammalian *Hox* genes have been deduced, in part, from circumstantial evidence. For example, all *Hox* genes examined display distinct temporally and spatially regulated patterns of expression in the developing embryo. Typically, expression is initiated during gastrulation in the mesoderm and ectoderm lineages and continues up to mid-gestation and early organogenesis (Holland and Hogan 1988). The majority of *Hox* genes have overlapping domains of expression in the neural tube and prevertebrae that extend from the caudal end of the embryo to a defined anterior limit. The anterior boundaries of expression appear to correlate (with few exceptions) to the linear order of the genes within a given cluster. Consequently, different members of the *Hox* clusters are expressed in discrete but overlapping regions along the anteroposterior axis of the embryo. It has been proposed from these observations that the specific combination of different *Hox* genes expressed in a particular axial region provides a unique genetic address specifying the characteristics, or identity, of a particular axial level (Kessel and Gruss 1991). Consistent with this hypothesis, grafting individ-

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ual somites that express the gene *Hoxa-7* (*Hox1.1*) to heterotopic positions in the anteroposterior axis of mouse embryo is not sufficient to respecify *Hoxa-7* gene expression (Beddington et al. 1992; Gruss and Kessel 1991).

Genetic approaches have been used to directly test the function of *Hox* genes in governing vertebral identity in the intact embryo. A number of dominant gain-of-function mutations have been reported in transgenic mice. The outcome of these experiments depends on the gene studied and the promoter elements used. Some transgenic strains show homeotic posteriorization analogous to that described in *Drosophila* following ectopic *HOM-C* gene expression (Balling et al. 1989; Lufkin et al. 1992; McLain et al. 1992). In contrast, other transgenes produced anterior transformations, a result more in keeping with phenotypes observed for loss of function of *Drosophila* homeotic genes (Jegalian and De Robertis 1992; Pollock et al. 1992). In cases leading to posteriorizing transformation, the morphological alterations appear to be limited to embryonic regions lying anterior to the normal domain of gene expression. One interpretation of these experiments is that ectopic expression of specific *Hox* genes in more anterior regions of the embryo is sufficient to cause the repatterning of anterior skeletal structures (Balling et al. 1989; Lufkin et al. 1992). In the case of transgenes that give anterior transformations, vertebral changes arise in the posterior regions of the embryo where the genes are normally expressed, suggesting that the relative quantitative level of expression of particular *Hox* genes has an important role in specifying vertebral identity (Jegalian and De Robertis 1992; Pollock et al. 1992). Thus, in both of these experimental situations, perturbing the pattern of expression of single *Hox* genes clearly has important consequences on the development of the embryo.

An alternative approach is to eliminate *Hox* gene function. Gene targeting in embryonic stem (ES) cells has allowed the production of strains of mice carrying predetermined mutations in a number of *Hox* gene loci (Chisaka and Capecchi 1991; Lufkin et al. 1991; Chisaka et al. 1992; LeMouellic et al. 1992; Ramirez-Solis et al. 1993). Loss of *Hoxa-3* (*Hox1.5*) and *Hoxa-1* (*Hox1.6*) function results in morphological defects largely confined to the more anterior domains of normal gene expression and, interestingly, does not influence the formation of axial structures (Chisaka and Capecchi 1991; Lufkin et al. 1991; Chisaka et al. 1992). In both mutants, the malformations appear to be structural deficiencies resulting from perturbations to neural crest populations rather than homeotic transformations and are therefore more analogous to phenotypes obtained from loss of function of the *labial* gene, the *Drosophila* homolog of *Hoxa-1* (Merrill et al. 1989). In contrast, mutation of the *Hoxc-8* (*Hox3.1*) and *Hoxb-4* (*Hox2.6*) genes that have more posterior boundaries of expression results in anterior transformation of skeletal segments that normally express these genes (LeMouellic et al. 1992; Ramirez-Solis et al. 1993). These abnormalities thus resemble those observed for some *HOM-C* mutations [such as

Antp, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*)] and suggest that subsets of *Hox* genes may contribute in determining the identity of axial structures. Intriguingly, the vertebral transformations observed in the *Hoxc-8* mutant mice appear to be similar to those obtained in transgenic animals overexpressing either *Hoxc-8* or *Hoxc-6* (*Hox3.3*). Collectively, these data argue that the precise quantitative contribution by specific *Hox* genes plays an important role in controlling vertebral identity (Jegalian and De Robertis 1992; Pollock et al. 1992).

We have been studying the *Hoxa-5* gene which, in common with other mammalian *Hox* genes, is expressed in a distinct domain along the anteroposterior axis of the developing embryo. In the 8-day-old embryo, *Hoxa-5* expression is restricted to the posterior presomitic mesoderm (Dony and Gruss 1987). One day later, *Hoxa-5* transcripts are detected primarily in the somites of the thoracic region, suggesting that *Hoxa-5* expression is confined to those cell populations that ultimately give rise to this region of the body (Dony and Gruss 1987). At 12.5 days of gestation, *Hoxa-5* transcripts are present in the prevertebral column, with expression extending from pv24 (which corresponds to the fourth lumbar vertebra) to a rostral boundary that coincides with pv3 (the third cervical vertebra) (Gaunt et al. 1988). The greatest abundance of transcripts is found in the region encompassing pv6 to pv14 (Gaunt et al. 1988, 1990). The gene is also expressed in the ribs and in other mesodermal derivatives such as metanephros, lung, stomach, and intestine (Dony and Gruss 1987; Gaunt et al. 1988). Within the developing spinal cord, the anterior limit of *Hoxa-5* expression coincides with the posterior floor of the myelencephalon (Gaunt et al. 1988). On the basis of protein sequence comparisons, *Hoxa-5* can be grouped in the same subfamily as *Hoxb-5* (*Hox2.1*) and *Hoxc-5* (*Hox3.4*). These three genes have been shown to have similar domains of expression along the anteroposterior axis (Gaunt et al. 1990).

The *Hoxa-5* gene encodes a nuclear phosphoprotein capable of binding to specific DNA sequences. The presence of *Hoxa-5*-binding sites has been reported in the putative *Hoxa-5* regulatory regions and in *cis*-regulatory elements of other *Hox* genes, transcription factor genes, and viral genes (Odenwald et al. 1989). Thus, *Hoxa-5* protein may act in both an auto- and a cross-regulatory fashion, similar to that described for a number of *Drosophila* loci (Hayashi and Scott 1990).

To examine whether the *Hoxa-5* gene has an essential developmental function and, more specifically, to determine any contribution by this homeo domain protein in the establishment of axial identity, we have used gene targeting in ES cells to generate a line of mice that carry a disrupted *Hoxa-5* gene (Jeannotte et al. 1991). Although mice that are heterozygous for the mutation are phenotypically normal, roughly half of those animals homozygous for the disrupted *Hoxa-5* allele die at birth or shortly thereafter. Analysis of the skeletal structures of homozygotes revealed consistent abnormalities affecting the region between the sixth cervical vertebra (C6) and

the first lumbar vertebra (L1). The most frequent morphological changes are the absence of tuberculi anterior on C6 and the presence of cervical ribs on the seventh cervical vertebra (C7) causing it to assume an appearance typical of a thoracic vertebra. Taken together, these results show that *Hoxa-5* plays a central role in specification of axial identity in the developing embryo.

Results

Inactivation of the Hoxa-5 gene in mice

We previously described the generation of ES cell lines in which the DNA-binding homeo domain of the *Hoxa-5* gene is interrupted by the introduction of a *neo* gene. (Jeannotte et al. 1991). Targeted clones were obtained by using either a promoterless gene targeting vector or by a positive-negative selection procedure. In both cases, the mutation generated will, at best, result in the production of a truncated *Hoxa-5* molecule lacking the final 58-amino-acid residues of the protein. This region contains the third helix of the homeo domain thought to confer DNA-binding specificity of Hox proteins (Otting et al. 1990; Gehring 1992). A correctly targeted ES cell clone (designated M-31-13), generated with the positive-negative targeting vector, gave germ-line chimeric mice. The resultant heterozygous *Hoxa-5* mutant mice were viable and indistinguishable from their wild-type littermates, and heterozygotes of both sexes proved fertile. *Hoxa-5* heterozygous F₁ animals were mated with MF1 strain partners to expand the pool of mice carrying the mutant allele.

Hoxa-5 inactivation is associated with a high rate of perinatal lethality

To assess the consequence of loss of *Hoxa-5* function, heterozygous *Hoxa-5* mice were intercrossed. The resulting offspring were genotyped at 3–4 weeks of age by

Figure 1. Southern analysis of offspring obtained from heterozygous crosses. Tail DNAs from 3- to 4-week-old pups derived from *Hoxa-5* m/+ intercrosses were digested with *EcoRV*, blotted, and probed with a *Hoxa-5* exon 2 *BglII*–*HindIII* probe. The genotype of each animal is indicated at the top of each lane. (+) Wild-type allele, lower band; (–) disrupted *Hoxa-5* allele, upper band.

Table 1. Ratios of genotypes in litters from crosses of heterozygous *Hoxa-5* mutants

Age	Number of litters	Number of pups	Genotype		
			+ / + (%)	+ / – (%)	– / – (%)
12.5 days p.c.	18	170	37 (22)	104 (61)	29 (17)
18.5 days p.c.	11	104	27 (26)	47 (45)	30 (29)
3–4 weeks	85	619	195 (31)	344 (56)	80 (13)

Southern blot analysis of genomic DNA digested with *EcoRV* (Fig. 1). Under these conditions, the wild-type gene is visualized as a 7-kb band and the mutant allele as an 8-kb band. From a total of 619 progeny analyzed, only 80 animals homozygous for the *Hoxa-5* mutation were obtained. This corresponds to some 13% of offspring instead of the expected 25%, assuming the mutation was inherited in a Mendelian fashion (Table 1). To determine at what stage the mutant animals were dying, heterozygous *Hoxa-5* animals were intercrossed and pregnant females were sacrificed at different times in gestation [the day at which the vaginal plug was found was designated 0.5 days postcoitum (p.c.)]. The embryos were dissected, and individual visceral yolk sac tissues were used as a source of genomic DNA for genotyping. As shown in Table 1, at 18.5 days p.c., the numbers of wild-type embryos, heterozygotes, and homozygotes were found to conform to the expected 1 : 2 : 1 ratio. No overtly abnormal embryos were observed. Curiously, at 12.5 days p.c., the relative proportion of the progeny deviates from the expected ratio of 1 : 2 : 1 ($P < 0.01$). This difference is difficult to explain because the expected ratios were recorded at 18.5 days p.c.. The absence of *Hoxa-5* function thus appears to lead to significant mortality between birth and weaning.

The fraction of homozygous mutant animals that survived to adulthood proved to be fertile and did not display any obvious phenotypic abnormalities. Matings were set up between heterozygous and homozygous animals (Table 2). At 18.5 days p.c., of 69 embryos retrieved for analysis, half proved to be homozygotes. Careful monitoring of litters during the first week of postnatal life showed that 61% of the homozygous pups died within 4 days of birth. Finally, homozygous animals were intercrossed. The survival rate of the resultant homozygous pups after 1 week was only 47% (of 99 mice born; data not shown), and the majority of pups died at birth or shortly thereafter. In general, homozygous pups that died during this time period appeared normal on the day of birth and were able to suckle, but subsequently displayed a general failure to thrive. Gross necropsy of selected animals did not reveal any overt tissue and organ defects that could account for their death.

Expression of wild-type and mutant Hoxa-5 alleles

Restriction analyses of the targeted *Hoxa-5* allele present

Table 2. Ratios of genotypes in litters from crosses of heterozygous *Hoxa-5* mutants with homozygous *Hoxa-5* mutants

Age	Number of litters	Number of pups	Genotype			
			+ / -		- / -	
			living (%)	dead ^a (%)	living (%)	dead ^b (%)
18.5 days p.c.	7	69	37 (54)	N.A. ^c	32 (46)	N.A. ^c
Birth	14	126	60 (48)	4 (3)	24 (19)	38 (30)

^aAnimals die within 2 weeks after birth.^bAnimals die at birth or within 4 days after birth.^c[N.A.] Data not available.

in clone M-31-13 confirmed that the intended replacement mutation had occurred (Jeannotte et al. 1991). To confirm that this mutation had affected the synthesis of *Hoxa-5* transcripts, Northern blot analysis was performed. RNA isolated from lung, kidney, and intestine of wild-type, heterozygous, and homozygous adult animals was assayed by hybridization with a *Hoxa-5* exon 2 probe. This probe derives from a region 3' of the homeo domain and detects four transcripts of approximately 2, 4, 8, and 9 kb in length in RNA samples from 12.5 day embryos and some adult tissues (Fig. 2; Odenwald et al. 1987). In contrast, in samples from homozygous mutant animals, the RNA species corresponding in size to the wild-type transcripts were absent, and only transcripts 1 kb longer than the wild-type *Hoxa-5* transcripts were present (Fig. 2). As expected, these novel transcripts

proved to contain *neo* sequences (data not shown), suggesting that they derived from the mutated allele with the size increase being attributable to the insertion, in the opposite transcriptional orientation, of the 1-kb MC1neo A⁺ cassette within the homeo box sequence (as described in Jeannotte et al. 1991). Thus, integration of the targeting vector eliminated expression of normal *Hoxa-5* mRNAs. In heterozygotes, both the wild-type and mutated forms of *Hoxa-5* transcripts were present, although expression of transcripts derived from the mutated allele appeared to be significantly reduced. This presumably resulted either from a reduction in the stability of these transcripts owing to the presence of the *neo* sequences or reduced transcriptional activity of the targeted allele.

Hoxa-5 mutants show homeotic transformations of the skeleton

To determine whether mutation of the *Hoxa-5* gene affected the formation of structures derived from tissues in which the gene is normally expressed, we examined the skeletons of perinatal animals (between 18.5 day p.c. embryos and birth). The majority of homozygous animals displayed a variety of skeletal abnormalities restricted to the cervical and thoracic regions (Table 3). The most predominant of these was a phenotypic alteration of C7 which, in many animals, was found to have associated rib structures. Indeed, 73% of the homozygous mice examined had extensive ribs on one or both sides of C7 (Figs. 3 and 4). In approximately one-third of cases, these ribs were fused ventrally with the ribs associated with the first thoracic vertebra (Fig. 3C). In some cases, cervical ribs made an independent contact to the sternum (Figs. 3D and 4A). On occasion, extra ribs were seen in wild-type animals, but in these cases they took the form of small anlagen (Table 3). In heterozygous embryos, the number of instances of small cervical ribs associated with C7 was markedly increased, but only rarely were animals found that possessed an extensive rib. This may indicate that the specification of this vertebra could be particularly sensitive to *Hoxa-5* gene dosage effects. Analysis of adult homozygotes revealed the same morphological change of C7, but the proportion of animals with pronounced long cervical ribs was reduced (only 3 of 21 animals analyzed; data not shown). Thus, the mu-

Figure 2. Expression of the *Hoxa-5* gene in *Hoxa-5* wild-type, heterozygous, and homozygous mutant animals. Total RNA (20 µg) was isolated from adult intestine, lung, and kidney tissues, fractionated by electrophoresis through a formaldehyde-agarose gel, transferred to Hybond membrane, and hybridized to a ³²P-labeled *Hoxa-5* exon 2 BglII-HindIII probe. One wild-type (+/+), one heterozygous (+/-), and three individual homozygous mutants (-/-) were analyzed. Poly(A)⁺ RNA (5 µg) from normal 12.5-day-old embryos was used as a control to illustrate the sizes of endogenous *Hoxa-5* transcripts. To demonstrate RNA integrity, the blot was rehybridized to a mouse α -actin probe.

Table 3. *Skeleton morphology according to Hoxa-5 genotypes*

	Genotype		
	+/+	+/-	-/-
Sternum			
number of sternebrae			
3	0	0	3
4	22	71	108
5	0	0	11
normal	20	56	89
abnormal (fusion, link, etc.)	2	15	33
Xiphoid process			
normal	19	57	78
perforated	0	11	40
fissured	3	2	7
Tuberculum anterior on C6			
present	22	69	26
absent	0	3	111
Ribs on C7			
absent	14	13	11
small	8	63	63
extensive	0	4	89
fused to sternum	0	0	7
fused to T1 ribs	0	0	28
unilateral	5	22	21
bilateral	3	35	90
Ribs on L1			
absent	21	64	99
small	1	6	20
extensive	0	0	3
unilateral	0	0	9
bilateral	1	6	13
Number of ribs			
13	21	59	32
14	1	10	79
15	0	0	10
Number of animals analyzed	22	71	122

tant animals that display a strong C7 phenotype represent a significant proportion of the class of homozygotes that die during the perinatal period.

A second frequent phenotypic change seen in the *Hoxa-5* homozygous mice was the absence of tuberculum anterior on C6, which occurred in more than 90% of the embryos and adult animals (Figs. 3 and 4). This was seen most easily in adults, following complete ossification of the tuberculum anterior (Fig. 4B). In transverse view, C6 becomes morphologically very similar to C3, C4, and C5, and this alteration does not interfere with the entry of the vertebral artery into the C6.

The phenotypic transformation of C7 into a thoracic vertebra confers a different vertebral distribution (C6/T14/L6) in the majority of the homozygotes. Additionally, the presence of ribs on the first lumbar vertebra was noted in 19% of homozygous embryos (Table 3; Fig. 5). This second transformation event, in combination with that affecting C7, leads to the generation of a small proportion of mutants (8%) that possessed 15 as opposed to 13 pairs of ribs. However, it is important to note that

while the identity of the vertebrae is altered in the *Hoxa-5* mutants, no cases were found in which the total number of presacral vertebrae was altered in comparison to control animals, indicating that the changes documented in mutant animals are homeotic.

A small proportion of homozygous animals were found to have defects of the sternum (Fig. 6). In wild-type and heterozygous animals, in addition to the manubrium sterni and the xiphoid process, 4 sternebrae were present in the intercostal region. The attachment sites of the first six ribs are normally separated by sternebrae, whereas the sixth and seventh ribs attach to the sternum at the same point. In 9% of the homozygotes examined, an extra sternebrae was present that appeared to form by fission of two of the manubrium sterni. This is illustrated in the animal shown in Figure 6B, in which the first ribs derived from C7 and were fused to the sternum at a position normally occupied by the first pair of thoracic ribs. A consequence of this displacement is that the first thoracic ribs joined the sternum in the center of the manubrium sterni, which appeared to be divided in these animals. In addition, the sternebra anterior to the xiphoid process (which normally corresponds to the fourth sternebra) was reduced in size and linked to the preceding sternebra. Overall, this resulted in a close juxtapositioning of the sites of attachment of the fifth, sixth, and seventh ribs. In a further three homozygous animals, fusion of adjacent sternebrae was observed resulting in a decrease in their number and production of irregularities in the sternocostal junctions. Figure 6C shows an example of a sternum in which the attachment of the ribs was asymmetric. In addition, ossification of the xiphoid process was altered in many mutants (Fig. 6B,C). The xiphisternum normally develops from two centers of ossification, which initially lie to either side of the ventral midline and which rapidly fuse together across the midline (Kaufman 1992). Thus, at 18.5 days p.c., a discrete hole is sometimes apparent in the central part of the xiphoid cartilage. However, in the case of the homozygous mutants animals, the frequency of holes and fissures was significantly higher than that found in wild-type and heterozygous animals (39% vs. 14% and 19%, respectively) and the malformations were not necessarily centrally located along the midline.

Analysis of a large sample of mutants showed that all of the skeletal transformations described above were often, but not always, found in the same animal. For example, it was noticeable that animals showing either anterior transformation of the first lumbar vertebra or the sixth cervical vertebra also consistently showed posterior transformation of the seventh cervical vertebra. Although these transformations varied in their physical extent between individual mutants, the penetrance of the transformation was highest in C6 and C7 and lowest between T1 and L1. Thus, the region encompassing C6 to L1 seemed to delineate the extent of skeletal transformations that occur in *Hoxa-5* mutant mice. The basis of the variable expressivity of the skeletal phenotypes that characterize *Hoxa-5* mutants is not known, but as the animals used in the current study were maintained in a

Figure 3. Skeletal transformations affecting the cervicothoracic region in *Hoxa-5* mutants. Skeletons of animals ranging in age from 18.5 day p.c. embryos to newborn animals were stained with alizarin red (visualizes the bones) and alcian blue (visualizes the cartilage). (A) Lateral view of a wild-type embryo showing the cervicothoracic transition region. Note the absence of ribs on the seventh cervical vertebra and the presence of tuberculum anterior on the sixth cervical vertebra. (B–D) Lateral view of *Hoxa-5* homozygous mutant embryos showing a rib on the seventh cervical vertebra. In C, the cervical rib appears to be fused ventrally with the first thoracic rib, while in D, cervical ribs make an independent contact to the sternum. In the three homozygous mutant embryos, the C6 vertebrae lack a tuberculum anterior. The arrowheads indicate the cervical ribs.

hybrid genetic background, subtle genetic differences may be a contributory factor, as has been documented recently for the *Hoxb-4* mutation (Ramirez-Solis et al. 1993).

Discussion

We have used gene targeting to generate a mutation at the *Hoxa-5* locus. In this mutation, the introduction of the *neo* gene into the homeo domain of the *Hoxa-5* locus is predicted to generate a loss-of-function allele. Our analysis of the resultant mutant mouse strain has shown that loss of function of the *Hoxa-5* gene results in a dramatic decrease in viability of homozygotes at birth. Moreover, the mutation leads to homeotic transformations of the vertebral column in homozygous mutants.

Expression studies are highly suggestive that mouse

Hox genes act in concert to specify positional information along the anteroposterior axis of the developing embryo in a manner analogous to their *Drosophila* counterparts. Mutations in *Drosophila* homeotic genes result in abnormal development of specific segments of the embryo (Akam 1987). According to the model proposed by Lewis (1978), loss-of-function mutations should transform segments into the likeness of more anterior ones while gain-of-function mutations are predicted to give transformations to more posterior segments. However, it is obvious that this model does not hold true because ectopic expression of either *Deformed* (*Dfd*) or *Sex combs reduced* (*Scr*) confers a more anterior identity to posterior epidermal cells (Wakimoto and Kaufman 1981; Merrill et al. 1987; Kuziora and McGinnis 1988). Similarly, lack of *Scr* function leads to a partial transformation of the labial segment to a maxillary segment and of

Figure 4. Transformation of cervical vertebrae in *Hoxa-5* mutants. Vertebrae were dissected from clearing preparations by cutting the intervertebral disc and the sternum from wild-type (+/+) and mutant (-/-) 18.5-day-old embryos (A) and adults (B). In homozygous animals, the tuberculum anterior (completely ossified in adults) is absent on the sixth cervical vertebra. The morphological appearance of C6 then becomes strikingly similar to C3, C4, and C5. Homozygous animals had ribs on the seventh cervical vertebra in some cases, which then closely resembles the first thoracic vertebra (A). The arrows indicate the tuberculum anterior; the arrowheads show the cervical ribs.

the prothoracic segment to the mesothoracic segment in the embryo, both of which represent posterior transformations (Wakimoto and Kaufman 1981). Complete elimination of *Scr* gene function reveals the function of the overlapping, more posterior *Antp* gene (Kaufman et al. 1990). In *Drosophila*, homeotic transformations that arise from loss-of-function mutations are only observed for those genes having expression domains that overlap those of other *HOM-C* genes. Thus, *Scr*, which belongs to the group of *HOM-C* genes that correspond the *Drosophila* homologs of *Hoxa-5*, differs from other homeotic genes in not conforming to the model proposed by Lewis (1978).

We have documented both categories of morphological transformations in *Hoxa-5* homozygous mutant mice. Anterior transformations were seen to affect C6, causing it to adopt a morphological appearance strikingly similar to the more anterior vertebra C5. Additionally, in a

smaller number of cases, the first lumbar vertebra was transformed into a thoracic vertebra based on the appearance of ribs. However, a very common observation in mutant animals was that C7 adopts a more posterior fate and is transformed into a thoracic vertebra. As mutations in *Scr* do not give the expected transformations, together with our results showing that *Hoxa-5* mutants display a posterior transformation, these data reinforce the hypothesis that both genes are members of the same subfamily. On the basis of sequence homology within the homeo domain, relative position within their respective gene clusters, and the type of transformations that accompany their mutation, a strong argument can be made that *Scr* is the true *Drosophila* homolog of *Hoxa-5*. Moreover, it was shown recently that ectopic expression of mouse *Hoxa-5* sequences in *Drosophila* results in *Scr*-like transformations and that *Hoxa-5* can participate in *Drosophila* regulatory networks by activating down-

Figure 5. Transformation of the first lumbar vertebra in *Hoxa-5* mutants. (A) Ventral view of a wild-type 18.5 day p.c. embryo showing the absence of ribs on the first lumbar vertebra. (B,C) Ventral view of *Hoxa-5* homozygous mutant embryos showing a pair of short ribs on the first lumbar vertebra (B) and more extensive ribs (C). The arrows indicate the first lumbar vertebra.

stream targets of *Scr* (Zhao et al. 1993). Collectively, these results strongly suggest that *Hoxa-5* and *Scr* are cognate genes. It will be interesting to analyze the phenotypic consequences of introducing mutations into the two mouse *Hoxa-5* paralogs.

In *Drosophila*, mutational studies have indicated that important functional roles of specific *HOM-C* genes seem to be confined to the subregions that accumulate the highest levels of expression within a more extensive expression domain (McGinnis and Krumlauf 1992). Generally the highest levels of mammalian *Hox* gene expression are found in tissues corresponding to the more anterior regions of expression. If quantitative differences in the absolute levels of gene expression are important functionally, it might be expected that these regions of the embryo would show the most severe perturbations (Hunt and Krumlauf 1992). In *Hoxa-5* mutant mice, the most frequent morphological changes are confined to a small region of the axis that includes the sixth and seventh cervical vertebrae. The highest level of *Hoxa-5* expression, as assessed by in situ hybridization, is in the region including pv6 to pv14 (C6 and T7, respectively), with lower levels of gene expression extending more caudally (Gaunt et al. 1988). The pv6–pv14 region includes the region of the thorax that participates in the formation of the sternum. Because *Hoxa-5* is expressed in the mesodermal condensation that gives rise to the sternum and ribs precursors, abnormalities at the level of the sternum can be attributed to the absence of functional *Hoxa-5* gene in this region of the embryo. In *Hoxa-5* mutant mice, the affected region of the body axis is thus entirely contained within the normal expression domain of the gene.

The posterior prevalence model does not adequately

explain the mode of action of the *Hoxa-5* mutation, as transformations of identity occur in axial regions that express *Hox* genes located 5' to the *Hoxa-5* gene in the cluster (namely genes that have a more posterior domain of expression). The basis of this model is that positional specification is largely achieved by the dominant action of the most posterior homeotic gene at a given axial level. Lack of one component should theoretically result in a group of several vertebrae at a specific axial level adopting a single fate, rather than a more extended series of vertebrae adopting the morphology of immediately adjacent ones (Hunt and Krumlauf 1992). The *Hoxa-5* mutant phenotype is thus better explained by models in which quantitative levels of expression have a critical role. Similar findings have been described in animals mutant for the *Hoxc-8* gene, which is also expressed in the trunk region (LeMouellic et al. 1992; Pollock et al. 1992). Thus, loss of function of either of these two genes results in abnormalities that span a more extensive region than that documented in mice mutant for the *Hoxa-1* and *Hoxa-3* genes expressed more anteriorly, which show defects confined to the branchial region (Chisaka and Capecchi 1991; Lufkin et al. 1991; Chisaka et al. 1992).

Presence of cervical ribs on C7 has been recorded in embryos exposed to teratogenic doses of retinoic acid, whereas anterior transformation of C6 into C5 was reported in RAR γ homozygous mutants (Kessel and Gruss 1991; Kessel 1992; Lohnes et al. 1993). Retinoic acid is known to modulate expression of *Hox* genes in both cultured cells and in vivo (Kessel and Gruss 1991; Papalopulu et al. 1991; Simeone et al. 1991; Kessel 1992). In embryos treated in utero with retinoic acid, an overall anterior extension of the normal domains of expression

Figure 6. *Hoxa-5* mutants show abnormalities of the sternum. (A) Ventral view of a wild-type 18.5 day p.c. embryo showing the sternum region. Normally, seven pairs of ribs are attached to the sternum, which contains four sternabrae in addition to the manubrium sterni and the xiphoid process. (B,C) Ventral views of the sternum region of mutant embryos. In B, the cervical ribs are fused to the sternum at the top of the manubrium sterni at the position normally occupied by the first thoracic ribs. The first thoracic ribs join the sternum in the middle of the manubrium sterni, which as a consequence appears to be split in two. The last two sternabrae before the xiphoid process are linked together, resulting in a close juxtaposition of the sites of attachment of the fifth, sixth, and seventh thoracic ribs. In C, fusion of adjacent sternabrae results in irregularities in the sternocostal junctions. Ossification of the xiphoid process also appears altered, as fissures and holes are observed (B,C). (m) Manubrium sterni; (x) xiphoid process; (arrowhead) cervical rib.

of specific *Hox* genes was reported that correlate with the induced alterations in morphology (Kessel and Gruss 1991; Kessel 1992; Marshall et al. 1992). One caveat is that it is not yet clear whether the morphological effects can be attributed solely to alterations in *Hox* gene expression.

In addition to expression in the spinal cord, *Hoxa-5* mRNA is localized to mesoderm structures derived from the somites, the lateral mesoderm, and the lateral plate. Gross analysis of homozygous pups did not reveal any major perturbations of tissues or organs that normally express the *Hoxa-5* gene. However, clearly the major phenotype presented by *Hoxa-5* mutant mice is a greatly reduced viability during the first few days of postnatal life. We note that approximately half of the mutant animals die within the first week of birth and that the reduction in viability is intrinsic to the mutant animals as the loss of pups occurs irrespective of the genotype of the mother. Thus, loss of *Hoxa-5* function in some way interferes with the correct physiological functioning of major organ systems. The *Hoxc-8* mutation also results in very marked postnatal lethality (~6% of mutants survived to adulthood), with the surviving animals showing neurological disorders and slight runting (LeMouellie et al. 1992). In contrast, the surviving *Hoxa-5* mutants appear indistinguishable from wild-type or heterozygous siblings in behavior, reproductive performance, and longevity. The variability in expressivity of the mutation

may result from segregation of "modifier" loci. This interpretation is reinforced by recent data showing that one of the *Hoxb-4* mutations results in high rates of postnatal lethality (~50% survive to adulthood) in a hybrid background and in a complete loss of homozygous animals in an inbred genetic background (Ramirez-Solis et al. 1993). We are currently backcrossing the *Hoxa-5* mutation into the inbred 129/Sv background to determine whether the penetrance of the phenotype is more pronounced.

Because of the genomic organization of the *Hox* cluster in vertebrates, it is difficult to assess whether the phenotype arises from a single gene mutation. One possibility is that owing to the close juxtaposition of genes within a cluster, the *neo* cassette might interfere with the correct expression of adjacent genes. The dynamic patterns of *Hox* gene expression are generated by a combination of positive and negative regulatory elements that differentially restrict the spatial, temporal, and tissue-specific domains (Zakany et al. 1988; Bieberich et al. 1990; Püshel et al. 1991; Tuggle et al. 1990; Whiting et al. 1991). The strong conservation of the genomic structure of the homeotic clusters between species such as human and mouse suggests that the precise overall organization of a given cluster is necessary for correct regulation and expression of the component genes. This is supported by the observation that long and interspersed transcripts, multiple promoters, shared regulatory re-

gions, and differential splicing are features associated with *Hox* gene complexes (Boncinelli et al. 1991; Sham et al. 1992). Introduction of subtle mutations destroying the coding potential of specific *Hox* gene transcripts provides an important alternative method by which to assess the completely unique contribution of individual *Hox* genes (Hasty et al. 1991; Valancius and Smithies 1991; Ramirez-Solis et al. 1993).

A final possibility is that the paralogous genes *Hoxb-5* and *Hoxc-5* may compensate in part for loss of *Hoxa-5* function. It is becoming increasingly clear that key regulatory molecules in development, such as growth factors or transcription factors, operate as networks of related family members and have overlapping spatial and temporal domains of expression. It will be interesting to compare the phenotypes of animals harboring single mutations with those carrying compound mutations of paralogous *Hox* genes.

To verify that the phenotype observed in the *Hoxa-5* mutant mice results solely from a mutation at this locus, it will be necessary to complement the mutation using a *Hoxa-5* transgene. However, one potential complication in using this approach is the necessity of defining the *cis*-acting regions required for the appropriate spatial and temporal regulation of *Hoxa-5* gene expression. A 912-bp region located upstream of the first exon of the *Hoxa-5* gene has been identified that is sufficient to direct *lacZ* reporter gene expression in a discrete rostrocaudal region of the central nervous system (Zakany et al. 1988; Tugle et al. 1990). However, the pattern of *lacZ* expression does not entirely recapitulate the endogenous *Hoxa-5* expression pattern described by *in situ* hybridization analysis. An additional complication is that, as for most other *Hox* genes, multiple transcripts of different sizes are derived from the *Hoxa-5* locus. It has not been determined whether these alternate transcripts encode different functional forms of the *Hoxa-5* protein as has been shown for the *Hoxc-6* gene (Oliver et al. 1988). Northern analysis shows that the *neo* insertion introduced in the *Hoxa-5* locus disrupts all four *Hoxa-5* transcripts and therefore destroys the DNA-binding domain of any protein (or proteins) that might result from translation of these transcripts (Fig. 2).

In summary, mutation of the *Hoxa-5* gene leads to developmental defects in those tissues that normally express the gene product. Moreover, the distribution of the abnormalities that characterize *Hoxa-5* mutant mice is in general agreement with a combinatorial expression model, in which the expression of multiple *Hox* genes is required to specify axial identity, with mutation of a single component being sufficient to influence the regional specification in the developing embryo.

Materials and methods

Generation of germ-line chimeras

Embryo manipulations were carried out as described (Schwartzberg et al. 1989). Ten to twelve ES cells from clonal ES cell lines carrying the *Hoxa-5* mutation were injected into individual blastocysts, derived from either C57Bl/6 or MF1 mice. Injected

blastocysts were cultured in ES cell medium for 1–2 hr and then transferred in groups of 6–10 into pseudopregnant female mice. Chimeric pups were identified by coat color chimerism. Chimeric males were bred to MF1 females to test for germ-line transmission of the dominant agouti coat color marker. Heterozygous progeny carrying the mutated allele were identified following Southern blot analysis of tail DNA obtained from resultant F₁ agouti animals.

Southern blot analysis

Genomic DNA was prepared from either tail tissue or embryonic tissue as described in Hogan et al. (1986). Purified DNA was digested with the indicated restriction endonuclease, fractionated by electrophoresis through 0.8% agarose gels, blotted onto N-Hybond membrane (Amersham), and hybridized to the 850-bp *Bgl*II–*Hind*III ³²P-labeled genomic probe containing the 3'-untranslated region of the second exon of the *Hoxa-5* gene.

Northern analysis

Total RNA from wild-type, heterozygous, and homozygous adult tissues was prepared as described in Chomczynski and Sacchi (1987), and Northern analysis was performed according to Maniatis et al. (1989). The 850-bp *Bgl*II–*Hind*III genomic fragment containing the 3'-untranslated region of the second exon of the *Hoxa-5* gene was used as a probe to detect endogenous *Hoxa-5* mRNAs as well as mutated transcripts. In addition, blots were also hybridized with a mouse α -actin probe (Minty et al. 1981) as well as with a *neo* probe.

Skeletal analysis

Specimens were prepared as described in McLeod (1980), except that staining with alcian blue 8GS and alizarin red S was performed for 7 days. Skeletons were then cleared in 2% KOH before restaining with 0.015% alizarin red S in 1% KOH for 24 hr to clearly identify ossified structures.

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