Gene Transpositions in the *HoxD* Complex Reveal a Hierarchy of Regulatory Controls

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Summary

Vertebrate *Hox* genes are activated following a temporal sequence that reflects their linear order in the clusters. We introduced two *Hoxd* transcription units, labeled with *lacZ*, to an ectopic 5′ position in the *HoxD* complex. Early expression of the relocated genes was delayed and resembled that of the neighboring *Hoxd-13*. At later stages, locus-dependent expression in distal limbs and the genital eminence was observed, indicating that common regulatory mechanisms are used for several genes. These experiments also illustrated that neighboring genes can share the same *cis*-acting sequence and that moving genes around in the complex induces novel regulatory interferences. These results suggest that high order regulation controls the activation of *Hox* genes and highlight three important constraints responsible for the conservation of *Hox* gene clustering.

Introduction

Vertebrate *Hox* genes are clustered in four genomic loci containing 9–11 genes (McGinnis and Krumlauf, 1992). They are transcribed from the same DNA strand, and sequence analyses have revealed the high conservation of this genetic system among animal species (Duboule and Dollé, 1989; Graham et al., 1989). The relationship between the clustered organization and the expression patterns of the genes has been studied extensively (e.g., see Gaunt, 1991) and showed that genes located at the 3′ extremities of the complexes are expressed with anterior boundaries while 5′-located genes are expressed in more posterior areas (Gaunt et al., 1988; Krumlauf, 1994). This type of colinearity is analogous to that observed in Drosophila (Lewis, 1978). In vertebrates and many other metazoan, another type of colinearity exists whereby a delay is observed in the appearance of transcripts encoded by more 5′-located genes. During development, transcripts from a given *Hox* gene are usually not detected before those produced by its 3′-located neighbor. Therefore, the physical order of the genes is reflected in the temporal sequence of their activation (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991). Temporal colinearity, initially reported with 5′-located genes, is also observed with more anterior genes (Dekker et al., 1993) and in embryonal carcinoma (EC) cells (Simeone et al., 1990). *Hox* gene activation starts at early gastrulation (Gaunt, 1987; Deschamps and Wijgerde, 1993) and is completed in mice in about 2 days, by the tail bud stage (Dollé et al., 1991a, 1991b). In fish, temporal colinearity is respected in spite of the acceleration of the activation process (van der Hoeven et al., 1996).

Gain- and loss-of-function experiments have shown that *Hox* genes are required to properly build specific body structures (e.g., see Krumlauf, 1994). The nature of the structure that emerges from a particular anterior–posterior (AP) level apparently depends on the specific combination of *Hox* products present at that level (e.g., see Kessel and Gruss, 1991). Coupling between the AP morphogenetic progression and the concurrent sequential *Hox* gene activation may be central to the mechanism that determines these combinations. It is therefore critical to understand the mechanistic bases of colinearities. Among various hypotheses, gene proximity may be necessary for a high order regulatory mechanism to either establish (Dollé et al., 1989) or maintain (e.g., see Peifer et al., 1987; Paro, 1990; Gaunt and Singh, 1990) the proper *Hox* expression patterns, for instance, through transitions in chromatin configurations.

We have used homologous recombination-mediated transgene insertions in embryonic stem (ES) cells to explore to what extent the position of a *Hox* gene in its complex is important for its regulation. We challenged temporal colinearity by inserting *Hox/lacZ* genes at the 5′ extremity of the *HoxD* complex, between *Evx-2* and *Hoxd-13* (Dollé et al., 1994). We separately transposed two genes at the same position. The first was derived from *Hoxd-11*. Upon random integration, it was expressed from prevertebra 27 (pv27) to the tail (Gérard et al., 1993). The second was derived from *Hoxd-9* and was expressed with a more anterior rostral boundary (pv20) (Renucci et al., 1992). We show that the expression of both transgenes was reprogrammed upon relocations. At early stages, the transposed genes were first silenced and then behaved like the nearby *Hoxd-13* gene. Later, some of their original features were nevertheless resumed. Moreover, moving regulatory elements around in the *HoxD* complex induced misregulation and severe developmental defects. From this, we conclude that *Hox* genes are subject to a hierarchy of controls by which the functionality of *cis*-acting gene-specific regulatory elements primarily depends upon high order mechanisms, giving way to more local regulatory interactions.

Results

Insertion of *Hoxd-11*

We first selected a transgene that mimicked important aspects of endogenous *Hoxd-11* regulation. In particular, expression in trunk was comparable with that of the chromosomal locus with an anterior boundary located near pv27 (Gérard et al., 1993). Expression of the transgene was observed in all three germ layers. In such

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Table 1. Genotypes of Mice Used in This Study

Locus	Fusion Gene	Integration	Homologous Arms	PGK*neo*	Orientation in *HoxD*	Glt[a] or Chimeras[b]
TgN[d-11/lac]Ge	*Hoxd-11/lacZ*	Random	−	−		Glt
TgN[d-11/lac/*neo*]Ge	*Hoxd-11/lacZ*	Random	+	+		Chimeras
TgH[d-11/lac/*neo*]Ge	*Hoxd-11/lacZ*	Targeted	+	+	*Hox*-like	Glt
TgH[d-11/lac]Ge	*Hoxd-11/lacZ*	Targeted	+	−	*Hox*-like	Glt
TgN[d-9/lac]Ge	*Hoxd-9/lacZ*	Random	−	−		Glt
TgN[d-9/lac/*neo*1]Ge	*Hoxd-9/lacZ*	Random	−	+		Glt
TgN[d-9/lac/*neo*2]Ge	*Hoxd-9/lacZ*	Random	+	+		Chimeras
TgH[d-9/lac/*neo*]Ge	*Hoxd-9/lacZ*	Targeted	+	+	*Hox*-like	Glt
TgH[d-9/lac/*neo*R]Ge	*Hoxd-9/lacZ*	Targeted	+	+	*Evx*-like	Chimeras

The names of loci (see Experimental Procedures section) are accompanied by short descriptions of their major features.
[a]Germ line transmission was obtained and lines were established.
[b]Animals were analyzed for *lacZ* as chimeras.
Abbreviations: Glt, germ line transmission.

lines (TgN[d-11/lac]Ge) (see Table 1 and the Experimental Procedures section for the nomenclature), however, staining in limbs was essentially absent. An insertion site was chosen in the middle of the *Evx-2/Hoxd-13* intergenic region (Figure 1A, *is*), between the two transcription start sites, after interspecies sequence comparisons had revealed no conservation. The transgene and PGK*neo* selection cassette were flanked by the two halves of this region (Figure 1, X and Y) so that transcription of the fusion gene was as for *Hoxd* genes (Figures 1B and 1C). Positive ES clones (7%) were verified by a combination of probes (Figure 1E). The PGK*neo* cassette was subsequently removed from this TgH[d-11/lac/*neo*]Ge locus to prevent potential interferences

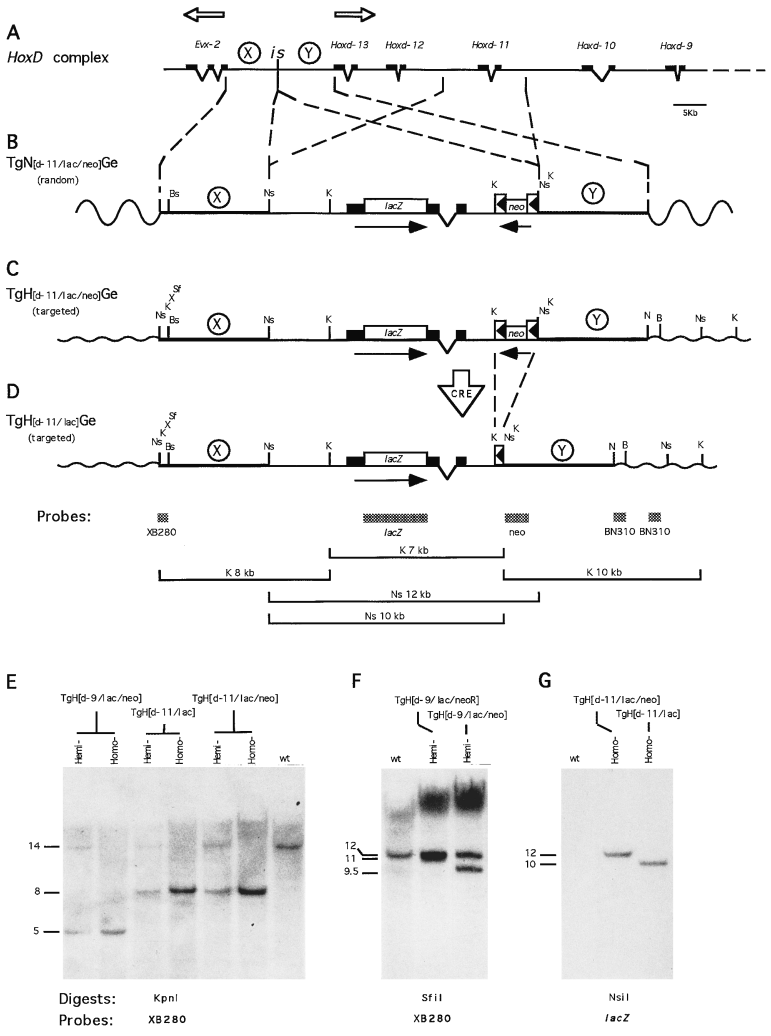

Figure 1. Transposition of *Hoxd-11*

(A) Map of the *HoxD* complex. Dashed lines indicate the origins of the fragments used in the targeting vector. Relocated *Hoxd-11* was fused to the *lacZ* gene. *is*, insertion site.

(B) Targeting vector integrated randomly in the genome (wavy line).

(C) Same vector after homologous recombination (small wavy lines).

(D) Same as under (C), after treatment with the Cre recombinase. PGK*neo* is excised and one *loxP* site is left behind. DNA probes used to verify these configurations are shown below together with sizes of informative restriction fragments.

(E)–(G) Southern blots showing homologous recombinations of both fusion genes.

(E) KpnI digests of DNA from either TgH[d-9/lac/*neo*]Ge animals (Figure 3D) or the two *Hoxd-11* constructs, with or without PGK*neo* (TgH[d-11/lac/*neo*]Ge and TgH[d-11/lac]Ge) (Figures 1C and 1D), probed with XB280.

(F) Same probe on a SfiI digest of animals hemizygous for *Hoxd-9* relocated in either orientation (TgH[d-9/lac/*neo*]Ge or TgH[d-9/lac/*neo*R]Ge) (Figures 3D and 3E).

(G) *lacZ* probe on a NsiI digest shows excision of PGK*neo* from TgH[d-11/lac/*neo*]Ge, producing the TgH[d-11/lac]Ge locus. wt, wild-type DNA.

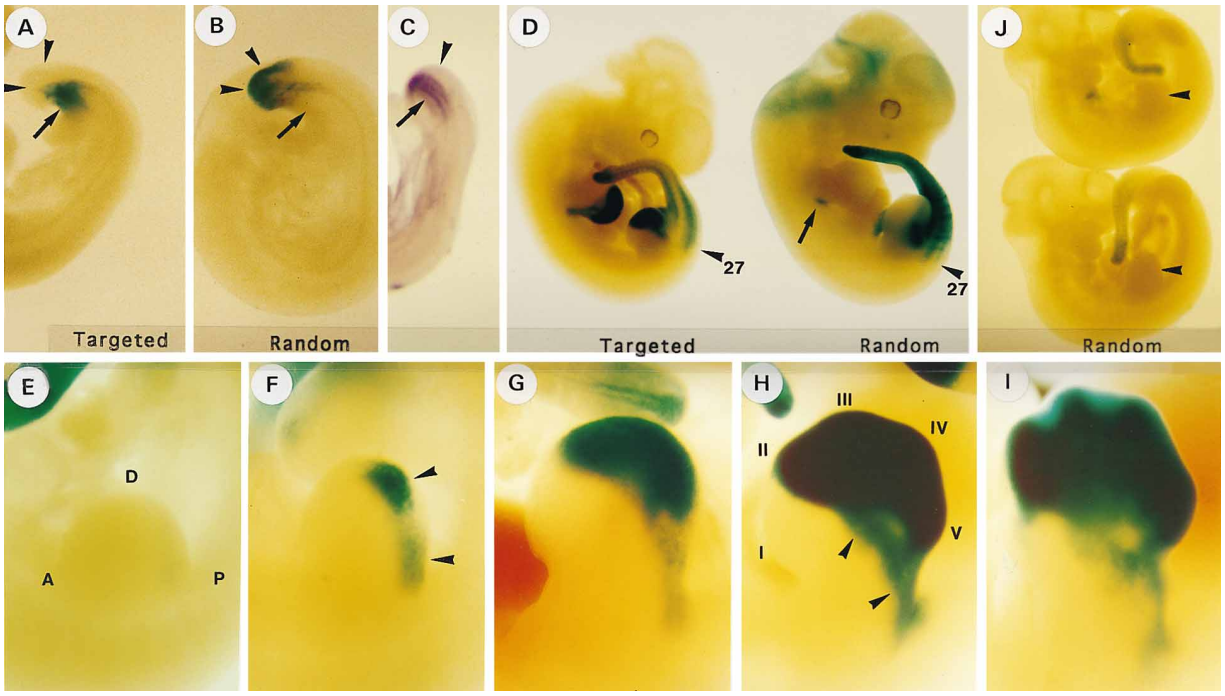

Figure 2. Expression of Relocated or Randomly Integrated *Hoxd-11*

(A) Early expression of relocated *Hoxd-11* (34 somites) in the ventral tail bud, surrounding the proctodaeum (arrow). Tail bud mesenchyme is negative (arrowheads).

(B) The random locus is expressed from the twenty-seventh somite stage throughout the tail bud (arrowheads), but not around the proctodaeum (arrow).

(C) The onset of endogenous *Hoxd-11* RNA expression is shown, by in situ hybridization, at the eighteenth somite stage, in the ventral aspect of the future tail bud (arrow).

(D) Day 11 fetuses expressing the relocated (left) or randomly integrated (right) *Hoxd-11* genes. Note the weaker trunk expression on the left and strong expression in limbs. The pattern of random *Hoxd-11* shows strong staining in trunk while limbs are virtually negative. The AP boundaries are nevertheless comparable (arrowheads).

(E)–(H) Expression of the relocated transgene in developing forelimbs at early day 10 (E), late day 10 (F), late day 11 (G), day 12 (H), and day 13 (I). Staining appears posteriorly (arrowheads) and becomes strong distally, with the persistence of a more proximal forearm domain (arrowheads in [H]).

(J) Two day 11 control chimeric animals (from different ES clones) showing transgene expression when flanked by the homologous arms and integrated randomly. No expression is recorded in either limbs or genitalia (arrowheads). A, anterior; P, posterior; D, distal; I, II, III, IV, and V in panel H point to presumptive digits.

between PGK*neo* and the regulation of neighboring *Hox* genes (Figure 1D, TgH[d-11/lac]Ge). ES cells carrying both versions (Figures 1C and 1D) of transposed *Hoxd-11/lacZ* were introduced in the germ line of chimeric mice, and homozygous animals were produced. As controls, three ES cell clones were isolated that had randomly integrated the targeting vector (Figure 1B, TgN[d-11/lac/*neo*]Ge) in order to evaluate the influence of the *Hoxd-13/Evx-2* intergenic DNA fragments used for recombination.

Expression of Relocated *Hoxd-11*

Lines with and without PGK*neo* showed similar β-gal staining, indicating that the PGK*neo* did not interfere with *Hoxd-11/lacZ* regulation. However, expression was markedly different from that of either the conventional transgene (TgN[d-11/lac]Ge) (Gérard et al., 1993) or the resident *Hoxd-11* gene. First, the earliest sign of relocated *Hoxd-11* expression was detected at the thirty-fourth somite stage, as a lateral stripe of cells between the hind limbs, over the proctodeal region (Figure 2A, arrow). At this stage, no staining was seen anywhere else, in contrast with the uniform posterior staining observed at the twenty-seventh somite stage in conventional transgenic embryos (Figure 2B, arrowheads). Expression of the relocated transgene at this stage was reminiscent of the pattern of resident *Hoxd-11*-specific RNA accumulation in the hindgut diverticulum, seen at an earlier stage (ca. the eighteenth somite) (Figure 2C). Expression was subsequently established in the trunk, primarily in the central nervous system, with an anterior boundary at ca. pv27, corresponding to both endogenous gene and transgenic constructs (Figure 2D, arrowheads).

Second, staining appeared in median and distal parts of developing limbs (Figures 2D–2I), unlike in conventional transgenic embryos (Figure 2D, arrow). The distal limb pattern was like the late domains of all three *Hoxd-11*, *Hoxd-12*, and *Hoxd-13* genes (e.g., see Figure 8). In contrast, the early (posterior) *Hoxd-11* limb domain (Sordino et al., 1995) was not detected (Figure 2E). Expression in the central (forearm) domain was further

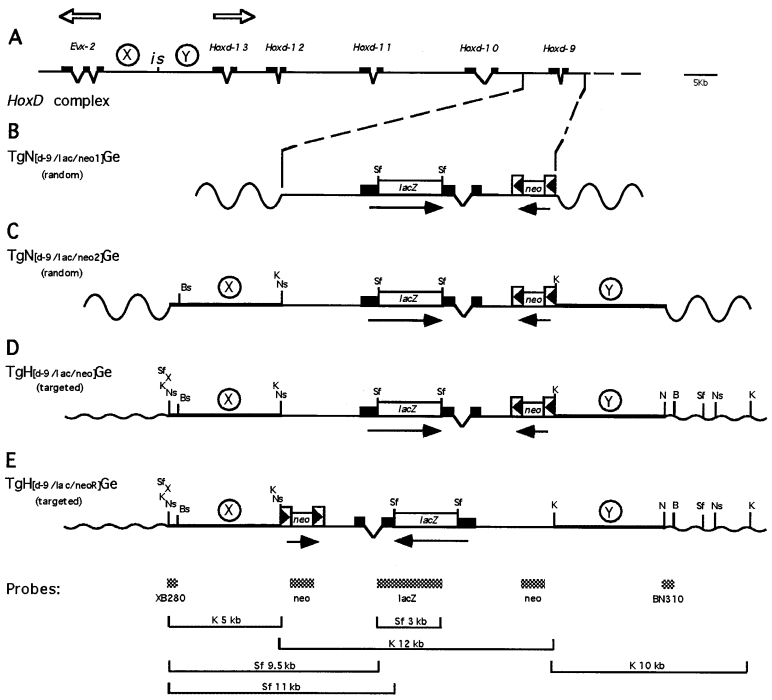

Figure 3. *Hoxd-9* Transpositions

(A) DNA from the *Hoxd-9* locus was flanked by the *loxP*–PGK*neo*–*loxP* cassette and inserted randomly to generate the TgN[d-9/lac/*neo*1]Ge locus (B). The same fragment was flanked by the homologous arms (X and Y) and inserted randomly (TgN[d-9/lac/*neo*2]Ge) (C). Homologous recombination of this latter construct gave rise to TgH[d-9/lac/*neo*]Ge (D). In (E), *Hoxd-9/lacZ* was recombined at the same locus but in the reverse orientation (TgH[d-9/lac/*neo*R]Ge) so that direction of transcription was as for *Evx-2*.

reinforced (Figures 2H and 2I, arrowheads) after the distal domain had been established. Therefore, early expression of relocated *Hoxd-11* resembled that of *Hoxd-13*. At the handplate stage, however, β-gal staining mimicked quite closely that of endogenous *Hoxd-11* (Figures 2H and 2I). Concomitantly, the genital eminence strongly stained, as for both *Hoxd-13* and *Evx-2* genes (Dollé et al., 1994), but unlike the conventional transgene that was expressed there laterally. In summary, expression of relocated *Hoxd-11* first resembled that of *Hoxd-13*. Nevertheless, several aspects of endogenous *Hoxd-11* expression were subsequently resumed.

ES clones with the targeting vector integrated randomly were analyzed in chimeric fetuses. Several animals were obtained for each clone and all gave the same β-gal pattern. While an apparent down-regulation in posterior trunk was observed, no staining in limbs was ever detected (TgN[d-11/lac/*neo*]Ge) (see Figure 1B, and Figure 2J, arrowheads). These experiments showed that the *Evx-2*/*Hoxd-13* intergenic region was not sufficient to drive expression in limbs and genitalia when placed outside of the *HoxD* complex, even though both *Hoxd-13* and *Evx-2* are expressed there.

Insertion of *Hoxd-9*

We next inserted a *Hoxd-9/lacZ* fusion gene for two reasons. First, the time delay between the activations of *Hoxd-9* and *Hoxd-13* (about 1 day) is important enough to observe with confidence a potential effect of the relocation. Second, *Hoxd-9* is expressed only weakly, if at all, in digits. *Hoxd-9/lacZ* was inserted at the same site (TgH[d-9/lac/*neo*]Ge) (Figure 3D) and three additional types of ES cell clones were used as controls. First, we used clones with randomly integrated *Hoxd-9/lacZ* without the homologous arms, but with PGK*neo* (TgN[d-9/lac/*neo*1]Ge) (Figure 3B). This single copy locus was transferred into germ line. Second, we used ES clones with randomly integrated targeting construct (Figure 3C) to assess the behavior of the transgene flanked by the homologous arms (as in Figure 1B). Finally, we investigated the importance of transcriptional orientation by using a targeting vector wherein both *Hoxd-9/lacZ* and PGK*neo* were in the reverse orientation (TgH[d-9/lac/*neo*R]Ge) (Figure 3E).

Expression of Relocated *Hoxd-9*

In many respects, expression of relocated *Hoxd-9* resembled that of relocated *Hoxd-11*. β-gal was detected in distal limbs (Figure 4A) while no trace of the proximal *Hoxd-9* pattern was observed. Expression was very weak (if there was any) in forearm and much reduced in trunk (Figures 4A and 4B). Nevertheless, an AP boundary was subsequently observed at the expected position for *Hoxd-9* (ca. pv20) (Figure 4A). As for *Hoxd-11*, relocation of *Hoxd-9* led to strong expression in the genital bud (Figures 4A and 4B, arrows). We observed that three ES clones carrying random integrations (see Figure 3C) gave reduced signal in trunk mesoderm, weak in neural tube, and nothing in limbs (Figure 4C, arrowheads), corroborating *Hoxd-11* control clones. Expression of *Hoxd-9* inserted in the reverse orientation (see Figure 3E) was analyzed at 13.5 days. At this stage, both orientations gave similar staining (Figures 4D and 4E), in particular in digits and genitalia.

Timing of Activation

The time of *Hoxd-9* activation was analyzed by using the TgN[d-9/lac/*neo*1]Ge locus (see Figure 3B). This line was similar to the conventional *Hoxd-9* transgenic line

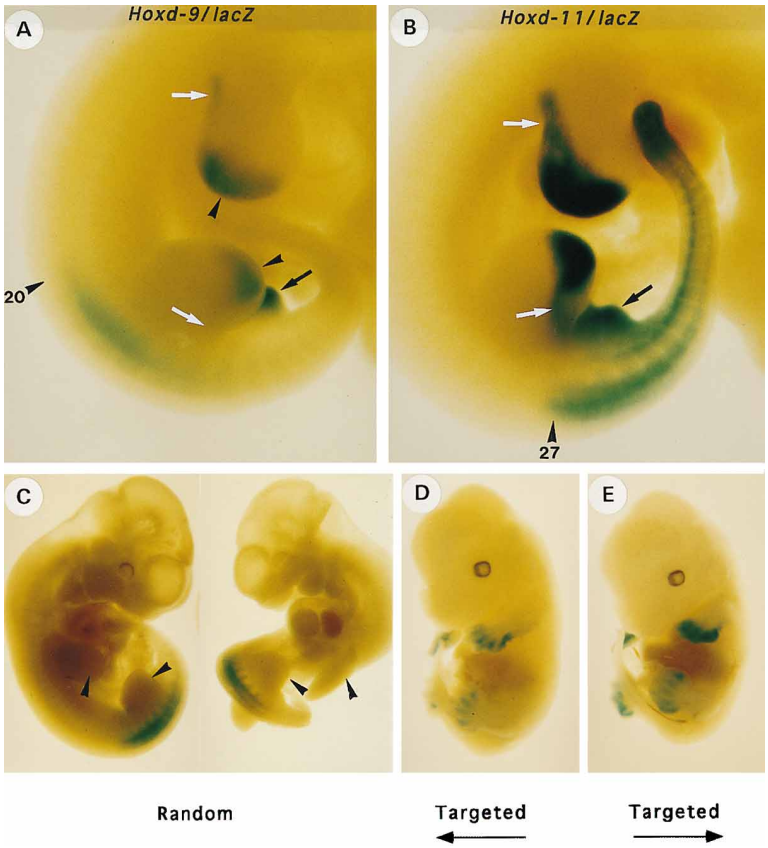

Figure 4. Expression of *Hoxd-9/lacZ* and *Hoxd-11/lacZ* in Different Genomic Configurations

Relocated *Hoxd-9* (A) and *Hoxd-11* (B) in day 11 fetuses. Expression in limbs is similar distally (arrowheads), while no clear staining is seen proximally for *Hoxd-9* (white arrows). In both cases, strong staining is detected in genital tubercles (black arrows).

(C) Two day 11 fetuses, chimeric for ES cell with the same construct as in (A) but integrated randomly (Figure 3C). Expression in the trunk is as for (A), but limbs and genitalia are negative (arrowheads).

(D) and (E) Expression of relocated *Hoxd-9* in both transcriptional orientations; the "*Hox*" orientation (E) or the "*Evx*" orientation (D). Stainings are virtually identical.

but carried a single copy flanked by the selection cassette. It was thus identical to the relocated locus, except for genomic locations, the former being outside the complex, the latter inside (see Figures 3B and 3D). In this random line, *Hoxd-9/lacZ* was expressed as in conventional lines (Renucci et al., 1992). Strong staining was present in trunk mesoderm and spinal cord posterior to pv19–20, extending to the developing tail. The limb mesenchyme was essentially negative (Figure 5C, left).

We assessed the times of activation by crossing the random and targeted lines. In one cross, females were homozygous for relocated *Hoxd-9* (TgH[d-9/lac/*neo*]Ge) and males hemizygous for a random copy (TgN[d-9/lac/*neo*1]Ge). F1 animals carried either one copy of each relocated and randomly integrated *Hoxd-9* (Figures 5A and 5B, random/targeted) or one copy of relocated *Hoxd-9* only (Figures 5A and 5B, targeted). β-gal reaction at early day 9 gave half of the litter stained with the random pattern (Figure 5A, left). Other littermates had no staining, even though they were hemizygous for relocated *Hoxd-9* (Figure 5A, right). Staining of similar litters 12–15 hr later showed that all animals were now stained, half with the relocated pattern (Figure 5B, right, arrow), the other half with the random pattern (Figure 5B, left). In a second cross, one parent was hemizygous for the random copy, the other hemizygous for the targeted copy. Comparison of littermates illustrated again the major differences in either the distal limb domain (arrows), the genital eminence (arrowheads), or the expression in trunk (Figure 5C).

Phenotypes

Mice homozygous for relocated *Hoxd-11* with PGK*neo* (see Figure 1C) displayed a digit phenotype. Digits II and V were abnormally short, owing to reduction or absence of the second phalange (P2) (Figures 6A–6C, arrowheads). As this resembled a *Hoxd-13* allele, we carried out a complementation test. Mice *trans*-hemi/heterozygous for relocated *Hoxd-11* and null *Hoxd-13* (TgH[d-11/lac/*neo*]Ge/o; *Hoxd-13*$^{+/-}$) showed a clear *Hoxd-13* phenotype, including truncations of P2 in digits II and V (Figure 6D, arrowheads), while *Hoxd-13*$^{+/-}$ animals were essentially normal (Figure 6E). We concluded that transposition of *Hoxd-11* produced a hypomorph *Hoxd-13* allele. The same digit phenotype was observed upon *Hoxd-9* relocation (data not shown).

Surprisingly, mice homozygous for transposed *Hoxd-11* without PGK*neo* (see Figure 1D) displayed a severe phenotype, not only in digits, but also in the carpus and forearm regions. Both radius and ulna were short and abnormal in shapes, precluding proper formation of the wrist and inducing lateral deflections of the hands (Figure 7). This forelimb-specific phenotype was similar to that of mice lacking three doses of group 11 *Hox* genes (Davis et al., 1995). Fetuses homozygous for relocated *Hoxd-11* were therefore analyzed for the expression of neighboring genes. Specimen from all genotypes had equivalent *Hoxd-11* RNA staining (Figure 8D), suggesting that the insertion of *Hoxd-11/lacZ* did not affect the resident *Hoxd-11* gene. In particular, expression in presumptive forearms and digits was indistinguishable from controls (Figure 8D, arrowheads), indicating that

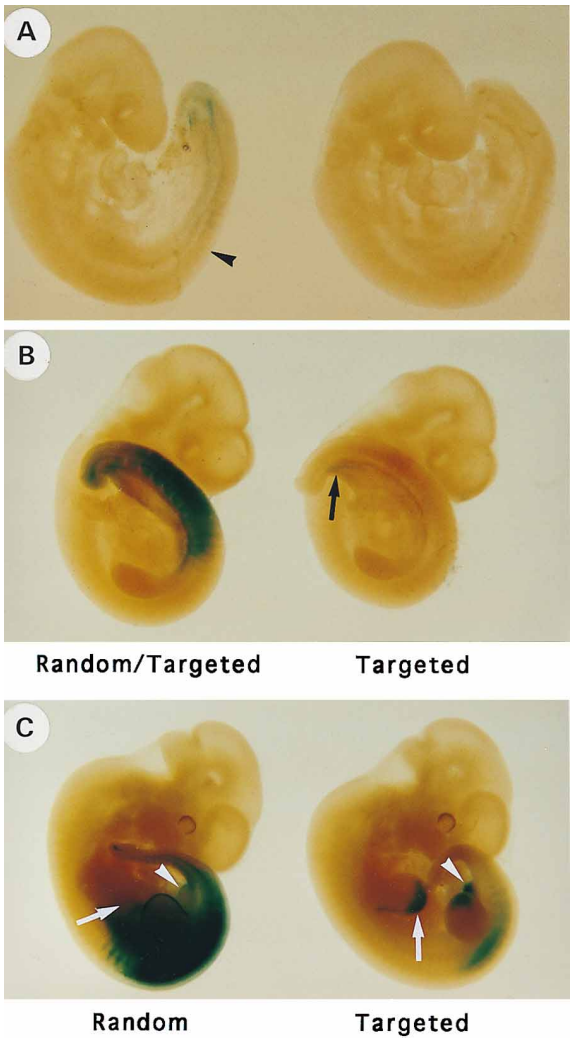

Figure 5. Temporal Regulation of Targeted Versus Random *Hoxd-9*

(A) and (B) Littermates that have segregated the two *Hoxd-9* configurations. Specimen in the left have both random and targeted copies (Figures 3B and 3D), while specimen in the right have only the targeted copy (Figure 3D). (A) Early day 9 and (B) late day 9. Expression of the random copy appears before day 9, from pv20 (arrowhead). In contrast, expression of the targeted copy is first observed at late day 9 (B, arrow), in the proctodeal area.

(C) Day 10 littermates with either one random (left) or one targeted (right) copy. At this stage, differences in expression in limbs and genitalia are spectacular.

the phenotype was not associated with loss-of-function of group 11 genes.

In contrast, *Hoxd-13* transcript distribution in fetuses homozygous for relocated *Hoxd-11* without PGK*neo* was importantly modified, as they displayed a robust ectopic domain in the zygopodium (Figure 8A, bottom, arrowheads), a region where *Hoxd-13* is normally not expressed but where group 11 genes are functional (Figure 8D). Interestingly, this ectopic domain was barely detected in fetuses homozygous for relocated *Hoxd-11* with PGK*neo* cassette (Figure 8A, middle). Therefore, a correspondence was established between the absence of PGK*neo*, an ectopic *Hoxd-13* expression in forearms, and the occurrence of the forearm phenotype. Altogether, this suggested that forearm alterations resulted from a *Hoxd-13* gain-of-function. These studies also revealed a decrease in the quantity of *Hoxd-13* transcripts in the distal limb domain of homozygous transgenic animals (Figure 8A), consistent with the *Hoxd-13* hypomorphic digit phenotype (see Figure 6).

Discussion

Hox Complex–Dependent Silencing Effect

We have used ES cell–based gene insertions as a tool for studying the mechanistic bases of colinearity. We report results from experiments wherein *Hox* genes were introduced at an ectopic position within their own complex. We discuss the implications of these observations with respect to the importance of high order regulatory control, *Hox* complex–dependent versus gene-dependent regulation, and the sharing of regulatory elements.

During gastrulation, vertebrate *Hox* genes are activated in a temporal sequence that reflects their positions on the complexes (Izpisúa-Belmonte et al., 1991). This progressive availability of novel *Hox* functions is likely a prerequisite for the correct establishment, in time and space, of a combinatorial system, and may thus be essential for specification of structures (Duboule, 1992, 1994). Mechanistic explanations of temporal colinearity fall into three nonexclusive categories. First, *Hox* genes may be activated following a cascade of controls exerted by more 3′-located genes. In this view, group 1 gene products are necessary to activate group 2 genes, et cetera. Second, *Hox* genes may respond differentially to a regulatory molecule in a colinear fashion, e.g., through a gradient of affinities. This (morphogen) proposal (Gaunt and Strachan, 1994; Grapin-Botton et al., 1995) may incorporate components of the first (reviewed by Langston and Gudas, 1994; Krumlauf, 1994). Finally, temporal colinearity may involve high order regulatory controls. In this latter case, *Hox* genes may either become sequentially available for transcription owing to the relief of a pre-existing barrier, e.g., through a transition from an inactive to active state of chromatin configuration (Dollé et al., 1989; Duboule, 1992), or be regulated by long-range interactions with a control region, in a way related to the globin complex (Hanscombe et al., 1991; Wijgerde et al., 1995). High order regulation does not exclude the first two mechanisms but makes them secondary, for they would be subordinated to a more global mechanism acting on the complex.

The delay observed in the activation of relocated versus random *Hoxd-9* suggests that neither of the first two proposals can solely account for temporal colinearity. In both cases, the presence of identical *cis*-acting sequences would predict the same expression patterns, regardless of position. We demonstrate here that while the *Hoxd-9* transgene carried in *cis* sequences is capable of early activation, the locus exerted a silencing effect whereby the function of these sequences was delayed. This could reflect the silencing effect of a discrete element located near the insertion site. Alternatively, such a silencing may result from a linear mechanism involving, e.g., transitions in chromatin configurations. In Drosophila, Polycomb group gene–dependent

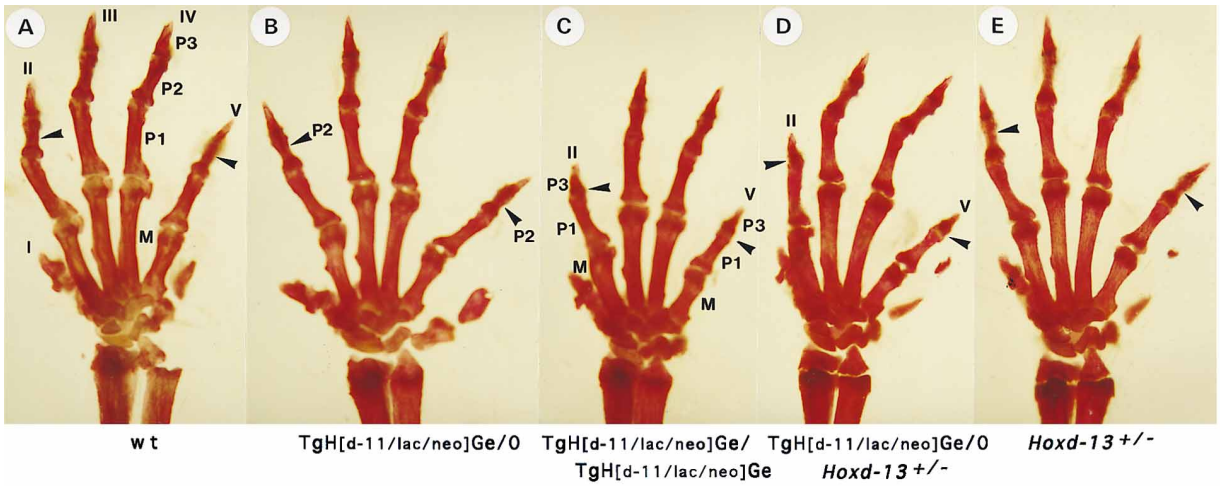

Figure 6. Digit Phenotype after *Hoxd-11* Relocation

(A) Wild-type hand.

(B) Hands hemizygous for relocated *Hoxd-11* appear normal. In contrast, animals with two copies of relocated *Hoxd-11* (C) have reduced and fused P2s in digits II and V (arrowheads).

(D) Noncomplementation between the TgH[d-11/lac/*neo*]Ge locus and the *Hoxd-13* null allele. Trans-hemi/heterozygous animals have abnormal skeleton, resembling a weak *Hoxd-13* mutant forepaw (loss of P2 in digit II and V; arrowheads).

(E) Hand heterozygous for the *Hoxd-13* allele (arrowheads). I to V; from thumb to minimus. P1, P2, P3, phalanges; M, metacarpals.

heterochromatin formation is thought to maintain the inactive state of Bithorax complex genes (e.g., see Paro, 1990; Simon et al., 1993). While related molecules may be involved in the regulation of vertebrate *Hox* genes (Pierce et al., 1992; Alkema et al., 1995; Van der Lugt et al., 1994; Yu et al., 1995; Müller et al., 1995), our results suggest that they would be used to serve an opposite strategy whereby vertebrate *Hox* complexes may undergo a "closed to open" transition, as opposed to the Drosophila "open to closed" process. We favor a view in which a progressive 3′ to 5′ transition in chromatin configuration would lead to stepwise accessibility of genes for transcription. Such a mechanism could account for the transitory silencing of relocated *Hoxd-9*-associated elements. Relocation of the same reporter gene at an intermediate position (e.g., the *Hoxd-11/10* intergenic region) may help to discriminate between these alternatives.

These experiments also have shown that a *Hox* gene activated late eventually established a rather anterior AP level of expression. Early expression of both relocated genes resembled that of *Hoxd-13*, yet late patterns contained some traits of the corresponding resident genes, suggesting that the position of the AP expression level does not necessarily depend upon the time of activation. The late appearance of these anterior shifts may indicate that the mechanisms responsible for temporal colinearity give way to other control mechanisms later in development. However, the precise time at which a *Hox* gene is expressed at a particular AP level may be of utmost importance, as for *Ubx* in the Drosophila thorax (Castelli-gair and Akam, 1995). It is also noticeable that some randomly integrated transgenes seem to conserve their timing of expression (e.g., see Whiting et al., 1991; Püschel et al., 1991), indicating that individual genes may carry in *cis* the necessary information (e.g., a target sequence for a factor expressed in a temporal fashion). In this respect, temporal colinearity may be required either to coordinate sequential activations or to act as a safety mechanism to prevent "late" genes from being turned on too early, a situation likely detrimental to the animals. Also, the importance of cross- and auto-regulation in transgenic animals has yet to be precisely assessed.

Hoxd Genes in Digits

Hoxd-10 to *Hoxd-13* are expressed in similar distal domains covering the presumptive digital area (Sordino and Duboule, 1996). This is of particular interest, since this domain is not established during development of teleost pectoral fins, and may thus be related to the neomorphic status of vertebrate digits (Sordino et al., 1995). Our transgenic analyses of the mouse *HoxD* locus has failed to identify regulatory element(s) for this distal domain (e.g., see Gérard et al., 1993). Nevertheless, when fusion genes were relocated near *Hoxd-13*, expression in distal limbs was induced. Transgenic limb expression did not result from the proximity of a *Hoxd-13* limb *cis*-acting element, as random integration in the presence of the intergenic region did not elicit such a regulation, suggesting that distal limb expression is primarily a locus-specific rather than gene-specific trait. This was further substantiated by the expression of PGK*neo* in both distal limbs and genitalia (Figure 8E), raising the possibility that from *Hoxd-10* to *Evx-2* expression in digits is regulated by one single control mechanism, in support of an evolutionary scenario by which *Hoxd* genes simultaneously evolved the same distal expression domain in parallel to the emergence of digits. Surprisingly, expression in digits and genital bud were always observed together, perhaps as a sign of shared developmental and evolutionary histories between these two specialties of higher vertebrates.

The expression pattern of *Hoxd-9* relocated in the

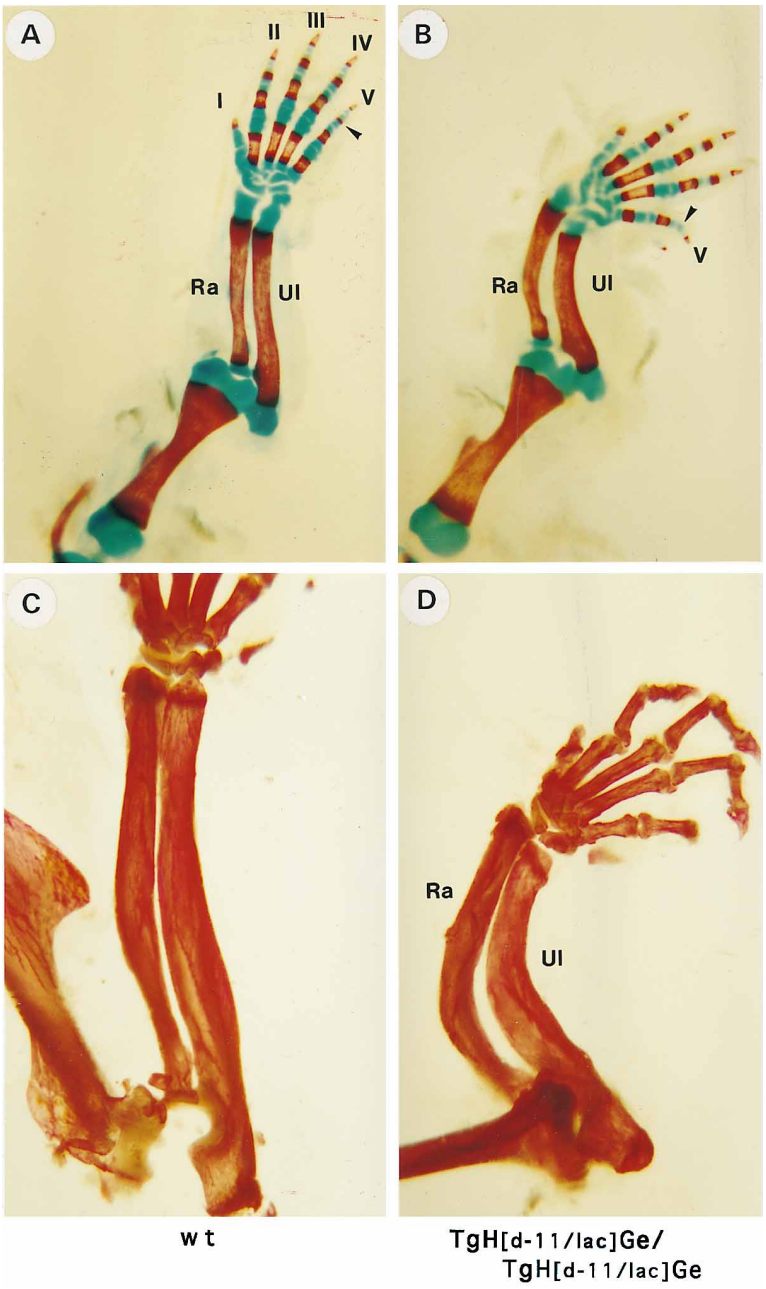

Figure 7. Forearm Phenotype of Mice Homozygous for Relocated *Hoxd-11* without PGK*neo*

Wild-type (A) and mutant (B) arms, 3 days after birth. An important deformation of forearm bones is visible. Both the radius and ulna are short and bent posteriorly. Reduction of the ulna induces an abnormal joint with the carpus, thus bending the whole hand by ~70°, posteriorly. In adult arms (C and D), the alterations are even stronger, up to 40% of normal size. More proximal parts of the forelimbs are normal. Ra, radius; Ul, ulna.

inverted transcriptional orientation argued against the presence of polycistronic or differentially spliced RNAs initiating from a master limb element between *Hoxd-13* and *Evx-2* to extend up to *Hoxd-10*. This further supports enhancer-sharing as a plausible mechanism.

Sharing Regulation and the Limb Phenotypes

A recessive digit defect was seen in all three lines that had inserts between *Evx-2* and *Hoxd-13*. We tested the TgH[d-11/lac/*neo*]Ge locus and showed that this was likely a hypomorph *Hoxd-13* loss-of-function allele. The corresponding reduced *Hoxd-13* transcription may reflect either a competition between the introduced transcription units and *Hoxd-13* for the distal limb regulatory element or a physical interference between the two genes. Expression in the presumptive forearm was observed only with relocated *Hoxd-11*, which indicated the presence of a corresponding *cis*-acting element in the transgene. However, as this was not seen with randomly integrated *Hoxd-11*, the environment of the complex may be functionally necessary. The presence of this element induced strong *Hoxd-13* ectopic expression in forearms when PGK*neo* was removed, indicating a possible insulator effect of this cassette (Figure 8). Simultaneous expression of both relocated *Hoxd-11* and resident *Hoxd-13* in forearms illustrated functional sharing of a vertebrate *Hox cis*-acting regulatory element (see Krumlauf, 1994). Ectopic *Hoxd-13* expression in forearms produced severe alterations of both the radius and ulna, similar to that described when two *Hox* group 11

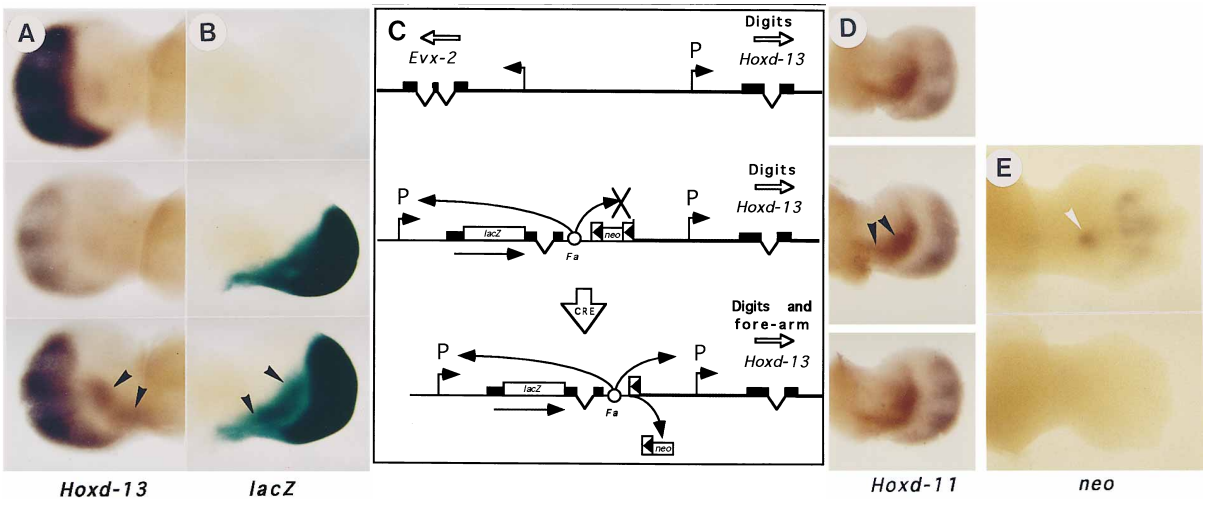

Figure 8. Expression of Posterior *Hoxd* Genes after Relocation of *Hoxd-11*

In situ analysis of *Hoxd-13* (A), *Hoxd-11* (D), and *neo* (E) RNAs and β-gal activity (B), in day 12 (A–D) or day 13 (E) forelimbs of animals from three genotypes: wild-type (top) and homozygous for relocated *Hoxd-11* with (middle) or without (bottom) PGK*neo* (see scheme). While expression of relocated *Hoxd-11* was not affected by PGK*neo* (B), expression of *Hoxd-13* was reduced distally in both PGK*neo* positive and negative variants (A). An ectopic *Hoxd-13* domain appeared in forearms of PGK*neo* negative animals (A, arrowheads), overlapping with *lacZ* expression (B, arrowheads). Resident *Hoxd-11* was not affected in either genomic configurations (D).

(C) Scheme of the loci with a potential explanation for *Hoxd-13* ectopic expression (see Discussion).

(E) Expression of *neo* is of the *Hox* type, with domains in digits and in the region of the wrist (white arrowhead). Specimen in (A) and (B) (top and bottom) were littermates processed together. Limbs in (A) and (B) are contralateral limbs. Anterior is up.

genes were inactivated (Davis et al., 1995), yet these mice had normal *Hoxd-11* RNA content. A possibility is that ectopic HOXD13 protein antagonizes the function of group 11 genes without affecting their transcriptions ("posterior prevalence") (Duboule and Morata, 1994), e.g., through competition for target sites or titration of cofactors, thereby inducing a phenocopy of group 11 deficiency in forearms. A similar phenotype was obtained by ectopic expression of *Hoxa-13* in chick wing buds (Yokouchi, et al., 1995), suggesting that group 13 proteins share this property. Ongoing crosses mixing one relocated *Hoxd-11* chromosome with two *Hox* group 11 null alleles generate forearm alterations, consistent with this hypothesis.

In summary, these gene transpositions have revealed three functional constraints linked to this peculiar clustered organization: first, the necessity for a high order complex-wide regulatory mechanism to coordinate expressions of closely located genes in a colinear fashion; second, the existence of enhancer elements controlling several genes at once, as shown by the distal limb and genital domains; and finally, sharing of *cis*-acting sequences between neighbor genes, involving more intimate structural conservation. At different levels of complexity, each of these constraints may have contributed to the remarkable structural and functional conservation of *Hox* clusters throughout evolution.

Experimental Procedures

Nomenclature

We have adapted the recommendations of Stewart (1995) and the genotypes are described in Table 1. They result from either random (N) or targeted (H) integration of *Hoxd-11/lacZ* and *Hoxd-9/lacZ* (d-11, d-9, lac) transgenes (Tg) together with the PGK*neo* selection cassette. Consequently, the lines should be described as, e.g., TgH[d-11/lac/*neo*]Ge/o for mice that have one copy (hemizygous) of the homologously (H) recombined *Hoxd-11/lacZ/neo* ([d-11/lac/*neo*]) construct (produced and kept at the University of Geneva, Ge). Other lines are named accordingly.

Recombinant DNA Techniques

For TgN[d-11/lac/*neo*]Ge and TgH[d-11/lac/*neo*]Ge, the *loxP*–PGK*neo*–*loxP* cassette was cloned 3′ to the *Hoxd-11/lacZ* fragment (Ns-E) (Gérard et al., 1993). This construct was inserted into the NsiI site of the 9.5 kb NotI fragment that covers the *Hoxd-13/Evx-2* intergenic region. For TgN[d-9/lac/*neo*1]Ge, TgN[d-9/lac/*neo*2]Ge, TgH[d-9/lac/*neo*]Ge, and TgH[d-9/lac/*neo*R]Ge, the *loxP*–PGK*neo*–*loxP* cassette was cloned 3′ to a *Hoxd-9/lacZ* fragment (p4.4*lacZ*) (Renucci et al., 1992). For TgN[d-9/lac/*neo*2]Ge, TgH[d-9/lac/*neo*]Ge, and TgH[d-9/lac/*neo*R]Ge, they were ligated in a KpnI linker introduced at the same NsiI site. For TgN[d-9/lac/*neo*1]Ge, the *loxP*–PGK*neo*–*loxP* cassette was cloned 3′ to the *Hoxd-9/lacZ* fragment. Targeted and random ES clones were tested with four DNA probes. We checked 5′ integration with XB280 (Figure 1), which gave the predicted 8 kb or 5 kb KpnI fragments. We verified 3′ integration with BN310, which gave a 10 kb KpnI fragment. The integrity of the constructs were controlled with *lacZ* and *neo* probes. Presence of *neo* was indicated by a 12 kb NsiI fragment, while its absence generated a 10 kb NsiI fragment. Mice were genotyped with tail DNA. 10 μg of DNA was digested, run onto an agarose gel, transferred to Hybond nylon membranes, and probed with DIG or radioactive labeled fragments.

ES Cells, Electroporation, and Microinjection into Blastocysts

ES cells (D3) (Doetschman et al., 1985) were cultured on embryonic fibroblasts with 1000 U/ml LIF produced by transfection of pC10–6R DIA/LIF. We electroporated 2×10^7 ES cells with 40 μg of linearized DNA (400V, 125 μF, for 2 s and 3 s). Cells were selected and amplified according to Joyner (1993). The *loxP*–PGK*neo*–*loxP* cassette was excised by electroporation of pMC–Cre (Gu et al., 1993). Microinjection of ES cells in C57/Bl6 blastocysts were carried out following standard procedures.

β-gal Staining, In Situ Hybridization, and Skeletal Preparations

β-gal staining of fetuses and in situ hybridizations were done as previously described (Zákány et al., 1988; Dollé et al., 1993). For skeletal preparations, adult or juvenile mice were processed according to Inouye (1976).

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