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Reference


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Genetic Analysis of a Conserved Sequence in the HoxD Complex: Regulatory Redundancy or Limitations of the Transgenic Approach?

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ABSTRACT Extensive sequencing in the HoxD complex of several vertebrate species has revealed a set of conserved DNA sequences interspersed between neighboring Hox genes. Their high degree of conservation strongly suggested that they are used for regulatory purposes, a hypothesis that was largely confirmed by using "classical transgenesis" or in vivo mutagenesis through the embryonic stem (ES) cell technology. Here, we show that this is not always the case. We report that the deletion of a conserved regulatory sequence located in the HoxD complex gives different results, depending on the transgenic approach that was used. In "conventional" transgenesis, this sequence was necessary for proper expression in a subdomain of the developing limb. However, a deletion of this sequence in complexo did not confirm this effect, thereby creating an important discrepancy between the classical transgenic and the ES cell-based, targeted mutagenesis. This unexpected observation may show the limitations of the former technology. Alternatively, it could illustrate a redundancy in regulatory circuits and, thus, justify the combination of parallel strategies. Dev. Dyn. 1998;213:1-11. © 1998 Wiley-Liss, Inc.

Key words: HoxD complex; embryonic stem cell technology; region X; targeted mutagenesis

INTRODUCTION

Vertebrate Hox genes are required for the proper organization and development of the body plan. Their correct function depends on the establishment in time and space of a precise pattern of partially overlapping expression domains in the embryo. The genetic mechanism(s) responsible for the sequential activation of these genes is largely unknown. However, it relies on a specific feature of this gene family, i.e., genes are controlled according to their genomic positions in the different complexes (colinearity; see Krumlauf, 1994, see references in van der Hoeven et al., 1996a,b). Whatever process underlies colinearity, it is likely that it involves some cis-acting regulatory sequences to mediate either specific interactions, in the case of upstream factors, or less specific types of controls, such as in the generation of high-order chromatin configurations. The characterization of such sequences, thus, may be an important step in our understanding of Hox gene regulation.

It is generally believed that DNA sequences that are highly conserved among different species and located outside transcribed regions serve some regulatory purposes (see, e.g., Burglin et al., 1987). Based on this assumption, the validity of which has been verified in many instances, we set out to sequence the "posterior" part of the HoxD complex in mammals, chick, and fish, i.e., the region containing AbdominalB (AbdB)-related genes (Izpisúa-Belmonte et al., 1991). Sequence comparisons led to the definition of several conserved regions that were further analyzed by using a "classical" reporter transgenic approach (Renucci et al., 1992; Gérard et al., 1993; Beckers et al., 1996; Hérault et al., 1998). Deletion or mutation of particular sequences within large reporter transgenes usually modified some aspect of the regulation, thus allowing the attribution of precise function to DNA sequences. In this way, short stretches of DNA were identified to play a role either in the position of the expression boundaries (see, e.g., Gérard et al., 1993; Vogel's et al., 1993), in germ layer specificity (Zákány et al., 1988; Kress et al., 1990; Püschel et al., 1991), or in expression domains linked to particular structures, such as the limbs (Schughart et al., 1991; Eid et al., 1993) or the hindbrain (Sham et al., 1993; Studer et al., 1994; Pöppler et al., 1995).

Although the transgenic approach has proven to be powerful, it also contains intrinsic problems that should not be overlooked. For example, the almost systematic production of multiple copy insertions through DNA injection may produce a transgenic locus that is not directly comparable to the endogenous locus. Also, autoregulation and cross regulation, which have been demonstrated to be important for labial- or Deformed-related genes (Pöppler et al., 1995; Chan et al., 1997;
Gould et al., 1997; Maconochie et al., 1997), may interfere with the interpretation of the results in the presence of both the endogenous locus and other interacting gene products. For these reasons, it is desirable that the final output of such transgenic approaches be controlled by a targeted approach within the endogenous locus in vivo. In the case of Hox genes, few cases have been reported so far in which both approaches were used. In each of those cases, however, the results that were obtained by using the targeted approach confirmed partially or entirely the transgenic results (Gérard et al., 1996; Dupé et al., 1997; Zákány et al., 1997a,b; Studer et al., 1998; Hérault et al., 1998; Gavalas et al., 1998).

Posteriors Hoxd genes are important determinants of limb development. During limb budding, these genes are expressed with proximodistal and anteroposterior specificities and follow temporal colinearity (Dollé et al., 1989). The expression domains of these genes in limbs are complex and are established through several phases of activation, which are potentially controlled by different enhancer sequences (Duboule, 1994; Nelson et al., 1996; Hérault et al., 1998). In our search for regulatory sequences that are active during limb development, we identified two regions of high sequence similarities between tetrapods. One region was localized between Hoxd-13 and Hoxd-12 and was shown by transgenesis to drive expression of a Hoxd-12 reporter transgene in a restricted posterior domain of the growing limb buds. Deletion of this region in vivo confirmed this result and showed that the corresponding limb expression domain of the Hoxd-12 gene was absent in mutant animals (Hérault et al., 1998). The second conserved sequence was isolated between Hoxd-11 and Hoxd-12 and was shown to drive expression of both Hoxd-11 (Beckers et al., 1996) and Hoxd-12 (Hérault et al., 1998) reporter transgenes in a proximal and posterior domain of the endogenous Hoxd-11 gene.

To evaluate the function of this sequence precisely in the regulation of posterior Hoxd genes, we set out to delete it from its endogenous context through an embryonic stem (ES) cell-based strategy. Mice were recovered at the expected Mendelian ratio. Surprisingly, no abnormal phenotype was detected in the absence of the neomycin selection cassette. Likewise, expression patterns of the surrounding Hoxd genes remained unchanged in homozygous mutants. We conclude that such sequence may drive expression of Hoxd-11 or Hoxd-12 in an as yet unidentified domain (in late fetuses or adults). Alternatively, it is possible that a regulatory redundancy prevents us from easily assessing the functional potential of this sequence.

**RESULTS**

**Targeted Deletion of Region X**

We previously identified a cis-acting regulatory element, region X (RX), located in the intergenic region between Hoxd-11 and Hoxd-12 and well conserved within tetrapods as well as in zebrafish. A detailed analysis of this element in Hoxd-11 and Hoxd-12 transgenes indicated that it was required to direct expression of reporter genes in a proximal posterior domain of the developing forelimb in mouse embryos (Beckers et al., 1996; Hérault et al., 1998). However, the combined loss of function of group II genes and Hoxd-12 did not induce any abnormal phenotype in this precise region (Davis et al., 1995; Davis and Capecchi, 1996). It was therefore unclear whether this expression domain reflected a genuine function of either Hoxd-11, Hoxd-12, or both genes. To reveal the function of RX, we deleted it from its endogenous position in the HoxD complex through homologous recombination in mouse ES cells (Fig. 1A). Internal as well as external DNA probes (Fig. 1B) were used to identify four clones that had recombined the targeting vector at the expected locus. Male germ line chimeras were generated from two clones. In the F2 progeny, wild type, heterozygous (HoxD<sup>RX<sub>neo</sub></sup>+/<sup>-</sup>) and homozygous (HoxD<sup>RX<sub>neo</sub></sup>neo/neo) animals were obtained at the expected ratio. Mice homozygous for the deletion of RX appeared outwardly normal. They were fertile and had life spans of at least 1 year.

**HoxD<sup>RX<sub>neo</sub></sup> Allele**

Analysis of RX in transgenic mice suggested a function in developing limbs (Beckers et al., 1996). We therefore investigated mutant mice for alterations of the limb skeletal pattern. Limb skeletons of homozygous mutants (HoxD<sup>RX<sub>neo</sub></sup>neo/neo) were indistinguishable from wild type litter mates (not shown). Because previous compound mutants revealed that AbdB-related Hoxd and Hoxa genes can compensate one another’s function (see, e.g., Davis and Capecchi, 1996; Zákány and Duboule, 1996), we genetically combined the absence of RX with different Hox loss-of-function alleles. When the HoxD<sup>RX<sub>neo</sub></sup> allele was added to a Hoxa-13/Hoxd-13 transheterozygous background, a strong enhancement of the limb skeletal phenotype was scored. Autopods (hands and feet) of Hoxa-13/Hoxd-13 transheterozygous mice regularly showed a loss of phalanges 2 (P2) in digit V (minimus) as well as a fusion of phalanges 1 and 2 in digit I (thumb). In the distal row of the carpus, the d3 and d4 bones, were occasionally fused, and an additional element (d5) could occur (Fromental-Ramain et al., 1996; see Fig. 2A). In triple mutants (Hoxa-13<sup>+/−</sup>, Hoxd-13<sup>+/−</sup>, HoxD<sup>RX<sub>neo</sub></sup>neo<sup>+/−</sup>), these alterations became fully penetrant. In addition, mice of this genotype displayed an additional posterior digit-like structure (Fig. 2B; V with asterisk) as well as a strong reduction of the fifth metacarpal bone (Fig. 2B). Likewise, a fusion of the proximal carpal bones, the navicular lunate and triangular bones, that occurred with incomplete penetrance in mice heterozygous for both Hoxa-11 and Hoxd-11 loss of function became fully penetrant in Hoxa-11<sup>+/−</sup>, Hoxd-11<sup>+/−</sup>, HoxD<sup>RX<sub>neo</sub></sup>neo<sup>+/−</sup> animals (not shown).

In addition, HoxD<sup>RX<sub>neo</sub></sup>RX<sup>neo</sub> mice displayed an anterior shift of the lumbar sacral transition in the vertebral column. Whereas wild type mice showed six lumbar
Deletion of RX Does Not Alter Hoxd Gene Expression

We subsequently excised the PGK-neo selection cassette from the mutagenized locus by using the Cre recombinase in the recombined ES cells in vitro (Gu et al., 1993). This excision process generated the HoxdRX allele, which was free of selection marker and harbored only a 261 base pair (bp) large deletion, including RX (Fig. 1C). This configuration was controlled by Southern analysis and was confirmed further at the nucleotide level. The sequence of polymerase chain reaction (PCR) in typing the offspring from established lines. Xh, XhoI; S, SmaI; Xb, XbaI; E, EcoRI; H, HindIII.

Vertebral transformation were present. In particular, the vertebral column of Hoxd13/-/- mice was normal (not shown). Therefore, the phenotypic alterations observed in association with the HoxdRX allele had been induced by the presence of the selection cassette in the locus.

We next assessed the regulatory function of RX by looking at Hoxd gene expression in HoxdRXRX mutant mice. We initially focused on Hoxd-11 and monitored its expression by using whole mount in situ hybridizations. We analyzed either 9.5-dpc embryos, a stage at which Hoxd-11 expression is first observed in the tail bud region, or 12.5-dpc embryos, a stage at which the expression pattern in limbs has developed fully. We did not observe any difference in Hoxd-11 expression be-
Fig. 2. Phenotypes of the HoxD\textsuperscript{RX neo} and HoxD\textsuperscript{RX} alleles when combined with Hoxa-13/Hoxd-13 transheterozygous mutant background. 

\textbf{A}: Forelimb autopod of a Hoxa-13\textsuperscript{1/2}/Hoxd-13\textsuperscript{1/2} mutant mouse. In digit I, phalanges 1 and 2 are fused (P1/P2), whereas, in digit V, P2 is missing. In the distal carpal row, d3 and d4 are fused (d3/d4). Occasionally, an additional carpal element occurred posteriorly (the arrow indicates an incomplete split of the d3/d4 bone). 

\textbf{B}: Forelimb autopod of a Hoxa-13\textsuperscript{1/2}/Hoxd-13\textsuperscript{1/2}/HoxD\textsuperscript{RX neo} mutant mouse. In addition to the alterations observed in the transheterozygous mutant (A), an additional digit-like structure is found posteriorly (V with asterisk), and the fifth metacarpal (mc5) is strongly abnormal. 

\textbf{C}: Forelimb autopod of a Hoxa-13\textsuperscript{1/2}/Hoxd-13\textsuperscript{1/2}/HoxD\textsuperscript{RX} mutant mouse. The genotype of this mouse is the same as that in B, except that the PGKneo cassette was removed. The autopod is indistinguishable from that of Hoxa-13/Hoxd-13 transheterozygous mutants (A). 

Fig. 3. The PGKneo cassette induced posterior transformations in lumbar and sacral vertebrae. 

\textbf{A}: Wild type vertebral column. The third lumbar vertebra (L3) is the first to show an accessory process (arrow). 

\textbf{B}: Vertebral column of a HoxD\textsuperscript{RX neo} mouse showing morphological posterior transformations. All vertebrae posterior to L5 adopt a morphology that resembles the posterior next vertebral element. For example, the normal L6 has features of the first sacral element (S1). Thus, the lumbosacral transition is shifted anteriorly. In addition, the second lumbar element now bears an accessory process (arrow). 

\textbf{C}: Wholemount in situ hybridization of a wild type embryo at 13.5 days postcoitus (dpc) using a Hoxd-11 probe. The arrow indicates the anterior limit of expression in the sclerotome. 

\textbf{D}: A HoxD\textsuperscript{RX neo} litter mate hybridized with the same probe. Hoxd-11 expression extends anteriorly in the sclerotome (arrow) but not in the central nervous system (CNS).
tween HoxD RX/RX mutants and their wild type litter mates. Unexpectedly, the proximal posterior domain of limb expression that had been associated with RX by using conventional transgenesis (LE1 in Beckers et al., 1996) was not lost upon deletion of this element from the endogenous locus (Fig. 4E,F). This indicated that RX is not critical to drive endogenous Hoxd-11 expression into this limb domain.

Because RX was equidistant from both Hoxd-11 and Hoxd-12, we investigated its potential influence either on this latter gene (Fig. 4C,D) or on more distantly located members, such as Hoxd-13 (Fig. 4A,B), Hoxd-10, and Hoxd-9. Wholemount in situ hybridizations did not reveal any modifications in the expression patterns of these genes in mutant fetuses. In particular, none of the alterations detected in trunk mesoderm or limb buds of HoxD RX neo mice were observed. We concluded that RX may not be required for the expression of AbdB-related Hoxd genes between 9.5 and 12.5 days of embryonic development.

Genitourinary System

Loss-of-function alleles of groups 10, 11, and 13 Hox genes have revealed their particular functions in the morphogenesis of the urogenital system. Uterine defects and homeosis of the vas deferens as well as cryptorchidism were associated with the inactivation of Hoxa-11 and Hoxa-10 (Hsieh-Li et al., 1995; Rijli et al., 1995; Satokata et al., 1995; Gendron et al., 1997). Notably, compound mutant mice for Hoxa-11 and Hoxd-11 loss-of-function alleles showed enhanced alterations in the male reproductive system, suggesting that Hoxd-11 is involved in the morphogenesis of this system. In addition, mice double homozygous for these two mutant alleles displayed severe renal malformations, leading to perinatal death (Davis et al., 1995). Likewise, mice mutant for Hoxd-13 displayed abnormal morphogenesis of the prostate (Podlasek et al., 1997).

We investigated the role of RX in HoxD gene expression in these urogenital structures by using in situ hybridization on sections of 19-dpc mutant embryos. Hoxd-11 and Hoxd-10 were expressed in the metanephros and ureter of wild type embryos (Fig. 5A,E), and their expression was indistinguishable from that of control mice (Fig. 5B,F). Similarly, Hoxd-10 was expressed in the prostatic primordia of both homozygous mutant and wild type embryos (Fig. 5A,D). In the urethra, Hoxd-11 again was expressed identically in both homozygous mutant and wild type litter mates (Fig. 5G,H). The situation was the same for Hoxd-12 (not shown). Therefore, in situ hybridization did not point to a potential role of RX in HoxD gene expression in the urogenital system, at least not at these stages.
TABLE 1. Genotypes of Offspring From Crosses Between Hoxa-11<sup>-11</sup>/HoxD<sup>RX/RX</sup> Males and Females<sup>a</sup>

<table>
<thead>
<tr>
<th>Genotypes from Hoxa-11&lt;sup&gt;-11&lt;/sup&gt;/HoxD&lt;sup&gt;RX/RX&lt;/sup&gt; intercrosses</th>
<th>Expected ratios (%)</th>
<th>Scored ratios (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.25</td>
<td>8.7</td>
</tr>
<tr>
<td>HoxD&lt;sup&gt;RX/RX&lt;/sup&gt;</td>
<td>12.5</td>
<td>16.7</td>
</tr>
<tr>
<td>Hoxa-11&lt;sup&gt;-11&lt;/sup&gt;</td>
<td>6.25</td>
<td>7.9</td>
</tr>
<tr>
<td>Hoxa-11&lt;sup&gt;-11&lt;/sup&gt;/HoxD&lt;sup&gt;RX/RX&lt;/sup&gt;</td>
<td>25.0</td>
<td>23.8</td>
</tr>
<tr>
<td>Hoxa-11&lt;sup&gt;-11&lt;/sup&gt;/HoxD&lt;sup&gt;RX/RX&lt;/sup&gt;</td>
<td>12.5</td>
<td>11.9</td>
</tr>
<tr>
<td>Hoxa-11&lt;sup&gt;-11&lt;/sup&gt;/HoxD&lt;sup&gt;RX/RX&lt;/sup&gt;</td>
<td>12.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Hoxa-11&lt;sup&gt;-11&lt;/sup&gt;/HoxD&lt;sup&gt;RX/RX&lt;/sup&gt;</td>
<td>6.25</td>
<td>7.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>One hundred twenty-six mice were collected and scored for their genotype at 2 or 3 weeks after birth. The ratio of each genotype is compared to the expected Mendelian ratio. RX, region X in the murine HoxD complex.

We also examined urogenital tracts from adult double-homozygous mutant males (Hoxa-11<sup>-11</sup>/HoxD<sup>RX/RX</sup>). Dissected reproductive systems of Hoxa-11 homozygous mutants were indistinguishable from those of double-homozygous mice. They showed comparable degrees of vas deferens malformation and cryptorchidism. The male urogenital system is comprised of a variety of ducts and glands that join in a defined sequence to form the urethra. These structures are tightly packed, hindering the assessment of morphological alterations by gross inspection. Urogenital systems of at least 6-week-old male mice from the four different genotypes (wild type, HoxD<sup>RX/RX</sup>, Hoxa-11<sup>-11</sup>/HoxD<sup>RX/RX</sup>, and Hoxa-11<sup>-11</sup>) were examined by histology in serial transverse sections. An as yet unrepeated alteration was associated consistently with the Hoxa-11 loss of function. In wild type mice, the ducts of the vas deferens joined at the midline before the vesicles of the glans ductus deferens (or ampullary gland) started to fuse to form two new ducts that ended on the vas deferens. Thus, they appeared as two tubes contained within one another. In Hoxa-11<sup>-11</sup> mutant animals, the two tubes of the vas deferens did not join medially. Instead, the vesicles of the ampullary glands had already fused to the vas deferens before the two ducts joined at the midline (not shown). This alteration was understood as a premature fusion of the ampullary gland to the vas deferens. However, this mutant phenotype was neither found in HoxD<sup>RX/RX</sup> mutants nor enhanced in urogenital tracts from double-homozygous mutants; hence, it is not documented here.

**DISCUSSION**

Sequence comparison between orthologous loci from different vertebrate species has been a powerful approach to identify regulatory sequences. The regulatory potential of such sequences was most often assayed by conventional transgenesis, i.e., by using a reporter gene construct with or without particular cis-acting DNA sequences. This approach has been widely used in the case of the Hox complexes, in which regions of orthology were easy to define and were rather small. Consequently, several control sequences were isolated in this way and were shown to be responsible for important aspects of Hox gene expression (see, e.g., Bieberich et al., 1990; Kress et al., 1990; Püschel et al., 1990; Tuggle et al., 1990; Whiting et al., 1991; Sham et al., 1992; Renucci et al., 1992; Zwartkruis et al., 1992; Charité et al., 1995; Gérard et al., 1993; Vogels et al., 1993; Marshall et al., 1994; Studer et al., 1994; Aparicio et al., 1995; Morrison et al., 1995, 1997; Pöpperl et al., 1995; Bradshaw et al., 1996; Chan et al., 1997; Gould et al., 1997; Maconochie et al., 1997). Yet, the deduced functional potential was rarely assayed in the endogenous context, within the Hox complex itself, due to the technical difficulty of the ES cell-based mutagenesis. However, in those cases in which regulatory sequences were studied by using both conventional and ES cell-based transgenesis, the results were usually compat-
ible, i.e., the phenotypic consequences of the modification in vivo corresponded to some extent to the expectation generated by the transgenic approach, even though in vivo modifications had systematically weaker effects than those predicted (Gérard et al., 1996; Dupé et al., 1997; Zákány et al., 1997a,b; Gavalas et al., 1998; Hérault et al., 1998; Studer et al. 1998).

Our extensive sequencing of the posterior part of the HoxD complex in mice, birds, and fish has revealed a rather low number of DNA sequences significantly conserved outside the RNA coding regions (Renucci et al., 1992; Gérard et al., 1993; van der Hoeven et al., 1996a,b; Hérault et al., 1998). These potential control elements were all studied by both conventional transgenesis, using lacZ as a reporter gene, and ES cell-based mutagenesis. The deletion of fine mutagenesis of these sequences in their endogenous contexts had different consequences. The study of RXI, a Hoxd-12 control region, showed that it was required in developing posterior limbs, as predicted from conventional transgenesis (Hérault et al., 1998). Likewise, mutagenesis of a nuclear receptor binding site downstream of Hoxd-11 confirmed the repressive function of another of these regions (RIX; Gérard et al., 1996). In contrast, the deletion of RVIII did not exactly reproduce the transgenic analysis. Although this region proved to be necessary for the activation of a Hoxd-11/lacZ transgene, its deletion in complexo did not prevent the activation of the endogenous Hoxd-11 gene, even though this activation did not occur properly (Zákány et al., 1997b).

Similarly, mutagenesis of retinoic acid response elements (RAREs) that are known to control the expression of Hoxb-1 and Hoxa-1 did not induce phenotypes as strong as the transgenic approach may have predicted (Dupé et al., 1997; Gavalas et al., 1998; Studer et al., 1998). Here, we report the deletion of RX, a sequence located between Hoxd-11 and Hoxd-12. In this case, a strong discrepancy was observed between the results obtained by using the two approaches.

Phenotypes

Mice deleted for RX and containing the PGK-neo selection cassette showed a clear phenotype in the vertebral column in which an anterior vertebral transformation correlated with a gain of function of Hoxd-11. In the limbs, alterations were detected only in the presence of additional null alleles either for Hoxd-13 and Hoxa-13 in the digits or for Hoxa-11 and Hoxd-11 in the carpus. Because a slight reduction in the expression of both Hoxd-11 and Hoxd-12 was scored in RX mutant embryonic limbs, the reinforcement of these phenotypes can be explained by a local and partial loss of function. The absence of phenotype in RX homozygous simple mutants likely results from the functional redundancy known to occur amongst Hox genes, in particular during limb development (Davis and Capecchi, 1996; Zákány et al., 1997a). Consequently, both gain-of-function and loss-of-function phenotypes were observed after deletion of RX.

However, these various phenotypic traits disappeared upon excision of the selection cassette. This cassette contained the PGK promoter and was shown in similar cases to induce either gain-of-function or loss-of-function of genes located at the vicinity of the insertion site (see, e.g., Fiering et al., 1995; Hug et al., 1996; McDevitt et al., 1997; Zákány et al., 1997b). It is thus clear that the deregulation in the expression of the neighboring Hoxd-11 and Hoxd-12 genes was caused by the presence of the PGK promoter. This raises once again the need for removing extra sequences from targeted loci, particularly when genes occur in tightly clustered configuration (see, e.g., Olson et al., 1996; Zákány et al., 1997b). It also suggests that some previously described Hox phenotypes may require further analysis.

No Phenotype Associated With RX Deletion

Mice homozygous for a deletion of RX showed no particular abnormality in their phenotypes, even after all major sites of expression of posterior Hoxd genes were carefully investigated. Surprisingly, the expression domain that had been associated systematically with RX in transgenic mice (proximal limb) was not affected in the deleted animals. Potential explanations for this unexpected observation are not trivial. One possibility is that multiple sequences either contribute to the establishment of this expression domain or are capable on their own of driving expression therein and that only one such sequence was located on the reporter transgene. In such a scheme, the presence of the gene in the complex would allow for a compensation of the regulation due to a sequence located nearby, whereas an externally localized transgene would be silent in this domain upon deletion of this sequence. It is also possible that RX is a target sequence for auto- or cross-regulation, whereas the activation of the gene is dependent on another control element located outside the transgene but within the complex. In this case, the absence of RX would suppress the transcription of the transgene in a particular domain, whereas the endogenous copy would still respond to the activating mechanism.

Another explanation is that the deletion of RX induced a phenotype that we did not detect. Although we analyzed all of the sites where either Hoxd-11 or Hoxd-12 loss-of-function mutations were reported to have an effect, we cannot rule out the possibility that these Hox genes have important functions in as yet unidentified tissues or organs, in particular during adult life. It is also possible that the functional redundancy observed for Hoxd-11, for example, in the forearms and kidneys, equally applies to these unidentified expression sites, making their detection even more difficult. This issue will be addressed by using large deficiencies covering the HoxD complex. In such configurations, RX might reveal its genuine functional potential.
Despite these tentative explanations, we would like to stress that RX is the largest sequence of such high sequence similarity reported so far in Hox intergenic regions. The conservation between zebrafish and mouse DNA (Beckers et al., 1996) suggested an important function that is shared by all vertebrates. The absence of a clear phenotype linked to its deletion is therefore an unexpected observation. This is especially surprising when one considers that RX is the only conserved sequence identified between Hoxd-12 and Hoxd-11.

Interestingly, none of the control elements located within the AbdB-related Hoxd genes (group 9–13) turned out to be critical for the major expression features of the neighboring genes. Instead, deletion or mutagenesis of these elements lead to alterations in the modulation of Hoxd gene expression, such as slight differences either in the expression timing (Zákány et al., 1997b), in the position of the AP boundary (Gérard et al., 1996), or in the regulation of a restricted and specific expression domain (Hérault et al., 1998). However, none of these modifications seriously challenged the major colinear activation of these genes in time and space. It thus appears that conserved regulatory sequences within the posterior HoxD complex are involved mainly in the refinement and secondary control of the expression patterns rather than in the original establishment of the transcript domains. This suggests that the control mechanism responsible for the sequential activation of these genes during development may not necessarily rely on classical cis-acting regulatory elements that are detectable by using our strategy.

**Transgenic vs. ES Cell-Based Approaches**

The deletion of RX from its endogenous context failed to reproduce the effect that had been observed previously on a transgenic context. This discrepancy, rather than pointing to the weakness of one particular system, may illustrate the need for both approaches in combination. On the one hand, the random integration of multiple copies of a partial genomic locus in the presence of a functional, endogenous counterpart is certainly very far from physiological conditions, in particular when clustered genes are analyzed individually. On the other hand, however, the clustering of regulatory sequences bearing some functional redundancy may prevent the dissection of their respective importance if
which was flanked by two loxP sites in the same orientation, was inserted as a blunted BamHI/XhoI fragment, in a reverse orientation with respect to the transcription of Hoxd genes, into a blunted HindIII site located 303 bp upstream of the deleted RX. The 5' fragment of homology extended over 5.7 kb from the selection marker insertion site up to a BamHI upstream of Hoxd-12. The 3'-flanking sequence covered 3.5 kb from RX to a BsmI site in the Hoxd-11 intron.

ES cells (D3; Doetschman et al., 1985) were cultured on embryonic fibroblasts in presence of LIF. ES cells (10^7) were electroporated with 40 µg of linearized targeting vector. G418 selection and amplification of the electroporated cells was done as described previously (Joyner, 1993). Excision of the selection cassette from cells that had homologously recombined the targeting constructs was carried out in vitro by electroporation of 10 µg of the pMC-Cre plasmid producing the Cre recombinase of bacteriophage P1 (Gu et al., 1993). ES cells were microinjected into C57Bl/6 blastocysts and transferred to pseudopregnant foster mothers by following standard procedures.

Recombinant clones were identified by Southern analysis. For an external probe, a PstI HindIII fragment (PH; Fig. 1B), in combination with a HindIII digest of the genomic DNA, was used. The excision of the PGK-neo cassette was controlled by the combination of an XbaI digest and the use of probe SF47 (Fig. 1B). Routinely, genomic typing of F2 mice from established lines was performed by PCR. The pair of primers is indicated by asterisks in Figure 1C. It covered the deletion as well as the loxP site left behind. With these primers, the HoxdRX allele was 440 bp, and the wild type was 670 bp large. PCR products were cloned and sequenced from adult mutant mice to finally control the experiment. Compound mutants were produced with previously published alleles, i.e., Hoxd-13 (Dolle´ et al., 1993), Hoxa-13 (Fromental-Ramain et al., 1996; a gift of P. Chambon), Hoxa-11 (Small and Potter, 1993; a gift of S. Potter), and the HoxdMD allele, a triple loss-of-function of Hoxd-13 to Hoxd-11 in cis (Zákány and Duboule, 1996).

**In Situ Hybridization**

Fixation, hybridization, and detection of Hoxd gene expression in wholemount embryos was carried out by using a probe concentration of between 10 ng/ml and 50 ng/ml. Detection of digoxigenin-11-UTP labelled riboprobes was performed by using an alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim, Indianapolis, IN). The probes used have been previously published: Hoxd-11 (Gérard et al., 1996); Hoxd-13 probe (Dolle´ et al., 1991), and Hoxd-12 (Izpisúa-Belmonte et al., 1991). The Hoxd-10 riboprobe was transcribed from a 1,138-bp cDNA fragment (Renucci et al., 1992). In situ hybridizations on cryosections were performed by using standard protocols. Briefly, 19.5-dpc embryos were collected, embedded in optimum cutting temperature medium, frozen without fixation, and further processed after typing of extraembryonic membranes. Antisense riboprobes were labelled with (αS35)-CTP, followed by DNase I digestion and partial alkaline hydrolysis. Riboprobes were purified using...
quick-spin columns. Embryos were sectioned at 10 µm at (20°C, mounted on gelatin/chrome alum-subbed slides, and stored at (80°C. For hybridization, slides were fixed in 4% formaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine buffer, pH 8. Without prehybridization, slides were incubated overnight at 52°C with 20,000–30,000 cpm/µl of denatured probe in hybridization solution containing 50% formamide. Slides were then washed several times at 55°C, treated with RNase A at 37°C, transferred to standard saline citrate buffer, and finally dehydrated. Slides were processed for emulsion autoradiography (NTB-2; Kodak, Rochester, NY) and developed and fixed after 2–3 weeks (D19 and AL4; Kodak). For histological examination, slides were stained in 0.02% toluidine blue solution.

**Histology and Skeletal Preparations**

Male urogenital tracts were collected from typed adult mice, fixed for 1 day in Bouin's solution, and washed in water for another day, dehydrated, cleared, and embedded in paraffin. Kidneys and testes were sectioned sagittally at 7–10 µm, whereas the remaining urogenital tracts were cut transversally. Sections were stained in hematoxylin/eosin. Skeletal staining was carried out as described by Inouye (1976).Typed adult mice were stained for 3–4 days in 2% KOH and 0.1% alizarin red. Soft tissues were then cleared in 2% KOH. Uteri were isolated, fixed in 4% paraformaldehyde, and stained with an X-galactosidase cocktail. Stained samples were imbedded in paraffin, sectioned at 7–10 µm, and stained with eosin following standard procedures.

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