Deletion of a *HoxD* enhancer induces transcriptional heterochrony leading to transposition of the sacrum

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A phylogenetically conserved transcriptional enhancer necessary for the activation of *Hoxd-11* was deleted from the *HoxD* complex of mice by targeted mutagenesis. While genetic and expression analyses demonstrated the role of this regulatory element in the activation of *Hoxd-11* during early somitogenesis, the function of this gene in developing limbs and the urogenital system was not affected, suggesting that *Hox* transcriptional controls are different in different axial structures. In the trunk of mutant embryos, transcriptional activation of *Hoxd-11* and *Hoxd-10* was severely delayed, but subsequently resumed with appropriate spatial distributions. The resulting caudal transposition of the sacrum indicates that proper vertebral specification requires a precise temporal control of *Hox* gene expression, in addition to spatial regulation. A slight time delay in expression (transcriptional heterochrony) cannot be compensated for at a later developmental stage, eventually leading to morphological alterations.

Keywords: gene targeting/*HoxD* complex/*loxP/Cre*/mouse development/regulatory mutation

Introduction

Tetrapod genomes contain four *Hox* complexes: *HoxA*, *B*, *C* and *D*. A total of 39 genes are distributed in these four loci which encode proteins playing an important function in the organization of the body plan (e.g. Krumlauf, 1994). This structural organization is reflected in the way genes are expressed, so that those *Hox* genes located closer to the 3' end of a given complex are expressed near the cephalic end of the body axis while genes located at the 5' end are expressed caudally. *Hox* genes belonging to all four complexes are silent until gastrulation (Gaunt *et al.*, 1986; Deschamps and Wijgerde, 1993) and become successively activated following a temporal sequence in which 3' genes are transcribed first, followed by more 5'-located genes (Izpisua-Belmonte *et al.*, 1991). Loss-of-

function mutations as well as gain-of-function approaches produce severe morphological alterations (e.g. Krumlauf, 1994), thereby illustrating both the importance of this gene family during axial morphogenesis and the necessity for precise mechanisms to coordinate *Hox* gene activation with the rostro-caudal progression observed during vertebrate ontogeny (e.g. Duboule, 1994).

While experiments involving conventional transgenesis have identified several sequences required for the proper spatial distribution of particular *Hox* transcripts (e.g. Whiting *et al.*, 1991; Gérard *et al.*, 1993; Vogels *et al.*, 1993; Knittel *et al.*, 1995; Shashikant and Ruddle, 1996), observations of either recombinant or partially deleted *HoxD* complexes have suggested that the temporal sequence of activation depends upon, or is safeguarded by, a high order control mechanism that prevents premature expression of individual genes (van der Hoeven *et al.*, 1996; Zákány and Duboule, 1996). Once properly activated, *Hox* gene expression needs to persist and be maintained in a faithful manner, a task that could be achieved in part by auto- and/or cross-regulatory mechanisms, as suggested by work carried out *in vitro* (Zappavigna *et al.*, 1991) or, most significantly, *in vivo* (Studer *et al.*, 1996; Gould *et al.*, 1997).

In a search for *cis*-acting elements involved in the activation of posterior *Hoxd* genes, we had previously defined a pair of non-coding sequence motifs located downstream of the *Hoxd-11* transcription unit (Gérard *et al.*, 1993). Both sequences were found to be highly conserved throughout vertebrates such that the mouse and fish versions can be virtually exchanged (M.Gérard *et al.*, submitted). One of these conserved blocks, region IX (RIX), acts as a transcriptional silencer which limits the anterior extent of expression of both *Hoxd-11* and the neighbouring *Hoxd-10* along the vertebral column, as revealed by *in vivo* targeted mutagenesis using embryonic stem (ES) cell technology (Gérard *et al.*, 1996). RIX is preceded by an even longer conserved sequence motif, region VIII (RVIII), which has been shown to be required for initial activation of *Hoxd-11-lacZ* reporter transgenes (Gérard *et al.*, 1993). In this latter case, a 7 kb genomic fragment was capable of eliciting expression at, and posterior to, pre-vertebra 25 (pv25). Removing RVIII from the transgene led to a loss of the *Hoxd-11* early expression pattern. Accordingly, when RVIII was placed in front of a heterologous promoter, expression in a broad posterior body region was observed at an early developmental stage (Gérard *et al.*, 1993).

In order to analyse whether RVIII plays a significant role in early *Hoxd* gene activation and to assess its full functional potential, we used an ES cell-based approach to delete it from its original context, within the *HoxD* complex. Mice homozygous for a deletion of RVIII displayed at high frequency a patterning defect localized

selectively at the lower lumbar and upper sacral vertebrae, at the level of the *Hoxd-11* anterior expression boundary. This alteration coincided with the absence of both *Hoxd-11* and *Hoxd-10* transcripts at embryonic day 9 (E9), a stage at which these genes are normally functional in pre-somitic precursors of the affected vertebrae. Twenty four hours later, however, accumulation of both genes' transcripts was restored in these precise pre-vertebral structures. This analysis of RVIII-deficient mice demonstrates an important role for this enhancer in the timely transcriptional activation of *Hoxd-11* along the main body axis, consistent with a potential involvement in the 3' to 5' activation mechanism acting upon the *HoxD* complex. It also illustrates that the precise moment in development at which a particular *Hox* gene is expressed, and not only its spatial domain of expression, is of great importance, since a correct but slightly delayed expression cannot compensate for an early expression deficit.

Results

Targeted deletion of region VIII

A targeting vector was engineered in which 330 nucleotides (nt) containing RVIII were deleted and replaced by the FRT site-specific recombination target sequence (Figure 1). A PGKneo selection cassette was introduced ~700 nt downstream, in the opposite transcriptional orientation with respect to that of *Hoxd* genes. This selection cassette was flanked by two similarly oriented *loxP* sites. D3 ES cell clones in which homologous recombination had occurred were isolated and characterized by Southern blotting (Figure 1). Positive clones were transferred into the germ line of chimaeric males, and mice homozygous for the deletion of RVIII were produced. The loss of RVIII was then verified further with additional restriction digests. This first allele was referred to as *Hoxd-11*^{RVIII^{Ge1}} (Figure 1). A second allele was generated by crossing *Hoxd-11*^{RVIII^{Ge1}} homozygous females to males expressing the *Cre* recombinase such that *loxP* site-specific recombination led to excision of the PGKneo selection cassette. The resulting *Hoxd-11*^{RVIII^{Ge2}} mice (Figure 1) were bred to homozygosity and maintained as a *Cre* recombinase-free stock. These two alleles were used to study both the function of RVIII and potential regulatory interferences produced by the selection cassette.

Phenotype of mice lacking region VIII

Upon visual inspection, mice homozygous for the deletion of RVIII (*Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}}) were indistinguishable from their wild-type littermates. However, skeletal preparations revealed that a significant proportion of them displayed a disturbed sacral morphology, with a sacrum starting one vertebra more posterior than in wild-type animals. Instead of the L6 (six lumbar vertebrae) vertebral type observed in both normal and heterozygous mice, mutant animals showed either an abnormal S1 (first sacral vertebra) in an otherwise L6 type, or a L7 (seven lumbar vertebrae) formula (Figure 2, Table I, *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}}). Thus, RVIII-deleted mice had a recessive defect in the vertebral column reminiscent of that seen in *Hoxd-11* loss-of-function alleles. In these latter cases, however, the prevalent vertebral type was L7, and

defects were also reported to affect limbs and male fertility (Davis and Capecchi, 1994, 1996; Favier *et al.*, 1995).

In order to confirm the allelism of this mutation with *Hoxd-11* as well as to better assess the role of RVIII, we combined the *Hoxd-11*^{RVIII^{Ge2}} allele with a variety of alleles involving either *Hoxd-11* or *Hoxa-11*, the paralogous gene on the *HoxA* complex. This was deemed necessary as these two genes, as well as other posterior *Hoxd* genes, had been shown to act mainly through quantitative balances of their products (e.g. Davis *et al.*, 1995; Davis and Capecchi, 1996; Kondo *et al.*, 1996; Zákány *et al.*, 1996). As for the case of *Hoxd-11* and *Hoxa-11* loss-of-function alleles (Davis *et al.*, 1995; Zákány *et al.*, 1996), we expected that the morphological alterations obtained upon deletion of RVIII would be more readily apparent by concurrently removing doses of the cooperating genes such as *Hoxa-11* and *Hoxd-11*–*Hoxd-13*. We therefore used loss-of-function alleles for either *Hoxa-11* (*Hoxa-11*^{Cin}; Small and Potter, 1993) or *Hoxd-11* (*Hoxd-11*^{Sir}; Favier *et al.*, 1995), as well as a deficiency removing the functions of all three *Hoxd-11*, *Hoxd-12* and *Hoxd-13* genes (*HoxD*^{Del}; Zakany and Duboule, 1996). The outcome of crosses involving these different alleles is summarized in Table I.

In the presence of two normal doses of *Hoxa-11*, more than half of *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}} animals had an abnormal L6, two of which showed an L7 formula. In wild-type control and heterozygous animals, only one out of 60 specimens showed a malformation of the first sacral vertebra, while no L7 specimens were observed (Table I). The expressivity of this phenotype dramatically increased when one or both copies of *Hoxa-11* were removed, such that seven out of 11 animals of the *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}}; *Hoxa-11*^{Cin/+} genotype had a transformation of S1 into L7, while all double homozygotes were L7 (Table I; Figure 2). Consistently, whenever the homozygous *Hoxa-11*^{Cin/Cin} background was used, deletion of one copy of RVIII led to full penetrance and expressivity of the L7 phenotype (six out of seven; Table I). These results were confirmed by the incidences of supernumerary accessory processes on L4 (Table I, L4). In summary, the phenotype of RVIII-deleted mice in the vertebral column mirrored that obtained upon inactivation of *Hoxd-11* (Favier *et al.*, 1995), though being somewhat hypomorph since double *Hoxd-11*/*Hoxa-11* mutant mice were of the L8 type (Davies *et al.*, 1995) while *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}}/*Hoxa-11*^{Cin/Cin} were L7.

This resemblance to the *Hoxd-11* knock-out phenotype was not found when limb skeletons were analysed. In contrast to *Hoxd-11* loss-of-function alleles, which led to severe forelimb defects when combined with either one or two *Hoxa-11* mutant alleles (Davis *et al.*, 1995), the *Hoxd-11* RVIII-deficient allele induced no limb defects when combined with *Hoxa-11*^{Cin/Cin} mice, other than those known to derive from *Hoxa-11* inactivation alone (Figure 3; Table I). The fact that *Hoxd-11* function in developing limbs was independent of the RVIII regulatory sequence was demonstrated further by using *HoxD*^{Del} mice, i.e. mice lacking *Hoxd-13*, *Hoxd-12* and *Hoxd-11* functions (Zákány and Duboule, 1996). While all animals trans-heterozygous for the deficiency and the *Hoxd-11* null allele (*HoxD*^{Del}/*Hoxd-11*^{Sir}) displayed strong alterations in digits and carpal bones, the latter being characteristic of *Hoxd-11*

Fig. 1. Targeted deletion of region VIII (RVIII) in the murine *HoxD* complex. (A) 5' region of the *HoxD* complex. Open arrows indicate transcriptional orientations. (B) Enlargement of the *Hoxd-11* to *Hoxd-10* locus; the black rectangle indicates the position of RVIII, and selected restriction sites are shown below. The dotted rectangles mark the positions of the 5'-flanking, internal and 3'-flanking genomic probes used to monitor homologous recombination events in ES cells as well as *Cre*-mediated deletion of the selection cassette in mice. The sizes of the various fragments are given above. P, *Pst*I; B, *Bam*HI; Xh, *Xho*I; H, *Hind*III; E, *Eco*RI; S, *Sma*I. (C) Structure of the locus after deletion of RVIII with the inserted PGKneo selection cassette (*Hoxd-11*^{RVIII^{Ge1}}). The PGKneo marker gene (*neo*) is in reversed transcriptional orientation and is flanked by two *loxP* sites (arrowheads in rectangles). The stars point to the extremities of the targeting vector and the open brackets indicate the deletion. The sizes of the newly generated restriction fragments are given above. (D) Structure of the RVIII-deficient locus after excision of the selection cassette (*Hoxd-11*^{RVIII^{Ge2}}). New restriction fragments are shown on the top and a unique *loxP* site is on the left. (E) Genomic blots of F2 mice. The same *Bam*HI blot of *Hoxd-11*^{RVIII^{Ge1}} individuals was probed sequentially with the 3' external (left) and the region VIII probe (middle). Note the absence of the region VIII-specific signal in the homozygous mutant (–/–) animal. On the right, an *Eco*RI blot of *Hoxd-11*^{RVIII^{Ge2}} animals, probed with the 5' external probe.

inactivation (essentially an abnormal fusion within the proximal row of carpal bones; see Davis and Capecchi, 1995; Favier *et al.*, 1995), none of the *HoxD*^{Del}/*Hoxd-11*^{RVIII^{Ge}} mice exhibited these alterations. Meanwhile, full penetrance and strong expressivity were recovered in the vertebral phenotype (Figure 3; Table I). Taken together, these genetic analyses demonstrated that: (i) RVIII deletion is allelic to *Hoxd-11* and (ii) RVIII is selectively involved in the specification of the vertebral column whereas it takes no part in the function of *Hoxd-11* during the development of the limbs and the urogenital apparatus. This latter point confirmed that the *Hoxd-11*^{RVIII^{Ge2}} allele resulted from a regulatory, rather than structural, mutation.

Hoxd gene expression in RVIII-deleted mice

In wild-type mid-gestation fetuses, *Hoxd-11* transcripts are normally detected mostly in limbs and in the genital bud, as well as along the main body axis. In the trunk, expression starts rather posteriorly since the anterior limit of expression in the spinal cord matches the 25th pre-vertebra while in paraxial mesoderm transcription starts at the 27th pre-vertebra (Dollé and Duboule, 1989; Dollé *et al.*, 1989, 1991; Izpisua-Belmonte *et al.*, 1991).

From the above genetic analysis, we expected *Hoxd-11* transcripts to be importantly down-regulated in the developing trunks of *Hoxd-11*^{RVIII^{Ge2}} mutant animals. Surprisingly, whole-mount *in situ* analyses of *Hoxd-11* tran-

script accumulation at E10 and subsequent stages in trunk paraxial mesoderm and neuro-ectoderm of mutant animals gave expression patterns identical to those of wild-type specimens. Examination of earlier developmental stages, however, led to a different conclusion: at E9, the mutant fetuses showed very reduced levels of *Hoxd-11*-specific signal at the anterior part of the expression domain, i.e. at the level of the 25–27th somites (Figure 4). Strikingly, at the 28th somite stage, no signal was detected between the 25th and 27th somites, while expression in the spinal

Fig. 2. Phenotypic changes at the lumbo-sacral transition in RVIII-deleted (*Hoxd-11*^{RVIII^{Ge2}}) adult mice. (A) Vertebral type observed in wild-type mice with six lumbar vertebrae (L6) and a normal first sacral vertebra (S1), corresponding to the 27th position. (B) Most frequent phenotype (indicated as L6 in Table I) detected in RVIII-deleted homozygous mice showing an S1 partially transformed into lumbar character at its anterior part (white arrow). S3 also shows some features of a wild-type S2 vertebra, with lateral processes completely orthogonal to the main axis (arrowheads in A and B). (C) Completely transformed S1 vertebra displaying a wild-type lumbar morphology (L7, white arrow). The first sacral vertebra is at the 28th position. The 29th vertebra (S3 in wild-type) is also transformed into an S2 (28th) type. The incidence of this vertebral formula (~10% among *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}} animals) increased to 100% in double mutant animals *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}}; *Hoxa-11*^{Cin/Cin}; see Table I for details).

cord was observed at the level of somite 26 and posteriorly. Expression in the emerging hindlimb buds and in the tail bud region was present in RVIII mutant embryos, even at these early stages (Figure 4), and no visible change of expression in limbs and genitalia of mutant animals were observed subsequently.

Since region VIII is located between *Hoxd-11* and *Hoxd-10* (Figure 1), and because the 3' immediately adjacent region IX has been shown to be shared by these two neighbouring genes (Gérard *et al.*, 1996), we analysed *Hoxd-10* expression in *Hoxd-11*^{RVIII^{Ge2}} mice. In early wild-type E9 embryos (<27 somites), expression of *Hoxd-10* was weakly detected in the 24th pair of somites and became progressively stronger at the 25th, 26th and 27th somite levels. The anterior limit of expression was the same in both spinal cord and paraxial mesoderm, and extended posteriorly into the tail bud region. The incipient forelimb field in lateral plate mesoderm was also strongly positive for *Hoxd-10*. RVIII mutant fetuses from this stage, as well as from earlier stages, displayed dramatically reduced accumulation of *Hoxd-10* transcripts in the limb field and tail bud (Figure 4). However, as seen with *Hoxd-11*, the *Hoxd-10* expression pattern at E10 was resumed and appeared identical when homozygous RVIII-deficient mice were compared with normal littermates.

Insertional regulatory mutagenesis at the *Hoxd-11* locus

The phenotypic analysis of *Hoxd-11*^{RVIII^{Ge1}} homozygous mice, i.e. mice without RVIII but still containing the PGKneo selection cassette (before exposure to the *Cre* enzyme; Figure 1), gave a related though clearly different result. Homozygous mice of this configuration displayed vertebral defects also at the lumbo-sacral transition, but both the expressivity and penetrance largely exceeded the figures obtained with the PGKneo excised allele. This difference was accentuated by the presence of a highly penetrant abnormal phenotype in the carpus, where proximal carpal bone fusions were scored in most cases

Table I. Patterning defects in mice deleted for RVIII in various genetic backgrounds

Genotypes ^a	No.	Mice with skeletal defects			
		Vertebral			Carpal
		L4	L6	L7	
<i>Hoxd-11</i> ^{+/+} ; <i>Hoxa-11</i> ^{+/+}	(29)	4	–	–	–
<i>Hoxd-11</i> ^{RVIII^{Ge2}/+} ; <i>Hoxa-11</i> ^{+/+}	(31)	2	1	–	–
<i>Hoxd-11</i> ^{RVIII^{Ge2}/RVIII^{Ge2}} ; <i>Hoxa-11</i> ^{+/+}	(19)	14	14	2	–
<i>Hoxd-11</i> ^{+/+} ; <i>Hoxa-11</i> ^{Cin/+}	(6)	1	–	–	–
<i>Hoxd-11</i> ^{RVIII^{Ge2}/+} ; <i>Hoxa-11</i> ^{Cin/+}	(11)	7	1	2	1
<i>Hoxd-11</i> ^{RVIII^{Ge2}/RVIII^{Ge2}} ; <i>Hoxa-11</i> ^{Cin/+}	(11)	11	4	7	–
<i>Hoxd-11</i> ^{+/+} ; <i>Hoxa-11</i> ^{Cin/Cin}	(9)	–	–	1	6
<i>Hoxd-11</i> ^{RVIII^{Ge2}/+} ; <i>Hoxa-11</i> ^{Cin/Cin}	(7)	6	–	6	4
<i>Hoxd-11</i> ^{RVIII^{Ge2}/RVIII^{Ge2}} ; <i>Hoxa-11</i> ^{Cin/Cin}	(2)	2	–	2	2
<i>Hoxd-11</i> ^{RVIII^{Ge2}/Del}	(5)	5	2	3	–
<i>Hoxd-11</i> ^{Str/Del}	(3)	3	–	3	3
<i>Hoxd-11</i> ^{RVIII^{Ge1}/RVIII^{Ge1}}	(22)	17	7	11	22
<i>Hoxd-11</i> ^{RVIII^{Ge1}/Str}	(7)	n.d.	3	2	7
<i>Hoxd-11</i> ^{RVIII^{Ge1}/+}	(34)	10	–	–	1

^aAllele symbols: the *Hoxa-11*^{Cin} allele is a loss-of-function allele produced in Cincinatti (Small and Potter, 1993) while the *Hoxd-11*^{Str} allele is a loss-of-function allele produced in Strasbourg (Favier *et al.*, 1995). The *Hoxd-11*^{RVIII^{Ge2}} and ^{Ge1} alleles are deletions of region VIII in the absence or presence of the PGKneo cassette, respectively (see Figure 1). n.d.; not determined.

Fig. 3. *Hoxd-11* function in limb development is independent of region VIII control. Skeletal preparations of adult forelimbs. (A) Mice homozygous for RVIII deletion (*Hoxd-11*^{RVIIIge2/RVIIIge2}). These hands were indistinguishable from those of wild-type animals. (B) Double homozygous *Hoxd-11*^{RVIIIge2/RVIIIge2}; *Hoxa-11*^{Cin/Cin} animals. The pisiform (pi) and pyramidal (py) bones were fused (arrowhead) and attached to the navicular-lunate (nl, arrow). The distal ends of both radius (r) and ulna (u) were abnormally broad. All these defects are characteristic of the *Hoxa-11*^{Cin/Cin} genotype alone (Table I). (C) Animals trans-heterozygous for RVIII deletion and a *HoxD* deficiency inactivating *Hoxd-13*, *Hoxd-12* and *Hoxd-11* functions (*Hoxd-11*^{RVIIIge2/Del}). Size reductions of the second phalanges (P2) of digits II and V were scored (arrowheads), typical of the defects routinely found in *Hoxd-11*^{Del/+} allele alone. (D) For comparison, an animal trans-heterozygous for both the same *HoxD* deficiency and a loss-of-function allele is shown (*Hoxd-11*^{Del/Str}). In such hands, the complete loss of the second phalanges of digits II and V (arrowheads) were combined with defects in the P2s of other digits, as well as with fusions of proximal carpal elements.

(Figure 5; Table I). Nevertheless, in contrast to the homeodomain disruption alleles (Davis and Capecchi, 1994; Favier *et al.*, 1995), involvement of the pisiform bone in these fusions was rare, and more distal structures remained unaffected. Likewise, RVIII mutant homozygous males containing the PGKneo cassette (*Hoxd-11*^{RVIIIge1}) were fully fertile while *Hoxd-11* mutant mice were hypofertile. Both the vertebral and limb defects were recovered in *Hoxd-11*^{RVIIIge1/Str} trans-heterozygote animals (Figure 5, Table I), suggesting allelism with the *Hoxd-11* locus. This recessive phenotype was thus hypomorphic and showed a large subset of the defects observed in mice lacking *Hoxd-11* function. These genetic studies suggested that the PGKneo transcription unit probably interfered with the proper regulation of *Hoxd-11*.

To verify this point, we analysed the expression of *Hoxd-11* in *Hoxd-11*^{RVIIIge1} homozygous mice by whole-mount *in situ* hybridization. RNA accumulation showed a strong difference with respect to either the wild-type or the RVIII-deficient, PGKneo-less, mice. In the trunk, *Hoxd-11* transcript accumulation was severely delayed (for longer than in PGKneo-deleted mice; not shown). An important reduction in transcript accumulation was observed also in limb buds at E10 (not shown), as well as in a region immediately proximal to the forelimb autopod at E13 (Figure 5). In this prospective wrist area, *Hoxd-11* transcripts were not detected in the expected anterior proximal domain (Figure 5, arrowhead). In contrast, hybridization using a *neo*-specific probe indicated that the PGK promoter was particularly active in this domain, as judged by accumulation of *neo* gene transcripts (Figure 5, arrowhead). In this case, the overall expression pattern of the *neo* gene was clearly reminiscent of that shown by the neighbouring *Hoxd-11* gene, further indicat-

ing that the PGK promoter responded to the adjacent *Hox* regulatory signals (Rijli *et al.*, 1994; van der Hoeven *et al.*, 1996). A precise correspondence was established in this domain between a strong expression of the *neo* gene, a down-regulation of *Hoxd-11* and the occurrence of a *Hoxd-11*-specific carpal phenotype. This suggested that part of the regulatory interactions leading to the expression of *Hoxd-11* in this particular part of the carpus had been shifted towards the PGK promoter complex. Therefore, in the lumbo-sacral region, the penetrance and expressivity of the RVIII-deleted phenotype substantially increased in mice with the PGKneo cassette, due to the combined effects of the two mutagenic mechanisms, i.e. the deletion of an activating element and promoter competition or interference caused by the insertion of the selective marker. In the forelimb, the latter component induced a transcriptional suppression and consistent limb defect.

Discussion

We report here the functional analysis of a *Hoxd-11* regulatory sequence, region VIII, which originally was defined as being required for transcriptional activation of a *Hoxd-11* transgene (Gérard *et al.*, 1993). Using ES cell technology, we have deleted this element *in vivo* and produced two different alleles, one in which the PGKneo selection cassette was left in place, the other in which it was excised. Most of the alterations observed in homozygous *Hoxd-11*^{RVIIIge1} mice (with PGKneo) were a consequence of the activity of the nearby located PGK promoter, probably through promoter competition with the flanking resident *Hox* promoter(s). The differences observed in the phenotypes of *Hoxd-11*^{RVIIIge1} and *Hoxd-11*^{RVIIIge2} mutant

Fig. 4. *Hoxd-11* transcript accumulation at E9 in RVIII-deleted mice, as detected by whole-mount *in situ* hybridization. (A) *Hoxd-11* expression in 20, 22 and 24 somite stage wild-type embryos. (B) *Hoxd-11* expression in corresponding *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}} embryos. In mutant embryos, the signal was more restricted at any given stage, and is absent from dorsal structures (arrowheads). (C) *Hoxd-11* expression in 20 somite stage wild-type (left) and *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}} (right) embryos. Note the strong hybridization signal in the entire tail bud and non-segmented mesoderm region, in wild-type embryo, while the signal was absent from most of the tail bud and from the entire paraxial mesoderm in mutant embryos. The signal in mutant tail bud was localized to the ventral mesoderm lining the posterior pole of the coelom. (D) *Hoxd-11* expression in 27 somite stage wild-type (right) and mutant (left) embryos. Note the absence of signal in the 25–27 somite domain (arrowheads). (E) *Hoxd-10* expression in 26 somite stage wild-type (right) and mutant (left) embryos. Note the reduced signal in the 26–27 somite domain (brackets). In the embryos shown in (D), the signals in forelimb buds were indistinguishable between mutants and wild-type. Likewise, mutant and wild-type embryos showed equally strong hybridization signals with both probes in the respective expression domains at E10 and later (not shown).

mice show that a foreign promoter can elicit abnormal regulation of the nearby genes, and emphasize the absolute need to eliminate selection cassettes, especially when regulatory mechanisms or functions are investigated in clusters of genes (see also Héroult *et al.*, 1996; Olson *et al.*, 1996; van der Hoeven *et al.*, 1996). In contrast, the *Hoxd-11*^{RVIII^{Ge2}} (PGK^{neo} minus) allele produced patterning defects, linked to specific alterations in *Hoxd-11* gene transcription. These regulatory mutations have revealed important aspects of *Hox* gene regulation, in particular concerning (i) the existence of discrete regulatory controls at work in trunk and in limbs, (ii) the multi-phasic aspect of *Hox* gene expression (activation versus maintenance), (iii) the functional importance of a precise time control and (iv) the potential impact of transcriptional heterochrony upon evolutionary variations in morphologies.

A *Hoxd* trunk-specific regulatory region

Over two-thirds of the RVIII mutant *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}} mice showed defective morphogenesis of the 24th and 27th vertebrae. The 24th (the fourth lumbar vertebra) displayed an additional accessory process on the posterior edge of the neural arch, thus suggesting an anterior transformation into an L3 type (a third lumbar) vertebra. This transformation of vertebral identity matched with the upper rostral skeletal alteration reported in animals lacking the full *Hoxd-11* function (Favier *et al.*, 1996; Zákány *et al.*, 1996). The 27th vertebra (the first sacral vertebra; S1) showed partial or complete transformation into a terminal lumbar type (L6), and the penetrance and expressivity of this transformation was much enhanced in a *Hoxa-11* mutant background. Again, this was reminiscent of the *Hoxd-11* loss-of-function alleles. Consistently, animals trans-heterozygous for both the RVIII mutation

Fig. 5. Alterations in the limbs of *Hoxd-11*^{RVIII^{Ge1}/RVIII^{Ge1}} mice (containing the PGKneo cassette). (A) Skeletal preparations of a juvenile *Hoxd-11*^{RVIII^{Ge1}/RVIII^{Ge1}} forelimb showing fusion of cartilage and ossification centres between the pyramidal and navicular-lunate (arrow in left panel). In the middle, a trans-heterozygous *Hoxd-11*^{RVIII^{Ge1}/Str} paw which shows similar (though partial) alterations, i.e. cartilage fusion of the same proximal carpal elements (arrow); for comparison, a homozygous *Hoxd-11*^{RVIII^{Ge2}/RVIII^{Ge2}} paw is shown on the right (indistinguishable from wild-type). (B) *Hoxd-11* down-regulation in presumptive carpal structures. Whole-mount *in situ* hybridization of *Hoxd-11* transcripts in E13 forelimbs of various genotypes. Wild-type (left), *Hoxd-11*^{RVIII^{Ge1}/+} (middle) and *Hoxd-11*^{RVIII^{Ge1}/RVIII^{Ge1}} (right). In the proximal autopod domain (arrowheads), transcript accumulation is reduced in the heterozygotes and is not detectable in homozygous animals. (C) *neo* gene expression is detected concomitantly with *Hoxd-11* down-regulation. Whole-mount *in situ* hybridization of *neo*-specific transcripts in a E13 heterozygous *Hoxd-11*^{RVIII^{Ge1}/+} animal. A clear signal is detected in the same proximal autopod domain where *Hoxd-11* transcription is silenced (arrowhead). In fact, down-regulation of *Hoxd-11* is observed wherever strong *neo* expression appears.

and a triple *HoxD* loss-of-function (*HoxD*^{Del}) showed a combination of the above vertebral transformations, indicating a significant loss of *Hoxd-11* function associated with the RVIII-deficient chromosome.

Interestingly, deletion of RVIII did not affect *Hoxd-11* function in the other domains where this gene is required, such as the limbs and the urogenital apparatus, even when this allele was present in combination with other mutant backgrounds. This detailed genetic analysis clearly indicated that RVIII is required mostly during the development of the vertebral column. Consequently, different regulatory circuits are probably responsible for the expression and function of *Hoxd-11* in other structures. This confirms and extends previous observations describing the existence of a global and remote enhancer element controlling *HoxD* gene expression in distal limbs (van der Hoeven *et al.*, 1996). In a phylogenetic context, this suggests that successive (late occurring) recruitment of novel sites of function for *Hoxd-11* in the course of evolution (e.g. the limbs or metanephric kidneys) were probably accompanied by the involvement of novel regulatory sequences (a modular

regulation), in contrast to the alternative use of pre-existing elements to achieve different purposes.

Hoxd gene expression analysis in RVIII mutant embryos conformed to this view, since loss of *Hoxd-11* transcript accumulation was detected exclusively in paraxial mesoderm, in a domain corresponding to the presumptive sites of the future vertebral alterations. *Hoxd-10* transcript accumulation was also reduced in paraxial mesoderm, thereby demonstrating that these two neighbouring genes share a RVIII-dependent regulation *in vivo*. The concomitant use of regulatory sequences between *Hox* genes in the context of the *Hox* complex has already been reported for the immediately adjacent region IX (Gérard *et al.*, 1996). Interestingly, however, region IX is involved in a shared repressive mechanism necessary to position the spatial expression boundaries correctly. In contrast, our deletion of region VIII suggests the sharing of enhancer activity required for the proper transcriptional timing of neighbouring genes. At this level of resolution, however, we cannot exclude the presence of two separate sub-elements, one used exclusively by *Hoxd-10*, the other by *Hoxd-11*, or,

alternatively, a primarily *Hoxd-10*-mediated effect, if that gene served as a principal activator of early *Hoxd-11* transcription. Sharing of *Hox* regulatory elements also has been documented in transgenic contexts (e.g. Gould *et al.*, 1997) and might be one of the factors responsible for the conservation of a tight clustering throughout vertebrate evolution (e.g. Duboule, 1992; Krumlauf, 1994).

Importance of temporal control

Expression analyses of *Hoxd-11* and *Hoxd-10* in RVIII mutant animals indicated that an important transcript deficit at an early stage was responsible for the subsequent alterations in the vertebral column. In this respect, RVIII can be qualified as an early acting enhancer element necessary for proper transcriptional onsets of at least *Hoxd-11* and *Hoxd-10*. However, the expected transcript domains for these two genes were resumed soon after, such that no difference could be observed between mutant and wild-type animals from day 10 onwards. This observation shed light on two interesting aspects of *Hox* gene function and regulation. First, it indicates that the observation of an appropriate spatial distribution of *Hox* transcripts at a given developmental stage does not necessarily mean that the complete function will be achieved. It demonstrates that a precise requirement exists for these transcripts to be there at the right time, i.e. at the time when the structures in which they normally exert their functions will need (or be receptive to) the information. The importance of the transcriptional timing of *Hox* gene expression in the specification of different segments has been shown previously with the *Drosophila Ubx* gene (Castelli-Gair and Akam, 1995). Our results emphasize the need for a strict control of expression timing, hence the key role played by temporal co-linearity (Izpisua-Belmonte *et al.*, 1991), a mechanism which may be used to coordinate the activations of *Hox* genes along their complexes (van der Hoeven *et al.*, 1996).

Secondly, these results further suggest that the establishment of late rostral expression boundaries in the trunk may not depend strictly upon the time of *Hox* gene activation (see also van der Hoeven *et al.*, 1996), thus arguing in favour of a multiphasic control of *Hox* gene activation and expression. In such a scheme, RVIII would be used to adjust precisely the transcriptional onset of genes which, in the absence of RVIII, would be activated in rather faithful spatial domains, but not at the proper time. It has been argued that an important role for transcriptional enhancers is to antagonize the formation of repressive chromatin structures (Walters *et al.*, 1996). Such a mode of action of RVIII could be compatible with our observation that an overall repressive mechanism is acting on the posterior region of the *HoxD* complex to maintain genes silent (van der Hoeven *et al.*, 1996). Once started, gene expression and maintenance may in turn depend upon general *Hox* activators (e.g. *cdx*, Subramanian *et al.*, 1995) and/or positively acting mechanisms of auto- and cross-regulations (Zappavigna *et al.*, 1991; Studer *et al.*, 1996). Among those genes which potentially are involved in the early regulation of *Hoxd-11*, and which may thus interfere with region VIII function, genes such as *bmi-1* and *MLL/HRX/ALL-1* are good candidates, as their inactivations affect the lumbo-sacral transition (van

der Lugt *et al.*, 1994; Alkema, *et al.*, 1995, 1997; Yu *et al.*, 1995).

Hoxd-11 and the lumbo-sacral transition

In the tail buds of normal mouse fetuses, *Hoxd-11* transcripts are first detected at the 14th somite stage, on embryonic day 9. Transcription lasts, at least in some cells, until complete ossification has occurred, long after birth. The deletion of region VIII and concurrent delay in gene activation showed that an absence of RNA accumulation during the first 24 h led to a patterning defect that could not be corrected for by the subsequent normalization of expression. Therefore, gene function was required up to 5 days before a morphological identification of the first sacral vertebra became possible (at ~E14). A transitory deficit in the *Hoxd-11* gene product in pre-somitic mesoderm and early somites was enough to change their developmental fates. This indicates that the functional domain of a particular *Hox* gene should not be inferred from its expression at a late (and convenient) developmental stage such as the appearance of the pre-vertebral column.

In fully transformed (L7 type) animals, the transverse processes of the 28th vertebra established contacts with a relatively more posterior section of the os ilium (Figure 2). Consequently, the capacity of a normal 27th somite to generate a pre-vertebra capable of responding to the ilium by enhanced growth of its transverse processes (to become the first sacral vertebra) may also depend upon the presence of a sufficient dose of the appropriate group 11 gene products, *Hoxd-11* in particular. The reduced *Hoxd-10* transcript accumulation is also likely to contribute to the defects in the lumbo-sacral transition in RVIII mutants, as mutations within genes from groups 9–11 were all reported to affect this region in a similar way (Favier *et al.*, 1996; Fromental-Ramain *et al.*, 1996). The analysis of *Hoxd-10* mutant alleles will be informative in this respect.

Transcriptional heterochrony and the evolution of morphologies

As a consequence of their functional importance in organizing body plans, *Hox* genes were proposed to have been instrumental in the evolution of morphologies (e.g. Duboule, 1994; Burke *et al.*, 1995; Carroll, 1995). With respect to the vertebral column, the correspondence between the combination of *Hox* genes expressed at a particular level and the morphology of the derived vertebra suggests that modifications in expression along the AP axis may have been the source of vertebral transposition, and thus be linked to the origin of the various vertebral formulae found amongst tetrapods (Gaunt, 1994; Burke *et al.*, 1995). The mutation of RVIII may provide an interesting paradigm in this context, since the AP level at which hindlimbs articulate, i.e. the numerical level of the first sacral vertebra along the column (level 27 in mice), has been subject to considerable remodelling throughout tetrapod evolution (Todd, 1922). While close relatives of extinct stem tetrapods had 30 pre-sacral vertebrae (Coates, 1994), extant turtles have 18 and mammals have between 36 and 23 (Lessertisseur and Saban, 1967). Our results suggest that one of the potential mechanisms acting to induce such morphological transpositions relied on

transcriptional heterochronies, i.e. slight variations in the timing of expression of *Hox* genes. In this particular case, activating group 11 genes too early or too late would certainly shift the lumbo-sacral transition by several vertebrae anteriorly (Gérard *et al.*, 1996) or posteriorly (Davies *et al.*, 1995; Zákány *et al.*, 1996), respectively.

The effect of RVIII deletion on vertebral structures and its lack of effect on limb morphology may also indicate why such evolutionary transpositions were not obligatorily realized at the expense of either the limb archetype or the pentadactyl formula. Even though *Hoxd-11*, together with *Hoxd-12* and *Hoxd-13* (Zákány and Duboule, 1996), is involved in the generation of a stable pentadactyl fore- and hindlimb pattern, the independence of the two regulatory mechanisms may have allowed the modification of one without dramatically affecting the other. Thus, posterior *Hoxd* genes may have been used differently in the trunk to help vertebral transposition while their functions in limbs could remain virtually unchanged. The pivotal role of regions VIII (this work) and IX (Gérard *et al.*, 1996) in controlling *Hoxd* genes in vertebrae without influencing their functions in limbs suggests that variations in these two regulatory regions could contribute to lumbo-sacral transposition in a rather efficient way.

Materials and methods

Targeting vector and generation of mutant mice

For the targeting vector used for homologous recombination, a *loxP*-PGKneo-*loxP* cassette was used (gift of P.Kastner) flanked by the 5.5 kb *EcoRI*-*XhoI* *Hoxd-11*-*Hoxd-10* intergenic fragment used as 3' homology region (Gérard *et al.*, 1996). The 5' homology region consisted of the 2 kb *SmaI*-*EcoRI* genomic *Hoxd-11* fragment, in which the 326 bp *PstI*-*HindIII* fragment, containing RVIII (Gérard *et al.*, 1993), was replaced by the 68 bp *HpaI*-*HindIII* sequence, derived from pGem-FRT-2/Flp (a gift of S.Fiering and M.Groudine). Ligating *HpaI* to blunted *PstI*, and the *HindIII* site to the *HindIII* site of *Hoxd-11* placed an FRT sequence with its polarity reversed with respect to the 5'-3' polarity of the *HoxD* complex. This construct was linearized with *XhoI* prior to electroporation into D3 ES cells (Doetschman *et al.*, 1985; gift of R.Kemler). ES cell cultures, electroporation, G418 selection and genomic DNA isolation were done as reported previously (e.g. van der Hoeven *et al.*, 1996). Two homologous recombinant clones (*Hoxd-11*^{RVIII^{Ge1}, clones 6 and 48) were injected into C57Bl/6Jlco blastocysts and were established as germ line chimaeras by crossing to C57Bl/6Jlco females. Agouti F1 offspring were genotyped, and positive individuals were interbred to produce F2 progeny. Homozygous females from the clone 6-derived line were crossed to homozygous males of the CMVcre deleter strain (gift of J.Brocad and P.Chambon). The F1 progeny was genotyped, and neo minus individuals (*Hoxd-11*^{RVIII^{Ge2}) were selected and intercrossed to obtain F2 progeny. Selected F2 individuals were bred further to establish the *Hoxd-11*^{RVIII^{Ge2} stock, free of the CMVcre transgene. After removal of the PGKneo selection cassette, the genomic locus still contained one FRT site and one *loxP* site at the positions of either the RVIII deficiency or the original insertion site of the selection cassette, respectively. We consider it unlikely that these sites severely interfere with transcription of the nearby genes, even though we cannot formally exclude such a possibility.}}}

Genotyping of animals

Genomic Southern strategies used to identify the homologous recombination events in ES cells and genotyping of F1 and F2 individuals are summarized in Figure 1. The 5' external probe was a *HindIII*-*BglII* 777 bp fragment; the internal probe a *PstI*-*HindIII* 326 bp fragment and the 3' external probe a *PstI* 830 bp fragment. The loss of RVIII was verified on blots of F2 individuals using a region VIII-specific probe (Figure 1E, left and middle panels). For routine genotyping, the *EcoRI* digest allowed simultaneous identification of the wild-type (7 kb), *Hoxd-11*^{RVIII^{Ge1} (9 kb) and *Hoxd-11*^{RVIII^{Ge2} (15 kb) genotypes with either the 5' external or shared internal probes. Half the total DNA isolated from}}

yolk sac of individual E9 embryos was sufficient for genotyping with the digoxigenin-labelled probes and the chemiluminescence detection protocol used throughout these experiments.

Skeletal analysis and whole-mount *in situ* hybridizations

Newborn and 5-week-old F2 individuals were analysed from both *Hoxd-11*^{RVIII^{Ge1} and *Hoxd-11*^{RVIII^{Ge2} stocks to detect alterations in skeletal patterning. Identical types and incidences of carpal and vertebral alterations were obtained with the two *Hoxd-11*^{RVIII^{Ge1} lines (clones 6 and 48). Analysis of embryos was carried out both on genotyped F2 embryos (*Hoxd-11*^{RVIII^{Ge1} *Hoxd-11*^{RVIII^{Ge2}) and staged embryos from wild-type and homozygous mutant parents (*Hoxd-11*^{RVIII^{Ge2}). Skeletal preparations were carried out according to standard procedures. Whole-mount *in situ* hybridizations were done as reported previously (*Hoxd-11* and *Hoxd-10*: Gérard *et al.*, 1996; *neo*: van der Hoeven *et al.*, 1996).}}}}}}

Mutant stocks and crosses

To control allelism with *Hoxd-11*, *Hoxd-11*^{RVIII^{Ge1}/Ge1 mice were crossed with *Hoxd-11* loss-of-function heterozygous mice (*Hoxd-11*^{Str}, Favier *et al.*, 1995), and skeletons were analysed 6 days after birth. To establish genetic interactions with *Hoxa-11*, we crossed compound heterozygotes of the *Hoxd-11*^{RVIII^{Ge2} allele with *Hoxa-11*^{Cin} (Small and Potter, 1993), and analysed the F2 progeny at 5 weeks. To control the absence of RVIII function in limb patterning further, we crossed *Hoxd-11*^{RVIII^{Ge2}/+ heterozygous males to homozygous and heterozygous *HoxD*^{Del} females (Zákány and Duboule, 1996) and analysed skeletons of 5-week-old progeny. *Hoxd-13*, *Hoxd-12* and *Hoxd-11* gene products are not produced from the *Del* chromosome and trans-heterozygote animals with mutant alleles of either of these three genes produce severe limb defects (e.g. *Hoxd-11*^{Str/Del} in Figure 3D). *Hoxd-11*^{RVIII^{Ge1}, *HoxD*^{Del} (Zákány and Duboule, 1996) and *Hoxd-11*^{Str} (Favier *et al.*, 1995) were established on the C57Bl/6J×129 hybrid background. The CMVcre deleter strain used in generating the *Hoxd-11*^{RVIII^{Ge2} was originally established on the C57Bl/6×SJL hybrid background (Dupé *et al.*, 1997). The *Hoxa-11*^{Cin} allele was originally established on a C57Bl/6×129×CF-1 hybrid background (Small and Potter, 1993). Both *Hoxa-11*^{Cin} and CMVcre deleter strains were crossed into the C57Bl/6Jlco×129 hybrid background to obtain the compound heterozygous mice used in the test crosses.}}}}}

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