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Hox Gene Expression in Limbs: Colinearity by Opposite Regulatory Controls

Yann Hérault, Johannes Beckers,[1] Matthieu Gérard,[2] and Denis Duboule[3]

Department of Zoology and Animal Biology, University of Geneva, Sciences III, Quai Ernest Ansermet 30, 1211 Geneva 4, Switzerland

Genes of the *HoxD* complex have a crucial role in the morphogenesis of vertebrate limbs. During development, their functional domains are colinear with their genomic positions within the *HoxD* cluster such that *Hoxd13* and *Hoxd12* are necessary for digit development, whereas *Hoxd11* and *Hoxd10* are involved in making forearms. Mutational analyses of these genes have demonstrated their importance and illustrated the requirement for a precise control of their expression during early limb morphogenesis. To study the nature of this control, we have scanned the posterior part of the *HoxD* complex with a targeted reporter transgene and analyzed the response of this foreign promoter to limb regulatory influences. The results suggest that this regulation is achieved through the opposite effects of two enhancer elements which would compete with each other for interacting with nearby-located promoters. The physical position of a given gene within this genomic interval of opposite regulations might thus determine its final expression pattern. This model provides a conceptual link between the morphology of the future limb and the genetic organization of the *Hox* gene cluster, a translation of a genomic context into a morphogenetic topology.

***Key Words:* *Hox* genes; limb development; colinearity; vertebrates.**

INTRODUCTION

Targeted mutagenesis of vertebrate *Hox* genes located in the "posterior" part of the *HoxD* complex (from *Hoxd9* to *Hoxd13*), either alone or in *cis*- and *trans*-combinations, has confirmed their essential roles in the making of a limb (*Hoxd9*, Fromental-Ramain *et al.*, 1996; *Hoxd11*, Davis and Capecchi, 1996; Davis *et al.*, 1995; Favier *et al.*, 1995, 1996; *Hoxd12*, Davis and Capecchi, 1996; Kondo *et al.*, 1996, 1997a; *Hoxd13*, Dollé *et al.*, 1993; Hérault *et al.*, 1996, 1998; Zákány and Duboule, 1996). For example, alterations in the functions of group 13 genes strongly affected the hands and feet, whereas inactivations of group 11 genes truncated the forearms (Davis *et al.*, 1995; Fromental-Ramain *et al.*, 1996; Kondo *et al.*, 1997a). Such a topological specificity of *Hox* genes for various segments of the limbs relies upon the establishment of precise expression profiles for each of these genes. These expression domains are organized as a result of a multistep process involving several phases of activation during development (Hérault *et al.*, 1998; Nelson *et al.*, 1996; Shubin *et al.*, 1997). In an early phase, *Hoxd* genes are activated as a nested set of transcript domains, in a colinear fashion such as *Hoxd9* is expressed first and in a domain larger than that of *Hoxd10* (Dollé *et al.*, 1989). Subsequently, a second phase of expression establishes definitive transcript domains in the forearm and digits, concomitantly for several posterior *Hoxd* genes. The result of this complex developmental control is the presence, at day 11 of fetal development, of three distinct domains of *Hox* expression in limbs; one located proximally in the presumptive arm, another one located medially in the presumptive forearm, and the third covering the presumptive digit area. Posterior *Hoxd* genes will be expressed in these different domains following their respective genomic location in the complex, thus reflecting the future proximodistal axes of our appendages (Dollé *et al.*, 1989). While *Hoxd13* will be transcribed exclusively in the digit domain, *Hoxd11* will be also expressed in the forearm domain. In contrast, *Hoxd9* transcripts will be absent from

[1] Present address: The Jackson Laboratory, Bar Harbor, ME.
[2] Present address: Cancer and Developmental Biology Laboratory, ABL Basic Research Program, NCI–Frederick Cancer Research and Development Center, Frederick, MD 21702.
[3] To whom correspondence should be addressed. Fax: 41 22 702 6795. E-mail: Denis.Duboule@zoo.unige.ch.

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the digit domain but present in the forearm as well as more proximally (see references above).

The mechanistic bases of this intriguing correlation between limb axial morphology and the organization of genes along a DNA segment remain elusive. Expression studies nevertheless suggest that this type of colinearity may be mechanistically different from that involved during the development of the major body axis as a temporal factor does not seem to be involved in the former process, while clearly present in the latter. It was previously suggested that distinct limb expression domains were controlled by specific and different regulatory sequences (e.g., Nelson *et al.*, 1996), as exemplified by the expression of several *Hoxd* genes in the most distal (digit) domain. In this case, expression of five consecutive genes was apparently driven by a single enhancer element located at a distance, likely upstream of the complex (van der Hoeven *et al.*, 1996). This enhancer was shown to have a rather broad spectrum of activity as different *Hox/lacZ* reporter transgenes, even those usually not expressed in digits such as *Hoxd9/lacZ*, were activated in the distal domain when placed at a more 5′ position in the *HoxD* complex, independently from their transcriptional orientation (van der Hoeven *et al.*, 1996). These results were confirmed by the analysis of the *Ulnaless* (*Ul*) mutant mice in which such a global regulatory element may be involved in the deregulation of several contiguous genes in these limb domains leading to important alterations in limb morphology (Hérault *et al.*, 1997; Peichel *et al.*, 1997).

To further investigate the nature of this regulation, in particular the promoter specificity and mode of action of these enhancer sequences, we used five different lines of mice which carried insertions of the same transgene containing the housekeeping phosphoglycerate kinase-1 (PGK) promoter driving either the neomycin (*neo*) or hygromycin (*hygro*) resistance genes (Adra *et al.*, 1987). These various transgene insertions were produced by homologous recombinations in ES cells and these specific lines of mice were selected based on the position of the integration sites in the *HoxD* complex. Earlier experiments had indicated that such foreign promoters introduced within a *Hox* complex tend to adopt the regulation of the nearby-located *Hox* gene and hence behave like a *Hox* gene with respect to their expression patterns (Beckers and Duboule, 1998; Rijli *et al.*, 1994; van der Hoeven *et al.*, 1996; Zákány *et al.*, 1997b). In our case, consecutive integrations of the same neutral promoter were used to scan the regulatory influences controlling the expression of posterior genes of the complex in developing limbs, from *Evx2* to *Hoxd11*, i.e., over a distance of ca. 30 kb (Fig. 1; Materials and Methods). In the five lines, the PGK promoters were in the same orientation, opposite to the transcriptional orientation of the surrounding *Hox* genes.

We report here that PGK promoters located at different positions in the *HoxD* complex are able to respond to the limb control elements in a colinear fashion. However, expression in limbs was not necessarily identical to that of the neighboring gene indicating that PGK promoters were likely not controlled by immediately adjacent, gene-specific regulatory sequences. These results suggest that during limb development, posterior *Hoxd* genes are regulated by opposite proximodistal influences whereby the position of a given gene in the complex will result in a specific balance between digit and forearm expression domains.

MATERIALS AND METHODS

Mouse lines. The mouse PGK promoter was used to drive drug selection marker genes (either *neo* or *hygro*). The insertion sites were previously described (see below). Neither these insertions nor the accompanying mutations affected expression of the flanking genes in the digit or forearm domains (see references below). The differences observed in stainings can therefore not be accounted for by the mutational events. Insertion sites were selected downstream of *Hoxd11* (I1, the $HoxD^{C6neo}$ allele; Gérard *et al.*, 1996), between *Hoxd11* and *Hoxd12* (I2, the $HoxD^{RXneo}$ allele; Beckers and Duboule, 1998), between *Hoxd12* and *Hoxd13* (I3, the $HoxD^{RXIneo}$ allele; Hérault *et al.*, 1998), and between *Hoxd13* and *Evx2* and within *Evx2* (I4 and I5, the EvD^{Ge1} and $Evx2^{Ge}$ alleles, respectively; Hérault *et al.*, 1996).

Whole-mount in situ hybridization. Whole-mount *in situ* hybridizations were performed on genotyped heterozygous embryos at different developmental stages following standard procedures. The *neo* and *hygro* probes contained the complete open reading frames of the corresponding genes and were of similar behaviors regarding their time of staining and intensities. Control embryos were hybridized with *Hoxd* gene riboprobes as reported previously.

RESULTS

We used five different lines of mice carrying a PGK promoter at various locations in the posterior part of the *HoxD* complex. The positions of the insertion sites are shown by arrowheads in Fig. 1A and are referred to as I1 to I5, from the most 3′-located (anterior) one to the most 5′ (posterior), respectively. All transgenes were transcribed in the reverse orientation with respect to *Hoxd* genes. As previously described, point mutations or small deletions were located near the insertion sites for I1, I2, and I3. However, these mutations did not alter the regulation of posterior *Hoxd* gene expression during limb development, even if the small deletion in I2 abolished expression of the neighboring *Hoxd12* gene in a restricted posterior subdomain of the limb (Beckers and Duboule, 1998; Gérard *et al.*, 1996; Hérault *et al.*, 1998; indicated as a dashed red line in Fig. 4). Therefore, the limb expression patterns observed in this study were not influenced by these targeted modifications. The I4 insertion coincided with the introduction of point mutations in the open reading frames of both *Hoxd13* and *Evx2* genes, whereas in I5 the PGK promoter was inserted inside the open reading frame of *Evx2* (Hérault *et al.*, 1996). In these latter cases, heterozygous animals did not display any mutant phenotype, nor did they show changes in limb expression.

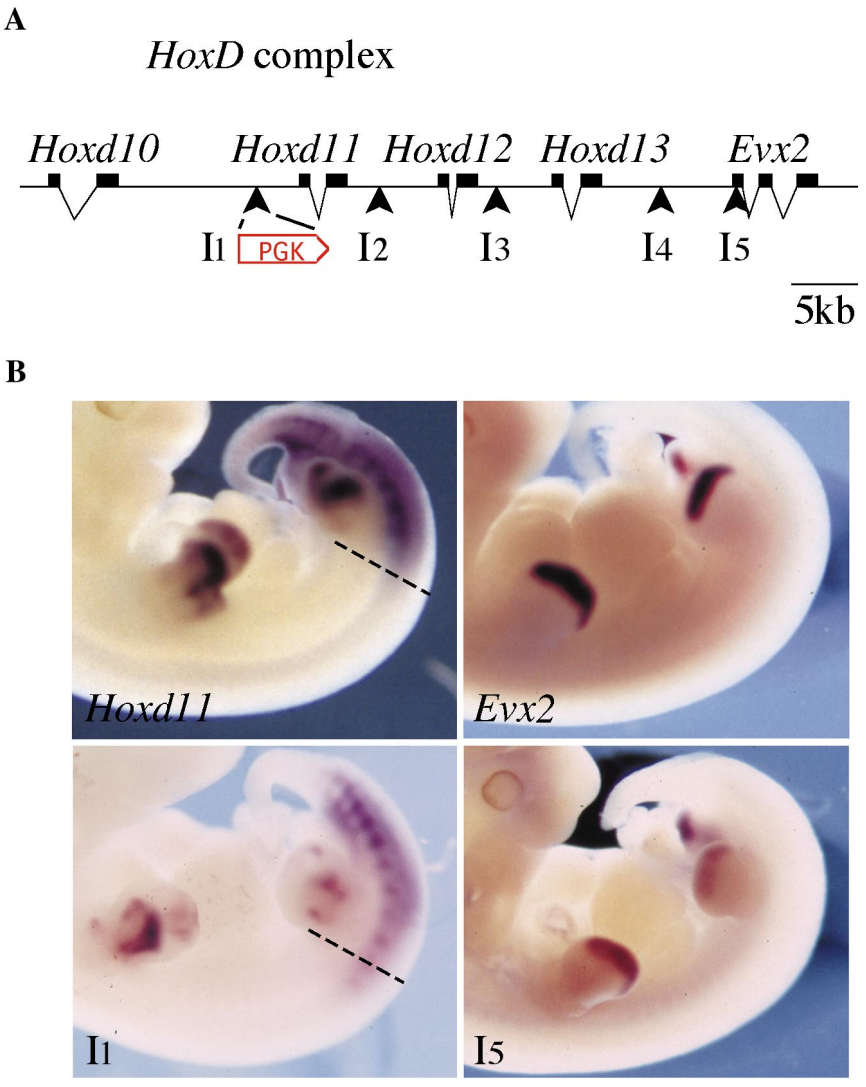


FIG. 1. Positions of the insertion sites and expression of the transgenes recombined within the *HoxD* complex. (A) Insertion sites of the transgenes (from I1 to I5) in the *HoxD* complex. The transgenes contain the PGK promoter followed by either the *neo* (A–D) or *hygro* (E) genes. The insertion sites were selected among lines of mice produced and kept in the laboratory (see Materials and Methods). Insertion sites were selected downstream of *Hoxd11* (I1), between *Hoxd11* and *Hoxd12* (I2), between *Hoxd12* and *Hoxd13* (I3), between *Hoxd13* and *Evx2* (I4), and within *Evx2* (I5). (B) Expression of the inserted transgenes and of the neighboring *Hoxd11* and *Evx2* genes. The expression of the PGK promoter in developing trunks, when located either downstream of *Hoxd11* (I1) or within *Evx2*, is similar to that of the neighboring genes *Hoxd11* and *Evx2*. The anterior expression limit of the transgene in I1 is slightly anteriorized, when compared to the endogenous *Hoxd11* gene, due to the point mutations introduced in the locus (dashed line; see Gérard *et al.*, 1996).

We first analyzed by *in situ* hybridization the distribution of *neo* or *hygro* mRNAs in the developing trunks of animals carrying the transgenes at different locations. Expression patterns driven by the PGK promoters were essentially indistinguishable from that of the neighboring genes. For example, the insertion within *Evx2* (I5) or within the intergenic region between *Hoxd13* and *Evx2* (I4) did not generate a clearly detectable pattern in the trunk, as observed for *Hoxd13* and *Evx2* mRNAs under usual experimental conditions (Figs. 1, 2F, and 2E, respectively). Likewise, insertion of the transgene close to *Hoxd11* (I1 and I2; Figs. 1, 2A, 2B, and 2D) or between *Hoxd12* and *Hoxd13* (I3; Fig. 2C) gave the expected trunk patterns for mice carrying these particular alleles. For example, while the $Hoxd11^{C6}$ allele showed a *neo* anterior expression boundary slightly anteriorized with respect to *Hoxd11* (I1; Figs. 1B, 2A, and 2D) as shown for *Hoxd11* itself in the C6 mutant background (Gérard *et al.*, 1996), both the $HoxD^{RX}$ and $HoxD^{RXI}$

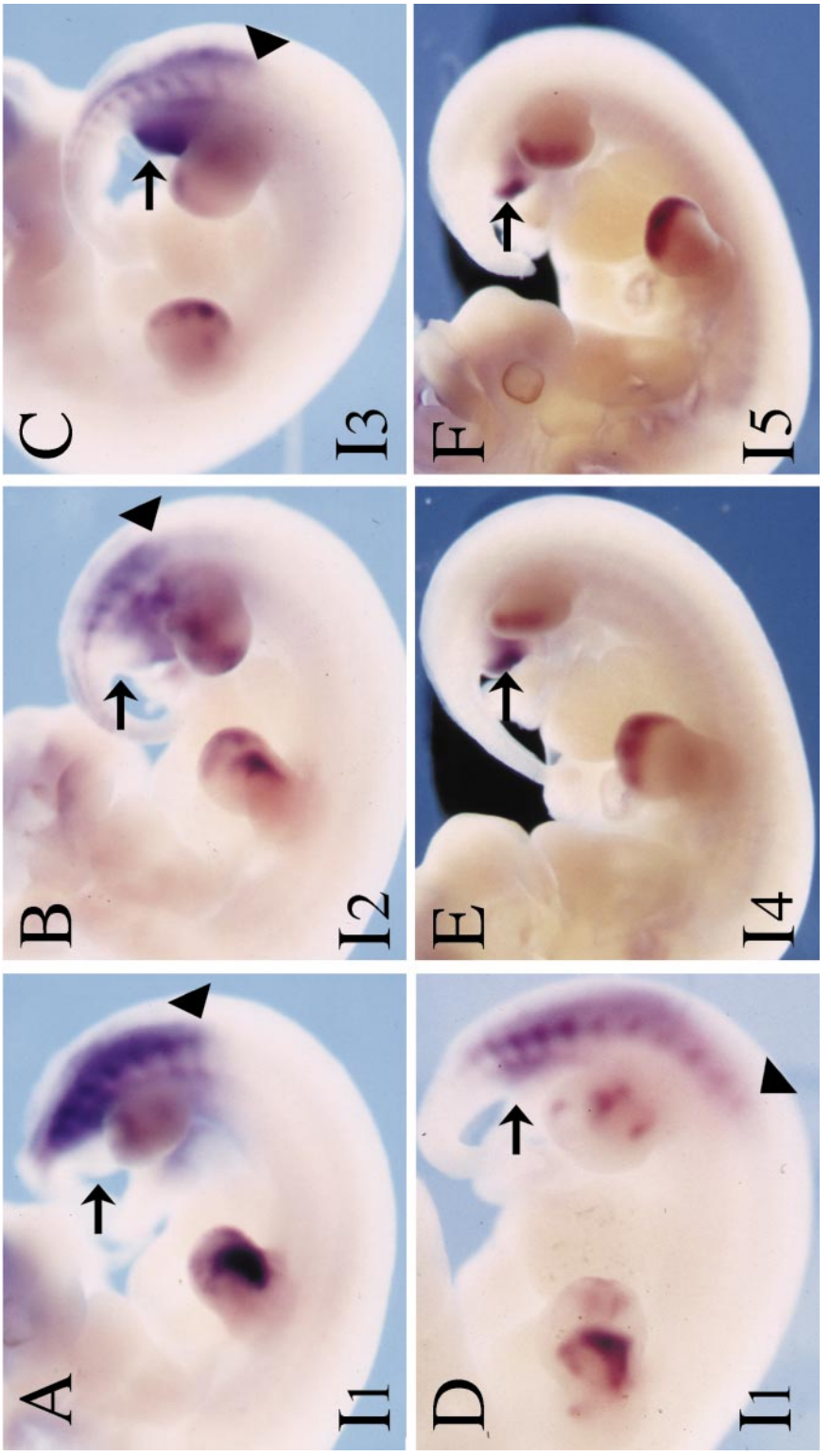


FIG. 2. Comparative expression of the transgene when introduced in I1 (A and D), I2 (B), I3 (C), I4 (E), or I5 (F). Transcript domains are revealed by *in situ* hybridization in either early (A) or late (B–F) 11.5 dpc fetuses. The transgene was expressed in the trunk, in I1 to I3, with an anterior limit located at the appropriate level for the neighboring genes (arrowhead in A–D), whereas the activation in the genital bud was only detectable in I3 to I5 (arrow in C, E, and F). Activation of the transgene in the genital domain was dependent upon the position of the transgene and expression was not resumed at a subsequent developmental stage such as at 12.5 dpc for I1 (D).

alleles displayed *neo* expression at the expected body levels for *Hoxd12* and *Hoxd13* (Figs. 2B and 2C, insertions I2 and I3, respectively).

Expression of the various recombined transgenes in developing limbs gave a slightly different picture. Whenever the reporter transgene was inserted at 5′ positions in the complex, e.g., within *Evx2* (I5) or between *Evx2* and *Hoxd13* (I4), expression patterns identical to those of the neighboring *Hoxd* genes were recovered, i.e., expression in the most distal part of the developing limbs corresponding to the presumptive digit domain (Figs. 3D and 3E). Insertion of the transgene at successively more 3′ positions showed that colinearity was maintained since a sustained expression domain appeared in the future forearm (Figs. 3A–3C, arrowheads). Concomitantly to the appearance of this forearm domain, expression in the distal domain was turned down (Figs. 3A and 3B, arrows). Therefore, insertion sites located between the two extreme positions (i.e., between I1 and I5) generated intermediate expression profiles with different ratios of the zeugopod (arrowhead) versus autopod (arrows) transcript domains (Fig. 3). A transition was thus observed from a forearm-specific type of expression (I1; Fig. 3A) toward a digit type of pattern (I5; Figs. 3D and 3E). Interestingly, induction of the PGK promoter was detected as a small restricted domain in the forearm at the I3 insertion site despite deletion of the RXI local control sequence (Fig. 3C, arrowhead). This confirmed that the action of the forearm enhancer was not mediated by this local sequence which was previously shown to have some function in limbs (Hérault *et al.*, 1998).

Surprisingly however, the respective importance of the two expression domains (forearm and digits) was somewhat different from that observed with the endogenous *Hoxd* genes (Fig. 4). While insertions of the transgene in the 5′ part of the complex (around *Hoxd13*) faithfully reflected the expression of the neighboring genes (Figs. 3D and 3E), insertion of the same transgene at more 3′ positions did not entirely recapitulate endogenous expression. In particular, the relative timing in the appearance of the two domains was modified and expression in digits was substantially delayed when compared to the endogenous situation (Figs. 3F–3J). For example, a PGK promoter located immediately upstream of *Hoxd11* showed no expression in the digit domain at day 10.5 dpc (Fig. 3H), a stage at which the endogenous *Hoxd11* gene is strongly expressed (Fig. 4A). Subsequently, only a weak signal appeared at day 11.5 (Figs. 3A and 3I). In this respect, the transgene inserted at the *Hoxd11* locus behaved somewhat like *Hoxd9*, rather than like *Hoxd11*, with a strong expression proximally and virtually no transcript in digits even though both endogenous *Hoxd11* and *Hoxd10* (the two surrounding genes) were still strongly expressed distally (not shown; Fig. 4A).

Therefore, even when inserted between *Hox* genes strongly expressed in digits, the PGK promoter was barely activated therein. In other words, while a colinear distribution of the forearm and digit domains was detected in our series of transgene insertions (by comparing PGK promoters), the transition in the relative distributions of these two domains, with respect to the genomic distance, was shorter than that observed with the surrounding endogenous genes

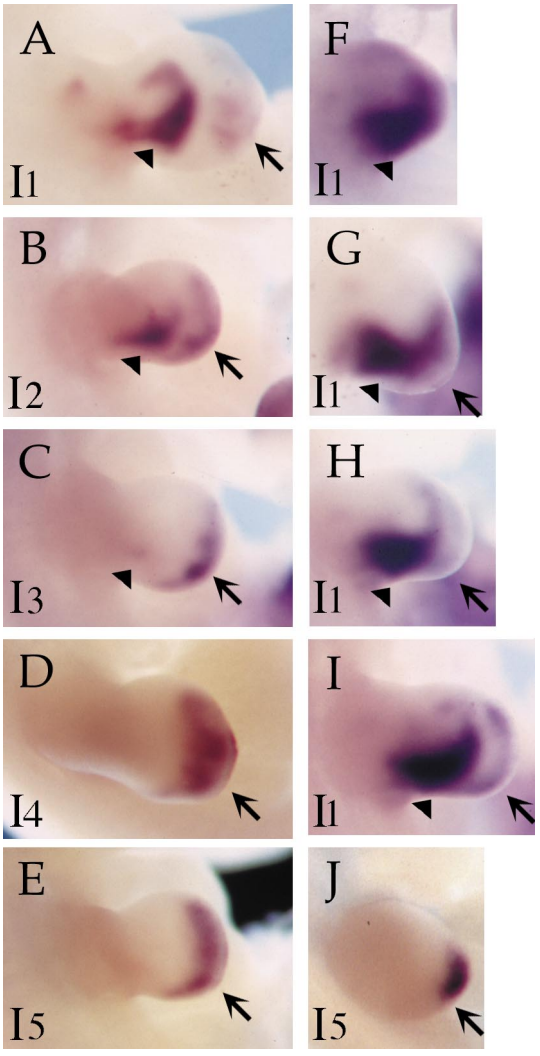
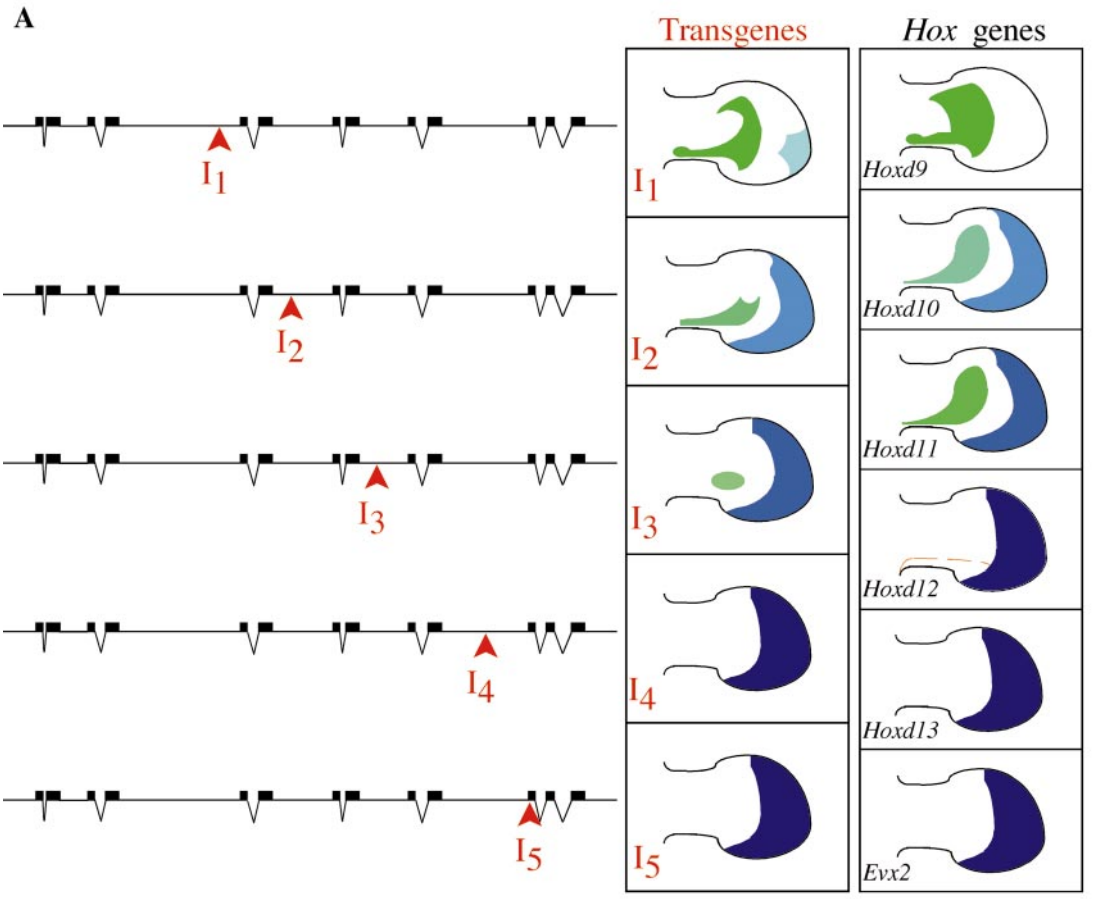
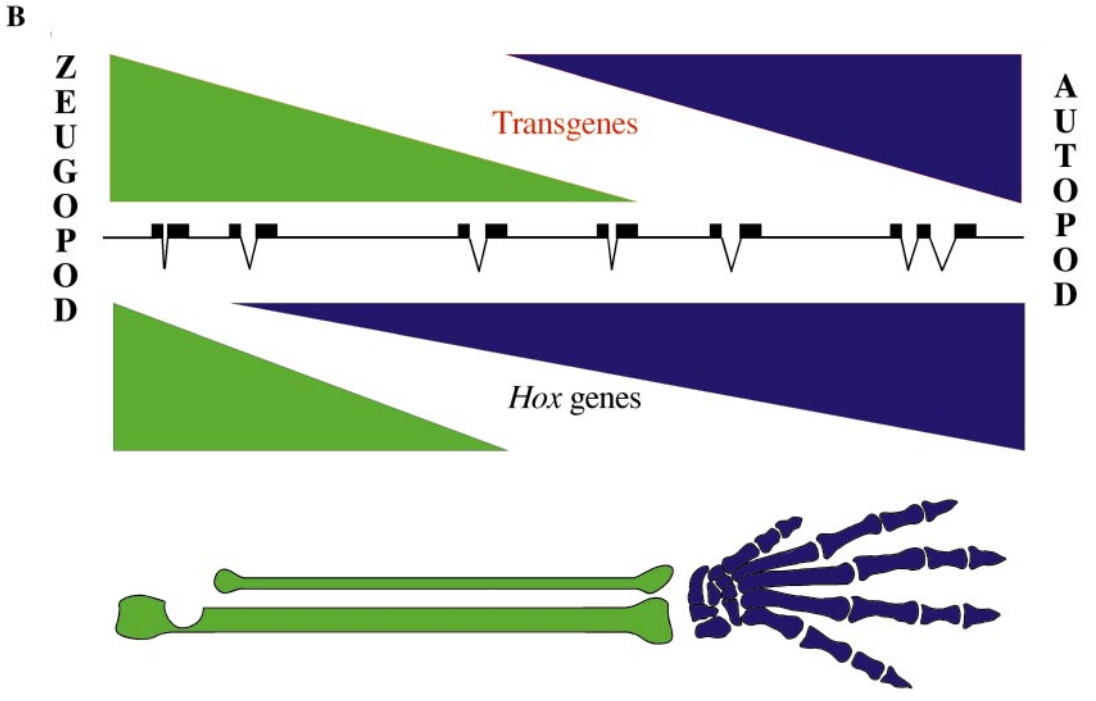


FIG. 3. Expression of the targeted transgenes during limb bud development. The expression patterns are observed by whole-mount *in situ* hybridizations of 11.5 dpc fetuses (A–E) using *neo*- or *hygro*-specific probes. The various insertion sites are indicated from I1 to I5. At the I5 position, the transgene is expressed in the forearm domain (A; arrowhead). The strength of this expression domain decreases along with moving the insertion sites from I1 to I4 (A–C, arrowheads). Alternatively, the activation of the PGK promoter in the distal domain is enhanced when the transgene is inserted closer to *Evx2* (arrow in B–E). This effect does not depend upon the timing of activation since the transgene, when located at the I1 locus, is activated in the forearm domain early on (F) and is maintained there in subsequent developmental stages (G–I). In the distal part, a weak expression is observed at this site of integration, when the autopodal region formed (G–I, arrow). The same reporter gene is already well activated in this distal domain when inserted in *Evx2* (I5, J).





(Fig. 4). A strong digit domain appeared only at the level of endogenous *Hoxd12,* whereas it already appeared at the level of *Hoxd10* in the wild-type situation. Consequently, there was no perfect continuity between the expression of the endogenous genes and that of the transgene insertions such that a break in colinearity was observed. The fact that colinearity, while respected among the engineered insertional events, was out of phase with respect to the endogenous situation demonstrated that the specificity of transgene expression in limbs was likely not mediated by endogenous *cis*-acting gene-specific elements, in contrast to other reported cases of neomycin expression during trunk development (Rijli *et al.,* 1994).

A similar difference was observed in the expression of the inserted transgenes in genital buds (Figs. 2A–2F). When inserted either at I1 (upstream of *Hoxd11;* Fig. 2A) or at I2 (between *Hoxd11* and *Hoxd12;* Fig. 2B), the PGK promoter was not activated in the presumptive genital bud, unlike the endogenous *Hoxd11* gene. However, a clear signal appeared when the transgene was located either at I3 or in more 5′ positions (Figs. 2C, 2D, and 2F). These latter observation confirmed that distal expression of *Hox* genes in limbs systematically correlated with expression in the genital bud (Fig. 2; Kondo *et al.,* 1997b; van der Hoeven *et al.,* 1996; Zákány *et al.,* 1997a). Indeed each time a PGK promoter was active in the digit area, it was found equally active in the genital eminence. These data reinforce the proposal that the same enhancer sequence is able to control expression in both structures, suggesting that ontogenic as well as evolutionary relationships may exist between these different appendices (Kondo *et al.,* 1997b).

DISCUSSION

During mammalian development, *Hoxd* genes related to the *Drosophila AbdB* gene (genes belonging to groups 9 to 13) are involved in the patterning of the body axis below the thoracic level. In addition, they are required for proper morphogenesis of the limbs (Dollé *et al.,* 1989). In the trunk, these genes are activated and maintained in a colinear fashion such that for example group 9 genes are switched on before those of group 10 and are expressed at more anterior positions. During limb budding and extension, colinearity is also observed for the same *Hoxd* genes, though with some important differences. In the course of limb budding, a nested set of expression domains appears, in a way reminiscent of the colinear mechanism at work in the trunk (Dollé *et al.,* 1989; Nelson *et al.,* 1996). At a later stage however, more distal transcript domains appear rather simultaneously for all these genes and display the same distribution, e.g., in the area covering the presumptive digits. This bipartite regulation suggests that different processes are at work to build up *Hoxd* gene expression patterns in limbs (Duboule, 1994; Nelson *et al.,* 1996). While an early phase of expression would follow a "trunk strategy" in the establishment of the transcript domains (sequential activation, nested set of domains), a late phase would subsequently rely upon the activity of global enhancers controlling the activity of several genes at once, hence leading to similar patterns in both time and space. Some evidence has been obtained which indicates that the almost identical digit expression domains of *Hoxd10* to *Hoxd13* are indeed controlled by the same regulatory sequences (van der Hoeven *et al.,* 1996).

In this work, we looked at the nature of these regulatory controls by scanning the posterior part of the *HoxD* complex with a set of transgene insertions containing the PGK promoter driving either the neomycin or hygromycin genes. In agreement with previously reported cases, insertions near a given *Hoxd* gene induced the PGK promoter to drive reporter expression in the trunk, in a way resembling that of the neighboring gene. These data confirmed that a foreign promoter introduced next to a *Hox* gene usually adopts the expression pattern of that particular *Hox* gene, in particular regarding its rostrocaudal expression boundary (e.g., Rijli *et al.,* 1994). This observation further emphasized the limited specificity of *Hox* gene promoters. It indicated that a generic *Hox* expression pattern seems to be imposed to those genes located in the complex and is subsequently modulated by additional mechanisms related to the gene's position. It is however not clear whether these similarities in expression were due to the presence of *Hox*-specific *cis*-acting regulatory elements located near the inserted

FIG. 4. (A) Schematic representation of the relative distribution of transcripts between the forearm (green) and digit (blue) presumptive domains. Expression of the inserted transgenes is shown in the left column, whereas the right column indicates the expression of the endogenous *Hox* genes. In addition, the wt expression pattern of *Hoxd12* in the posterior part of the limb bud is shown by a red line. Comparisons between the two columns reveal that colinearities among *Hox* genes and among the transgenes are slightly out of phase. For example, the transgene inserted near *Hoxd11* (I1) behaves somewhat like the endogenous *Hoxd9* and not like *Hoxd11,* as seen by the downregulation of the PGK promoter in the distal domain. (B) Scheme illustrating a potential model for colinearity of *Hox* gene expression in limbs. Two global regulatory influences (enhancers) control the activity of the endogenous *Hoxd* gene promoters (bottom). One such element controls the activation in the distal (digit) domain while the other controls activation in the forearm (colors are as in Fig. 1). As a results of this opposite regulation, *Evx2, Hoxd13, Hoxd12, Hoxd11,* and *Hoxd10* are expressed in the digit domain (blue), whereas *Hoxd9, Hoxd10,* and *Hoxd11* are active in the forearm. The respective distribution of these two domains for those genes located in this locus would thus result from a competitive interaction between these two influences. In the case of the inserted transgenes, the same regulations would apply but the PGK promoter would sense them in a slightly different way.

PGK promoter or to a more global type of regulation acting at the level of the complex. The fact that a given PGK-driven expression pattern faithfully recapitulated that of the neighboring gene nevertheless suggests that the former type of regulation may be at work as far as the trunk is concerned.

The results obtained in developing limbs for these different integration sites confirmed that a different mode of regulation was operating for *Hox* gene regulation in the appendicular skeleton. While insertion of the transgene near *Hoxd13* gave the expected pattern (*Hoxd13*-like), recombination of the PGK promoter at more 3′ positions did not generate expression patterns totally identical to those of the neighboring *Hox* genes. For example, insertion near *Hoxd11* gave a pattern more related to that of endogenous *Hoxd9*. This observation suggested that late limb expression domains are not controlled by nearby-located *cis*-acting elements which would be responsible for the gene-specific differences in patterns (e.g., between *Hoxd11* and *Hoxd13*) in the wild-type situation. In such a case, one would have expected the transgene to adopt the limb expression profile of its neighbor *Hox* gene. In contrast, these data confirmed that the local (gene-specific) environment may not significantly interfere with expression of *Hoxd* genes in developing limbs. This is in agreement with the previous proposal that regulation of *Hoxd10* to *Hoxd13* expression in the (most distal) digit domain was due to the presence of a single remote control enhancer sequence (a "digit element"). In this view, all four genes would be regulated at the same time, i.e., whenever the enhancer would become active, and in the same domain (van der Hoeven *et al.*, 1996). The presence of such a strong enhancer provides an explanation for the activity of the inserted PGK promoters in the presumptive digit area. However, since insertion of the transgene at the *Hoxd11* locus led to a regulation of expression which was intermediate between that of *Hoxd9* and *Hoxd10*, it may be that the digit enhancer element, located 5′ of the *HoxD* complex (see Hérault *et al.*, 1997, 1998; Peichel *et al.*, 1997; van der Hoeven *et al.*, 1996) was not equally sensed by either the *Hox* or the PGK promoters, at a given insertion site. The PGK promoter indeed appeared less sensitive to this remote regulation; while it fully reacted when placed in the *Evx2* locus, it remained almost silent in the *Hoxd11* locus, unlike the endogenous *Hoxd11* promoter.

These results suggest a model for the colinear spatial regulation of *Hoxd* genes in limbs whereby opposite regulatory influences, exerted by separate control elements, would compete for interactions with those *Hoxd* promoters located in between (Fig. 4B). In such a scheme, each *Hox* promoter would differentially integrate two influences, one controlling expression in digits and the other controlling expression in the forearm, depending upon its position within this genomic regulatory interval. In this view, the exact position of a gene in the complex would determine its expression balance between forearm and digits, hence the morphology of the resulting limb skeleton, a direct translation from genomic to morphogenetic topology (Fig. 4B). While strong evidence supports the existence of a remote limb enhancer element (see above), the presence of a counterpart for the forearm is less compelling. It is thus equally possible that expression in the forearm domain (zygopodium) is a groundstate pattern for these posterior genes and that this regulation would be competed out by a digit element located at a more 5′ position. In this latter case, forearm expression could result either from a long-acting enhancer or from separate *cis*-acting sequences.

This potential mechanistic explanation of colinearity in limbs, based on the balance between two regulatory influences, would make *Hox* gene expression in developing appendices entirely dependent upon their microgenomic locations. It predicts that any gene lying in this genomic environment would fall into the control of these limb enhancers and hence become expressed there in a "*Hox*" fashion. Such a regulatory strategy would allow limb morphology to evolve as a consequence of slight variations in the structural organization of the complex. As such, it would also participate in the evolutionary maintenance of this tight-clustered organization. We nevertheless do not wish to extrapolate this model to the major colinear distribution of *Hox* gene expression domains in the developing trunk. Instead, we favor a view whereby posterior *Hoxd* genes, in the course of tetrapod evolution, recruited their already established genomic organization to elaborate a novel kind of colinear mechanism together with the elaboration of complex limbs. In this view, the genomic organization was fixed and stabilized due to the colinear function of these genes in the developing trunk. This genetic topology subsequently allowed a limb-specific colinear system to emerge through the design of novel enhancers. An important difference is indeed observed between trunk and limbs in the timing of *Hox* gene activation. In the developing trunk, *Hoxd* gene activation follows a clear temporal sequence which may be due, in part, to the linear release of a repressive state (Kondo *et al.*, 1997a). By contrast, global limb enhancer sequences, once functionally available, are expected to exert their regulatory effect on all promoters simultaneously. This difference is supported by experimental observations suggesting that temporal colinearity cannot be clearly observed in the appearance of the digit domain for posterior *Hoxd* genes and that some aspects of colinearity are even reversed in the construction of distal limbs (Nelson *et al.*, 1996). This latter possibility could be reconciled with the present results as *Hoxd13*, for example, would start to be expressed in digits before *Hoxd11* due to an increased proximity/affinity to the digit enhancer.

The mechanism behind such opposite regulations is still elusive. It could be related to the flip-flop mechanism postulated for the globin complex (Dillon *et al.*, 1997; Wijgerde *et al.*, 1995) whereby the distance between the enhancer and the promoter would be an important factor in the frequency of interactions, hence in transcriptional efficiency. In the present case, interactions between the digit enhancer and the *Hoxd13* promoter would be favored and

thus compete out the interaction with the forearm element. The isolation, relocation, and subsequent deletion of these potential enhancer elements will shed light on this mechanism and may suggest at which step such a regulatory scheme appeared in the course of vertebrate phylogeny.

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