Functional cooperation between the non-paralogous genes Hoxa-10 and Hoxd-11 in the developing forelimb and axial skeleton

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SUMMARY

The Abdominal B-related Hoxa-10 gene displays similar expression patterns in the differentiating forelimbs and hindlimbs of the mouse, with preferential expression around the humeral and femoral cartilages and more diffuse expression in distal regions. We found that a targeted disruption of Hoxa-10 has almost no effect in the forelimbs, while it affects the proximal hindlimb skeleton. The alterations were located along the dorsolateral side of the femur (labium laterale), with an enlargement and distal shift of the third trochanter, a misshapen lateral knee sesamoid, a supernumerary ‘ligament’ connecting these structures and an occasional duplication of the femoral trochlea. Some Hoxa-10−/− mutant mice developed severe degenerative alterations of the knee articulation upon ageing. Viable Hoxa-10/Hoxd-11 double mutant mice were produced by genetic intercrosses. The compound mutation resulted in synergistic forelimb phenotypic alterations, consisting of: (i) an exacerbation of Hoxd-11−/− phenotypic traits in the carpal and digital region, e.g. more pronounced truncations of the ulna styloid, pyramidal and pisiform bones and of some metacarpal and phalangeal bones and (ii) marked alterations in a more proximal region which is nearly unaffected in Hoxd-11−/− single mutants; the entire radius and ulna were truncated and thickened, with deformations of the ulna proximal extremity. Thus, functional redundancy can occur even between non-paralogous Abdominal B-related Hox genes. The double Hoxa-10/Hoxd-11 mutation also conferred full penetrance to the sacral and caudal vertebrae transformations which are ~50% penetrant in Hoxd-11−/− single mutants, revealing that functional cooperation can also occur between non-paralogous Hox gene products in axial skeleton patterning.

Key words: Hox genes, gene disruption, pattern formation, functional redundancy, combinatorial code, Hoxa-10, Hoxd-11, mouse, axial skeleton, forelimb

INTRODUCTION

Among the 38 members of the murine Hox family, 14 genes related to the Drosophila Abdominal B gene and located at the 5′ extremity of the HOXA, HOXC and HOXD complexes are likely to be involved in limb morphogenesis. The Hoxa and Hoxd genes are activated sequentially, and are expressed in nested proximodistal (P-D) domains along the developing forelimb and hindlimb buds, the most 5′-located gene transcripts being restricted to the autopods (the footplates) of the limbs (Dollé et al., 1989; Haack and Gruss, 1993; Duboule, 1992). The expression patterns of Hox genes are different from those of their Hoxa and Hoxd paralogues, and also different in forelimbs and hindlimbs (Peterson et al., 1994). Targeted mutations of Hoxd-11 (Davis and Capecchi, 1994; Favier et al., 1995), Hoxd-13 (Dollé et al., 1993) and Hoxa-11 (Small and Potter, 1993) affect restricted regions of the distal forelimb and hindlimb skeleton, the alterations generated by Hoxa-11 and Hoxd-11 mutations extending more proximal than those of the Hoxd-13 mutation. All three mutations also produced distinct vertebral transformations in the lumbosacral region.

As a 3′-located Abdominal B-related gene, Hoxa-10 is expressed up to very proximal regions of the developing limbs (Haack and Gruss, 1993; see below). We (Rijli et al., 1995) and others (Satokata et al., 1995) have inactivated the Hoxa-10 gene and found that this resulted in thoracic and lumbar vertebral transformations, as well as specific genital abnormalities. We describe here the Hoxa-10 mutant limb phenotype, which is essentially restricted to proximal regions of the hindlimbs. In addition, to investigate whether the expression of Hoxa-10 up to the distal extremities of the developing limbs is functionally significant, we have produced mice mutated for both Hoxa-10 and Hoxd-11 by genetic intercrosses. We show that these two mutations act synergistically to produce marked alterations in the skeletal elements already affected by the Hoxd-11 mutation, and also in more proximal forelimb regions. Moreover, axial skeletal analysis revealed an increased penetrance in double mutant mice, of the lumbar and sacral transformations...
inconsistently found in both single Hoxa-10 and Hoxd-11 mutants.

MATERIALS AND METHODS

Hoxa-10 and Hoxd-11 gene targeting

The production of Hoxa-10+/− and Hoxd-11+/− mutant mice by homologous recombination in ES cells has been described elsewhere (Rijli et al., 1995; Favier et al., 1995). The Hoxa-10 gene was disrupted by insertion of a PGK promoter-neomycin resistance minigene into the XhoI site of the homeobox. The Hoxd-11 mutation was generated by inserting a pMC1 neomycin resistance minigene after deletion of the intron and part of the second exon of the gene. Both targeted mutations disrupt the coding sequence within and C-terminal to the homeodomain, thus preventing the synthesis of truncated gene products able to bind DNA.

Double Hoxa-10/Hoxd-11 mutants were produced by successive intercrosses both in a 129/Sv inbred and a C57BL/6-129/Sv hybrid genetic background. The occurrence of each compound genotype was checked at 3-weeks post-partum. The recorded numbers were compared to those expected from Mendelian inheritance by a χ² test.

Skeletal analysis of mutant mice

The alizarin red/alcan blue skeletal staining procedure has been described previously (Dollé et al., 1993). The length of the metacarpals, first and second phalanges of digit II, III, IV and V, radius, ulna, humerus, scapula, metatarsal III, tibia, femur and pelvic belt were recorded for 5 to 7 animals of each compound genotype, either by direct measurements or using a fixed magnification on a display monitor. Bone length reductions were calculated by comparison with the mean length of the left and right corresponding bones of a control animal (wild type (WT) or heterozygous for only one of the two mutations) from the same litter and killed at the same age (3-4 months). Initial comparisons showed no significant bone size alteration in single heterozygous animals compared to WT. The mean reduction rate was then calculated for each genotype. Less extensive data have also been collected for 1-, 3- and 6-day-old double mutant animals.

For the analysis of the vertebral skeleton, the limits of the transformed region was sometimes equivocal because (i) the natural individual variability in mammal vertebral morphology is greater in the lumbar region than at the cervical and the thoracic level (Barone, 1986); (ii) all lumbar vertebrae are very similar to each other, lateral and spinous processes (the more useful features for vertebral rank identification) having a smooth gradient of size and shape from the first lumbar vertebra to the last one, and (iii) these differences between normal lumbar vertebrae of different rank are more subtle than the ones distinguishing, for example, a normal T13 from a transformed T13+ (whose ribs have an abnormal caudal curvature). The identification of mutant lumbar vertebrae was based therefore upon the closest morphology of lateral and spinous processes seen in lumbar vertebrae of control skeletons (for iconography, see Fromental-Ramain et al., 1996). We have also used the following morphological landmarks, which showed little variation between control animals: (i) an L3 morphology was assigned to the most anterior vertebra exhibiting lateral processes extending beyond the level of the intervertebral articulation. This criterion was associated with vertebra #23 in 14/17 control skeletons, and twice with vertebra #24, and once with vertebra #22; (ii) an L4 morphology was assigned to the most posterior vertebra harbouring accessory processes. On the L4 vertebra, these processes do not have square extremities like their more anterior counterparts, but have a thin extremity or a small round shape. This criterion was associated with vertebra #24 in 13/17 control skeletons, and once with vertebra #23, twice with vertebra #25 and once unilaterally in vertebra #24 and #25. The four last thoracic, the sacral and the two first caudal vertebrae were easily recognized, using criteria found in anatomical literature (Barone, 1986; Popesko, 1992). Hoxa-10−/− and Hoxd-11−/− single heterozygous mutant skeletons were similar to WT ones and thus considered as control in litters lacking WT animals. For each litter, vertebrae of the mutant and control animals were examined for the presence of these landmarks, and their frequency was determined at each level, which allowed us to assign a vertebral phenotype to the various genotypes. As an example of natural variability, note that one of the WT skeletons had only 12 ribs, and 2 out of 17 controls had a T10 with an abnormal morphology. Moreover, whenever asymmetric morphology was recorded for a vertebra, be it from a control or a mutant animal skeleton, the left side had always the more anterior morphology.

RESULTS

Hoxa-10 and Hoxd-11 transcript expression in the differentiating limbs

The expression pattern of Hoxa-10 wasanalyzed in the differentiating limbs (12.5-14.5 days post-coitum, dpc), since previous studies were performed at earlier stages (9.5 to 12.5 dpc; Haack and Gruss, 1993). At these earlier stages, Hoxa-10 transcripts were preferentially expressed around the differentiating skeletal elements of the proximal segments of the limbs. By 13.5 dpc, strong expression was detected around the cartilaginous blastemas of the femur and the humerus (Fig. 1A). In contrast, Hoxa-10 transcripts were much more diffuse in distal regions of the limbs and decreased toward the footplates (Fig. 1A). At 14.5 dpc, Hoxa-10 expression was detected around the femur primordium, with higher transcript levels toward its proximal extremity in the area of the presumptive trochanter (Fig. 1B, open arrows), and around its distal extremity (Fig. 1B). Transcripts of the paralogous gene Hoxd-10 did not extend more proximally than the presumptive knee anlage (Fig. 1A,B, arrowheads) and Hoxa-10 transcripts were more widely distributed than Hoxd-10 transcripts in the mesenchyme of the developing knee region (Fig. 1B). Similar expression patterns were observed at 14.5 dpc in the developing forelimbs (data not shown; see below).

Hoxa-10 and Hoxd-11 transcript distribution was also compared on adjacent limb sections. In the forelimbs, Hoxd-11 transcripts were strongly expressed in the autopod and extended proximally along the posterior half of the zeugopod (Fig. 1C, 12.5 dpc), with a clear expression around the ulna anlage (Fig. 1D, 13.5 dpc). The proximal boundary was at the level of the presumptive elbow, around the distal extremity of the developing humerus (data not shown). Hoxa-10 was expressed at low levels in the forelimb autopod (Fig. 1C), and at higher levels in the zeugopod, but with a more diffuse distribution than Hoxd-11 transcripts (Fig. 1C,D) Hoxa-10 was also expressed around the differentiating humerus (data not shown). Very similar patterns of expression were observed in the hindlimbs, with Hoxd-11 transcripts extending along the posterior part of the zeugopod including the periphery of the fibula anlage, but not around the tibia anlage (data not shown).

Proximal hindlimb skeletal structures are selectively affected in Hoxa-10−/− mutant mice

The proximal part of the femur presents two apophyses which serve as attachment sites for some sublumbar (trochanter minor) and rump (trochanter major) muscles. These two
trochanters, as well as the femoral head, appeared normal in Hoxa-10\textsuperscript{+/+} mutants (compare Fig. 2A to B,C). However, the third trochanter (trochanter tertius), the insertion site of the m. gluteus superficialis, located along the dorsolateral side (labium laterale) of the femur of gliridae (rodents and rabbits) and equidae, was altered in 55% of the Hoxa-10\textsuperscript{−/−} mutants (Fig. 2B,C). The mutant third trochanter was enlarged, shifted distally and did not present a distinct proximal edge. This alteration was observed in homozygous mutants of both the inbred and hybrid genetic backgrounds (Fig. 2B,C, respectively).

The distal articular surface of the femur consists of two condyles articulating with the tibia, and a trochlea articulating with the patella (Fig. 2D). The femoral condyles were not altered in Hoxa-10\textsuperscript{−/−} mutant mice (Figs 2E, 3). The normal trochlea has a unique medial groove (Fig. 2D). In 2 out of 18 mutants with a hybrid genetic background, the trochlea appeared duplicated, with a medial crest separating two symmetrical grooves (Fig. 2E).

Mice have two sesamoid bones at the level of the knee, each facing the femoral condyles at the medial side (ms, Fig. 3A) or lateral side (ls, Fig. 3D). These sesamoids are embedded in the tendons of origin of each gastrocnemius muscle. The medial sesamoid bones of most Hoxa-10\textsuperscript{−/−} mutant mice showed variable degrees of size reduction and were unilaterally absent in 3 out of 27 hindlimbs analyzed (Fig. 3B, and data not shown). The lateral sesamoid bones of Hoxa-10\textsuperscript{−/−} mutants were misplaced proximally and misshapen, usually displaying a ‘heart shape’ due to an abnormal proximal protrusion (Fig. 3E, curved arrow). Supernumerary ossified structures were also evident in some specimens (Fig. 3F, curved arrow). There was no detectable alteration in the tibia, fibula or hindfeet of Hoxa-10\textsuperscript{−/−} mutants.

An abnormal fibrous structure (hereafter called ‘ligament’) was detected along the lateral side of the femur, between the altered third trochanter and the lateral condyle and encompassing the altered lateral sesamoid bone (Fig. 4B, double arrow; visible also in Fig. 3E,F, small arrows). The severity of the lateral sesamoid deformation correlated with the presence of a more pronounced ‘ligament’. We noticed a similar, but thinner and more fragile structure in some control hindlimbs and less affected mutants. Thus, this mutant ‘ligament’ may correspond to an abnormal thickening of the septum intermuscularis femoris lateralis, a duplication of the fascia lata rejoining the femur on its lateral side (Barone, 1986). The muscle gluteus superficialis attaches distally to the third trochanter. In Hoxa-10\textsuperscript{−/−} mutants with abnormal third trochanter, its distal tendon was shifted distally and some of its fibers merged into the ectopic ‘ligament’ (data not shown). This may create pathological tension within the ‘ligament’, since the gluteus superficialis muscle is a flexor of the hip, whereas the gastrocnemius, attached at the level of the lateral sesamoid, is an extensor of the tarsal joint, these two movements being antagonistic during locomotion. The resulting impairment in knee mobility may explain why several 8- to 12-month-old Hoxa-10\textsuperscript{−/−} mutant mice developed severe degenerative alterations of the knee with numerous signs of...
Fig. 2. The Hoxa-10<sup>−/−</sup> femoral phenotype. (A-C) Cranial views of a 3 month-old control (A) and two Hoxa-10<sup>−/−</sup> mutants in an inbred (B) or hybrid (C) genetic background. (B) The third trochanter (trochanter tertius, tt, indicated by a bracket) is longer than in the control, and its shape is mildly altered especially at the proximal edge. (C) The third trochanter is more affected, extending over the middle of the femur shaft and with no clear proximal edge. Braces show abnormal (intracapsular, intraligamentary) ossification of the knee articulation. Because of these, the femur does not have the exact same orientation as in A and B, and the trochanter minor is hidden. (D,E) Distal extremities of a WT (D) and a Hoxa-10<sup>−/−</sup> (E) femur. (D) On the anterior side is the trochlea, where the patella articulates. On the posterior side are the condyles which articulate to the tibia via the menisces. (E) The whole distal extremity of the femur appears enlarged with a duplicated trochlea, but the two condyles seem normal. c, condyle; ls, deformed lateral sesamoid; tj, trochanter major; tn, trochanter minor; tt, trochanter tertius; t, trochlea.

Fig. 3. Knee alterations in Hoxa-10<sup>−/−</sup> mutant mice. (A-C) Medial views of the knee skeleton of a WT (A) and two Hoxa-10<sup>−/−</sup>(B,C) specimens. (B) Reduction of the medial sesamoid, while other structures including the patella and the menisces appear normal. (C) The specimen is older (10 months old) and displays numerous osteophytes and ectopic intracapsular and intraligament ossified structures (indicated by braces). All other animals displayed in this figure are 3-4 months old. (D-F) Lateral views of the knee skeleton of a WT (D) and two Hoxa-10<sup>−/−</sup>(E,F) specimens. The lateral sesamoid is embedded in the tendon of the gastrocnemius muscle lateral caput. (E) The mutant lateral sesamoid has a heart shape and is displaced proximally along the femur shaft. The extra edge (curved arrow) is orientated proximally and is the insertion point of an abnormal ‘ligament’ still visible after the clearing procedure (three parallel arrows). (F) Notice the altered shape of the lateral sesamoid and the supernumerary sesamoid (curved arrow), embedded in the ‘ligament’ (three parallel arrows).
f, femur; ls, lateral sesamoid; me, meniscus; ms, medial sesamoid; p, patella; t, tibia.
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Fig. 4. A supernumerary 'ligament' in Hoxa-10+/− hindlimbs. Dissected muscles of the lateral side of a control (A) and a Hoxa-10+/− mutant (B) hindlimb. (A) The fascia lata was reclined, the muscle gluteus superficialis (gs) dissected and some caudal muscles removed to expose the gastrocnemius and the caudal side of the vastus lateralis. (B) The same preparation on a Hoxa-10+/− mutant revealed a novel ‘ligament’ (double arrow) extending from the third trochanter, where the gluteus superficialis is attached, to the lateral sesamoid, where the gastrocnemius is attached. g, gastrocnemius muscle; gs, gluteus superficialis muscle; vl, vastus lateralis muscle.

Synergistic effects of the Hoxa-10 and Hoxd-11 mutations on the generation of forelimb abnormalities

Double mutants were produced by genetic intercrosses. In the F2 progeny, all compound mutant genotypes were obtained in a Mendelian ratio at the age of three weeks ($\chi^2 = 7.16, 8$ degrees of freedom, out of 161 animals), and no increased lethality was observed thereafter. Thus, Hoxa-10+/−/Hoxd-11+/− double mutants of both sexes are fully viable. The abnormalities described below were similar in 129/Sv inbred and 129/Sv-C57BL/6 hybrid genetic backgrounds. Hoxa-10/Hoxd-11 double mutants of both sexes where hypofertile, to the same extent as previously described for Hoxa-10+/− male and female mutants (Satokata et al., 1995; Rijli et al., 1995), and for Hoxd-11+/− male mutants (Favier et al., 1995).

Neither Hoxa-10+/− nor Hoxd-11+/− single mutants (Favier et al., 1995) exhibited any external forelimb abnormality. In contrast, most of the double homozygous mutants and 25% of the Hoxa-10+/−/Hoxd-11+/− mutants presented shortened and twisted forelimbs, with the forepaws turned outwards (data not shown). These malformations clearly impeded the animal motility, as judged by ‘climbing tests’ showing that control animals could hang themselves for more than 2 minutes to the cage cover grid, whereas mutant animals whose skeleton was the most affected could not hang for more than 10 seconds or not at all.

Double homozygous mutants exhibited severe alterations of the skeletal elements in the forelimb zeugopod, which were not seen in the single mutants. All Hoxa-10+/−/Hoxd-11+/− mutants had shortened radius and ulna (20% shortening on average; Table 1, Fig. 5). However, the humerus and scapulae of the double mutants were not shortened, excluding a general growth deficiency (Table 1). Comparative measurements revealed that single Hoxd-11+/− mutants had a milder shortening of the radius and ulna (7 and 10%, Table 1) which was not reported in our initial study (Favier et al., 1995). In fact, the radius and ulna of Hoxd-11+/− mutants were morphologically normal, except in their distal extremities (Fig. 5B). In contrast, the radius and ulna of double homozygous mutants were truncated.
Table 1. Limb phenotype

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<td>23/3</td>
<td>14/0</td>
<td>8/4</td>
<td>25/7</td>
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Length reduction of limb bones

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Hox mutants

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<tr>
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d3, distal carpal 3; c, central carpal bone; ppsl, palmar process of the scapholunate.

(a) Control include Hoxa-10 +/- / Hoxd-11 +/-, Hoxa-10 +/- / Hoxd-11 +/-, and Hoxa-10 +/- / Hoxd-11 +/- animals.
(b) Displayed as the % of the length of the same bone in control littermate specimen. Mean values for each genotype were calculated out of 5-7 animals each.
(c) Metacarpal 5 and other phalanges were less reduced (>90% of normal length) in all mutant genotypes, and are not displayed in this table.
(d) Number of limbs with normal / abnormal morphology.
(e) Include an extra or a bigger than usual lateral sesamoid bone or a medial one (found in three double mutant limbs) or combination.
(f) Bigger sesamoids at the palmar and proximal end of the fourth metacarpal and/or presence of extra sesamoid bones at the palmar and proximal end of the third and/or the second metacarpal.
(g) In Hoxa-10 +/- / Hoxd-11 +/- and in Hoxa-10 +/- / Hoxd-11 +/- mutant limbs, when the ppsl is fused to the scapholunate, it often (~50%) presents evidence of secondary or even incomplete fusion to the main bone, which is not observed in control limbs.

and thicker (Fig. 5C). The abnormal angularity of the forepaw skeleton (compare Fig. 5C to A,B) probably results from both a greater size reduction of the ulna and a severe alteration of its styloid apophysis (see below).

The proximal region of the ulna was not clearly altered in single Hoxd-11 +/- mutants (Fig. 7B), but was severely affected in some double mutants, with extra bone ridges at the insertion site of the triceps brachialis and biceps brachialis muscles and an apparent truncation of the olecranon (Figs 7C, 5C). Several double mutants also had extra sesamoid bones or alterations of the shape of the normal elbow sesamoid (data not shown; Table 1).

The additional homozygous disruption of Hoxa-10 also increased the severity of the Hoxd-11 +/- autopal alterations. As previously described (Davis and Capecchi, 1994; Favier et al., 1995), Hoxd-11 +/- mutants display variable archipodium abnormalities which include slight alterations of the ulna and radius styli, pisiform/pyramidal and/or pyramidal/scapholunate bone fusions, truncation of the pisiform bone, separation of the palmar process of the scapholunate and occasional supernumerary sesamoids. The neopodium abnormalities essentially consist of size reductions of metacarpals II to V and of the second phalanx of digit II. In newborn double homozygous mutants, the autopod appeared similar to those of single Hoxd-11 +/- mutants (Favier et al., 1995) with a additional minor alteration of the pyramidal. The radius and ulna were only slightly reduced, and the ulna styloid appeared normal. The percentage reduction increased by 3 and 6 days post-partum and at the same time a gap appeared between the ulna styloid and the pyramidal bone (data not shown). In adults, the ulna styloid was much more severely truncated in Hoxa-10 +/- /Hoxd-11 +/- mutants (Figs 6B, C, 7F) than in single Hoxd-11 +/- mutants (Fig. 7E). Thus, there was no articular relationship between the ulna styloid and pyramidal bone (compare Fig. 6A to B,C). Some extra sesamoid bones were often present dorsally (Fig. 6B, brace) or laterally (Fig. 6C, thin arrow) to the pyramidal bone. These may result from an induced instability of the wrist (Fig. 6B, brace), or may represent unfused vestigia of the ulna styloid (Fig. 6C, thin arrow). Some double mutants also presented a wide gap (Fig. 6C, large arrow), sometimes associated with an abnormal exocurrence (Figs 6B, 7F, black arrows) at the radial side of the ulna. By contrast, the distal extremity of the radius was not more malformed than in Hoxd-11 +/- single mutants (Fig. 6B,C, white open arrows). The severity of the Hoxd-11 +/- alterations of the pyramidal and pisiform bones (the two posterior bones of the proximal row of the carpus) was also increased in double mutants. Not only were these bones always fused in the Hoxa-10 +/- /Hoxd-11 +/- mutants, but they were more truncated than in the single Hoxd-11 +/- mutants (compare Fig. 7H and I). Additional differences between the carpal alterations of double and single mutants included: (i) a reduced rate of fusion between the scapholunate and the pyramidal bone, (ii) an enlargement of the sesamoid bone at the palmar base of metacarpal 4 and/or extra sesamoids at the base of other metacarpals, (iii) a reduced rate
of separation of the palmar process of the scapholunate, (iv) a fusion between the central and distal three carpal bones (Table 1). This latter abnormality was also found in some Hoxd-11−/− single mutant littermates; a founder effect may explain why this fusion was not seen in our previous study (Favier et al., 1995).

The size reduction of metacarpals II and, to a lesser extent of metacarpals III and IV, was also more pronounced in double homozygous mutants than in single Hoxd-11−/− mutants (Table 1). Interestingly, the second phalanx of digit V of double mutants was both truncated and malformed, which was not observed in single Hoxd-11−/− mutants (not shown).

The Hoxa-10+/−/Hoxd-11−/− mutants displayed variable forelimb abnormalities. Whereas some mutants had malformations similar to those of Hoxd-11−/− single mutants, others exhibited wrist alterations which were as severe as in double homozygous mutants (data not shown). In contrast, Hoxa-10+/−/Hoxd-11−/− mutants had no forelimb abnormalities, except for the presence of a small sesamoid bone between the radius and the pisiform (already seen in some Hoxd-11−/− mutants, Favier et al., 1995) and, in some mutants, there were a mild alteration of the ulna styloid and slight size reductions of the ulna, radius and some metacarpal and phalangeal elements (data not shown; see Table 1).

The hindlimb phenotype of Hoxa-10+/−/Hoxd-11−/− double mutants was indistinguishable from that of single Hoxa-10+/− mutants. We did not detect size or shape alterations of the tibia, fibula or of any other hindfoot elements (data not shown; see Table 1).

**Increased penetrance of the vertebral transformations in Hoxa-10/Hoxd-11 double mutants**

Wild-type (WT) mice have 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and a variable number of caudal (Ca) vertebrae (7C,13T,6L,4S phenotype). As previously reported, about half of the Hoxd-11−/− mutants have 7 lumbar vertebrae (7C,13T,7L,4S phenotype), indicating that vertebra #27 was transformed from a S1 to a ‘L6-like’ morphology, all subsequent vertebrae being anteriorly transformed at least until vertebra #32 (Ca2, transformed into a Ca1* vertebra) (Favier et al., 1995; Fig. 8). Furthermore, in most of Hoxd-11−/− animals with 7 lumbar vertebrae, vertebra #25 (WT L5) had the accessory processes characteristic of an L4 identity (see Materials and methods), while spinous and lateral processes of vertebrae #25, #26 and #27 were similar to those of WT L4, L5 and L6 vertebrae, respectively (Fig. 8).

The Hoxd-11−/−/Hoxa-10+/− mutants showed the same altered phenotype as single Hoxd-11−/− mutants, but with a full penetrance: 15 out of 16 skeletons had seven lumbar vertebrae and the #27 vertebra of the last specimen was partially transformed from a S1 to a lumbar morphology; using the criteria described in Materials and methods, all vertebrae #25, #26 and #27 were anteriorly transformed (L4*, L5*, L6* in Fig. 8).

Most Hoxa-10+/− mutants have a 7C,14T,5L,4S phenotype, vertebra #21 being transformed from a L1 to a ‘T13-like’ morphology. These anterior transformations always extend down to vertebra #24 (L4 transformed into L3*) and, with an incomplete (50%) penetrance, down to vertebra #26 (L5 and L6 transformed into L4* and L5*) (Rijli et al., 1995). We noticed that in 4 out of 34 mutants, vertebrae #27 and #28 were also transformed (S1 and S2 transformed into L6* and S1*, these mutants having a 7C,14T,6L,3S phenotype). No increase in penetrance or expressivity of the Hoxa-10+/− thoracic transformations (Rijli et al., 1995) was detected in any Hoxd-11/Hoxa-10 double mutants. All but two Hoxa-10+/−/Hoxd-11−/− mutants displayed anterior transformations from vertebra #21 to #32 (Fig. 8). One specimen displayed anterior transformations from vertebrae #21 to #26 only (as some single Hoxa-10+/− mutants) and another from vertebrae #21 to #28 (as the most affected single Hoxa-10+/− mutants). Thus, Hoxa-10+/−/Hoxd-11−/− mutants exhibited transformations which were at least those of single Hoxa-10+/− mutants, but more frequently encompassed the domain of anterior transformation of both Hoxa-10 and Hoxd-11 mutations (Fig. 8). Note that heterozygous Hoxd-11+/− mutants did not display any axial skeleton abnormalities in this region.

All Hoxa-10+/−/Hoxd-11−/− mutants had a 7C,14T,6L,4S phenotype. Vertebrae #21 to #32 were anteriorly transformed as if the affected region would result from the reunion of the Hoxa-10−/− and the Hoxd-11−/− ones, but with a full penetrance (Fig. 8). Moreover, the accessory processes of vertebra #26 displayed an L4-like morphology in 10/13 cases. This could be considered as a partial anterior transformation of a L6 to L4 morphology (L4** in Fig. 8). In about 50% of the double homozygous mutants, and one Hoxa-10+/−/Hoxd-11−/− mutant, we found one abnormal posterior lumbar vertebra (#25, #26 or #27) which could not be easily ranked; these vertebrae were assigned a rank between those of its two neighbours.

Both Hoxd-11−/− and Hoxa-10+/− single mutants inconsistently showed abnormal fusions between spinous processes of two sacral vertebrae, S2 and S3, whether the sacrum was at its normal position (S2 and S3 are vertebrae #28 and #29) or was composed of anteriorly transformed vertebrae (S2* and S3* are then vertebrae #29 and #30, see Fig. 8, footnote e). These fusions were consistently found in the Hoxa-10+/−/Hoxd-11−/− and Hoxa-10+/−/Hoxd-11−/− mutants; in three of the double homozygous mutants, we even found fusion of three sacral spinous processes.

Taken together and in spite of some variability in the lumbar-sacral vertebral phenotype for a given genotype (higher in mutant than in control animals), our data indicate extensive anterior transformation of the lumbar and sacral vertebrae, with a maximum penetrance in the double mutants.

**DISCUSSION**

The Hoxa-10−/− mutation selectively affects structures of the hindlimb stylopod

The alterations detected in Hoxa-10−/− mutants are essentially limited to the hindlimbs, even though Hoxa-10 displays similar expression patterns in the developing forelimbs and hindlimbs. These hindlimb alterations are restricted to a proximal region which encompasses the stylopod and the knee region, but limited to the hindlimbs, even though Hoxa-10 displays similar expression patterns in the developing forelimbs and hindlimbs. These hindlimb alterations are restricted to a proximal region which encompasses the stylopod and the knee region, but excludes the femoral head, thus extending proximally up to the developmental expression boundary of Hoxa-10. The subtle and inconsistent alterations of the humeral deltoid crest may represent a mild counterpart of the hindlimb third trochanter abnormalities. In contrast, the Hoxd-11 mutation affects a limited region of the forelimb autopod, with only minor effects in hindlimbs (Davis and Capecci, 1994; Favier et al., 1995), and Hoxa-9/Hoxd-9 double mutations also selectively affect
Fig. 6. Carpal alterations in Hoxa-10<sup>−/−</sup>/Hoxd-11<sup>−/−</sup> mutants. Dorsal views of the carpus of a WT animal (A) and two Hoxa-10<sup>−/−</sup>/Hoxd-11<sup>−/−</sup> mutants (B,C). The mutant ulna styloids are severely truncated, especially in C. Note that the radius styloid is only slightly affected at its ulnar side (white open arrows), as seen in Hoxd-11<sup>−/−</sup> single mutants. (B), The thick arrow indicates an abnormal excrescence at the ulna extremity (see also Fig. 7F). The pyramidal is partly hidden by a de novo large sesamoid bone (brace) which is in continuity with extensor muscle tendons. (C) The distal extremity of the ulna is abnormally bent, generating a central gap (large arrow). The curved arrow indicates a truncated polygonal portion of the pyramidal bone which lies at the same level, but has no articular relationship with the scapholunate bone, the os hamatum and the ulna styloid. The small arrow points to a small bone that may correspond to an unfused vestigia of the ulna styloid. Note also the fusion between the central bone and the distal carpal 3 and the altered shape of the scapholunate, typical of some Hoxd-11<sup>−/−</sup> single mutants. c, os centrale; d3, os capitatum or distal carpal 3; d4, os hamatum or distal carpals 4/5; l, lunate portion of the scapholunate; r, radius; s, scaphoid portion of the scapholunate; sl, scapholunate; py, os triquetrum or pyramidal; py+pi, fused pyramidal and pisiform bones; u, ulna.

Fig. 7. Details of the forelimb skeleton in WT animals (A,D,G), single Hoxd-11<sup>−/−</sup> (B,E,H) and double Hoxa-10<sup>−/−</sup>/Hoxd-11<sup>−/−</sup> mutants (C,F,I). (A-C) Proximal portion of the ulna, medial views. The olecranon (tuber olecrani) is only slightly affected in the simple Hoxd-11<sup>−/−</sup> mutant (B). The thin arrow points to the fusion line between the diaphysis and the part derived from the proximal ossification center. (C) The olecranon and the entire articular region are strongly misshapen in this double Hoxa-10<sup>−/−</sup>/Hoxd-11<sup>−/−</sup> mutant and the fusion line cannot easily be identified. The thin arrow tentatively indicates such a line. The large arrows indicate abnormal ridges which were connected to the triceps brachialis, ancone and biceps brachialis muscle tendons before dissection. The arrowhead points to an abnormal process close to the articular surface. (D-F) Distal extremity and styloid of the ulna, dorsal views. The styloid of the single Hoxd-11<sup>−/−</sup> mutant (E) is mildly truncated with a round shape (open arrow). The distal aspect of the Hoxa-10<sup>−/−</sup>/Hoxd-11<sup>−/−</sup> ulna (F, open arrow) is almost flat. The styloid is difficult to recognize as there is no fusion line of its secondary ossification center (thin arrows in D and E). The curved arrow shows an abnormal process at the radial side of the ulna extremity. (G-I) Dissected pyramidal and pisiform bones, lateral views. Dorsal side is to the left, palmar side to the right. The pyramidal and pisiform of the single Hoxd-11<sup>−/−</sup> mutant (H) are fused and truncated, but the line of fusion is recognizable (thin arrow) and the pisiform still has a typical shape. In the double Hoxa-10<sup>−/−</sup>/Hoxd-11<sup>−/−</sup> mutant (I), the line of fusion cannot be identified and the pisiform part is severely truncated. pi, pisiform; py, pyramidal; py+pi, fused pyramidal and pisiform bones; u, ulna.
3-4 months. (b) This phenotype is not fully penetrant (Favier et al., 95). In the series analyzed in this study, 6/13 skeletons were normal and one presented an asymmetrical transformation of vertebra #26. The six remaining specimens had 7 lumbers, the anterior transformation extending to #25 (which acquired the L4 morphological criteria described hereabove). (c) Hoxd-11+/−/Hoxa-10+/− displayed the same phenotype as Hoxd-11+/−/Hoxa-10−/− mutants, but with a nearly full penetrance. Only one skeleton displayed a #27 vertebra with an intermediate morphology between S1 and L6. (d) In most Hoxd-11+/−/Hoxa-10−/− mutants, the anterior transformation (Rijli et al., 1995) occurred as illustrated down to vertebra #26, but occasionally extended down to #29 (which acquired an intermediate S2-S3 morphology) in 12% of the specimen. (e) This is the percentage of skeletons with abnormal fusion (no fusion was observed in control skeletons) between the spinous processes of two sacral vertebrae, usually S2 and S3 (or S2* and S3*). In three Hoxd-11+/−/Hoxa-10−/− mutants, a third spinous process (of S1* or S4*) was involved.

Whereas the Hox mutant limb phenotypes previously reported (Dollé et al., 1993; Small and Potter, 1993; Davis and Capecchi, 1994; Favier et al., 1995) correspond to bone truncations and fusions, i.e. to the lack of skeletal material, the present Hoxa-10 femoral phenotype is characterized by the presence of extra skeletal material along the lateral side (labium laterale) of the femur. The enlarged third trochanter may represent a primary effect of the Hoxa-10−/− mutation, since it correlates with a preferential expression of Hoxa-10 during fetal stages (13.5-14.5 dpc) around a portion of the differentiating femur. The lack of Hoxa-10 expression at these stages may alter the local production or recruitment of cells with a skeletogenic fate. We believe that the supernumerary ‘ligament’, which links the third trochanter to the lateral condyle, rather than being a de novo structure, is a thickening of the septum intermuscularis femoris lateralis. This ‘ligament’ has indeed the same anatomical relationships as the WT septum, including a histological continuity with the periosseum. The merging of part of the glutaeus superficialis tendon in the mutant ‘ligament’ reflects the WT situation in which this tendon is in continuity with the septum and the fascia lata at its site of insertion. The abnormal thickening might be the consequence of antagonistic tensions between the gastrocnemius and the glutaeus superficialis which is misplaced on the mutant third trochanter. The altered shape of the lateral knee sesamoid may in turn be a consequence of such mechanistic phenomena, since sesamoid bone morphologies are known to depend on pressure stress in connective structures during fetal development (Hall, 1986; Hinchliffe, 1994). However, two alternative possibilities cannot be ruled out. Firstly, the ‘ligament’ might derive from a distal subset of the cells which are abnormally recruited proximally to form the altered third trochanter. Secondly, an abnormal patterning of the glutaeus superficialis muscle, or at least of its distal insertion, might be a primary effect of the Hoxa-10−/− mutation. The expression of Hoxa-10 in presumptive limb muscle cells (Haack and Gruss, 1993) and in the femur periosteum at 14.5 dpc (this study) may support this hypothesis. Thus the primary effect of the Hoxa-10

![Fig. 8. Schematic representation of the lumbar and sacral vertebral transformations of Hoxa-10 and Hoxd-11 single and compound mutants. Thoracic vertebrae (T) are in blue, lumbar (L) in red, sacral (S) in yellow and caudal (Ca) in black. The transformed vertebrae are filled according to the penetrance of the transformation: light shading indicates a 30-80% penetrance, and full color a 90-100% penetrance. The thoracic transformations found in Hoxa-10 mutants are not represented. The bars on the left indicate the expression domain of ‘posterior’ Hoxa and Hoxd genes in the developing vertebral column, light grey indicating lower levels of expression. (a) Control animals were taken from the following genotypes: WT and single Hoxa-10−/+ or Hoxd-11−/+ heterozygous animals. No skeleton from other genotypes was analyzed without at least one control animal from the same litter, analysed at the age of the skeleton of the forelimb stylopod (Fromental-Ramain et al., 1996). The almost complete lack of forelimb alterations in Hoxa-10−/− mutants cannot be readily accounted for by functional redundancies between Hoxa-10 and its paralogous genes, since Hoxd-10 is not expressed as proximally as Hoxa-10 (our unpublished data) and Hoxa-10 is apparently not expressed in the presumptive limb muscle cells (Haack and Gruss, 1993) and in the developing vertebral column, light grey indicating lower levels of expression. (a) Control animals were taken from the following genotypes: WT and single Hoxa-10−/+ or Hoxd-11−/+ heterozygous animals. No skeleton from other genotypes was analyzed without at least one control animal from the same litter, analysed at the age of 3-4 months. (b) This phenotype is not fully penetrant (Favier et al., 95). In the series analyzed in this study, 6/13 skeletons were normal and one presented an asymmetrical transformation of vertebra #26. The six remaining specimens had 7 lumbers, the anterior transformation extending to #25 (which acquired the L4 morphological criteria described hereabove). (c) Hoxd-11−/+/Hoxa-10−/+ displayed the same phenotype as Hoxd-11−/+/Hoxa-10−/− mutants, but with a nearly full penetrance. Only one skeleton displayed a #27 vertebra with an intermediate morphology between S1 and L6. (d) In most Hoxd-11−/+/Hoxa-10−/− mutants, the anterior transformation (Rijli et al., 1995) occurred as illustrated down to vertebra #26, but occasionally extended down to #29 (which acquired an intermediate S2-S3 morphology) in 12% of the specimen. (e) This is the percentage of skeletons with abnormal fusion (no fusion was observed in control skeletons) between the spinous processes of two sacral vertebrae, usually S2 and S3 (or S2* and S3*). In three Hoxd-11−/+/Hoxa-10−/− mutants, a third spinous process (of S1* or S4*) was involved.

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function in limb development in which each paralogous articulation, generating abnormal tensions in the wrist extensor sesamoids may be a consequence of the instability of the wrist pisiform, but not to the scapholunate. The abundance of extra explain why, in double mutants, the pyramidal displays no tionship with other elements except the pisiform. This could carpus, such that the pyramidal primordium would lose its rela-

The malformations in the zeugopod: the truncation of the ulna to be more critical than forelimb morphogenesis. Part of the more severe ‘double mutant’ carpal phenotype might develop as a consequence of the truncations of the ulna styloid, pisiform and radius (zeugopod), as shown in the present study (at least in a

Partial functional redundancy between Hoxa-10 and Hoxd-11 in forelimb morphogenesis

Although Hoxa-10 is expressed in the developing forelimb, its inactivation generates almost no skeletal abnormalities. In contrast, Hoxd-11 inactivation affects a restricted skeletal region of the forelimb autopod, essentially the proximal row of the carpus and some metacarpal bones (Davis and Capecchi, 1994; Favier et al., 1995). Interestingly, Hoxa-10+/−/Hoxd-11−/− mutants exhibit a ‘synergistic’ forelimb phenotype, characterized by alterations which are milder in the Hoxd-11−/− mutants, such as the truncations of the ulna styloid, pisiform and pyramidal bones, metacarpal bones and second phalanx of digit V. In addition, there are striking alterations in a more proximal region nearly unaffected in single Hoxd-11−/− mutants which display only a subtle shortening of the radius and ulna. These alterations, which extend up to the most proximal expression domain of Hoxd-11 in the developing zeugopod, correspond to truncations and thickening of the radius and ulna, and deformations of the ulna proximal articular surface. As for the metacarpal bones in single Hoxd-11−/− mutants (Favier et al., 1995), these alterations appear only after birth. Further studies are necessary to investigate whether these late arising defects reflect a direct effect of Hoxa-10 or Hoxd-11 after birth or the consequences of underlying defects occurring prior to birth.

Thus, Hoxa-10 can exert a morphogenetic function in regions that are not affected in single Hoxa-10+/− mutants. Clearly, two non-paralogous Hox genes which are co-expressed in the developing zeugopod and autopod of the forelimb can have partly redundant functions, which are revealed only upon inactivation of both genes. Furthermore, there is a gene dosage effect, as Hoxa-10+/−/Hoxd-11−/− mutants display features of the double homozygous mutant phenotype, albeit with a lower penetration. However, Hoxa-10 or Hoxd-11 are not functionally equivalent as Hoxa-10+/−/ Hoxd-11−/− mutants display almost no forelimb abnormalities, except for slight bone size reductions. Thus, Hoxd-11 appears to be more critical than Hoxa-10 for zeugopodal and autopodal forelimb morphogenesis. Part of the more severe ‘double mutant’ carpal phenotype might develop as a consequence of the malformations in the zeugopod: the truncation of the ulna primordium may generate a post-axial gap in the developing carpus, such that the pyramidal primordium would lose its relationship with other elements except the pisiform. This could explain why, in double mutants, the pyramidal displays no recognisable articular surface and is systematically fused to the pisiform, but not to the scapholunate. The abundance of extra sesamoids may be a consequence of the instability of the wrist articulation, generating abnormal tensions in the wrist extensor and digit flexor muscle tendons.

Davis et al. (1995) have proposed a model of Hox gene function in limb development in which each paralogous Hox group 9 to 13 would specify, respectively, the scapulum, sty- lopodal, zeugopodal, archipodium autopodal and neopodium autopodal bones. The Hoxa-10+/− hindlimb phenotype is in agreement with this proposal, which, however, did not predict that Hoxa-10 can be functional in the developing ulna and radius (zeugopod), as shown in the present study (at least in a Hoxd-11−/− background). Davis et al. (1995) also proposed that the metacarpal and phalangeal neopodium alterations seen in Hoxd-11−/− mutants were secondary to the patterning defects of the zeugopod. We rather believe that the neopodium abnormalities seen in Hoxd-11−/− and Hoxa-10+/−/Hoxd-11−/− mutants reflect a primary morphological function (and not a secondary consequence of the more proximal phenotype) of Hoxd-11 and Hoxa-10 in this region for the following reasons: (i) the neopodium is a tetrapod neomorphic structure (Shubin and Alberch, 1986; Sordino et al., 1995), (ii) Hoxd-11 has two domains of expression in mouse limb buds, one of which corresponds to the neopodium (Sordino et al., 1995; Davis and Capecchi, 1994), (iii) the autopodal structures are differently affected by the Hoxa-10/Hoxd-11 mutations (The second carpal row is mainly unaffected and the metacarpals are more affected than most phalanges).

There is no synergistic effect of the double Hoxa-10+/−/ Hoxd-11+/− mutation on the generation of hindlimbs abnor-

malities. This further demonstrates that Hox genes, even though they are expressed in both limbs, may act differently during forelimb and hindlimb development. This lack of a synergistic effect between Hoxa-10−/− and Hoxd-11−/− mutations in the hindlimbs may reflect a functional redundancy of Hoxc-9, Hoxc-10 or Hoxc-11, which are expressed in the hindlimb, but not the forelimb mesenchyme (Peterson et al., 1994).

Taken together, our results demonstrate that during limb development: (i) a given Hox gene can act in a ‘specific’ domain (here Hoxd-11 in the autopod), where its function cannot be fulfilled by other Hox genes, and (ii) it can also act in a wider ‘redundant’ domain where the loss of its function can be compensated for by (an)other Hox gene product(s) (here Hoxa-10). There are several examples of functional redundancy between Hox paralogues during limb development: Hoxa-9 and Hoxd-9 in the developing forelimb stylopod (Fromental-Ramain et al., 1996), Hoxa-11 and Hoxd-11 in the forelimb zeugopod (Davis et al., 1995), and Hoxa-13 and Hoxd-13 in both limb autopods (our unpublished results). Importantly, we show here that functional redundancy exists even between non-paralogous genes which are co-expressed in a given region of the developing limbs, i.e. Hoxa-10 and Hoxd-11 in the forelimb zeugopod. Thus, in disagreement with a strict application of the posterior prevalence principle (Duboule, 1991, but see also Duboule and Morata, 1994), functional redundancy is not limited to members of the same paralogous group, and a given Hox gene can specify structures in a region where it is co-expressed with more 5’-located non-paralogous genes. However, one should stress in this respect that the functional redundancy which is revealed by the compari-

son of the phenotypes of single and double mutants may not reflect the physiological situation: the knock-out of a physiologically relevant Hox gene may create a new situation in which other paralogous or non-paralogous Hox gene(s) expressed in the same region, could ‘artefactually’ replace the relevant gene. In other words, genes that appear to be functionally redundant from knock-out studies may not be redundant in normal development (see also Fromental-Ramain et al., 1996).

Cooperation between non-paralogous Hox genes for vertebra specification

In the developing prevertebral column, the two non-paralogous
genes Hoxa-10 and Hoxd-11 are widely co-expressed caudally to prevertebra #24, while only Hoxa-10 is expressed in more anterior regions up to prevertebra #21. As expected from its expression domain, our results show that Hoxd-11 has no effect alone or in combination with Hoxa-10, rostral to vertebra #25, where the same phenotype of anterior transformations of vertebrae #21 to #24 is seen in Hoxa-10+/-, Hoxa-10+/+Hoxd-11+/- and Hoxa-10+/+Hoxd-11+/+ mutants (see Fig. 8).

Interestingly, the anterior transformation of vertebrae #25 and #26 becomes fully penetrant in Hoxa-10+/+Hoxd-11+/- and in Hoxa-10+/+Hoxd-11+/- double mutants, whereas it is only partially penetrant in Hoxa-10+/- and in Hoxd-11+/- single mutants. Thus, two non-paralogous genes belonging to different HOX complexes appear to be partially redundant for the specification of vertebrae #25 and #26. Whether this cooperation corresponds to a ‘quantitative’ effect (both gene products triggering the same molecular events) or to a ‘qualitative’ effect (each gene product triggering distinct ‘parallel’ events which are not absolutely required on their own) is unknown. We note that the present functional cooperation between two non-paralogous genes is reminiscent of that reported for Hoxb-5 and Hoxb-6, where Hoxb-5+/-Hoxb-6+/- transheterozygotes display a malformation also present in either single homozygote mutants (Rancourt et al., 1994). In any event, the Hoxa-10/Hoxd-11 functional cooperation is compatible with a combinatorial model of vertebra specification by at least some of the Hox genes which are expressed in a given prevertebral segment (Kessel and Gruss, 1991). The fact that Hoxd-11+/- mice exhibit with a partial penetrance an anterior transformation of vertebrae #29-32, even though Hoxd-11 is coexpressed in this region with Hoxd-12 and Hoxd-13 (see Fig. 8) further support a combinatorial model. Similarly, the observations that all vertebrae #27 to #32 were anteriorly transformed with a full penetrance in Hoxa-10+/-Hoxd-11+/-, Hoxa-10+/-Hoxd-11+/+ and Hoxa-10+/+Hoxd-11+/- are in agreement with a combinatorial model.

In all cases, the above partial functional redundancies between non-paralogous genes are inconsistent with the posterior prevalence model as initially formulated for limb development (Duboule, 1991), since this model stipulates that a given Hox gene would specify structures only in the region where there is no more posterior (5’-located) Hox gene expressed. This rule also postulates that “anterior structures would be produced by default, i.e. as a result of the non-expression of more posterior genes”. The present anterior transformations are in agreement with this proposal. However, it is noteworthy that, in view of the expression domains of the Hoxd genes (see Fig. 8), one might have expected to see all vertebrae #25 to #29 acquiring a L4 identity, which is clearly not the case. As argued by Duboule and Morata (1994), the interpretation of the effects of Hox gene loss-of-function mutation is made more difficult in vertebrates than in Drosophila, due to (i) the existence of paralogous genes, (ii) quantitative aspects of the posterior prevalence rule which are apparent even in Drosophila, and (iii) possible perturbing effects of the mutation on cross-regulation between Hox genes. The disruption of all genes whose anterior boundary of expression is posterior to vertebra #24 (including Hoxd-11) may be required to see all more posterior vertebrae acquiring an L4 identity. In this respect, we note that vertebrae #26 is converted from an L6 identity in WT to a L4 identity in Hoxa-10+/-Hoxd-11+/- double mutants.

Finally, it is striking that the anterior transformations of all vertebrae #25 to #32 are 50% penetrant in Hoxd-11+/- mutants and 100% penetrant in the Hoxa-10+/-Hoxd-11+/- double mutants. Even a ‘quantitative’ functional prevalence (Duboule and Morata, 1994) of Hoxd-12 and Hoxd-13 would require that the penetrance of the Hoxd-11 mutation decreases in the vertebrae #29-#32 region, where Hoxd-12 and Hoxd-13 are expressed. However we note that the penetrance of the anterior transformations decreases in Hoxa-10+/- mutants caudal to vertebral #25 where Hoxa-11 and Hoxd-11 are co-expressed. Thus, either there is no ‘quantitative’ functional prevalence of Hoxd-12 and Hoxd-13 over Hoxa-11, or one may speculate that the Hoxd-11 mutation generates a real duplication of vertebra #25 with a concomitant ‘reranking’ of the following vertebrae during their successive specification. This would assume that there is a recurrent mechanism for specification of vertebrae, which does not specify the identity of a given vertebra according to its absolute position, but rather according to the specification of its neighbour. Such a spatiotemporal control, which might be exerted at an early time during gastrulation, would of course make the interpretation of the axial skeleton phenotypes resulting from Hox gene loss-of-function mutations more difficult.

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