Altered Hox expression and increased cell death distinguish Hypodactyly from Hoxa13 null mice

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ABSTRACT Hypodactyly (Hoxa13Hd) mice have a small deletion within the coding sequence of Hoxa13 and a limb phenotype that is more severe than that of mice with an engineered null allele of Hoxa13. We used whole-mount in situ hybridization, Nile blue sulfate staining and genetic crosses to determine the basis for the phenotypic differences between these two mutants. Expression of Hoxd13 was unaffected in Hoxa13 -/- mice, but its domain was reduced at the anterior and posterior margins of the autopod in Hoxa13Hd/Hd limb buds. The maturation of Hoxd11 expression was delayed and expression of Hoxa11 failed to become restricted to the autopod/zeugopod junction in both Hoxa13Hd/Hd and Hoxa13 -/- limb buds compared to wild-type mice. Fgf8 expression was normal in both Hoxa13Hd/Hd and Hoxa13 -/- mice throughout limb development. A dramatic increase in cell death was observed in limb bud mesenchyme of Hoxa13Hd/Hd mice as early as E11.5 but not in mice homozygous for the null allele. Genetic background was excluded as the basis for the phenotypic differences. Compound heterozygotes (Hoxa13 -/Hd) displayed an intermediate phenotype relative to both homozygotes suggesting that Hoxa13Hd has an effect on the development of the autopod beyond that which may result from a loss of HOXA13 protein. These results show that Hoxa13Hd has a negative effect on the survival of the mesenchyme in the autopod, unlike the Hoxa13 null mutation, that cannot be explained by a failure of the AER to express Fgfs. In addition, at least one target of HOXA13 may be Hoxa11.

KEY WORDS: Hypodactyly, Hoxa13, cell death, limb development

Introduction

Numerous putatively null Hox gene mutations have been constructed using homologous recombination (e.g. Dolle et al., 1993; Small and Potter, 1993; Davis and Capecchi, 1994). Results from these studies and gain-of-function experiments indicate that Hox genes cooperate in regulating the growth and differentiation of specific mesenchymal condensations within their area of expression (Morgan and Tabin, 1994; Davis et al., 1995; Davis and Capecchi, 1996; Goff and Tabin, 1997).

Hoxa13 is one of two vertebrate Hox genes in which both induced and spontaneous mutations have been studied. Hypodactyly (Hoxa13Hd) is a spontaneous mouse mutation that was found to be a 50 base pair deletion within the coding sequence of exon 1 of the Hoxa13 gene (Mortlock et al., 1996). In this mutant, a stable mRNA is synthesized from the Hoxa13Hd allele; however, the 50-nucleotide deletion introduces a translational frameshift 25 amino acids after the putative initiator methionine that would likely preclude the synthesis of a protein containing the homeodomain. Mice homozygous for this mutation have a single digit on each paw; heterozygotes have absent or shortened first digits and other minor autopodal defects. Mice with an engineered deletion of Hoxa13 have a different, less severe limb phenotype (Fromental-Ramain et al., 1996). Homozygous null mutants have 4 digits on each paw and heterozygotes exhibit a mild alteration of the claw on the first digit.

Comparative phenotypic and molecular analyses were performed to understand the basis for the pronounced differences in the limb phenotype between mice with Hypodactyly or with the Hoxa13 engineered null mutation. We examined the effects of genetic background on the limb phenotype, examined skeletons of compound heterozygotes, explored the expression of Fgf8 and 5’ Hoxa and Hoxd genes in mutant limbs, and compared the amount of cell death in developing Hoxa13Hd/Hd and Hoxa13 -/- limb buds. Results from these experiments demonstrate that the Hoxa13Hd allele has a negative effect on survival of the limb mesenchyme and

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on Hoxd13 expression that cannot be explained solely by loss of HOXA13 protein.

Results

Genetic background does not account for phenotypic differences

One hypothesis for the differences observed between Hoxa13<sup>Hd</sup> mice and mice carrying the null allele is that genetic background (BL6/C3HFe for Hoxa13<sup>Hd</sup> and 129/Sv for Hoxa13<sup>-</sup>) modifies the morphogenetic consequences of each mutation. To test for effects due to strain differences, each mutation was crossed onto the alternate background and the skeletons of offspring from heterozygous F<sub>1</sub> intercross matings were analyzed.

The Hoxa13<sup>Hd</sup> phenotype has been reported previously (Hummel, 1970; Innis et al., 1996; Mortlock et al., 1996). Hoxa13<sup>Hd/</sup> mice have a variable shortening of the first digit (ranging from missing nail to loss of both phalanges), alterations in the timing of maturation of specific bones within the autopod, and fusions of the d1-d2/c carpals and cuneiform 3-naviculare tarsals. Hoxa13<sup>Hd/Hd</sup> mice have a single digit (corresponding to digit IV) and loss or hypoplasia of most carpals and tarsals at every stage examined. Skeletal stains of E14.5 heterozygous and homozygous Hoxa13<sup>Hd</sup> embryos on the alternate 129/Sv<sup>1</sup> background are shown in Figure 1A. On this alternate background, the Hoxa13<sup>Hd/</sup> mice exhibit shortening of the first digit compared with wild-type skeletons (arrows, n=6). In addition, the condensations corresponding to cuneiform 3 and naviculare tarsals are not clearly separated (arrowhead; 10 of 12 hindlimbs), in contrast to +/+ limbs at this stage, consistent with the potential for fusion between these two elements in Hoxa13<sup>Hd</sup> mice at a later stage of development. Hoxa13<sup>Hd/Hd</sup> skeletons have only one visible digital condensation and a reduced number of carpals and tarsals (n=8). These abnormalities are consistent with the previously reported phenotype of Hoxa13<sup>Hd</sup> mutant embryos on the BL6/C3HFe background (Hummel, 1970; Mock et al., 1987; Mortlock et al., 1996; Robertson et al., 1996), indicating little, if any, phenotypic modification by the 129/Sv<sup>1</sup> background.

The effect of the BL6/C3HFe alternate genetic background on the Hoxa13 null mutation was explored first by gross examination. On the parental background (129/Sv), the Hoxa13<sup>-/</sup> adult mice exhibit shortening and abnormal ventral bending of the nail of digit I,
symphalangism (fusion of the phalanges) of digit I, and fusion of the soft tissue of digits II and III (Fromental-Ramain et al., 1996). Hoxa13 -/- embryos form only a rudimentary condensation in the forelimb corresponding to digit I, no digit I condensation in the hindlimb, and the formation of condensations of the other digital anlagen is delayed. In addition, forelimb digits II and V and all hindlimb digits lack the second phalanx, and carpal and tarsal elements are poorly defined. The soft tissue defects between Hoxa13 +/- adults on the parental (129/Sv) or mixed BL6/C3HFe backgrounds were compared (Table 1). Soft tissue fusions were observed in 9/11 mice (82%) generated by intercrossing Hoxa13 +/- animals (parental background); the other 2 animals had a malformed claw of digit I only. Of the 9 animals with fusions, 7 were bilateral, 2 affected the left hindlimb only, though one of these unilateral cases included digit IV as well as II and III. When the Hoxa13 null mutation was crossed onto the alternate BL6/C3HFe mixed background, only 21/39 mice exhibited soft tissue fusions (54%). Of these, 13 were unilateral with a preference for involvement of the left limb (9/13). The limb phenotype in the remaining mice (18/39) was limited to a malformed claw of digit I. In all cases, the extent of fusion was variable and differences in the extent of left versus right side involvement were observed.

The skeletal phenotype of the Hoxa13 null mutation on the alternate BL6/C3HFe background was analyzed in embryos (Fig. 1B). Homozygous mutant embryos on the alternate background (n=2) lack digit I except for a small rudiment in the forelimb (asterisk, Fig. 1B), lack the second phalanx of digits II and V in the forelimb, and lack the second phalanx in all digits in the hindlimb. In addition, the carpals and tarsals were poorly defined. Finally, digit V in the hindlimb is thin and not clearly separated from the tarsals. This phenotype was similar to what was observed on the parental, 129/Sv, genetic background (L. Post, data not shown; Fromental-Ramain et al., 1996). There was no significant difference in the phenotype of Hoxa13 Hd/+ or Hoxa13 Hd/Hd mouse limbs when crossed to the 129/Sv genetic background. Hoxa13 -/+ adults exhibited less severe soft tissue fusions when placed on a mixed BL6/C3HFe background. This alteration was limited to heterozygotes, as the limb phenotype of Hoxa13 -/- mice on the BL6/C3HFe background was the same as that reported for the parental strain.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Parental background</th>
<th>BL6/C3HFe background</th>
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<tbody>
<tr>
<td>Soft Tissue Fusions, Digits II-III:</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Bilateral</td>
<td>7</td>
<td>8 (1)</td>
</tr>
<tr>
<td>Unilateral</td>
<td>2 (1)</td>
<td>13</td>
</tr>
<tr>
<td>Left</td>
<td>2</td>
<td>9 (5)</td>
</tr>
<tr>
<td>Right</td>
<td>0</td>
<td>4 (1)</td>
</tr>
<tr>
<td>Malformed Claw Only, Digit I:</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Total:</td>
<td>11</td>
<td>39</td>
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The number of animals with each defect is listed. All animals with soft tissue fusions also exhibited malformation of the claw of digit I. Numbers in parentheses indicate the number of animals who also had involvement of digit IV in the fusions.

### Fig. 2. Comparison of skeletal phenotype between Hoxa13 -/-Hd compound heterozygotes and Hoxa13 +/- mice.

The rudimentary condensation of digit I in the forelimb is missing in the compound heterozygotes (asterisk). In the hindlimb, digit V is reduced in size and articulates with metatarsal IV (arrow) rather than with the processus trochlearis (arrowhead). In addition, all hindlimb digits exhibit an anterior bending.

**Embryonic limb phenotype of compound heterozygotes**

Given that steady-state Hoxa13 Hd mRNA levels are normal in abundance (Mortlock et al., 1996), its translation would be predicted to lead to a frameshift resulting potentially in the production of a protein without a homeodomain. Therefore, Hoxa13 Hd would likely act as a functionally null allele of Hoxa13 and compound heterozygotes (Hoxa13 +/-Hd) should have a phenotype similar to Hoxa13 +/- mice.

As a test of allelism and to explore the phenotype of compound heterozygotes, Hoxa13 +/- males were bred with Hoxa13 Hd/+ females and embryos were examined for skeletal defects. From these matings, approximately 15% (8/55) of the embryos generated were compound heterozygotes as determined by genotype analysis. This is lower than the predicted 25%, but not unexpected given the fetal lethality associated with both Hoxa13 Hd and the Hoxa13 null...
Hox gene expression in mutants

Most single Hox gene null mutations result in alteration in patterning (e.g. Morgan et al., 1992; Condie and Capecchi, 1993); loss of structure is often observed when two or more null alleles are combined (Condie and Capecchi, 1994; Davis et al., 1995; Rijli et al., 1995). One explanation for the more severe Hoxa13Hd phenotype is that Hoxa13Hd causes functional loss or misexpression of other Hox genes necessary for proper formation of the autopod. To test this hypothesis, whole-mount in situ hybridization was performed to examine Hox gene expression in Hoxa13Hd and Hoxa13Hd E11.5 limb buds (Fig. 3). Sense controls for all probes were negative (data not shown). Hoxa13 expression was maintained in a normal distribution in Hoxa13Hd embryos at E11.5 (n=3).

The normal expression domain of Hoxd13 extends across the entire anteroposterior (AP) axis of the distal autopod at E11.5 (Dolle et al., 1991; Fig. 3). This domain was reduced at the anterior and posterior margins in Hoxa13HdHd limbs at (n=4) and remained smaller at E12.5 (n=4, data not shown) indicating that this reduction was not simply the result of a developmental delay in the establishment of the expression domain. Expression of Hoxd13 in Hoxa13Hd limbs is not significantly altered compared to wild-type embryos (n=3).

Hoxa11 expression in wild-type mice is first detected across most of the autopod except for a small area at the posterior distal tip and by E11.5 has become restricted to a narrow stripe corresponding to the autopod/zeugopod junction in both mutants resulting in increased expression in the anterior and distal autopod. There is a slight delay in the alteration of Hox11 expression from a single, broad domain to two stripes in both mutants. All images are dorsal views of right forelimbs. A, anterior; P, posterior.

Fig. 3. Whole-mount in situ hybridizations of E11.5 embryos with 5′ Hox genes. Right forelimbs hybridized with probes specific for Hoxa13, Hoxd13, Hoxa11 or Hoxd11. Hoxa13 transcripts are detected in Hoxa13HdHd embryos, possibly at higher levels. Hoxd13 expression is reduced at both the anterior and posterior margins in Hoxa13HdHd embryos, but is not altered in Hoxa13Hd embryos. Hoxa11 expression fails to become restricted to the autopod/zeugopod junction in both mutants resulting in increased expression in the anterior and distal autopod. There is a slight delay in the alteration of Hox11 expression from a single, broad domain to two stripes in both mutants. All images are dorsal views of right forelimbs. A, anterior; P, posterior.

allele at the late gestational ages we examined (E15.5 and E16.5; Post and Innis, unpublished observations; Fromental-Ramain et al., 1996). Furthermore, there was a direct correlation between defects and genotype for both homozygous mutants and for compound heterozygotes. Representative skeletons of two Hoxa13-/- embryos (E15.5) and several Hoxa13-Hd compound heterozygotes (1 embryo at E15.5, 3 embryos at E16.5) are shown in Figure 2. Skeletons of Hoxa13-/- and Hoxa13-Hd both fail to develop digit I, the second phalanx of digits II and V of the forelimb, and the second phalanx of all of the remaining four digits in the hindlimb. However, several morphological differences were observed in Hoxa13-Hd skeletons that were not observed in any Hoxa13-/- animals in our study. In the forelimb, the rudimentary condensation in Hoxa13-/- mice corresponding to digit I never formed in the compound heterozygote (asterisk, Fig. 2). Hoxa13-/- mice have a second phalanx in forelimb digits II and III; however, in Hoxa13-Hd mice, these condensations were not observed at both E15.5 and E16.5. Digit V is dramatically shorter in the hindlimbs of Hoxa13-Hd mice than in Hoxa13-/- (arrow, Fig. 2) and it articulates with the metacarpal of digit IV rather than the processus trochealis (arrowhead, Fig. 2). At E15.5, hindlimb digit II is thinner and the tarsals are smaller in Hoxa13-Hd mice. By E16.5 these bones in Hoxa13-Hd mice more closely resemble the E15.5 Hoxa13-/- limb, suggesting a further delay in growth in the compound heterozygote. Finally, hindlimb digits II and III in Hoxa13-/- mice exhibit a mild abnormal bending toward the anterior side at E14.5 and E15.5. In every compound heterozygote examined, all hindlimb digits exhibited this unusual anterior bending (n=4 mice). The similarities between Hoxa13-/- mice and the compound heterozygotes support our previous conclusion that Hoxa13Hd is an allele of Hoxa13 (Mortlock et al., 1996). The differences observed between Hoxa13-Hd and Hoxa13-/- are minor, yet are similar in degree to those seen between the individual heterozygous mutants (see Fig. 1).

AER function is unaffected in both mutants

Fibroblast growth factors from the apical ectodermal ridge (AER) are important for outgrowth of the limb bud along the proximal-distal axis (Summerbell et al., 1973; Niswander et al., 1993; Fallon et al., 1994). Another potential explanation for the loss of structure in Hoxa13Hd mice could be that the AER is not maintained during development leading to loss of expression of Fgfs. This hypothesis was tested by examining the expression of
Differences that result from two different mutations in the Hoxa13 paper, we have explored the basis for the dramatic phenotypic differences observed in Hoxa13 Hd/Hd limb buds in the mesenchyme under the AER (n=1) and Hoxa13 -/+ (n=3) mutants, Fgf8 expression is indistinguishable from wild-type embryos (Fig. 4A). At E14.5, low levels of Fgf8 expression are detected at the distal tips of the developing digits in the hindlimbs of wild-type mice (n=2). This expression pattern is maintained in the single digit of Hoxa13 Hd/Hd hindlimbs (n=2, arrowheads, Fig. 4B). Therefore, AER function as determined by Fgf8 expression is apparently unaffected in Hoxa13 Hd mice, suggesting that the digital loss cannot be attributed to a primary failure of the AER.

Nile blue sulphate staining reveals an increase in cell death in Hoxa13 Hd/Hd limb buds

Limb patterning is the result of growth coupled with selected programmed cell death (PCD). Several areas of the developing limb (anterior and posterior necrotic zones and the interdigital areas) are known to undergo PCD (Milaire, 1992, and references therein). Reduction in the amount of PCD may result in polydactyly; increased cell death may lead to loss of structures. To determine whether digital loss was secondary to increased cell death, Nile blue sulphate (NBS) staining of Hoxa13 Hd/Hd and Hoxa13 -/- embryos was performed. At E11.5, no observable staining is apparent in +/+ limb buds (n=3), but a narrow domain of stained cells is observed in Hoxa13 Hd/Hd limb buds in the mesenchyme under the AER (n=2, arrowheads, Fig. 5A). At E12.5, the anterior and posterior necrotic zones are visible in +/+ limb buds (n=5, Fig. 5B). However, the amount of cell staining in Hoxa13 Hd/Hd embryos has increased and extends to mesenchyme more proximal to the progress zone (n=1, Fig. 5B). Increased cell death was never observed in the AER. One area in the central portion of the autopod has fewer NBS-stained cells and may correspond to the tissue that will give rise to the single digit in these animals. Limb buds of Hoxa13 -/- mice at E12.5 (n=3) are indistinguishable from wild-type mice (Fig. 5B). Therefore, increased cell death in the autopod is responsible for the severe loss of tissue associated with the Hoxa13 Hd mutation.

Discussion

Analysis and comparison of the effects of multiple alleles are useful to obtain a better understanding of gene function. In this paper, we have explored the basis for the dramatic phenotypic differences that result from two different mutations in the Hoxa13 gene, Hoxa13 Hd and Hoxa13 null mice constructed by homologous recombination. The effect of genetic background on the phenotype was tested by crossing Hoxa13 Hd and the Hoxa13 null mutation onto the alternate backgrounds. Analysis of skeletons from these matings revealed no significant differences between the observed defects and those reported for each mutation (Fig. 1A and B). However, the soft tissue defects observed in Hoxa13 -/- animals appeared to be less severe on a mixed, 129/B6C3Fe background than on the original 129/Sv strain (Table 1). These data suggest that a reduced amount of HOXA13 protein can affect the final morphology of the digits, but that this activity can be influenced to a degree by other factors within the genetic background. However, in the complete absence of normal protein, the dramatic phenotypic differences observed between Hoxa13 Hd mice and Hoxa13 -/- mice cannot be explained by genetic background alone.

To test whether the Hoxa13 Hd mutation results in a null allele of Hoxa13, compound heterozygotes (Hoxa13 -/-) were generated (Fig. 2). The similarities observed between Hoxa13 -/- and Hoxa13 -/- skeletons confirmed that Hoxa13 -/- is clearly an allele of Hoxa13; however, the intermediate phenotype observed in the compound heterozygotes indicates that the Hoxa13 Hd mutation has an additional effect on limb development besides the loss of HOXA13 function.

Robertson et al. (1996) reported an increase in cell death of limb mesenchyme in Hoxa13 Hd embryos. In our studies, increased cell death was observed in limbs of Hoxa13 Hd embryos, but not Hoxa13 -/- embryos, at both E11.5 and E12.5 (Fig. 5) and encompasses most of the autopod except for a small area within the central core of the bud. This area of surviving tissue presumably gives rise to the single digit that later forms in Hoxa13 Hd mice. These results suggest that different mechanisms are responsible for the limb defects associated with each mutation. In Hoxa13 Hd embryos

Fig. 4. Whole-mount in situ hybridization of Fgf8 in Hoxa13 Hd/Hd and Hoxa13 -/- embryos. All images are dorsal views of right limbs except Hoxa13 Hd/Hd forelimb (left limb). (A) Fgf8 expression is not altered in either mutation at E11.5. (B) Expression is maintained at the distal ends of the digits in +/+ embryos at E14.5 and at the end of the single digit in Hoxa13 Hd/Hd embryos (arrowheads). Images are dorsal views of the hindlimbs. A, anterior; P, posterior.
embryos, tissue is formed early but undergoes cell death. In Hoxa13<sup>+</sup> embryos, loss of digit I and phalangeal elements are not caused by excessive cell death, but rather by a reduction in the proliferation of distal mesenchyme, leaving fewer cells available to undergo condensation and differentiation (Fromental-Ramain et al., 1996). The presumed loss of normal HOXA13 protein in Hoxa13<sup>Hd/Hd</sup> limbs may also contribute to poor digital development. The differential increase in cell death provides further molecular evidence that the Hoxa13<sup>Hd</sup> mutation has a deleterious effect on limb formation beyond a simple loss of function of HOXA13 protein.

Limb truncation can also occur when AER function is disrupted (Saunders, 1948; Summerbell, 1974). Fgf8 expression was examined as a marker for the AER and no significant alterations were observed between wild-type limb buds and those of Hoxa13<sup>Hd/Hd</sup> or Hoxa13<sup>-/-</sup> mice at E11.5 (Fig. 4). At later stages, Fgf8 expression, and therefore AER integrity, was unaltered in Hoxa13<sup>Hd/Hd</sup> limb buds compared to wild-type mice. Fgf4, which is downregulated prior to AER regression, was shown to be expressed longer in hindlimbs of Hoxa13<sup>Hd/Hd</sup> embryos compared with wild-type limb buds (Robertson et al., 1997). Taken together, these data suggest that growth factors are generated from the AER of both Hoxa13<sup>Hd</sup> and Hoxa13<sup>-/-</sup> mutants and are available to stimulate the underlying mesenchyme to proliferate. However, in Hoxa13<sup>Hd/Hd</sup> mice, there is an increase in cell death in the distal limb mesenchyme. It is possible that the growth factors are not properly received by the underlying mesenchyme, or the signal cannot be transmitted properly to the cells. Robertson et al. (1996) observed gaps between the AER and the underlying mesenchyme in some Hoxa13<sup>Hd/Hd</sup> limb buds, suggesting that the ectoderm and mesoderm may lose direct contact in these mutants. Alternatively, the growth factor receptors or interacting extracellular matrix molecules needed for growth factors to bind their receptors are not expressed properly in the mesenchyme of Hoxa13<sup>Hd/Hd</sup> limbs.

BMP function is directly involved in cell death in the limb (Ganan et al., 1996; Yokouchi et al., 1996; Macias et al., 1997). In addition, BMPs have recently been demonstrated to promote AER regression (Pizette and Niswander, 1999). The prolonged maintenance of the AER in Hoxa13<sup>Hd</sup> mice found by Robertson et al. (1996) suggests that BMP or mesenchymal function in relation to AER regression may be impaired in these animals.

Alterations in the expression patterns of the 5’ Hox genes described may also contribute to the severe phenotype in Hoxa13<sup>Hd</sup> mice. The expression of Hoxd13 was differentially altered between the two mutations at E11.5. In Hoxa13<sup>-/-</sup> embryos, the pattern of Hoxd13 expression is largely normal and spans the entire AP axis of the distal autopod. However, in Hoxa13<sup>Hd/Hd</sup> mice, there is a restriction to the central expression domain. This restriction is maintained at E12.5 indicating that it is not just the result of a developmental delay. This central expression domain roughly corresponds to the small area of surviving cells in these limbs. However, it is not clear whether the survival of this tissue allows the expression of Hoxd13 in this area inhibits cell death. Hoxa13 expression is normal in Hoxa13<sup>Hd/Hd</sup> mutant limb buds even in areas undergoing cell death, so the mere presence of increased cell death is not necessarily sufficient to explain the...
Comparison of Hypoactyly and Hoxa13 null mice

narrower Hoxa13 domain. Hoxa11 expression fails to become restricted in both Hoxa13"del/del" and Hoxa13"+/-" mutants to a similar degree (Fig. 3). The effect of prolonged Hoxa11 expression in the autopod is unknown. These results suggest that HOXA13 is necessary for the exclusion of Hoxa11 from the distal autopod and provides evidence in support of Hoxa11 as a potential transcriptional target of Hoxa13. Hoxa13 RNA (mutant form) is expressed in Hoxa13"del/del" limb buds in a normal domain, but at higher levels compared with wild-type mice. The increase in expression, albeit minor, was confirmed by Northern analysis (data not shown). Therefore, HOXA13 may negatively regulate its own steady-state expression levels.

In the complete absence of the limb-specific group 13 Hox genes (Hoxa13"+/-", Hoxd13"+/-"), embryos have a single, long condensation extending from the ulna but nothing resembling additional carpals (Fromental-Ramain et al., 1996). Hoxa13"+/-" mice have four digits (Fromental-Ramain et al., 1996) and even mice heterozygous for Hoxa13, but lacking Hoxd11, Hoxd12, and Hoxd13 (Hoxd"del/del"/Hoxa13"+/-", Zakany et al., 1997) have four digits, although they are short and poorly defined. Therefore, one copy of Hoxa13, even in the complete absence of 5' Hoxd genes, is sufficient to form carpals, tarsals, and digital elements. How, then, does Hoxa13"+/-" lead to such a severe reduction in the formation of these same elements? Given that RNA is produced from the Hoxa13"del" allele, a mutant protein could be generated that may contribute to the deleterious effects of this mutation on limb patterning. Work is in progress to explore this hypothesis.

Materials and Methods

Breeding and skeletal staining

Hypoactyly mice were obtained from The Jackson Laboratory where the mutation is carried on a BL6/C3HFe F2 hybrid. It has been carried in our laboratory by intercrossing Hoxa13"del/+" mice; consequently, the contribution of BL/6 and C3HFe varies in each mouse. Despite this mixed background, the Hoxa13"del" phenotype in these animals remains very similar. The engineered null mutation of Hoxa13 (Hoxa13"-/-") was generated in 129/SvJ mice and used in our experiments. Control mice have four digits (Fromental-Ramain et al., 1996) and even mice heterozygous for Hoxa13, but lacking Hoxd11, Hoxd12, and Hoxd13 (Hoxd"del/del"/Hoxa13"+/-", Zakany et al., 1997) have four digits, although they are short and poorly defined. Therefore, one copy of Hoxa13, even in the complete absence of 5' Hoxd genes, is sufficient to form carpals, tarsals, and digital elements. How, then, does Hoxa13"+/-" lead to such a severe reduction in the formation of these same elements? Given that RNA is produced from the Hoxa13"del" allele, a mutant protein could be generated that may contribute to the deleterious effects of this mutation on limb patterning. Work is in progress to explore this hypothesis.

DNA isolation and PCR analysis

DNA isolation and genotyping for the Hoxa13"del" allele was performed for comparison previously (Miller et al., 1988; Mortlock et al., 1996). Genotyping for the engineered Hoxa13 null mutation required separate reactions to detect the wild-type and mutant alleles. The reaction mixture for the wild-type allele included 100 ng DNA, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 200 µM dNTPs, 150 ng primer #4695H (5'-TCGTGGCAAAGAGCAATCAG-3'), 150 ng primer #4695L (5'-TTGGTGGTTTGGCCTGATGAGTC-3'), 2.5 µg BSA, and 1.5 U Taq polymerase (Gibco-BRL). The reaction mixture for the mutant allele was identical except that primer #4694H (5'-TGATAGGCGTGGTTAGCAATCAG-3') was replaced in place of #4693H. Cycling conditions were 94°C, 1 min; 62°C, 1 min; 72°C, 2 min for 2 cycles, then 30 cycles as above except the denaturation temperature was reduced to 92°C. A final extension step was performed at 72°C for 8 min. Amplified products of 620 bases (wild-type allele) or 500 bases (null allele) were visualized by 2% agarose gel electrophoresis.

Whole-mount in situ hybridization

Embryos assigned gestational ages were fixed in 4% paraformaldehyde for 4 h at 4°C, washed 3 times in FBS (1×PBS:0.1% Tween-20), and dehydrated through increasing concentrations of methanol. Whole-mount in situ hybridizations with digoxigenin-labeled RNA probes were carried out as described (Conlon and Hermann, 1993). The antisense probe specific for Hoxa13 is 900 base pair bases (Nco I-Pst I fragment) of the 3' untranscribed region between the homeobox and first polyadenylation signal (Mortlock et al., 1996). Hoxa11 and Hoxa13, Hoxa11, and Fgf8b probes were gifts of D. Duboule, S. Potter, and G. Dressler respectively.

Analysis of cell death

Cell death was assayed by Nile blue sulphate staining. E11.5 and E12.5 embryos from Hoxa13"del/del" or Hoxa13"+/-" heterozygous matings were dissected in PBS pre-warmed to 37°C. Yolk sacs were removed for genotype analysis. Embryos were rinsed twice in pre-warmed PBS, and then incubated in 0.005% Nile blue sulphate (Sigma) in PBS at 37°C for 30 min. They were then rinsed once in pre-warmed PBS and incubated overnight at 4°C. Limbs were photographed the following day using a Leica MZ8 dissecting microscope and camera.

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