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# Genetic analysis of a *Hoxd-12* regulatory element reveals global versus local modes of controls in the *HoxD* complex

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## SUMMARY

**Vertebrate *Hoxd* genes are essential determinants of limb morphogenesis. In order to understand the genetic control of their complex expression patterns, we have used a combined approach involving interspecies sequence alignments in parallel with transgenic analyses, followed by in vivo mutagenesis. Here, we report on the identification of a regulatory element that is located in the vicinity of the *Hoxd-12* gene. While this element is well conserved in tetrapods, little sequence similarity was scored when compared to the cognate fish DNA. The regulatory potential of this region XI (RXI) was first assayed in the context of a *Hoxd-12/lacZ* reporter transgene and shown to direct reporter gene expression in posterior limb buds. A deletion of this region was generated by targeted mutagenesis in ES cells and introduced into mice. Analyses of animals homozygous for the $HoxD^{RXI}$ mutant allele revealed the function of this region in controlling *Hoxd-12* expression in the presumptive posterior zeugopod where it genetically interacts with *Hoxa-11*. Downregulation of *Hoxd-12* expression was also detected in the trunk suggesting that RXI may mediate a rather general function in the activation of *Hoxd-12*. These results support a model whereby global as well as local regulatory influences are necessary to build up the complex expression patterns of *Hoxd* genes during limb development.**

Key words: *Hoxd-12*, Limb development, Mouse, Transgene

## INTRODUCTION

Tetrapod limbs are built up around three different segments: the most proximal piece, the stylopod, containing the humerus or the femur; the intermediate segment, the zeugopod (forearm and foreleg), containing the ulna and radius, or tibia and fibula, and the distal piece, the autopod (hand and foot), including the carpus or tarsus as well as digits and toes. The patterning of these various segments is in part controlled by gene members of the *HoxA* and *HoxD* complexes. These genes are those related to the *Drosophila AbdominalB* homeotic gene and are classified in five distinct groups of paralogy (from 9 to 13). Targeted gene inactivations have confirmed the requirement of *Hox* gene function for proper limb development and have pointed to the role of these gene products in controlling the establishment of the prechondrogenic condensation pattern and of their subsequent extension (*Hoxa-9* and *Hoxd-9*, Fromental-Ramain et al., 1996a; *Hoxa-10*, Favier et al., 1996; *Hoxa-11*, Small and Potter, 1993; *Hoxd-11*, Davis and Capecchi, 1994; Favier et al., 1995; Davis et al., 1995; *Hoxd-12*, Kondo et al., 1996, 1997; Davis and Capecchi, 1996; *Hoxd-13*, Dollé et al., 1993). Animals mutant for these different genes displayed alterations of the bony pattern restricted along defined parts of the proximodistal axis, usually corresponding to the most proximal expression domain of the mutated gene (e.g. Davis et al., 1995). It thus appears that the distribution of the functional domains of these genes is colinear with their genomic position within their *Hox* complexes. Accordingly, *AbdB*-related genes positioned more 3′ in their complexes, such as *Hoxd-9* or *Hoxa-9*, play a role in patterning the stylopod (Fromental-Ramain et al., 1996a) whereas genes located at the 5′ extremities (*Hoxd-13*; *Hoxa-13*) are expressed subsequently in distal domains and have a major function in the formation of the autopod (Fromental-Ramain et al., 1996b; Dollé et al., 1993).

The partial overlap in the expression domains of these genes added to their recent common phylogenetic origin contribute to the important functional redundancy of this system (see Rijli and Chambon, 1997 and refs. therein). However, genes with partially redundant functions are not fully equivalent and particular paralogy groups tend to control the morphogenesis of distinct pieces of the limb. For example, while at least four *Hox* genes genetically interact during zeugopod patterning, the essential contribution comes from *Hoxa-11* and *Hoxd-11* as double mutant mice have virtually no forearms (Davis et al., 1995). In the autopod, the situation is also complex as most posterior *Hox* genes can efficiently participate in the formation of digits depending upon the presence or absence of neighbouring gene functions (Davis and Capecchi, 1994; Favier et al., 1995; Davis et al., 1995; Davis and Capecchi, 1996; Kondo et al., 1996, 1998; Dollé et al., 1993; Hérault et al., 1996;

Zákány and Duboule, 1996; Zákány et al. 1997b). In this latter case, group 13 genes have nevertheless the most important role. It thus appears that a functional hierarchy exists between *Hox* gene products such that, in the presence of multiple gene products, those encoded by the more posterior genes have stronger functional impacts. This 'posterior prevalence' (see Duboule and Morata, 1994) has been observed in a variety of contexts and is particularly apparent in gain-of-function configurations (Peichel et al., 1997; Hérault et al., 1997).

It is therefore important that the temporal and spatial control of *Hox* gene expression be accurately regulated to prevent severe alterations in the morphogenesis of various structures. This is well exemplified by the ectopic expression of *Hoxd-13* in forearms, either artificially induced (van der Hoeven et al., 1996; Goff and Tabin, 1997) or following a mutation at the *Ulnaless* locus (Hérault et al., 1997; Peichel et al., 1997), where important defects in the formation of the corresponding bony elements were scored. Likewise, timing of activation may interfere with the proper onset of *Hox* gene expression, as has been described for *Hoxd-11* in the body axis. Mutagenesis of regulatory sequences has shown that a slight delay in transcriptional initiation of *Hoxd-11* led to the posterior transposition of the sacrum (Zákány et al., 1997a), while premature transcriptional activation shifted the sacrum anteriorly (Gérard et al., 1997).

The combined use of both interspecies DNA sequence comparison and transgenesis had been helpful in identifying enhancer sequences responsible for the regulation of *Hox* genes expression during development (e.g. Gérard et al., 1993, 1996; Aparicio et al., 1995; Frasch et al., 1995; Gould et al., 1997; Haerry and Gehring, 1996; Knittel et al., 1995; Marshall et al., 1994; Morrison et al., 1997; Popperl et al., 1995; see Maconochie et al., 1997 and refs therein). We used this approach to characterize two regions in the vicinity of *Hoxd-12*, RX and RXI, which were found conserved in different tetrapod species. Previous transgenic analyses of RX showed that it could control expression of a *Hoxd-11/lacZ* reporter transgene in proximal limb buds (Gérard et al., 1993; Beckers et al., 1996). In this paper, we report that the second region, RXI, for which no teleost counterpart could be clearly defined, is able to drive *Hoxd-12* reporter gene expression in a posterior domain extending from the presumptive zeugopodal area to the handplate. Using ES cell mediated targeted mutagenesis, we further introduced a deletion of this regulatory sequence at its resident location *in vivo*. Animals carrying this mutant allele displayed a downregulation of *Hoxd-12* expression in the corresponding area, while other posterior *Hoxd* genes remained unaffected. The combination of this mutation with a null allele of *Hoxa-11* (Small and Potter, 1993) genetically demonstrated a loss of function of *Hoxd-12* in both forearms and legs as well as in posterior trunk. Expression and function of *Hoxd-12* was nevertheless not modified in the handplate, confirming that other regulatory elements are involved in *Hoxd* regulation in digits. A model of *Hoxd* gene regulation during limb development is discussed which involves the combined action of global and local regulatory influences.

## MATERIALS AND METHODS

### DNA sequences and *lacZ* transgenic mice

The DNA sequence extending from the second exon of *Hoxd-13* to the first exon of *Hoxd-11* was established using subclones derived from genomic libraries of either mouse, chicken or zebrafish. Sequence alignments were carried out using conventional software. For conventional transgenic analyses, a 7.7 kb *Xho*I-*Sal*I mouse genomic DNA fragment containing *Hoxd-12* was subcloned. *lacZ* reporter sequences were inserted in frame at the *Sfi*I site of exon I to generate the *XSd-12/lacZ*$^{wt}$ transgene (Fig. 1D). Deletion of the conserved sequence X (RX) in the context of a *Hoxd-11* transgene was previously described (Beckers et al., 1996). Deletion of this particular sequence in the context of the *XSd-12/lacZ* transgene generated the *XSd-12/lacZ*$^{del(RX)}$ construct (Fig. 1D). Deletion of the RXI sequence from the same reporter transgene (*XSd-12/lacZ*$^{del(RXI)}$), was achieved using the *Bam*HI and *Nco*I sites flanking the mouse RXI sequence (Fig. 1D). Transgenes were injected into C57Bl/6j×CBA fertilized eggs and transferred into pseudopregnant females. Expression analyses of the different transgenes were carried out at 12.5 days post coitum (dpc) using β-galactosidase staining reaction on fixed embryos according to an established procedure. For the RX deletion construct, three additional fetuses were collected at days 9.5 (2) and 10 (1). While one expressed β-galactosidase throughout the body, the two others had expression patterns related to that of the *XSd-12/lacZ*$^{wt}$ construct. However, the difference in developmental stages prevented a clear comparison being established with the other constructs, hence these three animals do not appear in the final numbers. For the *XSd-12/lacZ*$^{wt}$ construct, three independent transgenic lines were established, two of which were used to precisely follow the expression profile of the transgene during development (data not shown).

### Deletion in ES cells and generation of the *HoxD*$^{RXI}$ alleles

The targeting vector used for the deletion of RXI in vivo was produced following the insertion of a PGK*neo* selectable cassette flanked by two loxP sites into the *Bam*HI-*Nco*I sites located at the vicinity of the RXI sequence, within the 7.7 kb *Xho*I-*Sal*I DNA fragment containing *Hoxd-12*. The linearized targeting vector was electroporated into D3 ES cells (Doetschman et al., 1985; gift of Rolf Kemler) to generate the *HoxD*$^{RXIneo}$ allele (Fig. 1E). Culture and electroporation of ES cells were as previously described. Five out of 144 ES cell clones were recovered which had recombined the targeting vector at the expected locus. Both 3′ and 5′ ends of the targeted locus were checked for integrity by Southern blot analysis. The XE and SE probes gave either a 2.5 kb and a 5.5 kb *Xba*I band, or a 8.5 kb and 10.5 kb *Xho*I fragments, indicating the targeted and wild-type loci, respectively.

### Mouse mutant lines

Chimaeric mice were obtained after microinjection of ES cells into C57Bl/6j blastocysts and germline transmission of the deleted allele was monitored by Southern blot analysis. Individual breeding colonies were established for two independent mutant ES clones. In order to prevent potential transcriptional interference due to the presence of the PGK promoter in the recombined *HoxD*$^{RXIneo}$ locus (Olson et al., 1996), we excised the PGK*neo* selection cassette by breeding with a *Cre* deleter strain (Dupé et al., 1997, gift of P. Chambon; Fig. 1E, *HoxD*$^{RXI}$). Animals without the selection cassette were typed, isolated and intercrossed to generate an homozygous line. PCR was used to genotype $F_2$ progenies, using RXI-specific upstream (5′-CCGACTTAAAA-CTTCTGCCTCCTCCCACCTACC-3′) and downstream (5′-GGTGACTATTCCCTACTAGCCAAGCTCCTAGCC-3′) primers. Amplified bands for the three allele (wt, *HoxD*$^{RXIneo}$ and *HoxD*$^{RXI}$) were of different sizes, 0.61 kb, 2.3 kb and 0.41 kb, respectively. The *Hoxa-11*$^{Cin}$ null mutant allele was a gift of S. S. Potter (Small and Potter, 1993). Other *Hoxd* alleles used in this work were described previously (*Hoxd-12*$^{lac}$, Kondo et al., 1996; *HoxD*$^{Del}$, Zákány and Duboule, 1996).

### Whole-mount in situ hybridization and skeletal analyses

Whole-mount in situ hybridizations were performed on genotyped embryos following standard procedures. The probes (*Hoxd-13, Hoxd-12, Hoxd-11* and *Hoxd-10*) were as previously described (Gérard et al., 1996; Hérault et al., 1997). For skeletal analyses, mice were

collected at the age of 2 months, processed and stained with alizarin red (Inouye, 1976).

## RESULTS

### A conserved sequence upstream of *Hoxd-12*

The comparison between mouse, chicken and zebrafish genomic DNA sequences located between the second exon of *Hoxd-13* and the first exon of *Hoxd-11* identified two blocks of sequence conservation, outside the transcription units. Regions X and XI (RX; RXI) were 227 bp and 200 bp in size, respectively, and positioned on either sides of the *Hoxd-12* gene (Fig. 1B). The distances between RX and RXI and the *Hoxd-12* transcription unit were also found conserved in the three different species (Fig. 1B). RX had previously been characterized in the context of a *Hoxd-11/lacZ* transgene and

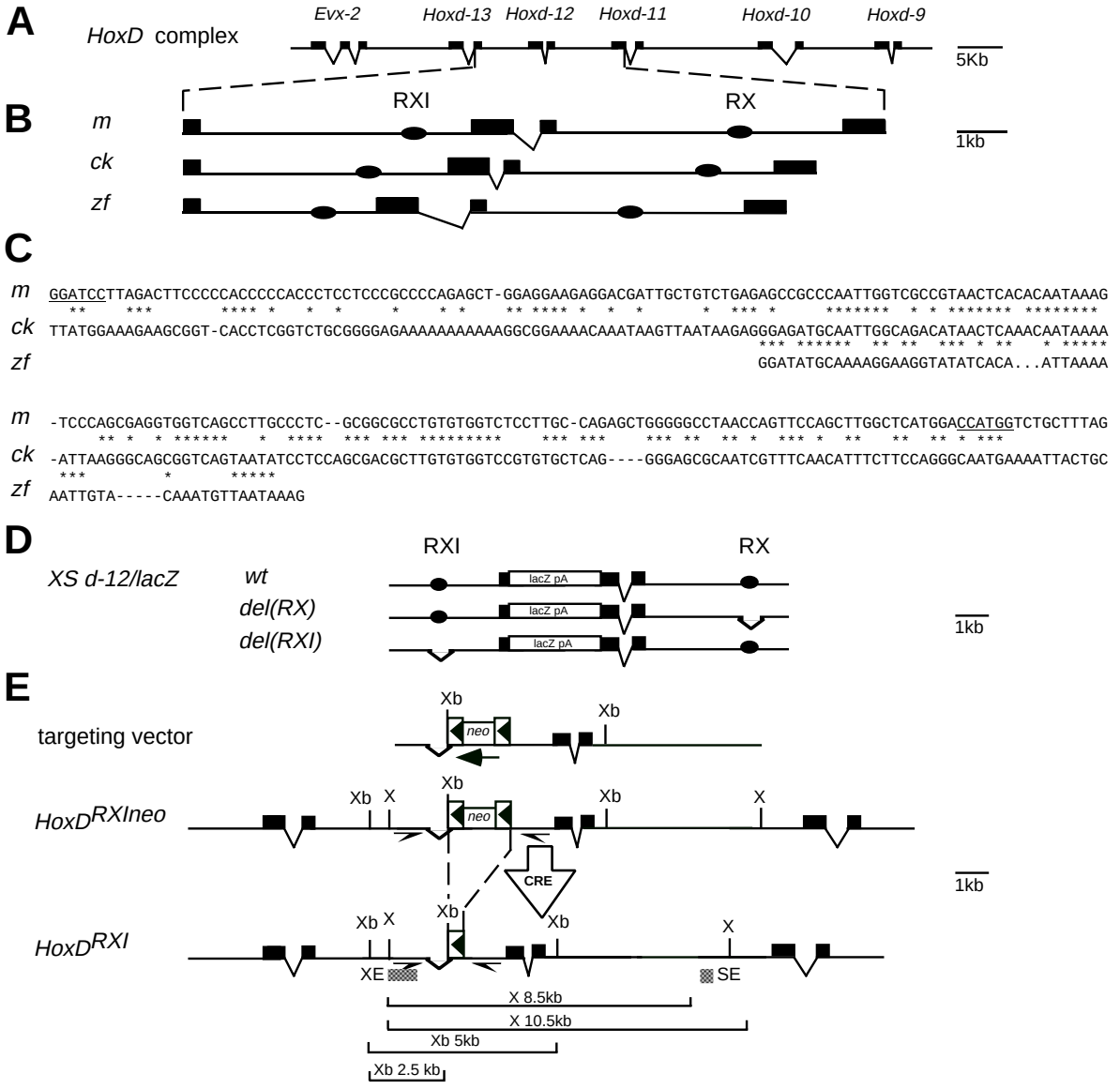


**Fig. 1.** Characterization of the RX and RXI conserved sequences. (A) 5′ part of the *HoxD* complex, from *Hoxd-9* (right) to *Evx-2* (left). (B) Genomic organization of the two intergenic regions flanking *Hoxd-12* in mouse (m), chicken (ck) and zebrafish (zf). Transcribed regions are shown in black rectangle and the respective positions of RX and RXI are indicated as filled black circles. (C) Nucleotide sequence comparison between the avian RXI (ck, middle line) and related sequences found at the same respective positions in either the mouse (m, top) or the zebrafish (zf, bottom) complex. The gap in the fish sequence indicates a gap of 21 bp. (D) Constructs used for the transgenic analysis. The *XSd-12/lacZ*$^{wt}$ transgene is the original *Hoxd-12* locus with an insertion of *lacZ* sequences in frame within the first exon. In the *XSd-12/lacZ*$^{del(RX)}$ construct, RX was deleted whereas in the *XSd-12/lacZ*$^{del(RXI)}$ construct, RXI was deleted. (E) Deletion of RXI in vivo and production of the *HoxD*$^{RXI}$ mutant locus. The targeting vector is depicted (top line) with the RXI deletion, and the PGK*neo* selection cassette flanked by two loxP sites. After homologous recombination, the *HoxD*$^{RXIneo}$ mutant locus was transferred into mice (middle line). Exposure of these mice to the *Cre* recombinase led to the excision of the selection cassette to generate the *HoxD*$^{RXI}$ locus (bottom line). X, *Xho*I; Xb, *Xba*I. Probes used for Southern blot are indicated (XE, SE) as well as the positions of primers for PCR.

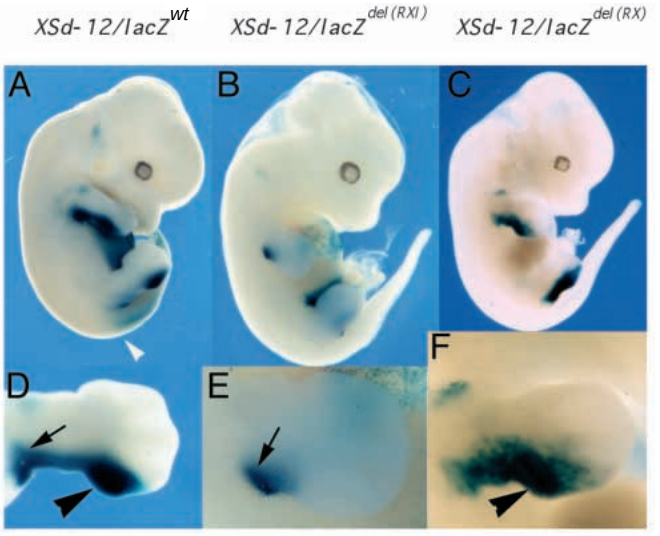


**Fig. 2.** Analyses of fetuses carrying the *XSd-12/lacZ* transgene with or without RXI and RX. β-gal staining of fetuses carrying either the *XSd-12/lacZ*$^{wt}$ (A,D), the *XSd-12/lacZ*$^{del(RXI)}$ (B, E), or the *XSd-12/lacZ*$^{del(RX)}$ (C,F) constructs. β-galactosidase activity was assayed in 12.5 dpc transgenic embryos. The *XSd-12/lacZ*$^{wt}$ transgene was expressed in the trunk, with an anterior limit located at the level of the 27th to 29th prevertebrae (white arrowhead in A) as well as along the posterior margin of developing limbs (D). In *XSd-12/lacZ*$^{del(RXI)}$ transgenic fetuses, β-galactosidase activity was restricted to the posterior and proximal limb domain (E, black arrow), whereas *XSd-12/lacZ*$^{del(RX)}$ fetuses expressed *lacZ* in a domain extending from the median to the distal part of the limb bud (F, black arrowhead). These two domains did not entirely overlap and were observed in the wild-type transgene (D, arrow and arrowhead).

shown to be necessary for expression in a proximal domain of the budding limbs (Beckers et al., 1996). RXI was found 1.3 kb upstream of the translation start site of *Hoxd-12* in the mouse genome. Its DNA sequence was well conserved with the avian counterpart; 55% of the nucleotides were identical over 200 bp. This conservation, however, was less pronounced between tetrapods and fish; alignment between the chick and zebrafish homologous DNA sequences revealed only a 60% identity over 56 bp (Fig. 1C). Interestingly, this weak similarity between chick and fish concerned sequence motifs that were not recovered in the mouse/chick comparison, suggesting that this DNA sequence had evolved slightly differently in the tetrapod lineage.

### *Hoxd-12/lacZ* transgenic analyses

We assessed the function of both RX and RXI in the context of a *Hoxd-12*-derived transgene. This transgene (*XSd-12/lacZ*$^{wt}$; Fig. 1) was constructed by an in-frame fusion of *lacZ* reporter sequence within the *Hoxd-12*-coding sequence and contained the flanking genomic DNA including both RX and RXI. Expression analyses of this chimeric gene from established transgenic lines revealed β-galactosidase staining at 12.5 dpc in the neural tube, with an anterior limit of expression located at the level of prevertebrae 27-29 (Fig. 2A), as well as in genital and anal areas (not shown). The transgene was also expressed with high incidence in budding limbs (6 out of 6 expressing fetuses), in a domain extending from proximal to distal, with a clear restriction to the posterior part at 12.5 dpc (Fig. 2D). While this limb expression profile was somewhat reminiscent of that of endogenous *Hoxd-12*, important differences were noticed, such as the absence of staining in all digits and a sustained expression in the zeugopod (compare Figs 2D and 3D).

In order to evaluate the importance of both RX and RXI in the control of this expression pattern, we produced two deleted versions of the *XSd-12/lacZ* transgene (Fig. 1D). First, the *XSdD-12/lacZ*$^{del(RX)}$ construct was obtained by removing the RX sequence from the *Hoxd-12* context. Secondly, the *XSd-12/lacZ*$^{del(RXI)}$ construct was produced by deleting RXI (Fig. 1D). Interestingly, the deletion of either one of these two sequences abolished expression in neural tube (Fig. 2B,C) suggesting that both RX and RXI may contribute to the establishment of *Hoxd-12* transcript domain in the developing trunk.

In addition to this significant effect in the trunk, important changes were observed in budding limbs where each of these regions appeared to have a specific regulatory property. Deletion of RX destabilized expression of the *Hoxd-12* transgene in the proximal/posterior domain, similar to the deletion of this same region from the *Hoxd-11* reporter gene (Beckers et al., 1996). In the case of *Hoxd-12*, however, the requirement for RX was not absolute as only 5 out of 9 fetuses showed the selective absence of this expression domain. With this transgene, the posterior expression domain extending up to the distal end of the limb bud was unaffected (Fig. 2C,F). Interestingly, 10 other fetuses carrying this deletion showed various ectopic expression profiles unrelated to the original *Hoxd-12/lacZ* pattern.

By contrast, the posterior expression domain (Fig. 2D, arrowhead) was removed upon deletion of RXI from the transgene and β-galactosidase activity was detected only in the proximal/posterior limb bud at 12.5 dpc (Fig. 2B,E, compare with D). Consequently, RX and RXI showed enhancer properties complementary to each other in the regulation of transgene expression in posterior limb domains. While RX facilitated transgene expression in a proximal part of the limb bud (Fig. 2E), RXI was required in a domain extending from the intermediate segment (the future zeugopod) to the distal end, the handplate (Fig. 2F).

### Targeted deletion of RXI in vivo

Since these transgenic studies suggested a potential involvement of RXI in the control of *Hoxd-12* expression, we investigated the role of this sequence in its endogenous context, i.e. within the *HoxD* complex. To this end, we generated a targeted deletion of RXI by homologous recombination in ES cells (Fig. 1E). A targeting vector was used to replace RXI region by a loxP-PGKneo-loxP cassette in ES cells and mice carrying this modified locus (the *HoxD*$^{RXIneo}$ allele; Fig. 1E) were produced. The PGK*neo* selection cassette was then removed by crossing *HoxD*$^{RXIneo}$ mice with an animal from a *Cre*-expressing 'deleter' strain (Dupé et al., 1997; Fig. 1E). The resulting locus (*HoxD*$^{RXI}$) was thus devoid of foreign promoter and expression of *Hoxd-12* and *Hoxd-13* could be assessed in the absence of artificially induced transcriptional interferences.

Adult mice homozygous for the deletion (*HoxD*$^{RXI/RXI}$) were obtained in a Mendelian ratio and did not exhibit visible morphological alterations. Mutant and wild-type embryos were recovered from *trans*-heterozygous crosses and analyzed by

whole mount in situ hybridization for the expression patterns of the four neighbouring *Hoxd* genes (from *Hoxd-10* to *Hoxd-13*). This series of analyses revealed that in *HoxD*$^{RXI/RXI}$ fetuses, *Hoxd-10*, *Hoxd-11* and *Hoxd-13* genes were expressed as in wild-type animals (Fig. 3B,C,G). If anything, a weak reinforcement of *Hoxd-11* expression was observed in some instances (e.g. Fig. 3C). In contrast, a marked reduction of *Hoxd-12* transcripts accumulation was noticed in both trunk and limbs of mutant specimens (Fig. 3A). In the trunk, *Hoxd-12* expression was significantly reduced when compared to wild-type or heterozygous animals (Fig. 3A,F) and became almost undetectable (Fig. 3F). In limbs, *Hoxd-12* transcripts were absent from the intermediate segments, the presumptive zeugopods, in both forelimbs and hindlimbs (Fig. 3D-F, arrowheads). However, *Hoxd-12* expression remained unaffected both in the proximal/posterior domain as well as in the future digit area (Fig. 3E,F; stars and white arrowheads, respectively). This abnormal *Hoxd-12* expression profile was already apparent in 10.5 dpc forelimbs of mutant embryos (Fig. 3D) and clearly matched the change in *lacZ* reporter gene activity observed upon deletion of RXI (see above).

## Skeletal analyses

Despite this clear difference in *Hoxd-12* expression in limb buds, skeletal defects were not detected either in the forearm or in the foreleg of homozygous *HoxD*$^{RXI/RXI}$ adult animals (Fig. 4A,B,G,H). This was not unexpected as the phenotype of two previous null alleles of *Hoxd-12* did not show obvious defects in the zeugopods, probably due to compensation by other *Hox* genes, in particular by the *Hoxa-11* and *Hoxd-11* gene products (Kondo et al., 1996; Davis and Capecchi, 1996). We therefore combined the *HoxD*$^{RXI}$ allele with a loss-of-function allele of *Hoxa-11* (*Hoxa-11*$^{Cin}$, Small and Potter, 1993) to produce compound heterozygous animals, which were subsequently interbred. Such *trans*-heterozygous animals had apparently normal radius and ulna, the two bones that constitute the adult forearm (Fig. 4C). In contrast, *Hoxa-11*$^{Cin/Cin}$ mice showed reduced and misshapen distal parts of both radius and ulna (Fig. 4D; Small and Potter, 1993), and an abnormally thick shape to the ulna (Fig. 4D). *Hoxa-11*$^{Cin/+}$;*HoxD*$^{RXI/RXI}$ animals showed comparable alterations of the forearms (Fig. 4E), as predicted for a *Hoxd-12* loss of function in the zeugopod (Davis and Capecchi, 1996). The severity of this forearm phenotype was importantly increased in double homozygous *Hoxa-11*$^{Cin/Cin}$;*HoxD*$^{RXI/RXI}$ mutant animals, leading to an abnormal bending of both radius and ulna towards the posterior side (Fig. 4F; arrowhead). Again, this phenotypic trait was similar to that reported for *Hoxd-12/Hoxa-11* null mutant animals (Davis and Capecchi, 1996), further confirming that deletion of RXI induced a loss of function of *Hoxd-12* in posterior forearm.

While hindlimbs of mice carrying these different alleles were affected in a less severe manner, they still showed comparable gene-dosage effects (Fig. 4G-L). The major alteration observed in *Hoxa-11*$^{Cin/Cin}$ hindlimbs was the absence of fusion between the tibia and the fibula, distally (Small and Potter, 1993; Fig. 4J). While animals heterozygous for the *Hoxa-11*$^{Cin}$ allele or double heterozygous for the *Hoxa-11*$^{Cin}$ and *HoxD*$^{RXI}$ alleles showed a clear defect at the junction between tibia and fibula, in no case were these two bones found separated (Fig. 4I). Yet this separation between tibia and fibula re-appeared upon introduction of another *HoxD*$^{RXI}$ allele (Fig. 4K) indicating that the *HoxD*$^{RXI}$ allele induced a complete loss of function of *Hoxd-12* in the intermediate segment of the hindlimbs.

To investigate whether this loss of function of *Hoxd-12* in forelimb and hindlimb zeugopods was accompanied by a loss of function in the most distal expression domain (the presumptive digits), we crossed *HoxD*$^{RXI}$ mice with a line

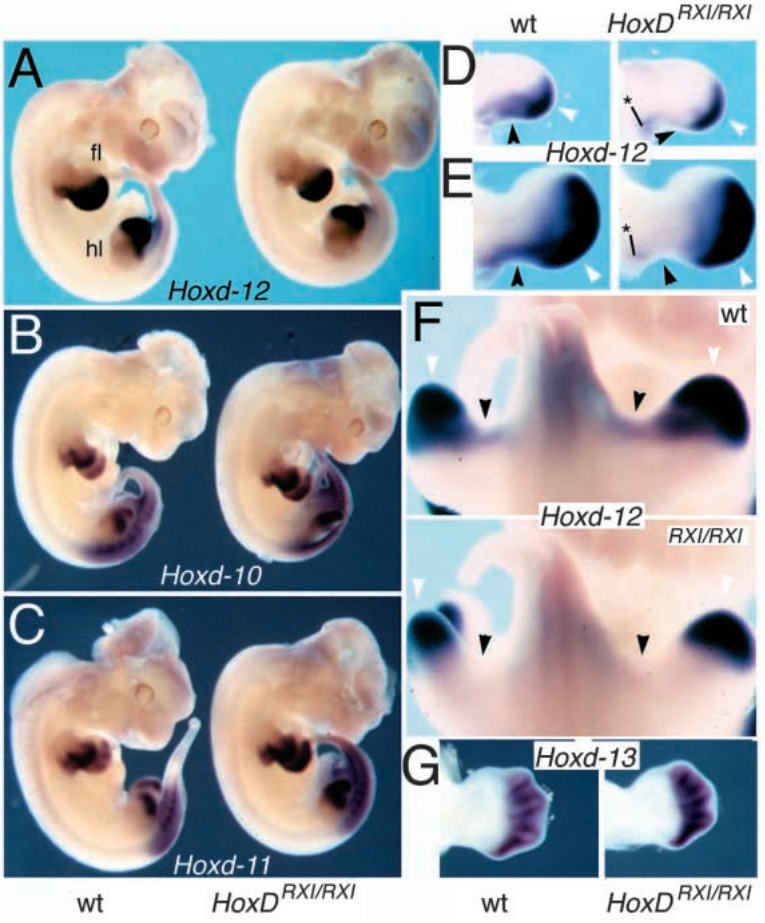


**Fig. 3**. Expression of posterior *Hoxd* genes in wild-type and RXI mutant mice. Whole-mount in situ hybridizations using *Hoxd-10*, *Hoxd-11*, *Hoxd-12* and *Hoxd-13* probes on *HoxD*$^{RXI/RXI}$ mutant fetuses. Comparison of the distribution of *Hoxd-12* (A,D,E,F), *Hoxd-10* (B), *Hoxd-11* (C) and *Hoxd-13* (G) transcripts in either wild-type (A-E,G left; F, top) or *HoxD*$^{RXI/RXI}$ (A-E,G right; F, bottom) 11.5-day-old fetuses. While no change was noticed for *Hoxd-10*, *Hoxd-11* or *Hoxd-13*, *Hoxd-12* expression was downregulated in both the trunk and in the posterior margin of limb buds (A,D-F). *Hoxd-12* loss of function in the presumptive zeugopods was best detected in 11.5 dpc homozygous *HoxD*$^{RXI/RXI}$ foetuses, in both forelimb (E) and hindlimb buds (F) (arrowheads). Downregulation was already seen at 10.5 dpc (D, black arrowhead), whereas both the proximal (D, E, asterisks) and the digit (D,E, white arrowhead) expression domains remained unaffected.

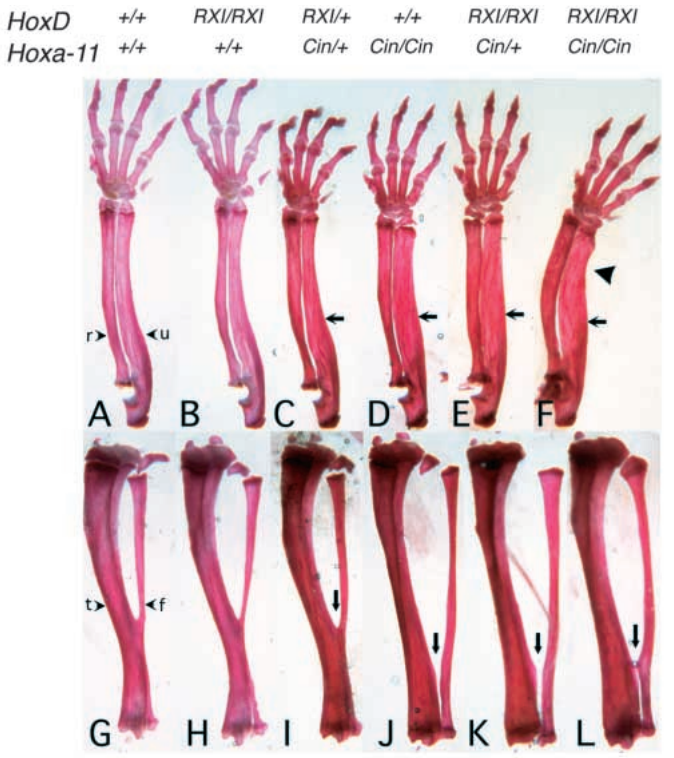


**Fig. 4.** Skeletal alterations in the limbs of adult RXI mutant animals. Comparison between the arms (A-F) and the legs (G-L) of specimens from different genetic constitutions (indicated on the top of the figure). Strong malformations of both radius (r) and ulna (u) were observed in *Hoxa-11*$^{Cin/Cin}$, *Hoxa-11*$^{Cin/+}$; *HoxD*$^{RXI/RXI}$ and *Hoxa-11*$^{Cin/Cin}$;*HoxD*$^{RXI/RXI}$ arms. In particular, the ulna of *Hoxa-11*$^{Cin/+}$;*HoxD*$^{RXI/RXI}$ animals (E) resembled that of *Hoxa-11*$^{Cin/Cin}$ simple mutants (D) whereas the ulna of *Hoxa-11*$^{Cin/+}$;*HoxD*$^{RXI/+}$ (C) was normal, indicating that the RXI mutation was linked to a loss of function of *Hoxd-12* in the forearm. In addition, the *Hoxa-11*$^{Cin/Cin}$;*HoxD*$^{RXI/RXI}$ animals displayed an abnormal bending of their zeugopods, near the junction with the autopod (F, arrowhead). In hindlimbs, the tibia (t) and fibula (f) were not fused distally (J-L, arrows) and the bones appeared thicker in double homozygous animals (L). The same phenotype is clearly visible in the ulna of the corresponding animals (C-F, arrows).

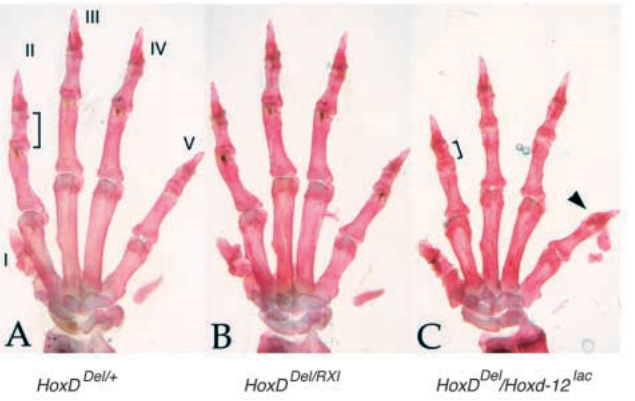


**Fig. 5.** Skeletal preparations of the hands of mice from the *HoxD*$^{Del/+}$ (A), *HoxD*$^{Del/RXI}$ (B) or *HoxD*$^{Del}$/*Hoxd-12*$^{lac}$ (C) genotypes. The *HoxD*$^{Del}$ allele is a triple inactivation of *Hoxd-13*, *Hoxd-12* and *Hoxd-11*, whereas the *Hoxd-12*$^{lac}$ allele is a loss of function of *Hoxd-12*. Mice of the *HoxD*$^{Del}$/*Hoxd-12*$^{lac}$ genotype showed a reduced second phalanges on both digits II (brackets) and V (arrowheads). I to V refer to digit number (from thumb to minimus, respectively); M, metacarpus; P1 to P3 are phalanges.

carrying a triple-inactivation in *cis* of *Hoxd-13*, *Hoxd-12* and *Hoxd-11* (*HoxD*$^{Del}$). When the *Hoxd-12*$^{lac}$ null allele was combined with *HoxD*$^{Del}$, a severe digit phenotype appeared as characterized by the absence of the second phalange (P2) of digit 5 and reduction of P2 of digit 2 (Fig. 5C). In contrast, *trans*-heterozygous *HoxD*$^{Del/RXI}$ animals displayed hand and foot skeletal patterns identical to those of *HoxD*$^{Del/+}$ specimen (Fig. 5A,B). This observation genetically demonstrated that the dose of functional *Hoxd-12* gene product, in the presumptive digit domain, was unaltered in the *HoxD*$^{RXI}$ allele as expected from RNA expression studies (Fig. 3E,F). Indeed, even a partial loss of function of *Hoxd-12* in digits would have induced a phenotype in digit 5, if combined with the *HoxD*$^{Del}$ deficiency (T. K. and D. D., unpublished). Therefore, the loss of function observed for *Hoxd-12* in the forearms of *HoxD*$^{RXI/RXI}$ mutant mice was exclusively confined to this part of the limbs and did not extend, even as a hypomorphic trait, to the autopod domain, illustrating the distinct regulatory controls at work in these different areas of developing appendages.

Skeletal alterations were not restricted to the limbs, as anticipated from the expression analyses. In the vertebral column, deletion of RXI induced vertebral transformations. The main defect was found at the lumbosacral transition (Table 1). In our crosses, wild-type mice had 13 thoracic and 6 lumbar vertebrae (T13L6). Often however, they displayed only 5 lumbar vertebrae (T13L5). In these particular genetic backgrounds, 20% of wild-type mice were of the T13L5 type. When one copy of the *Hoxa-11*$^{Cin}$ allele was introduced, all mice were of the T13L6 or T12L0L6 type. No L7 type was recovered from this genotype. When one or two copies of the RXI-deleted allele were added to this genotype, however, 10 and 30% of the mice, respectively, showed a L7 phenotype. Finally, while 20% of *Hoxa-11*$^{Cin/Cin}$ mice were of the L7 type, this number increased up to 60% when combined with two copies of the RXI allele. These numbers clearly pointed to a loss of function of *Hoxd-12* in the trunk, in association with the deletion of RXI.

## DISCUSSION

Vertebrate *Hoxd* genes related to the *Drosophila* gene *AbdB* are required during the development of axial structures such as the trunk and the limbs. Their expression is tightly regulated in time and space and mis-regulation of this precise control leads to severe alterations in developmental patterning. We set out to isolate and study regulatory sequences involved in such transcriptional controls, based on the hypothesis that they should be evolutionary conserved amongst different vertebrate species. Here, we report on the functional analysis of region XI, a short DNA fragment located upstream the *Hoxd-12* transcription unit.

**Table 1. Vertebral formulae in mice with different genetic combination of the *HoxD*$^{RXI}$ and *Hoxa-11*$^{Cin}$ alleles**

| Genotype | | | Vertebral formulae† (%) | | | |
|---|---|---|---|---|---|---|
| Hoxa-11 | HoxD | n* | T13L5 | T13L6 | T12L0L6 | T12L0L7 |
| +/+ | +/+ | 22 | 23 | 77 | | |
| | +/*RXI* | 34 | | 97 | 3 | |
| | *RXI*/*RXI* | 13 | | 100 | | |
| +/*Cin* | +/+ | 20 | | 20 | 80 | |
| | +/*RXI* | 31 | | 13 | 77 | 10 |
| | *RXI*/*RXI* | 11 | | | 73 | 27 |
| *Cin*/*Cin* | +/+ | 9 | | | 78 | 22 |
| | +/*RXI* | 32 | | | 66 | 34 |
| | *RXI*/*RXI* | 10 | | | 40 | 60 |

*The number of adult skeleton is indicated.

†The formulae has been established by considering the 21st vertebra as L1, because most often in heterozygous and homozygous *Hoxa-11*$^{Cin}$ allele, the 20th vertebra loses its ribs, being transformed into a L0 with lumbar type. The scoring of the cervical and sacral vertebrae is not indicated as no change has been observed in the various genetic constitutions.

## Region XI in transgenic mice

Sequencing of the 5′ parts of the *HoxD* complex in three different vertebrate species allowed us to identify a set of DNA stretches conserved between mammals, birds and fishes. In the case of region X, located upstream *Hoxd-11*, sequence conservation was obvious amongst all three species and the fish DNA was shown to control close-to-normal expression of *Hoxd-11* in transgenic mice (Beckers et al., 1996). A second region of high interspecies sequence similarity, RXI, was found after comparison between mouse and chicken DNA regions separating *Hoxd-13* from *Hoxd-12*. Surprisingly, this rather short DNA stretch was the only area of substantial sequence conservation throughout this entire intergenic region. Comparison with the homologous fish sequence revealed only a very restricted conservation with the chicken sequence, whereas only few matches were scored with the mouse counterpart. Therefore, it seems that RXI was already functional in early vertebrates and may thus have been involved in the control of an ancient *Hoxd-12* function. The extended similarities of RXI between the two tetrapod species indicated that it likely served an important function in this lineage and hence did not tolerate a high rate of base substitution.

Transgenic analyses of RX, in the context of the *Hoxd-12* transgene, revealed a functional potential equivalent to that observed using a *Hoxd-11* reporter transgene (Gérard et al., 1993; Beckers et al., 1996). In both cases, the presence of RX led to transgene expression in a proximal-posterior domain of the developing limbs. Taken together, these data suggest that the RX element may work on different promoters, at least in these various transgenic contexts, similar to enhancer elements known to control anterior genes in the *HoxB* complex (Gould et al., 1997).

Deletion of the second region (RXI) from the *Hoxd-12* transgenic context led to a very reproducible modification in the regulation of the transgene, i.e. the disappearance of an extended posterior domain of expression in both forelimb and hindlimb buds. As the affected transcript domain formed part of the endogenous *Hoxd-12* expression profile, it is likely that RXI is a genuine regulatory element involved in the transcriptional control of *Hoxd-12* during limb development. Interestingly, this particular transgene was unable to elicit expression of *Hoxd-12* either in the distal (digit) domain, or in the central zeugopod domain. This is similar to what was observed for either *Hoxd-11* or *Hoxd-13* (Gérard et al., 1993; Y. H., J. Zákány and D. D., unpublished data), further indicating that expression in these two limb domains require the presence of the target gene in the *HoxD* complex. Deletion of RXI from the *Hoxd-12* transgenic context did not only affect β-galactosidase staining in posterior limbs since expression in trunk disappeared as well. Altogether, these observations

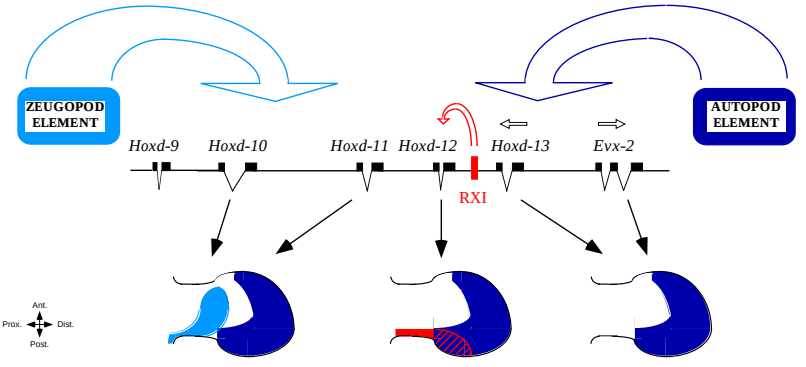


**Fig. 6.** Scheme illustrating the various regulatory influences acting on *Hoxd-12* to control its expression in developing limbs. Expression of *Evx-2*, *Hoxd-13*, *Hoxd-12*, *Hoxd-11* and *Hoxd-10* in the digit domain (dark blue) is regulated by a remote enhancer element (van der Hoeven et al., 1996). Likewise, expression of *Hoxd-10* and *Hoxd-11* in the zeugopods (light blue) may be controlled by another global regulatory element. The respective importance of these two domains, for every gene located in this genomic locus, results from a competitive interaction between these two influences perhaps due to a distance effect. Thus, displacing promoters within this locus would change their response to these two regulations (van der Hoeven et al., 1996). In addition to these global regulations, gene-specific control elements, not necessarily devoted to limb development, will contribute to part of the final pattern, such as RXI (red) in the case of *Hoxd-12*. Functional inactivation of RXI will therefore not affect the large digit and zeugopod domains.

indicated that RXI is of importance for a general *Hoxd-12* transcriptional control. These different points were addressed in vivo, by the production of mice lacking RXI.

### The RXI deficiency

The targeted deletion of RXI in vivo led to comparable effects on the expression of the endogenous *Hoxd-12* gene. First, no transcripts were detected in the posterior region of the developing zeugopod, whereas expression in the presumptive digit area remained unaffected. Secondly, a drastic reduction in expression was observed in the trunk, where *Hoxd-12* RNAs became hardly detectable. Likewise, abnormal phenotypes were scored in these two structures that clearly reproduced those alterations derived from a loss of function of *Hoxd-12*. However, this was not immediately apparent as the complete inactivation of *Hoxd-12* did not produce any obvious phenotype until the genetic background was sensitized by removing additional *Hox* functions. Two different null alleles of *Hoxd-12* have been produced, both inducing only a weak digit phenotype in homozygous animals (Davis and Capecchi, 1996; Kondo et al., 1996). The function of *Hoxd-12* in patterning the autopod was nevertheless confirmed by a double-inactivation in *cis* of *Hoxd-13* and *Hoxd-12*, which demonstrated that *Hoxd-12* can play an important role in developing limbs in the absence of the HOXD13 protein (Kondo et al., 1998).

We analyzed the effect of the RXI deletion in different genetic contexts. When brought over the $HoxD^{Del}$ allele, a triple inactivation of *Hoxd-13*, *Hoxd-12* and *Hoxd-11* in *cis* (Zákány and Duboule, 1996), the RXI allele did not enhance the digit phenotype, thus confirming that the presumptive digit expression domain of *Hoxd-12* was not affected by the $Hoxd\text{-}12^{RXI}$ allele. In contrast, when the RXI deletion was combined with a loss-of-function allele of *Hoxa-11*, the resulting $Hoxa\text{-}11^{Cin/+}$;$HoxD^{RXI/RXI}$ animals showed a forearm phenotype reminiscent of that observed in $Hoxa\text{-}11^{Cin/Cin}$ mice (Small and Potter, 1993). Under these conditions, the phenotype associated with the deletion of RXI was comparable to that obtained in $Hoxa\text{-}11^{Cin/Cin}$;$Hoxd\text{-}12^{-/-}$ animals, (Davis and Capecchi, 1996), further indicating that the limbs of our $Hoxd\text{-}12^{RXI}$ animals had a loss of function of *Hoxd-12* in the posterior halves of their zeugopods.

Various combinations between the $Hoxa\text{-}11^{Cin}$ and $HoxD^{RXI}$ alleles also revealed that the transcriptional downregulation of *Hoxd-12* in the trunk of $Hoxd\text{-}12^{RXI/RXI}$ animals was accompanied by the expected phenotype in the vertebral column. Stepwise removal of *Hoxd-12* function, through the RXI allele, from fetuses carrying or not carrying the $Hoxa\text{-}11^{Cin}$ allele significantly correlated with a progressive posterior shift of the sacrum resulting from the transformation of the first sacral vertebra into a lumbar type. Thus, more than half of $Hoxa\text{-}11^{Cin/Cin}$;$HoxD^{RXI/RXI}$ animals displayed an additional lumbar vertebra and 30% of $Hoxa\text{-}11^{Cin/+}$;$HoxD^{RXI/RXI}$ were of the same L7 type. In contrast, a L7 formula was never scored amongst $Hoxa\text{-}11^{Cin/+}$ progenies. This indicated that RXI is involved in the transcriptional control of *Hoxd-12* in the developing trunk, in addition to its function in limbs. These functional domains represent all of the *Hoxd-12* limb expression sites, with the exception of both the distal (digit) and part of the forearm limb domains. It is noteworthy that the alterations in the position of the lumbosacral vertebral transition are reminiscent of those obtained when modifying the activity of the neighbouring gene *Hoxd-11*, thus suggesting that RXI could regulate this gene as well. While we cannot entirely rule out this possibility, the fact that the expression of *Hoxd-11* is not qualitatively modified in homozygous RXI mutant foetuses makes it unlikely.

### Different regulations for *Hoxd-12* in limbs

Our results support a model whereby the complex expression profiles of posterior *Hoxd* genes in developing limbs are set up through multiphasic regulatory controls. Discrete phases of transcription are observed, which are driven by separate regulatory elements (Shubin et al., 1997). These elements can be classified into two groups; those acting locally as gene-specific control elements and those acting at a distance and capable of controlling several genes at once. In this latter category, two enhancer sequences appear to regulate the expression of the *Hoxd-10* to *Evx-2* genes in either the presumptive digit or forearm regions, respectively. These two regulations (large open arrows in Fig. 6) are likely achieved by two separate enhancer elements located at different positions and acting over several genes. These global regulatory units are specific for the *HoxD* complex and their evolutionary origins thus probably postdate the large scale genomic amplification that gave rise to the four *HOX* clusters (Holland et al., 1994). The fact that such regulatory controls do not seem to occur during the development of paired fins suggests that the corresponding enhancer sequences were added rather recently in the course of vertebrate evolution, perhaps in connection with either the elaboration of more complex limbs (van der Hoeven et al., 1996) or the improvement of an already well-organized ancestral tetrapod appendage. In this respect, global recruitment of the *HoxD* and *HoxA* complexes in limbs may have occurred at different times to generate stepwise but different innovations (Zákány et al., 1997b).

In contrast, particular *Hoxd* genes can refine or complement these generic limb expression domains by using additional elements acting locally, as illustrated in this work by RXI (Fig. 6). It is therefore likely that the final expression profiles of *Hoxd* genes in limbs results from the concomitant action of multiple long- and short-distance-acting regulatory sequences. However, we have shown here that the RXI element is also involved in the expression of *Hoxd-12* during posterior trunk development, which suggests that RXI is necessary for a rather general regulatory function and is not restricted to the limbs. It is thus possible that expression of *Hoxd* genes in developing limbs is established as a result of two separate mechanisms. The first suggestion is a limb non-specific mechanism relying on the general activation of *Hoxd* genes in their major site of expression, i.e. in the trunk. In this case, *Hoxd* gene expression in posterior limbs may respond to the same activating signals as those acting in the trunk. This second suggestion is a limb-specific mechanism involving a regulatory balance between various enhancers sequences acting at distances and responsible for controlling expression in forearm and digits. In this context, it is interesting to note that the expression specificity driven by RXI, both in transgenic configurations and in its endogenous context, is reminiscent of the expression profile found for *Hoxd* genes during the development of the pectoral and pelvic fins in zebrafish, i.e. a restriction to the posterior margin along the entire length of the fin bud (Sordino et al., 1995). This observation may further reflect the linkage between that particular transcript domain and a rather general

activation of *Hoxd-12* in the developing trunk. In this view, the poor conservation observed at the level of the sequence when fish and chicken DNA were compared would still indicate an ancestral function for this particular DNA stretch in mediating the activation of *Hoxd-12* during embryogenesis.

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