Developmental Cell *10*, 93–103, January, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2005.11.014

Control of *Hoxd* Genes' Collinearity during Early Limb Development

Basile Tarchini[1] **and Denis Duboule**[1,*]
[1] Department of Zoology and Animal Biology and
National Research Centre "Frontiers in Genetics"
University of Geneva
Sciences III
Quai Ernest Ansermet 30
1211 Geneva 4
Switzerland

Summary

***Hoxd* genes are essential for limb growth and patterning. They are activated following a complex transcriptional regulation, leading to expression domains that are collinear in both space and time. To understand the mechanism(s) underlying collinearity, we produced and analyzed a set of mouse strains containing systematic deletions and duplications within the *HoxD* cluster. We show that two waves of transcriptional activation, controlled by different mechanisms, generate the observed developmental expression patterns. The first wave is time-dependent, involves the action of opposite regulatory modules, and is essential for the growth and polarity of the limb up to the forearm. The second phase involves a different regulation and is required for the morphogenesis of digits. We propose that these two phases reflect the different phylogenetic histories of proximal versus distal limb structures and discuss the biological relevance of these collinear patterns, particularly for the origin of the anterior-to-posterior limb polarity.**

Introduction

Clustered genes belonging to the *Hox* family encode transcription factors necessary for proper development along the major body axis. In mesoderm derivatives, as well as in the spinal cord, various combinations of HOX proteins instruct cells as to their morphological fates, depending on their anterior-to-posterior (AP) positions along the trunk axis. Slight variations in these protein combinations can lead to important alterations in the resulting morphologies; hence, the distribution of such proteins has to be precisely orchestrated. Most of this control occurs at the transcriptional level, since *Hox* mRNAs already display precise combinatorial distributions.

This task is achieved in part through an intrinsic property of the system whereby clustered genes are activated in time and space by following their genomic topography; genes at the 3′ end of the clusters are activated first, early on and in the most anterior parts of the developing embryo, whereas genes located at progressively more 5′ positions are activated subsequently and in more posterior areas (Kmita and Duboule, 2003; Deschamps and van Nes, 2005). This phenomenon, referred to as collinearity, was originally described by Lewis (1978) while studying the genetics of the fruit fly *Bithorax* homeotic gene complex (BX-C), and it was subsequently extended to vertebrates (Gaunt et al., 1988). In the course of vertebrate evolution, this collinear regulation was coopted several times along with the emergence of structures developing from secondary axes, such as external genital organs and the limbs.

In mammals, both *HoxA* and *HoxD* gene clusters play a major growth-promoting function during early limb development, as shown by the effect of their partial or full combined inactivation leading to severe truncations (Davis et al., 1995; Kmita et al., 2005), rather than to homeotic transformations. In contrast, neither the *HoxB* nor the *HoxC* complexes seem to have a similar role, based on their expression patterns and the minor effects, if any, of their deletions in vivo (Suemori and Noguchi, 2000; Medina-Martinez et al., 2000). While expression of *Hoxa* genes suggests a function in the definition of proximal-to-distal domains, their *Hoxd* counterparts display asymmetric patterns as well along the posterior-to-anterior axis, indicating an additional role in the establishment of this critical skeletal polarity. This was confirmed by experiments in which a uniform expression of *Hoxd* genes led to limbs with bilateral symmetry (Zakany et al., 2004).

Hoxd genes are activated in limb buds by following multiple collinear strategies. Early on, in the incipient limb buds, genes are activated in a time sequence starting with the most 3′-located members, such as *Hoxd1* and *Hoxd3*. These genes are expressed throughout the emerging bud, a rather homogeneous expression observed until *Hoxd9*, which still displays this uniform pattern. Starting from *Hoxd10*, however, the expression domains become progressively restricted to successively more posterior limb cells, until *Hoxd12* and *Hoxd13*, as a set of nested patterns (Dollé et al., 1989; Nelson et al., 1996). Therefore, two collinear processes can be observed in the early limb bud, in time and space, the former hypothetically controlling the latter.

This restriction of the most 5′-located *Hox* gene expression in the posterior part of the developing bud is essential to trigger and/or maintain expression of the gene *Sonic hedgehog* (*Shh*) at the posterior margin, as shown by both gain- and loss-of-function experiments (Charité et al., 1994; Knezevic et al., 1997; Zakany et al., 2004; Kmita et al., 2005). In turn, likely by antagonizing the repressive effect of the *Gli3* gene product (Litingtung et al., 2002; te Welscher et al., 2002), *Shh* will modulate the second wave of *Hoxd* gene expression in the presumptive digit domain. Expression in digits will be restricted to those five genes located at the centromeric (5′) end of the cluster, with a 5′ to 3′ progressive collinear decrease in transcriptional efficiency. The posterior location of the *Shh* signal induces an AP asymmetry in the expression of *Hoxd* genes in emerging digits, which will be translated into the skeletal AP polarity observed in our hands and feet.

Therefore, *Hoxd* gene expression in developing limbs comes in two waves, before and after *Shh* signaling

*Correspondence: denis.duboule@zoo.unige.ch

(Zakany et al., 2004). While in both cases the underlying molecular mechanisms rely on gene topography, they appear to be quite distinct from each other. Expression of 5′-located *Hoxd* genes in digits is controlled by a global enhancer sequence located centromeric to the cluster and embedded within a *g*lobal *c*ontrol *r*egion (GCR), a region rich in enhancer sequences (Spitz et al., 2003). This regulatory region shows a tropism for the centromeric end of the cluster, partly due to both distance- and sequence-specific effects leading to preferential targeting toward the *Hoxd13* promoter. Regulation of the *Hoxd12*, *Hoxd11*, and *Hoxd10* promoters is observed with decreasing efficiencies, likely due to a proximity-dependent leakage of the system (Kmita et al., 2002), thus providing a mechanistic basis for a quantitative type of collinearity in this context.

By contrast, the collinear mechanism(s) underlying the first wave of *Hoxd* gene activation, in time and space, remained to be characterized. A previous deletion of the cluster indicated that the main corresponding regulatory sequence(s) were localized outside the cluster itself (Spitz et al., 2001). Subsequently, an engineered inversion of the same gene cluster revealed that the *Hoxd13* promoter, when placed at the position of *Hoxd1*, was expressed throughout the early limb bud, in a pattern related to this latter gene (Zakany et al., 2004). These results indicated that the mechanism at work is promoter independent and suggested that the progressive posterior restriction depends on the mere position of a transcription unit within the cluster. They also led to the hypothesis that a critical element required for this collinear activation was located at the telomeric (3′) side of the cluster, i.e., opposite the GCR (ELCR; Zakany et al., 2004).

To gain insights into this elusive early collinear mechanism, we report here on the production and analysis of a set of mouse strains carrying a variety of deletions and duplications of parts of the *HoxD* cluster. These alleles were produced by using the targeted meiotic recombination strategy (TAMERE; Hérault et al., 1998), starting with a set of parental lines such that breakpoints are readily comparable between various configurations. In these mice, gene topography is reorganized in many different ways, leading to important reallocations in their transcriptional controls during early limb budding. The analysis of such regulatory reallocations indicates that *Hoxd* gene collinearity in early limb buds is the result of two antagonistic regulations, implemented from either side of the cluster, which together establish the observed nested expression patterns in time and space. We also show that this early collinear event is subsequently translated into an anterior-to-posterior patterning system for the zeugopod (forearm). We conclude that two mechanistically distinct collinear phases of *Hoxd* gene expression are required for proper limb development and discuss the biological and evolutionary relevance of this observation.

Results

Serial Deletions and Duplications at the *HoxD* Locus

In order to produce serial deletions and duplications within the *HoxD* cluster, we used strains of mice carrying a single *LoxP* site positioned between the transcription units, from *Hoxd13* to *Hoxd4* (Figure 1, top). These stocks were intercrossed, and the *Sycp1-Cre* transgene was introduced, to induce interchromosomal meiotic recombinations (TAMERE; Hérault et al., 1998; Figure 1). A first set of scanning deletions was produced by associating the 5′-most *LoxP* site located upstream from *Hoxd13* (Figure 1; L1; see also Table S1 in the Supplemental Data available with this article online) with five other available *LoxP*-containing strains (Figure 1; L4–L8). The resulting allelic series is made of a set of nested deletions, referred to as "5′ nested deletions" (Figure 1). The shortest of these deletions removed the DNA interval from *Hoxd11* to *Hoxd13* (*HoxD*$^{Del(11–13)}$) and is referred to as Del(11–13) throughout the paper for sake of simplicity. The largest of these deletions (Del(i–13)) removed from *Hoxd8* to *Hoxd13*, including the intergenic region "i," between *Hoxd4* and *Hoxd8*, which is devoid of genes and flanked by the L7 and L8 *LoxP* sites. Intermediate-sized deletions removed from *Hoxd8*, *Hoxd9*, or *Hoxd10* to *Hoxd13* (Del(8–13), Del(9–13), and Del(10–13), respectively). Smaller deletions starting at the L1 position and removing *Hoxd13* alone or in combination with *Hoxd12* are described elsewhere (Kmita et al., 2002).

A second set of deletions (Figure 1; "nested internal deletions") included all alleles in which both breakpoints were internal to the cluster. Such deletions left native *Hoxd* genes on either sides of the deleted DNA interval. By crossing the L2 *LoxP* strain with either L5 or L6, two deletions were produced (Figure 1; Del(10–12) and Del(9–12)), which maintained *Hoxd13* at the 5′ end of the *HoxD* cluster. Additional internal deletions were generated by using systematic combinations between the L4 and L8 strains, with the aim of recovering all possible deletions involving the central part of the *HoxD* cluster (from *Hoxd10* to region "i"). Accordingly, ten additional deletions that removed either a single gene (Figure 1; Del(i–8), Del(8), Del(9), Del(10)), two consecutive genes (Figure 1; Del(i–9), Del(8–9), Del(9–10)), or three consecutive genes (Figure 1; Del(8–10), Del(i–10)) were isolated. Deletion of the region "i" alone was also obtained, and this deletion did not remove any *Hoxd* gene (Del(i)).

Finally, our *Sycp1-Cre* transgene-driven meiotic recombination system also generated DNA duplications, resulting from reciprocal recombination events. Two "nested" duplications were considered in this work. The first of these is the duplication of the DNA interval encompassing region "i" to *Hoxd9* (Figure 1; Dup(i–9)). The second of these is a larger duplication including region "i" up to *Hoxd10* (Figure 1; Dup(i–10)). A detailed account of these mouse strains can be found in Table S1.

Effects of Internal Deletions on Temporal Collinearity

We used this collection of alleles to look at the expression of genes flanking the breakpoints during early forelimb bud development. In situ hybridizations revealed consistent modifications in both the timing of gene activation and the spatial distribution of transcripts. First, we analyzed expression of the genes located immediately 5′ to the breakpoint of our set of nested internal deletions in order to monitor the potential regulatory consequence of bringing a gene closer to the telomeric end of the cluster. We used E9.0–E9.5 embryos and looked at the expression of *Hoxd10*, *Hoxd11*, and *Hoxd13*. For each of these genes, two distinct deleted

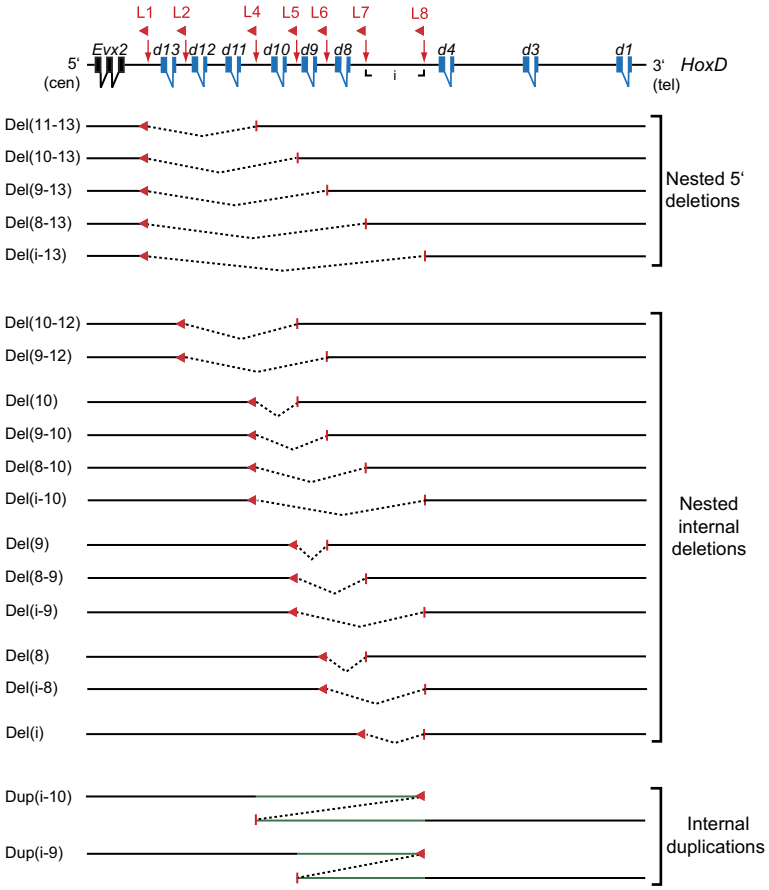

Figure 1. Deletion and Duplication Stocks Used in This Work

The line on the top shows the *HoxD* cluster with the positions of the various *LoxP* sites (L1–L8; red triangles), each of them representing an independent mouse strain. L7 and L8 flank the region "i," which is devoid of any *Hoxd* genes (in blue). A total of 17 deletion and 2 duplication strains of mice, as produced by the TAMERE recombination strategy, are shown below (Hérault et al., 1998). Breakpoints are identical at comparable positions (red triangle and bars). The deleted and duplicated DNA fragments are shown in dashed and plain, green lines, respectively. The strains are referred to as, e.g., Del(i–10) or Dup(i–10) for a deletion or a duplication, respectively, and they span the region "i" to *Hoxd10* inclusively ($HoxD^{Del(i–10)}$; $HoxD^{Dup(i–10)}$). Deletion strains are ordered according to the centromeric (5′, left) to telomeric (3′, right) positions of their 5′ and 3′ breakpoints along the chromosome. The five deletions at the top, all starting at the L1 breakpoint, are referred to as "nested 5′ deletions." Other deletions flanked by at least one *Hoxd* gene on each side are referred to as "nested internal deletions." The two duplication lines are shown at the bottom, with a *LoxP* site left in between the duplicated DNA fragments, which are drawn as superimposed such that duplicated genes are aligned with the rest of the figure. (cen) and (tel); centromeric and telomeric sides, respectively.

strains sharing the same 5′ breakpoint are shown as representatives of the results obtained (Figure 2). In this set of deletions, premature activation of the gene located 5′ to the deletion breakpoint was systematically observed when compared with age-matched control littermates.

For instance, while E9.0 control forelimb buds did not express *Hoxd10*, expression of this gene was clearly observed in embryos carrying either the Del(8–9) or Del(9) deletions, i.e., whenever *Hoxd10* was at the expected positions for either *Hoxd8* or *Hoxd9*, respectively (Figures 2C and 2F). The same was true for the expression of *Hoxd11* in either the Del(i–10) or the Del(9–10) strains; this gene was clearly activated prematurely in the incipient limb bud (Figures 2B and 2E), as was the case for *Hoxd13* in either the Del(9–12) or the Del(10–12) configurations. In this latter case, *Hoxd13* was relocated at the native positions for *Hoxd9* and *Hoxd10*, respectively, and as such was transcribed in the early bud, unlike in the wild-type situation where *Hoxd13* transcripts are not detected before E9.5–E10 (Figures 2A and 2D).

Such premature activations were not restricted to those genes immediately adjacent to the deletion breakpoints, but activations were also scored for genes located 5′ further away, showing that a global, rather than a locally restricted deregulation in the expression timing had occurred. For example, premature activation of *Hoxd10* was scored (along with *Hoxd9*) in Del(8) forelimb buds, i.e., in the presence of the *Hoxd9* gene in between the 5′ deletion breakpoint and *Hoxd10* (Figure 2I). Likewise, early activation of *Hoxd11* was observed in Del(i–9) (Figure 2H). Even genes located three transcription units away from the deletion breakpoint, such as *Hoxd13* in the Del(8–10) configuration, displayed a clear deregulation of their times of activation (Figure 2G), somewhat adopting the timing of the gene that would normally occupy this genomic position (*Hoxd8* in the case of Del(8–10)).

Finally, premature activation was also noticed for a gene normally expressed before the budding stage. In the Del(8) configuration, for example, *Hoxd9*, i.e., the gene adjacent to the deletion breakpoint, was activated too early in the presumptive limb territory of the lateral plate mesoderm (Figure 2P).

Effects of Internal Deletions on Spatial Collinearity

Analysis of the same set of nested internal deletions, but at subsequent developmental stages, i.e., in E10.5–E11.0 forelimb buds, led to another consistent observation concerning the spatial distribution of transcripts. In all cases, posterior genes located immediately 5′ of the various deletion breakpoints were ectopically expressed in the anterior region of the bud and therefore lost their characteristic posterior restriction observed in wild-type buds. This general observation is exemplified independently for *Hoxd10*, *Hoxd11*, and *Hoxd13* (Figure 2), for two distinct deletion stocks sharing the same 5′ breakpoint (Figure 2).

Hoxd10 mRNAs are normally absent from the anterior-proximal quadrant of the bud. However, in both Del(9) and Del(8–9) mutants, the *Hoxd10* signal expanded within this region (Figures 2L and 2O). Likewise, *Hoxd11* expression was clearly stretched out toward the anterior

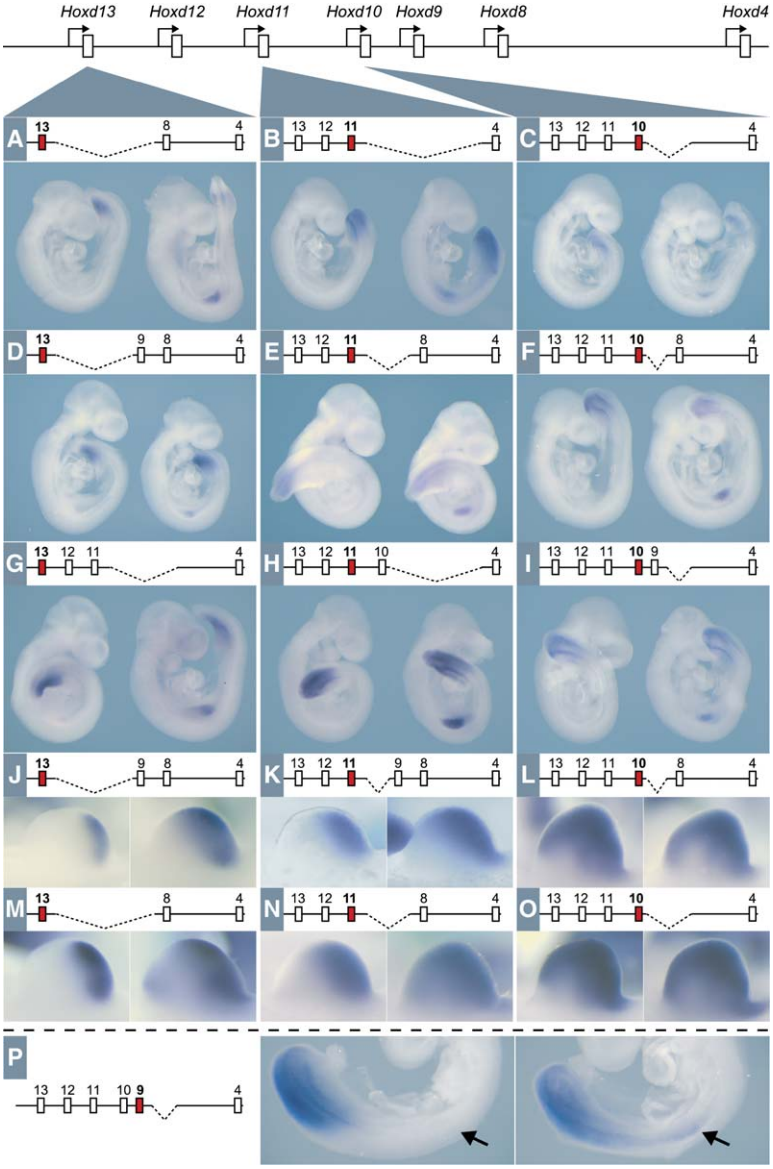

Figure 2. Expression of 5′ *Hoxd* Genes Flanking Internal Deletions

(A–P) The *HoxD* cluster is shown on the top, and the large, gray triangles indicate the gene whose expression is analyzed in the panels below. For the sake of clarity, each panel is accompanied by a schematic of the deletion (on the top), and the position of the gene analyzed is emphasized in red. (A–I) E9.0–E9.5 wild-type (left) and mutant (right) littermate embryos hybridized with *Hoxd13*, *Hoxd11*, or *Hoxd10* probes (first, second, and third columns, respectively). For each probe, three deletion strains are used. In two different cases, the monitored gene immediately neighbors the deletion. (A–F) In one case, the gene analyzed is lying further away in the 5′ direction. (G–I) In all cases, expression of the gene considered is detected in mutant forelimb buds, but not in age-matched control buds, indicating premature transcriptional activation in the deleted strains. (A) Del(9–12). (B) Del(i–10). (C) Del(8–9). (D) Del(10–12). (E) Del(9–10). (F) Del(9). (G) Del(8–10). (H) Del(i–9). (I) Del(8). (J–O) E10.2–E10.7 wild-type (left) and mutant (right) forelimb buds, hybridized with the same set of probes. All buds are oriented with anterior to the left. For each probe (in red), two deletion strains are shown in which the analyzed gene is located immediately 5′ of the breakpoint. In all cases, the expression domain in the mutant forelimb bud expanded anteriorly, when compared to age-matched controls. (J) Del(10–12). (K) Del(10). (L) Del(9). (M) Del(9–12). (N) Del(9–10). (O) Del(8–9). (P) In Del(8) mutant embryos, *Hoxd9* expression is discernible in the presumptive forelimb region at early day 9 (right; arrow), whereas it is absent in the presumptive forelimb region in wild-type littermate (left; arrow).

part of the limb bud in both Del(10) and Del(9–10) strains (Figures 2K and 2N). In this case, expression was almost uniform throughout the bud, in contrast to the typical posterior restriction of the *Hoxd11* pattern in age-matched forelimb buds. An anteriorization of the expression pattern was also scored for *Hoxd13* in the Del(10–12) strain (Figure 2J), whereas a visible ectopic anterior domain appeared for this gene in Del(9–12) mutant buds (Figure 2M). From this data set, we concluded that the temporal and spatial specificities of activation during forelimb bud outgrowth are likely determined by the relative position of a given *Hoxd* gene within the cluster. The closer to the telomeric end of the cluster a gene is naturally or artificially positioned, the earlier it shall be activated, and the more its expression will involve anterior limb bud cells.

Effects of Internal Duplications on Temporal and Spatial Collinearities

We confirmed this conclusion by analyzing mice carrying internal duplications of *Hoxd* genes. Genes located immediately 5′ to the duplicated DNA segment were indeed repositioned apart from the telomeric end of the cluster; hence, opposite types of regulatory reallocations were expected. We looked at the expression of both *Hoxd11* and *Hoxd12* in mice carrying the Dup(i–10) chromosome, i.e., a 36 kb duplicated fragment containing from region "i" to *Hoxd10* (Figure 1). In such mice, *Hoxd11*, *Hoxd12*, and *Hoxd13* were located at their normal positions with respect to the centromeric end of the cluster, yet their distance to the telomeric end was increased by 36 kb, with three additional transcription units in between (Figures 1 and 3A–3F). In E10.0 forelimbs, while *Hoxd11* and *Hoxd12* were both transcribed in posterior parts of control buds, transcripts were either not scored or were weakly detected at best in age-matched mutant buds (Figures 3A and 3B).

This transcriptional delay was also observed for a shorter duplication starting from the same 3′ point but without including the *Hoxd10* gene (Dup(i–9); Figure 1). Only two genes were duplicated along with this 27 kb DNA fragment, from region "i" to *Hoxd9*. In this

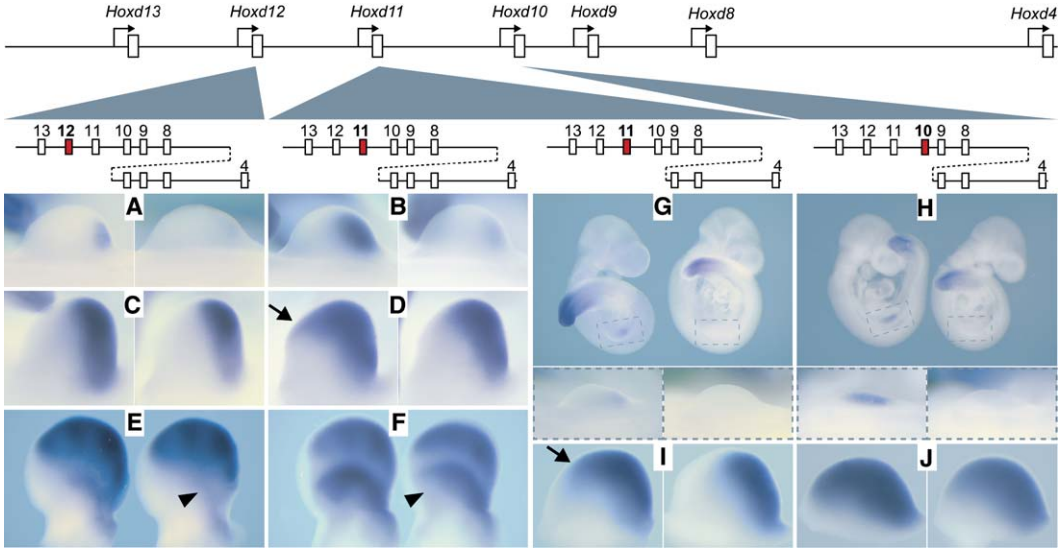

Figure 3. Expression of *Hoxd10*, *Hoxd11*, and *Hoxd12* in Two Internal Duplications

Schemes and colors are as for Figure 2. Age-matched controls are always on the left, and the limb buds are oriented with anterior to the left.
(A and B) E10.0 control (left) and Dup(i–10) (right) forelimb buds hybridized with either (A) *Hoxd12* or (B) *Hoxd11* probes. While control buds show the expected signal at the posterior aspect, mutant buds are either devoid of transcripts (*Hoxd12*) or severely depleted (*Hoxd11*).
(C and D) E11.0 buds hybridized with the same two probes. Both *Hoxd12* and *Hoxd11* transcripts are found abnormally restricted to the posterior bud when compared to controls.
(E and F) At E12.0, the proximal (forearm) expression domain of *Hoxd12* is barely visible ([E]; arrowhead), whereas that of the *Hoxd11* pattern is deprived of its anterior-most aspect ([F]; arrowhead; ventral view).
(G–J) Comparable observations were made with Dup(i–9) limbs. (G and H) E9.5 control (left) and Dup(i–9) (right) embryos hybridized with either (G) *Hoxd11* or (H) *Hoxd10* probes. Higher magnifications of the forelimb regions are shown below. Both genes are expressed in control buds, whereas they are undetected in mutant limb buds. (I and J) At E10.0–E10.5, both *Hoxd11* and *Hoxd10* transcripts are restricted to a posterior portion of the Dup(i–9) bud when compared to control patterns, showing that, upon internal duplications, the expression of 5′ *Hoxd* genes is generally compromised in the anterior-most normal domain. These results indicate that the transcriptional onset of a given 5′ *Hoxd* gene was delayed whenever located 5′ of an internal duplication. Furthermore, its expression was posteriorized.

case, neither *Hoxd10* nor *Hoxd11* mRNAs could be detected in E9.5 mutant buds, in contrast to controls (Figures 3G and 3H). Altogether, these results indicate that genes moved farther away from the telomeric end of the cluster are delayed in their activation during forelimb bud outgrowth, consistent with data obtained with the deletion lines. In these latter cases, premature activations were associated with the anteriorization of the limb bud expression patterns. We thus looked for a possible opposite effect in the duplication lines.

Such an effect was clearly observed as *Hoxd* genes located 5′ from the duplicated fragment adopted more posteriorly restricted expression patterns; their transcripts disappeared or were strongly depleted from the anterior-most domains of the forelimb buds. In the Dup(i–10) mutant stock, *Hoxd11* expression was more posteriorly restricted at E11.0 (Figure 3D), with the disappearance of the specific anterior extension of the pattern as seen in control specimen (arrow). This was also visible, though less obvious, with *Hoxd12*, as the expression of this gene is already tightly restricted to the posterior margin in the control buds (Figure 3C). A similar posteriorization effect was seen with the expression of both *Hoxd11* and *Hoxd10* in limb buds carrying the shorter duplication (Dup(i–9); Figures 3I and 3J). While this was particularly clear with *Hoxd11*, whose transcripts were absent from the anterior halves of E11.5 forelimb buds, unlike in the wild-type counterpart (Figure 3I; arrow), a similar tendency was detected for the expression of *Hoxd10* (Figure 3J).

Therefore, the comparisons between regulatory reallocations affecting genes located 5′ from either internal deletions or duplications revealed opposite transcriptional heterochronies associated with the concurrent modifications of the expression domains in the developing forelimb buds. Precocious activations corresponded to an anteriorization of the domain, whereas delayed transcription was followed by a posteriorization of the pattern. We assessed the consequences of these regulatory modifications on later stages of limb development and examined *Hoxd* gene expression in internal duplications in E12.0 forelimbs.

The signal in the autopod region was unaltered, consistent with the existence of a separate regulatory mechanism controlling this late phase of activation (see below). In contrast, the more proximal expression domains characteristic of *Hoxd10*, *Hoxd11*, and *Hoxd12* appeared weaker and/or truncated in its anterior extension. For instance, in Dup(i–10) limbs, the staining in the posterior-limited *Hoxd12* proximal domain appeared abnormally weak (Figure 3E; arrowhead). Likewise, the broad proximal domain of *Hoxd11* was deleted from its anterior-most portion (Figure 3F; arrowhead). These results indicated that the restriction of transcripts to posterior bud regions at E10.5, possibly due to delayed activation in the early bud, was maintained and affected

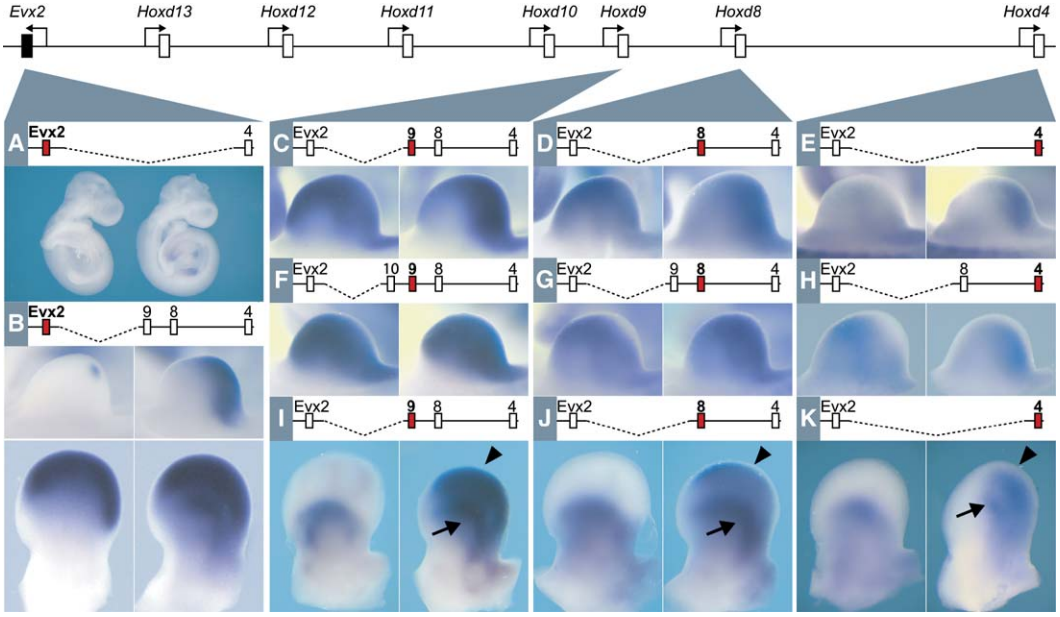

Figure 4. *Evx2* and *Hoxd* Gene Regulation in Nested 5′ Deletion Stocks

(A and B) *Evx2* adopts a 5′ *Hoxd*-like expression profile when positioned closer to the 3′ end of the *HoxD* cluster. (A) *Evx2* expression is never detected in the wild-type E9.2 forelimb bud (left), whereas it is transcribed in the forelimb bud in Del(i–13) embryos (right). (B) While *Evx2* mRNAs start to be detected in control E10.5 buds (upper panel; left; distal and posterior patch), it shows a broad posterior distribution in several 5′ deletions (e.g., Del[10–13], right), resembling that of *Hoxd11*. In wild-type E12.0 limb buds, *Evx2* is expectedly expressed in the autopod region (bottom panel; left). In Del(10–13) buds, however, the digit domain is complemented by a proximal/posterior expression reminiscent of the *Hoxd11* or *Hoxd12* pattern (right).

(C–K) Nested 5′ deletions induce drastic regulatory reallocations of *Hoxd* genes located 3′ to the breakpoint. (C–H) E10.5 wild-type (left) and mutant (right) limb buds hybridized with the (C and F) *Hoxd9*, (D and G) *Hoxd8*, and (E and H) *Hoxd4* probes. For each probe, two deletion strains in which the monitored gene (red) is either the first (top) or second (bottom) gene 3′ of the breakpoint are analyzed. At both positions, the gene considered is downregulated in the anterior half of the bud, whereas it is upregulated in the posterior half of the bud. (C and G) Del(10–13). (D and H) Del(9–13). (E) Del(8–13). (F) Del(11–13). (I–K) E12.0 limb buds hybridized with the same probes as for (C)–(H). One deletion in which the monitored gene is located immediately 3′ to the breakpoint is shown. (I and J) In the proximal region, *Hoxd9* and *Hoxd8* patterns have an opposite anterior-posterior symmetry with respect to wild-type controls, with strong reenforcement in the posterior part, (K) whereas the *Hoxd4* signal changes from a rather central spot to a posterior specificity. In addition, all genes are now expressed in the presumptive digit domain (arrowheads). All of these regulatory modifications are reminiscent of the wild-type *Hoxd10* to *Hoxd12* patterns. (I) Del(10–13). (J) Del(9–13). (K) Del(i–13).

the E12.0 late proximal domain as well, i.e., within the presumptive forearm.

Opposite Regulatory Mechanisms

These results confirmed that an early limb bud-activating element is located telomeric of the *HoxD* cluster (ELCR; Zakany et al., 2004) and indicated that the various onsets of transcriptional activation depend on the relative positions of genes with respect to this element. Genes located 3′ of the various deletions are not expected to change their time of activation, as their distance to this 3′ regulatory element remained unchanged. Accordingly, expression of *Hoxd10* in the smallest of the 5′ nested deletion strain, Del(11–13), revealed a normal onset of transcriptional activation in E9.2 buds (not shown). Unexpectedly, however, striking spatial redistributions of transcripts were scored for these 3′-located genes in E10.5 mutant forelimb buds, which were most obvious in the set of 5′ nested deletions.

In wild-type specimens, *Hoxd4*, *Hoxd8*, and *Hoxd9* share an expression pattern in which the anterior region of 10.5-day-old forelimb buds shows the highest steady-state level of mRNAs (Figures 4C–4H, left). Whenever these latter genes, however, were positioned at the 5′ end of the complex, after deletion of 5′-located genes, a strikingly different pattern of mRNA distribution was observed: it accumulated predominantly in the posterior limb bud, and there was a loss of anterior staining, clearly reminiscent of the expression of more 5′-located *Hoxd* genes at similar stages. For instance, in both Del(8–13) and Del(9–13) strains, *Hoxd4* was completely posteriorized, thus resembling the wild-type *Hoxd11* or *Hoxd12* patterns (Figures 4E and 4H). This was observed even when *Hoxd4* was separated from the deletion breakpoint by the *Hoxd8* transcription unit in the Del(9–13) allele. The same redistribution of transcripts was seen for *Hoxd8* in both the Del(9–13) and Del(10–13) configurations (Figures 4D and 4G). In the latter case, again, *Hoxd8* was separated from the breakpoint by the *Hoxd9* gene. *Hoxd9* itself behaved in the same way in both Del(10–13) and Del(11–13) forelimb buds (Figures 4C and 4F).

At E12.0, comparable regulatory reallocations were scored in the proximal domain, i.e., the presumptive forearm region. For example, at this stage, *Hoxd4* is normally expressed as a proximal and rather central spot. In the Del(i–13) limb buds, however, this domain was polarized posteriorly and evoked the proximal domain of 5′ *Hoxd* genes such as *Hoxd12* (Figure 4K; arrow). This unexpected effect was confirmed when looking at both *Hoxd8* and *Hoxd9* expression in either Del(9–13) or

Del(10–13), respectively. In the former case, the central and anterior domain of *Hoxd8* was transformed toward a posteriorly skewed domain, similar to that of *Hoxd10* (Figure 4J; arrow). In the latter case, a complete inversion in pattern polarity was seen for *Hoxd9*, again reminiscent of either the *Hoxd10* or *Hoxd11* wild-type pattern (Figure 4I; arrow). Altogether, these results indicated that the effects of the deletions on the expression of 3′-located genes in early budding forelimbs prefigured subsequent changes in the presumptive forearm domains at day 12.0; in both instances, a posteriorization of the staining was observed.

In addition, 5′ nested deletions invariably induced the remaining genes to be expressed in the more distal domain (autopod) due to reallocation of the digit enhancer located centromeric to the cluster. While such regulatory reallocations were previously shown for 5′-located *Hoxd* genes (Kmita et al., 2002), the capacity of *Hoxd* promoters that normally do not respond to this enhancer (e.g., *Hoxd8* or *Hoxd4*) to adopt this regulation remained elusive. This result shows that most *Hoxd* genes can respond to the digit enhancer, provided they are located at the appropriate position, demonstrating the quasi-absence of promoter specificity in this regulatory process. While the *Hoxd4* ectopic signal was limited to a posterior digital region in Del(i–13) buds (Figure 4K; arrowhead), both *Hoxd8* and *Hoxd9* transcripts occupied a broader region typical of 5′ *Hoxd* genes (e.g., in Del(9–13) and Del(10–13), respectively, Figures 4I and 4J; arrowheads).

Regulation of *Evx2*

Evx2, a homeobox-containing gene orthologous to the *Drosophila* gene *even skipped* (*eve*), is located 9 kb centromeric from *Hoxd13*, thus immediately flanking the 5′ end of the *HoxD* cluster. In wild-type specimens, this gene responds to the digit enhancer (Kmita et al., 2002; Spitz et al., 2003). Accordingly, *Evx2* transcripts appear in a posterior-distal spot at E10.5, which will subsequently expand to label the presumptive digit domain (Dollé et al., 1994; Figure 4B, upper left panel), where it will be expressed along with 5′-located *Hoxd* genes. In the early bud, however, *Evx2* transcripts remain undetected (Figure 4A). Subsequently, transcription of *Evx2* is confined to the digital plate and is never observed in the forearm domain (Figure 4B, bottom left panel), similar to *Hoxd13*.

We monitored *Evx2* transcription in the set of 5′ nested deletions, i.e., the allelic series in which this gene is immediately neighboring the L1 breakpoint. In all mutant forelimb buds, *Evx2* expression was clearly premature, and transcripts were found as early as day 9.0 in Del(i–13) buds (Figure 4A). While shorter deletions like Del(10–13) or Del(11–13) did not elicit transcription at this stage, they led to a comparable transcriptional heterochrony in E10.0 forelimb buds (not shown). Therefore, much like *Hoxd* genes, *Evx2* displayed an earlier onset of expression after its relocation closer to the 3′ end of the cluster. In E10.5 forelimbs, at the time normal *Evx2* expression starts in distal and posterior mesenchyme, all deletion stocks displayed a broad staining posteriorly, like *Hoxd10* or *Hoxd11* (e.g., Del(10–13); Figure 4B, upper right panel). At day 12.0, *Evx2* misregulation was constant amongst the various strains, with a conspicuous ectopic domain located at a proximal/posterior position, whereas expression in digits was expectedly normal (i.e., Del(10–13); Figure 4B, bottom right panel). Even though this proximal domain was reminiscent of the *Hoxd10* or *Hoxd11* patterns, it was more limited and transient, as no signal was left by day 13.0 (not shown).

Altogether, when flanking 5′ nested deletions, *Evx2* adopted a global forelimb expression profile typical of 5′-located (posterior) *Hoxd* genes such as *Hoxd10* or *Hoxd11*, responding to both temporal and spatial collinearities. The modification of the *Evx2* expression pattern in this set of deletions provided yet another example of the tight correspondence between pattern variations in the early limb bud domain, on the one hand, and in the subsequent proximal (forearm) domain, on the other hand, suggesting that the latter readily derive from the extension, in time and space, of the former.

Discussion

Ever since the observation that vertebrate *Hox* genes, much like those of flies, are expressed in a collinear fashion (Gaunt et al., 1988; Krumlauf, 1992), the molecular mechanism(s) underlying this enigmatic property has been sought (reviewed in Kmita and Duboule, 2003). In this paper, we report on the use of a systematic deletion/duplication approach in the *HoxD* cluster to understand the logic of this process during early limb bud development. Our strategy allowed us to readily compare various deleted and duplicated configurations, as they all originate from the same set of breakpoints, giving rise to a series of progressive modifications. Thus, a particular regulatory output, observed on one given configuration, was considered significant only if observed on all related configurations as well, leading to the description of general tendencies rather than particular cases. We believe this approach allows us to report on a general mechanism at work, as observed through an objective experimental design, rather than to elaborate on distinct data sets containing intrinsic experimental bias.

In a previous work (Kmita et al., 2002), a first set of 16 configurations was used to describe the mechanism underlying two particular aspects of the collinear process at work during digit development. The remote centromeric position of a "digit enhancer" (Spitz et al., 2003) thus accounts for the fact that only the most 5′-located *Hoxd* genes (at the extremity of the cluster) are expressed in developing digits (at the extremity of the limb), after a progressive decrease in transcriptional efficiency (see below). In this work, we use another 19 stocks of mice to address the question of temporal and spatial collinearity during the early phase of limb bud development, before digit morphogenesis. We conclude that the collinear mechanism is different from that regulating expression in digits and involves the antagonistic effects of positive and negative regulations driven by either side of the cluster.

Rather than being specific for mouse forelimb buds, we believe that the conclusions reached in this work can also be applied to hindlimb buds, since both types of limbs share the same general plan and are derived from similar basic processes, despite subtle differences. Likewise, our current knowledge of *Hox* gene function and expression in nonmammalian species, in particular

in birds, suggests that the conclusions of this work can be generalized to all vertebrates bearing limbs.

Two Waves of *Hoxd* Gene Expression Occur during Forelimb Bud Development…

All genetic configurations analyzed here showed that whatever spatial modification in transcript pattern was observed in the incipient limb bud, as a result of either a deletion or a duplication, the same variation of the expression pattern was translated at later stages into the presumptive zeugopod region (forearm). For example, whenever a given *Hoxd* expression domain was posteriorized in the early outgrowing bud, the same posteriorization was scored subsequently in the forearm domain. Altogether, these correlations indicate that expression of *Hoxd* genes in the presumptive forearm territory is a mere expansion of the expression patterns in early forelimb buds (Figure 5), which explains why these two transcript domains were always modified in concert with each other in the numerous lines of recombined mice produced over the past few years at this locus. The progressive expansion of the early bud domain into a zeugopod domain may reflect patterns of cell proliferation and lineages. The zeugopod domain is clonally derived from the early bud; however, some distal de novo transcriptional activation or proximal transcriptional decay could participate to the observed transition. We thus conclude that the forearm domain (Figure 5, bottom line in green) does not involve any regulatory control drastically distinct from that at work to establish the early forelimb bud patterns.

More importantly, this demonstrates that only two waves of *Hoxd* gene transcriptional activation are necessary for limb development. During the early phase, genes are expressed as a set of nested patterns in the incipient bud, subsequently translated into a zeugopod pattern. This phase mechanistically unifies "phases I and II" previously described by Nelson et al. (1996) and is required for proper development of the distal stylopod (humerus) and of the zeugopod (radius and ulna) (Davis et al., 1995; Kmita et al., 2005). One consequence of this early phase is the posterior confinement of some *Hox* gene expression, which, in turn, leads to the localization of *Shh* transcription at the posterior margin of the developing bud (Zakany et al., 2004).

Subsequently, the second wave of transcriptional activation occurs in the presumptive digit territory (Figure 5, black domain). Activation originally overlaps with the most distal part of the early domain, making its earliest detection difficult. This overlap is not observed with *Evx2* due to *Evx2*'s inability to respond to the first regulation. This second phase is modulated by *Shh* signaling, leading to the anterior-posterior asymmetry of the distal limb. The *Shh* signal is nonetheless not mandatory for the late transcriptional activation of *Hox* genes to occur since both early and late phases are observed in mice lacking *Shh* and *Gli3* (Litingtung et al., 2002; te Welscher et al., 2002).

…and Are Implemented by Distinct Collinear Mechanisms

In spite of sharing collinear features, the two waves of activation are driven by different mechanisms. The mechanism involved in the second wave has been described in some detail (Kmita et al., 2002) and relies on the activity of a poorly gene-specific digit enhancer (Spitz et al., 2003), located centromeric (5′) to the *HoxD* cluster. This process leads to the preferential and concomitant activation of the most 5′-located genes (*Evx2* and *Hoxd13*), whereas genes located farther 3′ are progressively less transcribed. The mechanism underlying the early wave of activation, uncovered by this work, is more complex and appears to depend on two opposite regulations. The first aspect of this mechanism (temporal collinearity) is the time-dependent activation of *Hox* genes in the incipient limb bud. Our data further suggest that this step is controlled by an as yet unidentified regulatory element located telomeric (3′) to the cluster, previously proposed as the Early Limb Control Regulation (ELCR; Zakany et al., 2004). Using both deletions and duplications, we show here that the time of activation is a function of the relative distance to this element (e.g., expressed as the number of promoters), whereas the distance to the centromeric end of the cluster is irrelevant (see Figure 5). Whether the temporal parameter is directly controlled by an enhancer/promoter type of mechanism, via distance-dependent probability of contact, or relies on progressive structural modifications of the chromatin or global chromosome architecture (Chambeyron et al., 2005) remains to be determined.

Notably, the progressive restriction of *Hoxd10–Hoxd13* transcription to posterior cells of the developing bud (spatial collinearity), leading to the observed set of nested transcript patterns, does not appear to depend solely on the relative position to the ELCR. Instead, this second aspect of the mechanism is triggered by an element localized centromeric to the cluster (Figure 5), as shown by the patterns of 3′-located genes, which are posteriorized despite maintaining their topographic relationships with respect to the telomeric side in 5′-nested deletions. Therefore, spatial collinearity in the early bud is an outcome of a balance between the time of activation (relative distance to the telomeric end) and position with respect to a 5′-located inhibitory regulation. While this restrictive element has not yet been identified, several DNA regions in this interval show high sequence conservation when compared with other vertebrate genomes, suggesting potential candidate sequences. They are currently assessed by functional approaches to address their importance in that respect. Any underlying molecular mechanism must involve a transcriptional repression of posteriorly expressed *Hoxd* genes in anterior limb bud cells. A candidate for mediating such an effect is the repressor form of the *Gli3* gene, whose inactivation leads to expression of these *Hoxd* genes in the anterior part of the limb bud (Zuniga and Zeller, 1999; te Welscher et al., 2002). In addition, GLI3 can directly complex with HOXD proteins (Chen et al., 2004). The overall process appears to be largely promoter-independent, and any *Hoxd* gene placed at a given position with respect to both extremities of the cluster will eventually respond to these two regulatory influences in a predictable fashion.

Virtual Collinearity

Examination of day 11.5–12.5 forelimb buds, i.e., once both waves of *Hoxd* gene activation are completed, immediately reveals a collinear transcript distribution along the proximal-to-distal (PD) limb axis (Dollé et al.,

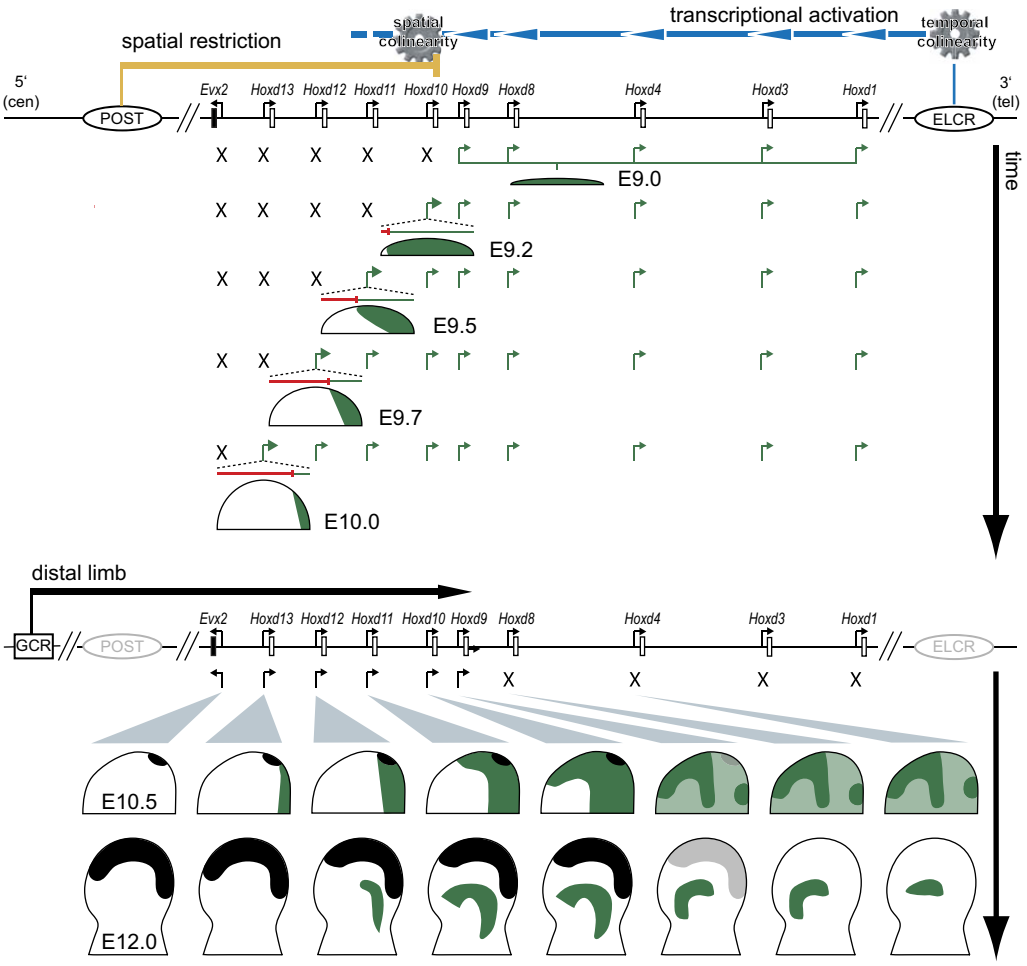

Figure 5. Model of Collinear Regulations during Limb Bud Development

This model is based on two distinct waves of expression, controlled by different mechanisms acting after various temporal parameters. The first mechanism acts during limb budding, is time-dependent (temporal collinearity), and involves two regulatory influences originating from either side of the cluster (top; blue arrow and yellow bar). This early mechanism generates expression patterns (in green) along with forelimb bud outgrowth. Temporal collinearity (blue arrows) in the limb is implemented by regulatory sequences located telomeric to the cluster (ELCR), and the transcriptional onset for a given gene depends on its relative distance to it, i.e., its position within the sequence of genes. Active genes are shown with a green arrow, whereas genes silent at a given time are labeled with an "X." Generic spatial restriction of transcripts in the posterior bud (yellow bar) is implemented by a distinct regulatory mechanism relying on sequences located centromeric to the cluster (POST). Spatial collinearity is the outcome of both influences; the temporal aspect likely plays a secondary role in defining progressive anteroposterior expression boundaries for successive genes. Consequently, posterior restriction is maximal for those genes located close to this regulatory sequence (e.g., *Hoxd13*), and it becomes progressively less efficient with increasing relative distance, leading to a minimal anterior repression for *Hoxd10* (red bars). The second wave of expression, independent of the former, is controlled by the GCR (Spitz et al., 2003), which drives expression of five contiguous genes concomitantly in the presumptive digit domain. This presumptive domain (black) appears at day 10.5 and is fully expanded, labeling the future autopod, by day 12 (bottom). At the same time, the early collinear domain progresses such that, at day 10.5, 3′-located genes (e.g., *Hoxd8* or *Hoxd4*) are still expressed throughout the bud (light green), though more strongly in an anterior domain as well as in a restricted posterior patch (green). At the same time, 5′-located genes keep the same collinear arrangement (green), derived from the early wave of expression. Subsequently, at day 12, a proximal domain is observed at the level of the zeugopod (green). The shape and polarity of this domain, for each gene, is directly derived from that observed at day 10.5 (compare the green domains in the bottom two lines), indicating that the zeugopod domain at day 12 is merely the product of the domain observed at day 10.5, which is itself derived from the early collinear mechanisms acting on the forelimb bud between days 9 and 10. During the second phase, the two opposite early enhancers are depicted in gray. They may continue to regulate expression in the zeugopod domains, or, alternatively, a maintenance system could substitute. These two independent waves of activation are necessary and sufficient to provide for the full *Hoxd* gene patterns during early limb development. (cen) and (tel); centromeric and telomeric sides, respectively.

1989; Kmita et al., 2002). A smooth transition is observed from a proximal expression (e.g., *Hoxd8*; *Hoxd9*) to a distal expression (*Hoxd13*), with *Hoxd10*, *Hoxd11*, and *Hoxd12* showing both proximal (distal stylopod and zeugopod) and distal (autopod) domains (depicted in Figure 5, bottom line). Interestingly, this collinear distribution of transcripts along the PD axis, which was the original flagship of collinearity in limbs (Dollé et al., 1989), does not rely by itself on any intrinsic molecular mechanism. It results from the combination of two independent collinear processes—one organizing the proximal domain (the first wave described in this work), and the second involved in controlling expression in digits (Kmita et al., 2002). This obvious, yet virtual, collinearity

is nonetheless indicative of the general topographic organization of these regulations whose control sequences are located at both extremities of the cluster. On the one hand, the location of the GCR favors distal expression of the 5′-located genes. On the other hand, the double activation-posterior restriction mechanism controlling the first wave is detrimental for proximal-anterior expression of these same genes, thus leading to this intuitive perception of highly organized PD patterns, a regulatory illusion.

The Obligation of Collinearity in Tetrapod Limbs

DNA sequences involved in the control of *Hoxd* gene transcription in developing limbs are mostly located outside the cluster (Spitz et al., 2001; this work). Understandably, collinearity was already at work during the development of the major body axis (the trunk) much before limbs emerged; hence, the de novo evolution of "limb enhancers" within the cluster itself may have proved difficult due to the tight organization of this gene cluster and its high density in transcription units (ca. one gene every 10 kb). The mere fact of having global enhancers positioned outside a gene cluster is a source of collinear regulation, as it introduces an asymmetry in whichever manner this enhancer will work. In this context, collinearity in limbs may be viewed as an obligatory readout of the system, rather than as an exquisite strategy that specifically evolved due to the associated output in appendage morphology.

Concerning the origins of these enhancer sequences, two alternative schemes—not exclusive from each other—can be considered. First, a novel limb enhancer sequence may have emerged and been selected outside the cluster. Alternatively, preexisting regulatory modules, positioned outside *HoxD*, may have been coopted for yet another functional output in parallel with limb evolution. As far as the digit enhancer is concerned, the first hypothesis likely applies, even though the emergence of this element occurred within a region already prone to confer global enhancer activity (GCR; Spitz et al., 2003).

Interestingly, however, the hereby-described regulatory strategy underlying the first wave of activation may instead derive from the second kind of scenario. Several aspects of the phenomenon reported in this paper are indeed related to what is observed during the formation of the major body axis, raising the possibility that part of this ancient trunk collinear regulation was recruited into the context of the newly growing limbs. In particular, the existence of two types of collinearities, temporal and spatial, which can be somehow disconnected from each other (reviewed in Kmita and Duboule, 2003), suggests that the collinear strategy used during trunk development relies on opposite mechanisms, much like that described above, for the early wave of activation in limbs. This is supported by the preliminary survey of the effects of our set of deletions/duplications on the timing and place of *Hoxd* and *Evx2* gene expression in the developing trunk, which suggests the same type of regulatory reallocations (e.g., see Figure 4A). A detailed analysis of this particular aspect will be informative in this respect and may shed light on this fundamental mechanism.

The existence of distinct regulatory processes for the two waves of *Hoxd* activation in limbs is coherent with the proposal that the proximal and distal parts of our limbs have different phylogenetic histories (Sordino et al., 1995; Shubin et al., 1997). In this context, it is noteworthy that the mechanisms resembling those implemented during the development of the trunk may control the early and proximal *Hoxd* gene expression, i.e., at a time and in places where *Hox* genes are necessary to build the "ancient" proximal part, whereas an apparently newly evolved enhancer accompanied the emergence of digits, i.e., of a rather recent evolutionary novelty. In this view, the various kinds of regulatory innovations, and their distinct mechanisms of cooption, may tell us about the phylogenetic history of the structure (Duboule and Wilkins, 1998).

AP Polarity and Genomic Topography

One important effect of the early phase of collinear activation is the restriction of *Shh* signaling to the most posterior margin of the limb bud (Zakany et al., 2004; Kmita et al., 2005). Since *Shh* signaling is a major factor in the establishment of the limb AP polarity (Riddle et al., 1993), this polarity appears to be the morphological translation of the asymmetry in the expression of some *Hox* genes, as a result of their early collinear expression. Consequently, the limb AP polarity may reflect nothing but a particular type of gene topography and its associated asymmetric regulations. Yet, the major function of *Hox* genes in limb development is not to AP pattern the structure, but rather to trigger its growth, as the absence of *Hox* function leads to very severe truncations along the proximodistal axis (Davis et al., 1995; Kmita et al., 2005). This apparent paradox suggests that the mechanism underlying the limb AP polarity did not evolve separately from, or in parallel with, the growth of the limbs. Instead, this mechanism was likely imposed as a collateral effect of the regulatory processes recruited to promote limb emergence and outgrowth.

In this view, an AP-polarized limb is the expected consequence of using asymmetrically located enhancer sequences to control *Hox*-dependent outgrowth. The cooption of this genetic system (as well as of other components) and its obligatory collinear regulatory strategy to promote limb development led to the morphological impossibility to produce symmetrical limbs, due to the regulatory constraints imposed by the essential function of this gene family during trunk development.

Experimental Procedures

LoxP Mouse Strains and Targeted Meiotic Recombination

The alleles described in this work were all obtained in vivo by breeding two mouse lines carrying distinct intergenic *LoxP* sites in the presence of the *Sycp1-Cre* transgene, which provides recombinase activity during male meiosis (TAMERE; Hérault et al., 1998). A detailed account of the strains is provided in Table S1. For this work, the following nine strains were produced: Del(10-12), Del(9-12), Del(i-10), Del(8-9), Del(8), Del(i-8), Del(i), Dup(i-10), and Dup(i-9). Embryos carrying a duplication were either homozygous and isolated after a genotyping procedure described previously (Figures 3I and 3J; Kmita et al., 2002), or they carried in *trans* a balancer deletion removing the gene of interest such as to monitor only transcription from the duplicated chromosome (Figures 3A–3H).

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridizations were carried out on fetuses between 9.0 and 12.0 days, by using standard procedures and

previously described probes. *Evx2*, (Dollé et al., 1994); *Hoxd13*, (Dollé et al., 1991); *Hoxd11*, (Gérard et al., 1993); *Hoxd10*, (Renucci et al., 1992); *Hoxd9*, (Zappavigna et al., 1991); *Hoxd8*, (Izpisua-Belmonte et al., 1990); *Hoxd4*, (Featherstone et al., 1988).

Supplemental Data

Supplemental Data including a table with the details of the various stocks of mice reported in this work are available at http://www.developmentalcell.com/cgi/content/full/10/1/93/DC1/.

Acknowledgments

We are grateful to Thi Hanh Nguyen Huynh for technical assistance and to the members of the laboratory for sharing reagents and discussions. We thank Marie Kmita for discussions and helpful comments on the manuscript. This work was supported by funds from the canton de Genève, the Louis-Jeantet Foundation, the Swiss National Research Fund, the National Center for Competence in Research (NCCR) "Frontiers in Genetics," and the EU programs "Eumorphia" and "Cells to Organs." This paper is dedicated to the memory of Ed B. Lewis.

Received: October 4, 2005
Revised: November 16, 2005
Accepted: November 18, 2005
Published: January 9, 2006

References

Chambeyron, S., Da Silva, N.R., Lawson, K.A., and Bickmore, W.A. (2005). Nuclear re-organisation of the Hoxb complex during mouse embryonic development. Development *132*, 2215–2223.

Charité, J., de Graaff, W., Shen, S., and Deschamps, J. (1994). Ectopic expression of Hoxb-8 causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures. Cell *78*, 589–601.

Chen, Y., Knezevic, V., Ervin, V., Hutson, R., Ward, Y., and Mackem, S. (2004). Direct interaction with Hoxd proteins reverses Gli3-repressor function to promote digit formation downstream of Shh. Development *131*, 2339–2347.

Davis, A.P., Witte, D.P., Hsieh-Li, H.M., Potter, S.S., and Capecchi, M.R. (1995). Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. Nature *375*, 791–795.

Deschamps, J., and van Nes, J. (2005). Developmental regulation of the Hox genes during axial morphogenesis in the mouse. Development *132*, 2931–2942.

Dollé, P., Izpisua-Belmonte, J.C., Falkenstein, H., Renucci, A., and Duboule, D. (1989). Coordinate expression of the murine Hox-5 complex homoeobox-containing genes during limb pattern formation. Nature *342*, 767–772.

Dollé, P., Izpisua-Belmonte, J.C., Boncinelli, E., and Duboule, D. (1991). The Hox-4.8 gene is localized at the 5′ extremity of the Hox-4 complex and is expressed in the most posterior parts of the body during development. Mech. Dev. *36*, 3–13.

Dollé, P., Fraulob, V., and Duboule, D. (1994). Developmental expression of the mouse Evx-2 gene: relationship with the evolution of the HOM/Hox complex. Dev. Suppl. 143–153.

Duboule, D., and Wilkins, A.S. (1998). The evolution of 'bricolage'. Trends Genet. *14*, 54–59.

Featherstone, M.S., Baron, A., Gaunt, S.J., Mattei, M.G., and Duboule, D. (1988). Hox-5.1 defines a homeobox-containing gene locus on mouse chromosome 2. Proc. Natl. Acad. Sci. USA *85*, 4760–4764.

Gaunt, S.J., Sharpe, P.T., and Duboule, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. Development *104*, 169–179.

Gérard, M., Duboule, D., and Zakany, J. (1993). Structure and activity of regulatory elements involved in the activation of the Hoxd-11 gene during late gastrulation. EMBO J. *12*, 3539–3550.

Hérault, Y., Rassoulzadegan, M., Cuzin, F., and Duboule, D. (1998). Engineering chromosomes in mice through targeted meiotic recombination (TAMERE). Nat. Genet. *20*, 381–384.

Izpisua-Belmonte, J.C., Dolle, P., Renucci, A., Zappavigna, V., Falkenstein, H., and Duboule, D. (1990). Primary structure and embryonic expression pattern of the mouse Hox-4.3 homeobox gene. Development *110*, 733–745.

Kmita, M., and Duboule, D. (2003). Organizing axes in time and space; 25 years of collinear tinkering. Science *301*, 331–333.

Kmita, M., Fraudeau, N., Herault, Y., and Duboule, D. (2002). Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs. Nature *420*, 145–150.

Kmita, M., Tarchini, B., Zakany, J., Logan, M., Tabin, C.J., and Duboule, D. (2005). Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. Nature *435*, 1113–1116.

Knezevic, V., De Santo, R., Schughart, K., Huffstadt, U., Chiang, C., Mahon, K.A., and Mackem, S. (1997). Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. Development *124*, 4523–4536.

Krumlauf, R. (1992). Evolution of the vertebrate Hox homeobox genes. Bioessays *14*, 245–252.

Lewis, E.B. (1978). A gene complex controlling segmentation in *Drosophila*. Nature *276*, 565–570.

Litingtung, Y., Dahn, R.D., Li, Y., Fallon, J.F., and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature *418*, 979–983.

Medina-Martinez, O., Bradley, A., and Ramirez-Solis, R. (2000). A large targeted deletion of Hoxb1-Hoxb9 produces a series of single-segment anterior homeotic transformations. Dev. Biol. *222*, 71–83.

Nelson, C.E., Morgan, B.A., Burke, A.C., Laufer, E., DiMambro, E., Murtaugh, L.C., Gonzales, E., Tessarollo, L., Parada, L.F., and Tabin, C. (1996). Analysis of Hox gene expression in the chick limb bud. Development *122*, 1449–1466.

Renucci, A., Zappavigna, V., Zakany, J., Izpisua-Belmonte, J.C., Burki, K., and Duboule, D. (1992). Comparison of mouse and human HOX-4 complexes defines conserved sequences involved in the regulation of Hox-4.4. EMBO J. *11*, 1459–1468.

Riddle, R.D., Johnson, R.L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. Cell *75*, 1401–1416.

Shubin, N., Tabin, C., and Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. Nature *388*, 639–648.

Sordino, P., Van der Hoeven, F., and Duboule, D. (1995). Hox gene expression in fins and the origin of vertebrate digits. Nature *375*, 678–681.

Spitz, F., Gonzalez, F., Peichel, C., Vogt, T.F., Duboule, D., and Zakany, J. (2001). Large scale transgenic and cluster deletion analysis of the HoxD complex separate an ancestral regulatory module from evolutionary innovations. Genes Dev. *15*, 2209–2214.

Spitz, F., Gonzalez, F., and Duboule, D. (2003). A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell *113*, 405–417.

Suemori, H., and Noguchi, S. (2000). Hox C cluster genes are dispensable for overall body plan of mouse embryonic development. Dev. Biol. *220*, 333–342.

te Welscher, P., Zuniga, A., Kuijper, S., Drenth, T., Goedemans, H.J., Meijlink, F., and Zeller, R. (2002). Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. Science *298*, 827–830.

Zakany, J., Kmita, M., and Duboule, D. (2004). A dual role for Hox genes in limb anterior-posterior asymmetry. Science *304*, 1669–1672.

Zappavigna, V., Renucci, A., Izpisua-Belmonte, J.C., Urier, G., Peschle, C., and Duboule, D. (1991). HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. EMBO J. *10*, 4177–4187.

Zuniga, A., and Zeller, R. (1999). Gli3 (Xt) and formin (ld) participate in the positioning of the polarising region and control of posterior limb-bud identity. Development *126*, 13–21.