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Induces Premature Transcription and Anterior Shift of the Sacrum

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The precise activation, in space and time, of vertebrate *Hox* genes is an essential requirement for normal morphogenesis. In order to assess for the functional potential of evolutionary conserved *Hox* regulatory sequences, a phylogenetically conserved bipartite regulatory element necessary for proper spatial and temporal activation of the *Hoxd-11* gene was replaced by its fish counterpart in the *HoxD* complex of mice, using an ES cell-based targeted exchange. Fetuses carrying this replacement activated *Hoxd-11* transcription prematurely, which led to a rostral shift of its expression boundary and a consequent anterior transposition of the sacrum. These results demonstrate the high phylogenetic conservation of regulatory mechanisms acting over vertebrate *Hox* complexes and suggest that minor time difference (heterochronies) in *Hox* gene activation may have contributed to important morphological variations in the course of evolution.

Murine *Hox* genes located in the posterior half of the *HoxD* complex are necessary for the proper organization of both the vertebral column and the limbs (Krumlauf, 1994; Dollé *et al.*, 1989). In particular, *Hoxd-11* is required to correctly position the lumbosacral transition, as shown by null or regulatory mutations of this gene which lead to transposition of this crucial morphological landmark along the rostrocaudal axis (Davis and Capecchi, 1994; Favier *et al.*, 1995; Zákány *et al.*, 1996). Using conventional transgenesis, several regulatory elements located at the vicinity of various *Hox* genes have been shown to be necessary for the positioning of anteroposterior expression boundaries in fetuses (e.g., Deschamps and Wijgerde, 1993; Gérard *et al.*, 1993; Knittel *et al.*, 1995; Shashikant and Ruddle, 1996; Whiting *et al.*, 1991; Marshall *et al.*, 1994). Within the *Hoxd-11/Hoxd-10* intergenic region, a bipartite enhancer element, consisting of regions VIII (RVIII) and IX (RIX), is required for precise activation and positioning of the *Hoxd-11* expression limit at the lumbosacral transition (prevertebral level 27). As shown by *in vivo* targeted deletion or subtle mutagenesis, removing region VIII delayed *Hox* gene activation (Zákány *et al.*, 1997) while mutations within RIX mispositioned the rostrocaudal boundaries of both *Hoxd-11* and *Hoxd-10* (Gérard *et al.*, 1996).

In zebra fish (*Danio rerio*), in the absence of a lumbosacral transition, *Hoxd-10* and *Hoxd-11* are also expressed in developing sclerotomes. However, in agreement with the relatively anterior position of the cloaca (level 17), which appears to always match the most posterior *Hoxd* gene's boundary, *Hoxd-10* and *Hoxd-11* are detected already from levels 9 and 11, respectively, i.e., about 15 metamers more anteriorly than their murine counterparts (van der Hoeven *et al.*, 1996). The timing of *Hox* gene activation is also rather different between the two species, as expected from the rapid developmental strategy of *Danio.* While sequential activation of the *HoxD* complex takes about 2 days in mice, it is achieved within a few hours in fish. In order to look at potential molecular bases for these species differences of *Hoxd* expression in time and position, we cloned and sequenced the posterior part of the fish *HoxD* complex. Sequence alignment with the mouse led to the definition of fish regions VIII and IX. To assess the functional potential of these conserved regions, we replaced the mouse enhancer by the fish version *in vivo*, using ES cell targeted exchange. While we show that the fish sequence can substitute for the function of the mouse counterpart, this replacement

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leads to a slightly premature activation of *Hoxd-11* which consequently shifts its expression boundary anteriorly, thus moving the sacrum toward the rostral direction.

MATERIALS AND METHODS

DNA Constructs and Analyses

For the conventional transgenic *lacZ* mice, the mouse *Xba*I–*Eco*RI (900 bp) genomic fragment, containing region IX and part of region VIII was substituted (by conventional cloning) by the corresponding zebra fish *Xba*I–*Eco*RI (575 bp) fragment. The two *lacZ* transgenes were therefore identical to each others except the mouse (Mo Ns-E/*lacZ*) or fish (Mo-Fi Ns-E/*lacZ*) origin of regions VIII and IX (Fig. 1B). To replace murine RVIII and RIX by their fish counterparts, the *Xba*I–*Eco*RI genomic fragment containing RIX and part of RVIII was substituted for the same *Xba*I-*Eco*RI zebra fish fragment which was tested with the help of the reporter transgene described above (Fig. 1C). The targeting construct included 3 kb of 5′ homology, the exchanged DNA, a *loxP*PGK*neoloxP* selection cassette in the reverse orientation with respect to *Hoxd-11*, and 5.5 kb of 3′ homology. The targeting vector was linearized by *Xho*I prior to electroporation. Southern blots were used to identify homologous recombination events, with a NsiI digest for the 5′ external probe 1, a *Bam*HI digest for the 3′ external probe 2, and a *Bam*HI digest for a mixture of internal probes 3 and 4 (see Fig. 1). The *Cre*-treated allele was identified using a similar strategy.

Generation of Transgenic Animals and Mutant Stocks

For the conventional transgenesis, transgenic fetuses were produced by DNA injection into fertilized eggs according to established procedures. Pregnant foster mothers were sacrificed and fetuses were stained for the activity of the *lacZ* reporter transgene. ES cells were from the D3 line (Doetschman *et al.*, 1985; gift of R. Kemler). They were kept on feeder cells, electroporated, and selected following published procedures (see Joyner, 1993). ES cells were injected into C57Bl6 blastocysts. Positive mice were bred with mice expressing the *Cre* recombinase as in Dupé *et al.* (1997). Interbreeding of *neo* minus *Hoxd-11*$^{Fi/+}$ animals allowed for the production of F2 progeny and *Hoxd-11*$^{Fi/Fi}$ animals were also used for establishing homozygous breeding stocks, in order to screen large numbers of individuals to assess the incidence of less penetrant hindlimb patterning defects.

Skeletal Analyses and Whole-Mount RNA Hybridization

Skeletons of adult *Hoxd-11*^{Fi} F2 and homozygous progeny from *Hoxd-11*$^{Fi/Fi}$ breeding pairs were isolated and stained according to standard Alizarin red S staining protocol. Whole-mount *in situ* hybridizations were carried out using established protocols. The *Hoxd-11*-specific antisense probe was transcribed from a 777-bp-long partial embryonic cDNA clone (Gérard *et al.*, 1996).

RESULTS

Cloning and sequencing of the posterior (5′-) parts of the fish, mouse and chick *HoxD* complexes allowed us to extensively compare for sequences conserved throughout these three vertebrate species. Among those best conserved sequences were region VIII and IX which localized at the same relative positions between fish and mouse *Hoxd-11* and *Hoxd-10* (Fig. 1A). Sequence conservation was high for RIX, particularly for motifs which were previously shown to be of importance for *Hoxd-11* and *Hoxd-10* regulation (Gérard *et al.*, 1996). In contrast, a large section of RVIII was more divergent, when the fish was compared to both mouse and chick sequences (Fig. 1D). As a first approach, we assessed the functional potential of the fish sequences to drive expression of the mouse *Hoxd-11* transgene during fetal mouse development. Eight transgenic fetuses containing the fish sequences expressed the *Hoxd-11* transgene posteriorly, as expected, but with a clearly anteriorized boundary when compared to the control full mouse transgene (Figs. 2A and 2B). This anteriorization was most prominent in the spinal cord, at Day 11, where the CNS boundary was systematically observed at the level of prevertebra 23 (pv23), i.e., three to four somites more anterior than in control animals. However, in axial and paraxial mesoderm, as well as in dorsal root ganglia, expression was consistenly weaker and not anteriorized with respect to the control mouse transgene. To know if such an anterior shift was linked to an earlier activation of the transgene, we stained transgenic fetuses at earlier stages. While β-galactosidase activity was detected at an early stage (Fig. 2C), a strict comparison with either the mouse transgene or the mouse endogenous gene was made difficult by the experimental conditions, which required the comparison between different transgenic contexts (integration sites, copy number) to conclude on a few hours difference in the timing of activation. This point was therefore addressed using an *in vivo* exchange strategy (Fig. 1C).

A targeting vector was designed to exchange most of mouse RVIII and all of RIX for the corresponding fish DNA fragment, starting at a conserved restriction site so that the replacement was conservative (Fig. 1C, red arrows). A selection cassette with *loxP* sites was introduced further apart and subsequently removed following exposure to the *Cre* recombinase to prevent transcriptional interferences with the PGK promoter driving the *neomycin* gene (Fig. 1C). Mice homozygous for the fish enhancer (*Hoxd-11*$^{Fi/Fi}$) appeared normal, except for a weakly penetrant phenotype in hindlimbs (see below). However, the analysis of skeletal preparations revealed that more than half of these animals had a rostrally shifted sacrum with five lumbar vertebrae (L5) instead of the six (L6) normally observed in *Hoxd-11*$^{+/+}$ mice. In addition, 35% of heterozygous *Hoxd-11*$^{Fi/+}$ animals showed a complete L6 to L5 transformation as well (Table 1), suggesting that an anterior gain-of-function had occurred for *Hoxd-11* (Zákány *et al.*, 1996). A detailed investigation

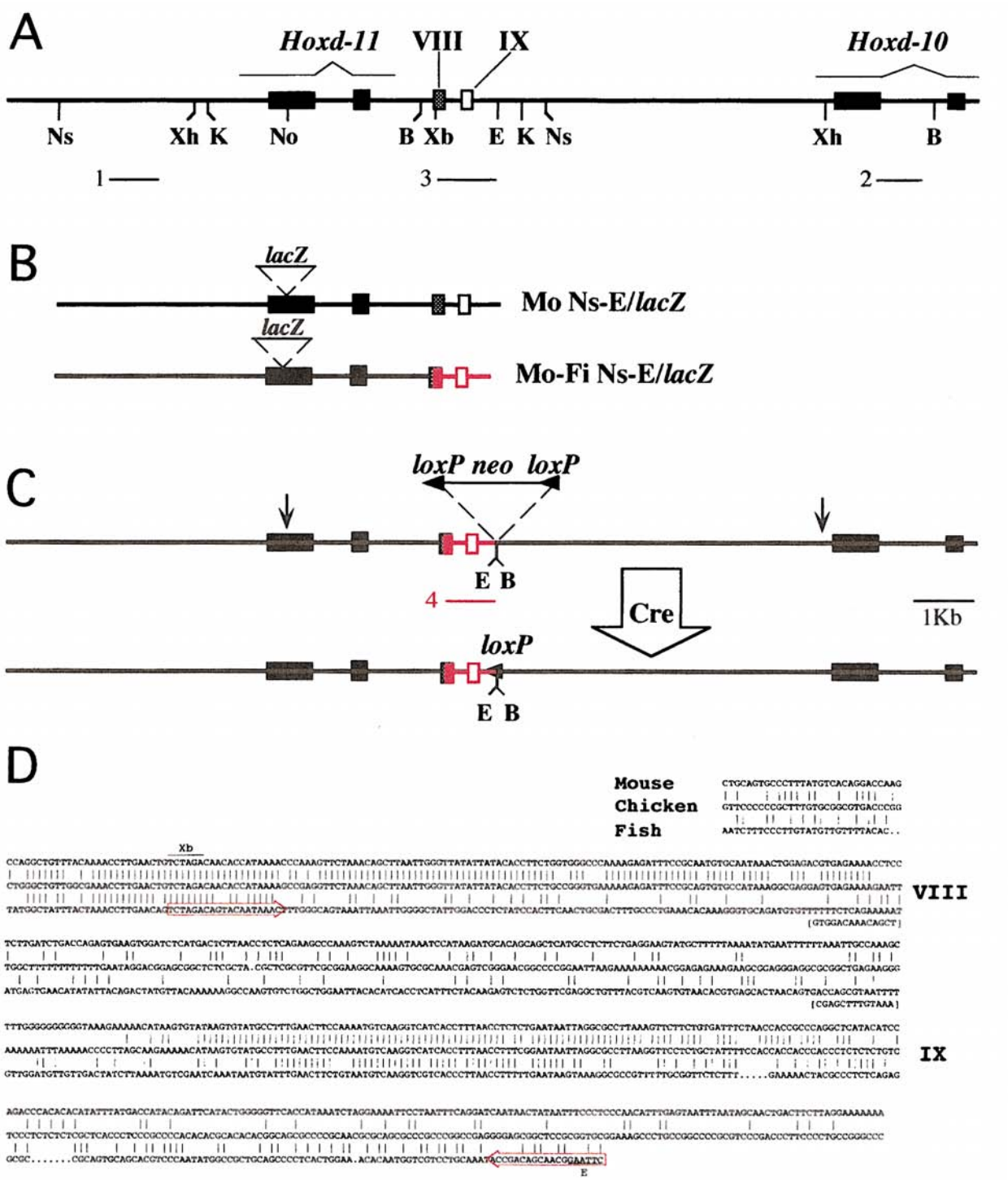

FIG. 1. (A) Schematic of the mouse *Hoxd-11* to *Hoxd-10* locus. The position of the bipartite conserved element (VIII; IX) is indicated 5′ of the *Hoxd-11* transcription unit. (B) Schematic of the two constructs used as transgenic *lacZ* reporters. On the top, the mouse transgene with the *lacZ* sequences inserted in the first exon. On the bottom, the same transgene with fish sequences in 3′, covering part of RVIII

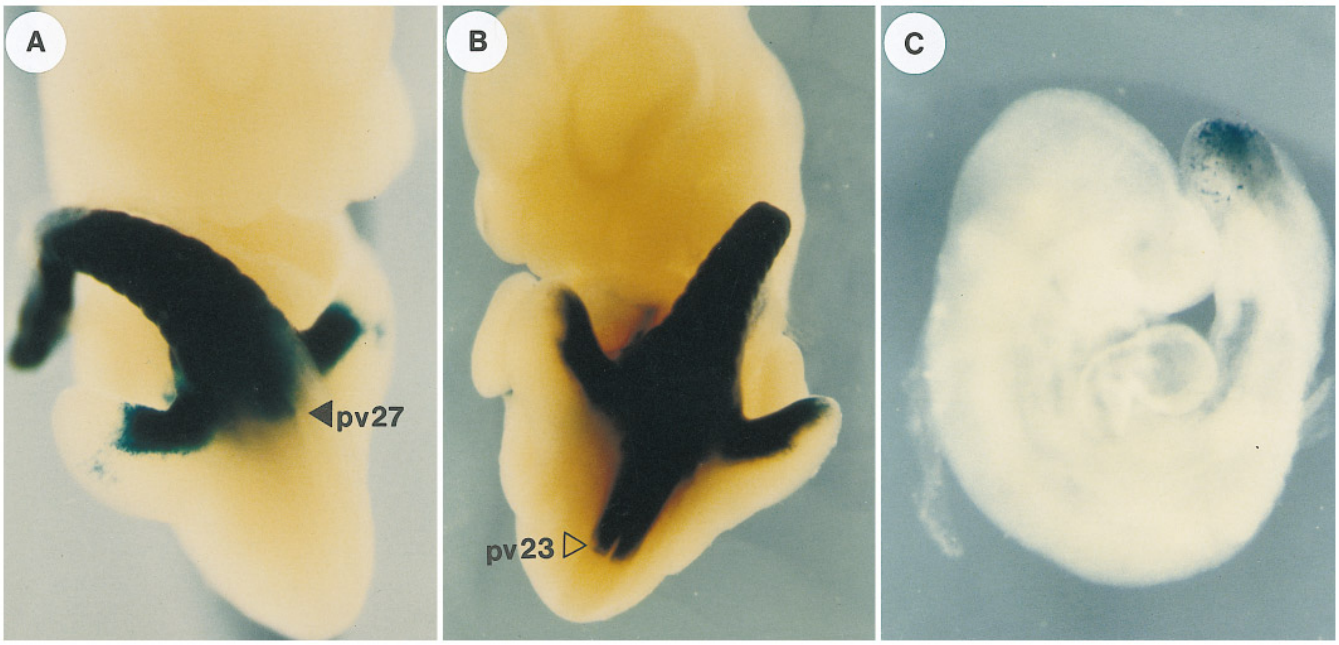

FIG. 2. Chimeric mouse/fish *Hoxd-11/lacZ* transgene expression during fetal development. (A) The mouse Ns-E transgene is expressed in a restricted domain along the anterior to posterior axis, with an anterior limit at the level of prevertebra 27 (arrowhead). (B) The chimeric transgene (Mo/Fi, right), in which the early embryonic enhancer region was replaced for the corresponding zebrafish genomic fragment (see Fig. 1), displayed a related posterior expression pattern with a novel, more anteriorly located limit in the spinal cord, at prevertebra 23. (C) Expression of the Mo/Fi transgene was seen as early as Embryonic Day 9, with a similar spatial restriction. Fetuses in A and B are at E11.5 of development. Black arrowhead shows the control pv27 limit along the main body axis; open arrowhead shows the novel pv23 anterior limit observed with the Mo/Fi transgene.

of the appendicular skeleton also showed occasional fusions between two small bones of the tarsus, the naviculare, and cuneiform 3 (Table 1). To further analyze the origin of these alterations, as well as to assess the effect of the fish enhancer on *Hoxd* gene transcription, we carried out whole mount *in situ* hybridizations on *Hoxd-11*$^{Fi/Fi}$ fetuses.

Whole-mount *in situ* hybridization using a *Hoxd-11* probe showed that *Hoxd-11*$^{Fi/Fi}$ embryos switched on *Hoxd-11* transcription prematurely. This was most visible by staining several homozygous or wild-type litters for *Hoxd-11* transcript accumulation under identical conditions. At early day 9, *Hoxd-11* was detected in the tail buds of *Hoxd-11*$^{Fi/Fi}$ animals, while systematically absent from controls (Fig. 3A, arrowheads). This difference in timing, accentuated soon after when the wild-type allele was activated (Fig. 2B; arrowheads), correlated with a clear-cut anteriorization of the expression boundary in mutant embryos, either at mid Day 9 (Fig. 3D; compare arrowheads on dorsal and ventral sides), or late Day 9 (Fig. 3C). This anterior shift of *Hoxd-11* expression domain was maintained in both spinal cord and paraxial mesoderm until Day 10 of development (Fig. 4; compare A to D and B to C; white arrowheads). In older mutant *Hoxd-11*$^{Fi/Fi}$ animals, however, *Hoxd-11* expression in mesoderm derivatives became undistinguishable from that of wild-type littermates (not shown). Therefore, the early activation of *Hoxd-11* led to an anterioriza-

and all of RIX. (C) Scheme of the recombined locus containing the fish enhancer element and one *loxP* site at a distance of 0.6 kb (bottom). The targeting construct is indicated by the two arrows on the top. Treatment with the cre recombinase gave the final configuration (bottom). The different probes used for typing are indicated under A (1, 2, 3). Ns, *Nsi*I; Xh, *Xho*I, K, *Kpn*I; No, *Not*I; B, *Bam*HI; Xb, *Xba*I; E, *Eco*RI. (D) Sequence alignment between the mouse (top), chick (middle), and fish (bottom) regions VIII (RVIII) and IX (RIX). The red arrows indicate the exact positions of the exchange DNAs as schematized under C and the positions of RVIII and RIX are shown on the right. While the extensive homology between mouse and chick regions VIII was not observed in the fish, region IX was better conserved throughout the three species. For region VIII, even though slightly better conservations could be observed when gaps were introduced, the overall picture was not importantly modified. Sequences in brackets correspond to additional fish DNA found between RVIII and RIX.

TABLE 1
Occurrence of Phenotypic Alterations in Percentage

	Lumbar vertebrae[a]			Fusion of tarsal bones			Exra-digit extra-phalange		
	L6		L5	+		−	+		−
Hoxd-11$^{Fi/Fi}$	44	(52)	56	11	(52)	89	2.5	(210)[b]	97.5
Hoxd-11$^{Fi/+}$	65	(14)	35	0	(14)	100	—[c]		—
Hoxd-11$^{+/+}$	95	(19)	5	0	(19)	100	—		—

Note. Total number of skeletons are in parentheses.
[a] Only complete L6 to L5 transformations were scored. Partial transformations were considered as wild type.
[b] Extra elements in hallux were scored in skeletons or by inspection on an estimated Mendelian distribution of 210.
[c] This phenotype was never observed in our colony.

tion of expression and subsequent rostral transposition of the sacrum. In this case, premature activation was translated into anterior gain-of-function.

Hoxd-11 rostral gain-of-function was not restricted to vertebrae and spinal cord but was also visible in lateral plate mesoderm, such that expression was also anteriorized in hindlimb buds. While *Hoxd-11* was never expressed in most anterior parts of wild-type developing limb buds (Figs. 4C and 4D; black arrowheads), expression of this gene in *Hoxd-11*$^{Fi/Fi}$ fetuses was strong throughout the buds, thereby resembling *Hoxd-10* or *Hoxd-9* patterns at similar stages (Figs. 4A and 4B; black arrowheads). Therefore, as for the trunk, premature activation of a posterior *Hox* gene modified its expression toward a more anterior type of pattern. This ectopic expression of *Hoxd-11* in anterior hindlimb buds correlated with a patterning defect detected in the hindpaws. Most notably, a weakly penetrant phenotype was observed on the anterior digit, the hallux. Five animals showed duplications of some parts of this digit (Figs. 4E–4H) with one triphalangeal hallux (Fig. 4F), a phenotype clearly reminiscent of *Hoxd-11* gain-of-functions in chick leg buds (Morgan *et al.*, 1992; Goff and Tabin, 1997).

DISCUSSION

Interspecies comparison of regulatory sequences is a powerful tool to investigate the conservation of functions in the course of evolution. The combination of this approach with conventional transgenesis has been very informative in the context of *Hox* gene regulation of expression (e.g., Gérard *et al.*, 1993; Marshall *et al.*, 1994; Knittel *et al.*, 1995). The use of the ES cell technology to modify (delete, mutate, transpose) such regulatory sequences in their endogenous contexts allows one to better control the experimental protocol, to circumvent position effects, and to obtain functional data under the best possible physiological conditions (Gérard *et al.*, 1996; Dupé *et al.*, 1997; Zákány *et al.*, 1997). Here, a mouse enhancer sequence was replaced by its fish counterpart *in vivo* in order to evaluate its level of functional conservation between these two species separated by about 800 million years of divergent evolution.

This interspecies DNA exchange has shown that a regulatory element essential for proper activation of *Hoxd-11* transcription could be replaced by its fish counterpart, even though sequence conservation was far weaker than among more closely related tetrapod species such as mouse and chick. While mice devoid of RVIII have a delayed expression of *Hoxd-11* (Zákány *et al.*, 1997), the fish sequence was able to provide for early *Hoxd-11* transcriptional activation, thus reflecting the profound conservation of regulatory mechanisms acting over vertebrate *Hox* complexes. However, *Hoxd-11* transcription, as controlled by the fish enhancer, was clearly premature. This acceleration in *Hoxd-11* transcription may reflect the faster transcriptional activation of *Hoxd* genes in fish. It is thus possible that such enhancer sequences themselves contain information regarding the speed of the activation process. Differential affinities for binding factors or for generating high order chromatin structures could for example account for this observation. Alternatively, the presence of the fish sequence in the murine genomic context might have led to abnormally rapid activation due to the actual nucleotide sequence divergence and differences between the corresponding mouse and fish binding proteins. The more extensive conservation of this regulatory region between mouse and chick suggests that other tetrapod-specific functions may be linked to this element as well. In the presence of the fish control element, alterations of these functions might be either too subtle to be detected in our mutant animals or compensated for by paralogous or neighboring genes.

Transcriptional Heterochrony

The relationship between transcriptional heterochrony (defined as the relative modification of the time of transcription of a given gene with respect to the other components of the genetic system in which this gene is active) and morphological variations is of importance in an evolutionary context (Fig. 5). During development, *Hox* genes are acti-

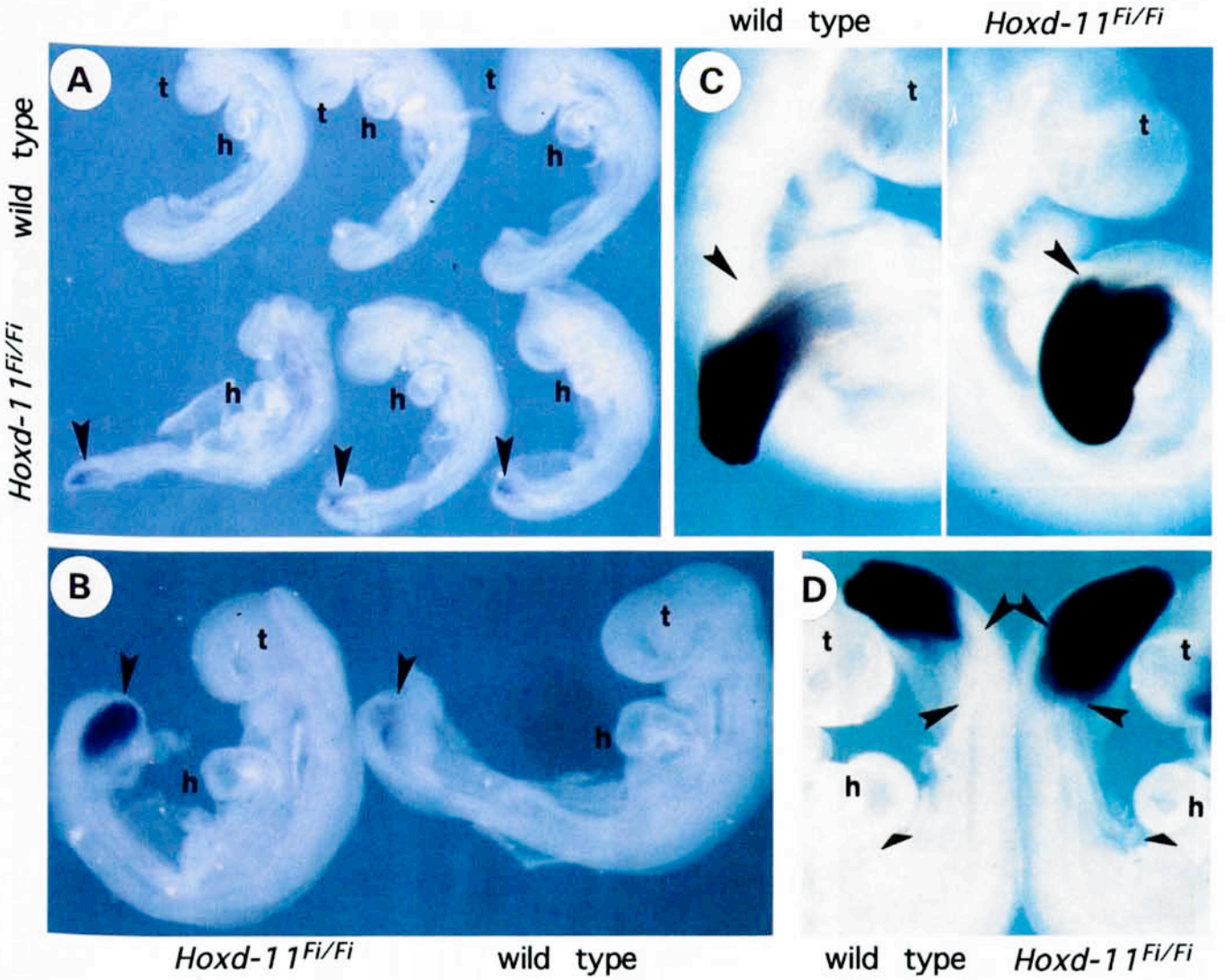

FIG. 3. Expression of *Hoxd-11* in *Hoxd-11*$^{Fi/Fi}$ and control mice. (A) In early Day 9 embryos, mutant animals (bottom) systematically expressed *Hoxd-11* in the tail buds while control specimen (top) did not. (B) A few hours later, expression of the wild-type *Hoxd-11* allele was detected (right); meanwhile, mutant embryos showed strong accumulation of *Hoxd-11* transcripts (left). In A and B the expression domains are indicated by arrowheads and the developing telencephalic vesicles (t) and hearts (h) are shown for direct size comparison. C and D are older fetuses, late and mid Day 9, respectively, showing the resulting anterior shift in expression, both ventrally and dorsally (arrowheads).

vated following a temporal sequence which is colinear with the genes' order in the complexes (temporal colinearity; Izpisua-Belmonte *et al.*, 1991; Gaunt and Strachan, 1996). This sequential activation parallels the progressive rostral to caudal morphogenesis of the vertebrate embryo and may thus be essential for the proper distribution of *Hox* transcripts along the anterior–posterior axis (Duboule, 1994). While the mechanism underlying this intriguing phenomenon remains unknown (discussed in van der Hoeven *et al.*, 1996), changing the speed of *Hox* gene activation, and hence their expression domains, may have been a rich source of morphological evolution (Duboule, 1994). In various vertebrates, specific combinations of *Hox* proteins along the AP axis correlate with homologous vertebral transitions (Gaunt, 1994; Burke *et al.*, 1995), suggesting that evolutionary transpositions of these morphological landmarks along the AP axis may have resulted from variations in *Hox* gene expression. Accordingly, experimental modifications of

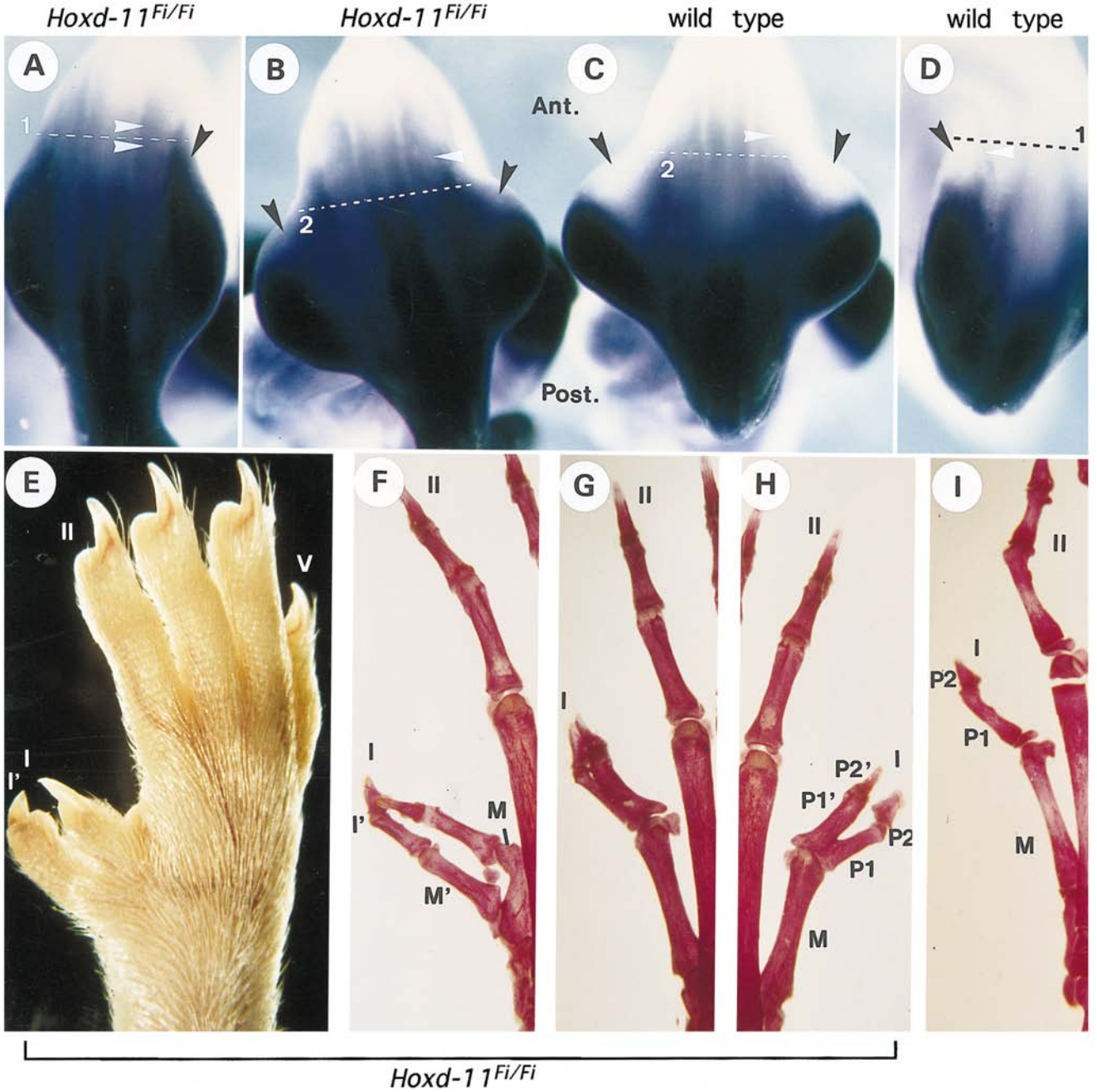

FIG. 4. Expression of *Hoxd-11* in early Day 10 (A, D) and late Day 10 (B, C) *Hoxd-11*$^{Fi/Fi}$ (A, B) or control (C, D) fetuses. Dashed lines (1 and 2) were drawn at the anterior most parts of hindlimb buds for comparing rostrocaudal levels. The anterior shift is clearly visible in the trunk (white arrowheads) and in limb buds, where the most anterior parts, normally free of *Hoxd-11* transcripts (black arrowheads in C and D), are stained in mutant specimen (black arrowheads in A and B). (E–H) Weakly penetrant phenotype observed in the feet of *Hoxd-11*$^{Fi/Fi}$ animals. The anterior digit, the hallux, was duplicated, either entirely (F) or partially (G, H). (I) A wild-type hallux is shown as control. I, II, and V refer to digit number, with I being the most anterior (hallux). I′, supernumerary digit I. M, metatarsal bones; P1, P2; phalanges 1 and 2, respectively. P′, supernumerary phalanges.

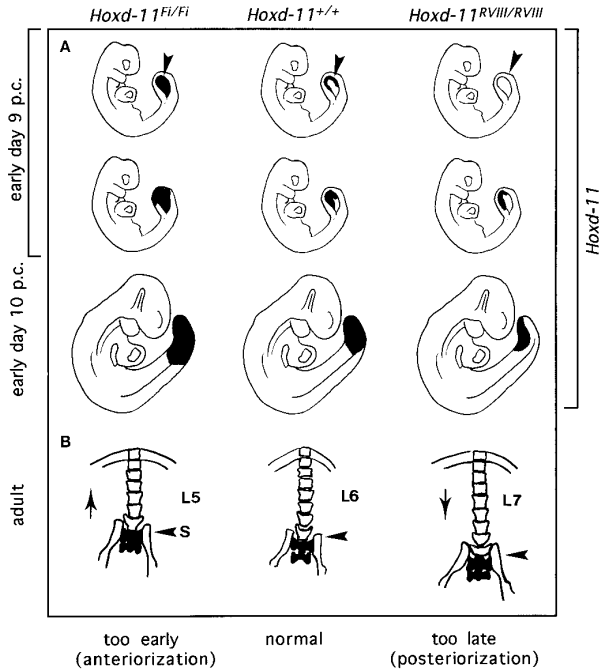

FIG. 5. The effect of heterochronic transcription of *Hoxd-11* (A) upon vertebral morphology in the lumbosacral transition (B). (Middle) Wild-type activation of the gene (shown in black) leads to its appropriate rostrocaudal expression boundary and 6 lumbar vertebrae (L6). (Left) The *Hoxd-11*$^{Fi/Fi}$ allele in which activation is premature, leading to an anterior shift of the expression domain and 5 lumbar vertebrae (L5). (Right) The *Hoxd-11*$^{RVIII/RVIII}$ allele (a deletion of region VIII; Zákány *et al.*, 1997), where activation is delayed leading to a posteriorized expression domain and 7 lumbar vertebrae (L7). The first sacral vertebrae are shown in black and the lumbosacral transition with a black arrowhead. This scheme illustrates that slight differences in the onset of *Hoxd* gene expression have major consequences upon the adult vertebral formula. In this case, changes in time lead to changes in morphology.

Hox gene expression patterns can induce anterior or posterior shifts of the corresponding morphologies, exemplified here by *Hoxd-11* and the lumbosacral transition (Davis and Capecchi, 1994; Favier *et al.*, 1995; Davis *et al.*, 1995; Zákány *et al.*, 1996). Here, we show that premature activation of *Hoxd-11* results in an anterior displacement of both its anterior limit of expression and the sacral morphology, thereby linking the timing of gene function to its topology. In contrast, postponed activation of the same gene was shown to shift the sacrum caudally (Zákány *et al.*, 1997; see Fig. 4). Consequently, changes as subtle as a few hours difference in the activation of a single *Hox* gene can have significant effects on the morphology of the animal. We believe that this clearly illustrates the importance of temporal precision in *Hox* gene activation and of the potential consequences of transcriptional heterochrony. We suggest that temporal colinearity has been a powerful and simple way to evolve morphologies without the need to design novel and complex regulatory sequences.

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