Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 13695–13700, December 1997
Developmental Biology

Regulation of number and size of digits by posterior *Hox* genes: A dose-dependent mechanism with potential evolutionary implications

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Edited by Mario R. Capecchi, University of Utah School of Medicine, Salt Lake City, UT, and approved September 29, 1997 (received for review July 11, 1997)

ABSTRACT **The proper development of digits, in tetrapods, requires the activity of several genes of the *HoxA* and *HoxD* homeobox gene complexes. By using a variety of loss-of-function alleles involving the five *Hox* genes that have been described to affect digit patterning, we report here that the group 11, 12, and 13 genes control both the size and number of murine digits in a dose-dependent fashion, rather than through a *Hox* code involving differential qualitative functions. A similar dose–response is observed in the morphogenesis of the penian bone, the baculum, which further suggests that digits and external genitalia share this genetic control mechanism. A progressive reduction in the dose of *Hox* gene products led first to ectrodactyly, then to olygodactyly and adactyly. Interestingly, this transition between the pentadactyl to the adactyl formula went through a step of polydactyly. We propose that in the distal appendage of polydactylous short-digited ancestral tetrapods, such as Acanthostega, the *HoxA* complex was predominantly active. Subsequent recruitment of the *HoxD* complex contributed to both reductions in digit number and increase in digit length. Thus, transition through a polydactylous limb before reaching and stabilizing the pentadactyl pattern may have relied, at least in part, on asynchronous and independent changes in the regulation of *HoxA* and *HoxD* gene complexes.**

Posterior (*AbdB*-related) *Hox* genes belonging to both the *HoxD* and the *HoxA* complexes are necessary for the proper organization and development of tetrapod digits (for review, see ref. 1). In mice, genetic analyses have revealed that multiple *Hox* functions cooperate in a rather nonspecific fashion to elaborate the normal sequence of bony elements that compose the appendicular skeleton. For example, the contribution of either *Hoxd-13* (2) or *Hoxa-13* (3) is required for digit development with double homozygous mutant animals lacking digits entirely (3). Additionally, both the number and the morphogenesis of digits are strongly affected in animals lacking three *HoxD* functions *in cis* (*Hoxd-11*, *Hoxd-12*, and *Hoxd-13*; ref. 4), more severely than in the absence of *Hoxd-13* alone, indicating that genes belonging to paralogous groups 11 and 12 may participate to these processes as well (see also refs. 5–7). Yet, the combined loss of *Hoxd-11* and *Hoxa-11* function had only incidental effects on digit morphology. Instead, it drastically truncated the intermediate pieces of both limbs (7), suggesting either that hierarchical relationships exist between *Hox* functions such that the function of group 11 genes in digits is negligible as long as group 13 proteins are present, or that group 11 and 12 genes can have a function in digits that is not necessarily required in wild-type animals (1, 5).

In *Hox* mutant strains that show selective distal limb defects, the most commonly observed digit alterations involve reduction in the size of skeletal elements and loss of phalanges. These alterations, sometimes combined with diversions from the pentadactyl formula, were considered to be possible atavisms (2, 3). Recently, similar digit defects also were observed in human patients carrying mutations either in the *HOXA13* or in the *HOXD13* genes (8, 9). Conversely, the forced ectopic expression of more than the normal amounts of various *Hox* products in limb buds induced surplus digital material (10–13), which is consistent with a quantitative role of *Hox* products in the making of a limb.

Although most extant tetrapods have a pentadactyl digit formula, an analysis of the fossil record indicated that ancestral stem tetrapods had polydactylous extremities with seven to eight digits (14). It therefore is assumed that the evolutionary transition between limbs without digits (adactylous), such as in *Panderychtis*, and limbs with five digits (pentadactylous) involved an intermediate stage of polydactyly, a state that perhaps was linked to the aquatic status of these primitive ancestral tetrapods (15). It is indeed conceivable that the crawling locomotion required by an aquatic environment, favored individuals with multiple and short digits, resulting in paddle-like autopods, rather than a more restricted number of longer digits, which, by contrast, may be advantageous for locomotion in a terrestrial environment. The functional importance of *Hox* genes in these evolutionary remodelings of the autopods remains speculative. However, three sets of evidence may support a scenario in which at least some of these modifications were driven, or paralleled, by changes in *Hox* gene regulation and expression. First, phenotypes induced by loss-of-function mutations of posterior *Hox* genes systematically present a strong atavistic character (2), therefore suggesting that these phenotypes somehow illustrate phylogenetic features or potentialities of the autopod. Second, the expression of *Hoxa* and *Hoxd* genes during teleost fin development is compatible with a role for these genes in the fin to limb transition (16, 17). And finally, the observation that the transcriptional regulation of up to five posterior *HoxD* complex genes active in the embryonic digits act through a shared regulatory mechanism makes it plausible that several *Hoxd* genes were recruited simultaneously during digit evolution to provide a sufficient dose of *Hox* proteins (18).

In this work, we used a variety of loss-of-function alleles of murine *HoxD* and *HoxA* complex genes expressed in digits to analyze the quantitative and qualitative aspect of this regulation, as well as to investigate the concurrent phenotypic

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transformations. We show that in compound mutants both the size and number of digits varied in response to quantitative modifications of *Hox* gene function, and that this response was linear and rather independent of the specific gene involved. A progressive decrease in dosage of functional *Hox* genes in developing digits induced a graded reduction in the length of the digits and was accompanied by a concomitant transition from pentadactyly to polydactyly, then to oligodactyly, finally reaching the adactyl condition. The relevance of these results is discussed in the context of an evolutionary process involving successive recruitments of the *HoxA* and *HoxD* complexes in developing appendages.

MATERIALS AND METHODS

Description of the Alleles. Four loss-of-function alleles, induced by gene inactivation in murine embryonic stem cells through homologous recombination, were used in the course of this work. The *Hoxd-13* and *Hoxa-13* mutant alleles were homeodomain disruptions, in which the TK*neo* selection cassette was inserted into the homeobox of either *Hoxd-13* (2) or *Hoxa-13* (3). For the *Hoxa-11* mutant allele, the gene was disrupted by replacing the homeobox containing genomic region with the PGK*neo* selection cassette (19). The $HoxD^{Del}$ mutant allele was produced as a *loxP*/*cre* induced deletion of a genomic region encompassing *Hoxd-13* and *Hoxd-12,* plus *lacZ* reporter sequences were inserted in-frame in the first exon of the *Hoxd-11* gene. This $HoxD^{Del}$ allele thus represents a triple *Hoxd-13*, *Hoxd-12*, and *Hoxd-11* loss of function, with a *Hoxd-11*/*lacZ* fusion product allowing for histochemical detection of β-galactosidase activity (4). All four alleles were generated in 129/Sv-derived embryonic stem cell line (D3 embryonic stem cells; ref. 20, gift of R. Kemler, Max-Planck-Institute of Immunobiology, Freiburg, Germany). *Hoxd-13*, *Hoxa-13*, and $HoxD^{Del}$ were maintained in a 129/Sv and C57BL/6 mixed genetic background. The *Hoxa-11* allele originally was established in the 129/Sv and CF-1 mixed genetic background, but, in the course of our experiments, it was bred into the 129/Sv and C57BL/6 mixed genetic background for at least four consecutive generations before it was used in the test cross.

Genetic Crosses. Genetic interactions between the *Hoxd-13*, *Hoxa-13*, and $HoxD^{Del}$ null alleles were established by using the following crosses. First, $Hoxd\text{-}13^{-/-}$ females were crossed to $HoxD^{Del/+}$ males to obtain $Hoxd\text{-}13^{-}/HoxD^{Del}$ transheterozygous animals. Second, compound heterozygotes for $Hoxa\text{-}13^{+/-}$;$Hoxd\text{-}13^{+/-}$ females were crossed to $HoxD^{Del/+}$ males to obtain $Hoxa\text{-}13^{+/-}$;$Hoxd\text{-}13^{-}/HoxD^{Del}$. Finally, $HoxD^{Del/+}$;$Hoxa\text{-}13^{+/-}$ males and females were intercrossed to obtain all possible genotypes issuing in the F_2 generation. Consistent with previous analyses, we found the $Hoxa\text{-}13^{-/-}$ constitution to be embryonic lethal (3), whereas the $Hoxa\text{-}13^{+/-}$;$Hoxd\text{-}13^{-/-}$, $Hoxa\text{-}13^{+/-}$;$Hoxd\text{-}13^{-}/HoxD^{Del}$ and $Hoxa\text{-}13^{+/-}$;$HoxD^{Del}/HoxD^{Del}$ configurations were semilethal with only occasional animals surviving to adulthood. Genotyping of $HoxD^{Del/+}$;$Hoxa\text{-}13^{+/-}$ F_2 progeny was carried out by using yolk sac-derived DNA, according to the Southern blotting protocols reported in the original descriptions of the alleles. Skeletal preparations were performed according to standard procedures (2). To establish genetic interactions with *Hoxa-11*, we crossed compound heterozygotes of the $HoxD^{Del/+}$;$Hoxa\text{-}11^{+/-}$ genotypes and analyzed the F_2 progeny either at birth, at 5 days or at 5 weeks after delivery. The $Hoxa\text{-}11^{-/-}$;$HoxD^{Del/Del}$ constitution was semilethal as well. All genotypes were present at birth following a Mendelian distribution, yet five of six $Hoxa\text{-}11^{-/-}$;$HoxD^{Del/Del}$ animals died by the fifth postnatal day because of kidney agenesis, consistent with the phenotype of $Hoxa\text{-}11^{-/-}$;$Hoxd\text{-}11^{-/-}$ compound homozygots (7).

Calculation of the *Hox* Dose and Digit Length. Based on previously reported strengths of phenotypes in digits, one wild-type haplotype of *Hoxd-11* together with *Hoxd-12* was considered to contribute one unit (henceforth *Hoxd-11*/*Hoxd-12*) whereas one wild-type allele of both *Hoxd-13* and *Hoxa-13* were considered to contribute two units of *Hox* function each. In this way, the wild-type *Hox* dose is the sum of two units (*Hoxd-11*/*12*), plus four units (*Hoxd-13*) plus four units (*Hoxa-13*), hence an arbitrary total of 10 units (Fig. 1*A*). In previous experiments, the most refractory indicator of genetic interactions between *Hoxd* genes were digits III and IV (see e.g., refs. 3 and 6) and the novel genetic constitutions isolated in the course of this work also confirmed the stability in the size of these digits (Figs. 1 and 2). Five-week-old animals' forelimb and hindlimb skeletons were observed under a dissecting microscope and the length of digit IV from the proximal end of the metatarsal bone to the tip of the claw was measured with 0.25-mm precision on a millimeter scale. The arithmetic mean of the left and right digits' length was expressed as a percentage of that observed in age matched controls, siblings whenever possible. The mutant vs. sibling control percentage values proved similar at the 11th day and at 5 weeks of age and were thus entered into the same plot (Fig. 1*H*). Each entry represented one animal. The entry for a single-dose unit was based on the observation that no digit developed in $Hoxd\text{-}13^{-/-}$;$Hoxa\text{-}13^{-/-}$ animals by embryonic day 16.5 (3), the oldest stage at which animals of this genotype can be recovered alive. The entry for two units represented four $HoxD^{Del/Del}$;$Hoxa\text{-}13^{+/-}$ animals; the entry for three units represented one $Hoxd\text{-}13^{-}/HoxD^{Del}$;$Hoxa\text{-}13^{+/-}$ animal; the entries for four units represented an $Hoxd\text{-}13^{-/-}$;$Hoxa\text{-}13^{+/-}$ and four $HoxD^{Del/Del}$ animals; the entries for five units represented an $Hoxd\text{-}13^{-}/HoxD^{Del}$ and three $HoxD^{Del/+}$;$Hoxa\text{-}13^{+/-}$ animals; the entry for six units represented one $Hoxd\text{-}13^{-/-}$ animal, and the entries for seven units represented two $HoxD^{Del/+}$ animals. The linear regression fit of forelimb digit IV length (Fig. 1 *H*) was calculated by using Cricket Graph software; the regression coefficient was 0.95. The validity of the plot can be controlled by projecting the digit length and *Hox* doses of other compound genotypes of either *Hoxd-11*, *Hoxd-12*, and *Hoxd-13* (6) or $HoxD^{Del}/Hoxd\text{-}11^{-}$ (21), which were not included in the present curve.

RESULTS AND DISCUSSION

Digit Length. The most refractory indicator of genetic interactions between *Hoxd* genes in distal limbs was digit IV. Digit IV is derived from a very posterior position in the developing limb bud, a region where the posterior *Hox* genes all are expressed early on (36). It is also the first digit to condense as the digital arch forms, which makes it a good indicator of quantitative interactions between gene products, as revealed by its length (Fig. 1). For example, $HoxD^{Del/+}$ mice, in which the function of one haplotype of *Hoxd-11*, *Hoxd-12*, and *Hoxd-13* were inactivated simultaneously, showed phalanx 2 defects in digits II and V in the forelimb (Fig. 2*B*). The size of digit IV nonetheless remained essentially unaltered. However, when one dose of group 13 gene was further removed, either in $HoxD^{Del/+}$;$Hoxa\text{-}13^{+/-}$ (Fig. 1*C*) or in $HoxD^{Del}/Hoxd\text{-}13$ mice (Fig. 1*D*), animals had severe defects in every digit, the length of digit IV being also reduced to about 70% of wild-type length (Fig. 2 *A*, *C*, and *D*). Subsequent elimination of the wild-type alleles, for example in specimen of the $Hoxd\text{-}13^{-/-}$;$Hoxa\text{-}13^{+/-}$ (Fig. 1*F*) or $HoxD^{Del/Del}$;$Hoxa\text{-}13^{+/-}$ (Fig. 1*G*) genotypes, generated an accentuated reduction in digit length, leaving only 30% and 20% of normal size, respectively.

A linear relationship was observed between, on the one hand, the additive dose of *Hoxd-11*/*Hoxd-12* taken together, *Hoxd-13* and *Hoxa-13*, and on the other hand, the adult size of forelimb digit IV (see *Materials and Methods* and Fig. 1). In this context, $Hoxd\text{-}13^{-}/HoxD^{Del}$ mice, for example, had a similar

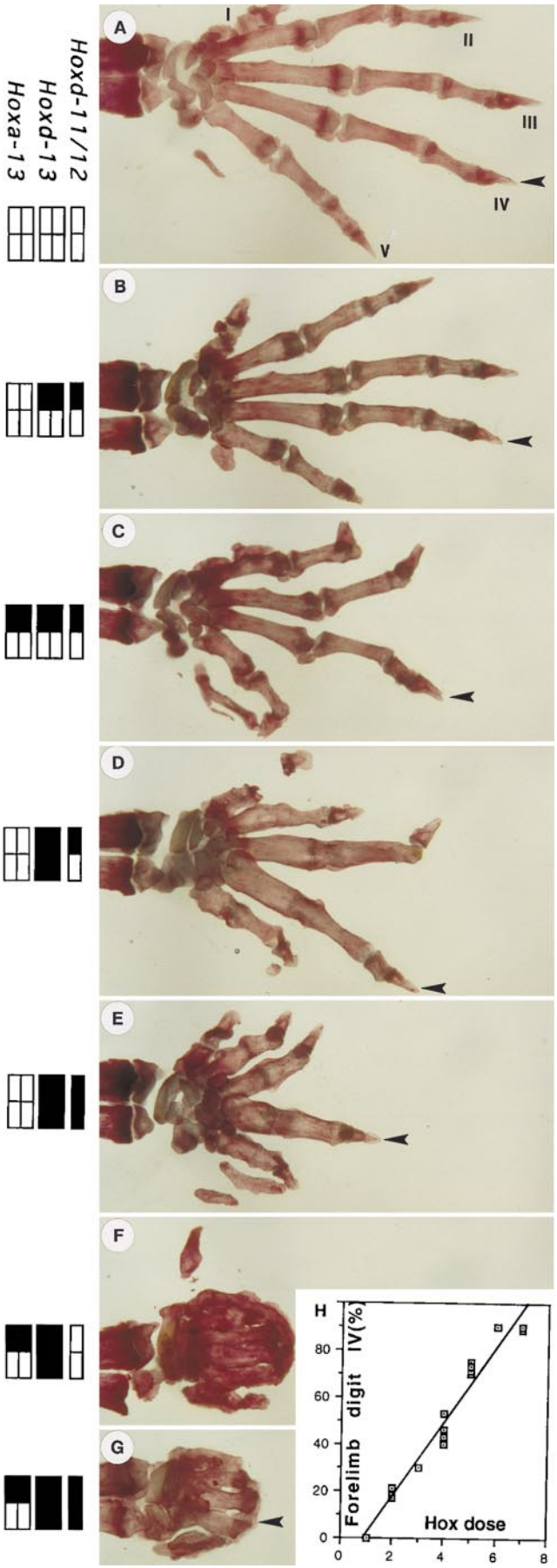

FIG. 1. Digit length is additively determined by the dose of *Hoxd-11*, *Hoxd-12*, *Hox-13*, and *Hoxa-13* gene products. The length of forelimb digit IV (arrowheads), including the metacarpal bone, was taken as a reference measure in mice of different genotypes. Compound mutant genotypes are indicated on the left, black rectangles indicating the loss of one dose of the corresponding gene (shown at the top). In this view, the wild-type hand (*A*) had 10 doses of active products. From *B* to *G* doses were progressively removed by using the corresponding genotypes: (*B*) $HoxD^{Del/+}$; (*C*) $HoxD^{Del/+}$;$Hoxa\text{-}13^{+/-}$; (*D*) $Hoxd\text{-}13^{-}/HoxD^{Del}$; (*E*) $HoxD^{Del/Del}$; (*F*) $Hoxd\text{-}13^{-/-}$/;$Hoxa\text{-}13^{+/-}$; (*G*) $HoxD^{Del/Del}$;$Hoxa\text{-}13^{+/-}$. (*H*) The length of digit IV was measured, expressed as fraction of wild-type digit length, and plotted against the *Hox* dose (see *Materials and Methods* for the calculation of the respective dose per gene). In this way, the length of digit IV varied as a linear function of the dose, regardless of the nature of the combination. (*A–G*) Anterior is up, posterior is down. I–V indicate digit number with, by convention, digit I being the thumb. In mutants with more or less than five digits, the phalanx pattern makes individual homologization impossible, but the digit found at the position corresponding to wild-type digit IV was always the longest.

"*Hox* dose" to $HoxD^{Del/+}$;$Hoxa\text{-}13^{+/-}$ (five doses; Fig. 1 *D* and *C*). Correspondingly, they generated very similar sizes for digit IV. In hindlimbs, the same linear relationship was observed between seven and four dose units, although at lower doses involving $Hoxd\text{-}13^{-/-}$ and $Hoxa\text{-}13^{+/-}$, further reducing the *Hoxd-11*/*Hoxd-12* dose did not importantly reduce hindlimb digit size, which stayed at about 30 percent of wild type (Fig. 2 *D* and *E*).

These results suggested that the function of posterior *Hox* genes during digit growth is exerted mostly at the quantitative level and does not involve precise qualitative combinatorial information. It is, however, impossible to completely rule out a scenario in which only the group 13 genes would be critical for digits. In such a view, the absence of part or all group 13 function would allow more 3′ genes to exert an "artificial" function in digit development, i.e., a function that they normally would not carry out in the presence of group 13 functions (1, 5). This possibility cannot be assessed by using the available alleles, and the genetic approach also may have to be complemented by biochemical evidence.

Digit Number. In forelimbs and hindlimbs of mice triple homozygous mutant for *Hoxd-11*, *Hoxd-12*, and *Hoxd-13* (Fig. 2*C*; $HoxD^{Del/Del}$), as well as in forelimbs of animals compound homozygous for the *Hoxd-13* mutation and heterozygous for *Hoxa-13* (ref. 3 and Fig. 1*F*), up to seven digit vestiges were found. Therefore, starting from a *Hoxd-13* homozygous mutant background, the additional elimination of either the *Hoxd-11*/*Hoxd-12* locus, or one allele of *Hoxa-13*, led to an increase in digit number. When *Hoxd-11*/*Hoxd-12* and *Hoxa-13* levels were reduced simultaneously from the same *Hoxd-13*-deficient background, novel polydactylous constitutions were found combined with more severe digit size reductions. However, the two extreme genotypes generated by combining these various alleles, i.e., $HoxD^{Del/Del}$; $Hoxa\text{-}13^{+/-}$ (four digits, Figs. 1*G* and 2*E*) and $HoxD^{Del/+}$;$Hoxa\text{-}13^{-/-}$ (three digital condensations visible only at embryonic day 14.5, not shown) displayed oligodactyly, with very small digit vestiges.

In $Hoxa\text{-}13^{-/-}$ simple mutant animals, i.e., with the normal complement of functional *Hoxd* genes, oligodactyly also was observed because of the loss of digit I (3). This observation was, however, consistent with the poor expression of *Hoxd* genes at the anterior edge of the autopod (in digit I primordium), leading to a strong dependence of this digit on the remaining *Hoxa-13* function (3). This particular case was thus different from the low *Hox* dose genotypes reported here, where changes in digit number involved also the domain that normally would give rise to digits II–V. In the $HoxD^{Del/Del}$;$Hoxa\text{-}13^{+/-}$ genotype, oligodactyly was observed in the presence of one *Hoxa-13* wild-type allele and thus digit I is presumed to be present (Fig. 2*E*, arrowhead). Likewise, in $HoxD^{Del/+}$;$Hoxa\text{-}13^{-/-}$ animals, which have only three digit condensations at

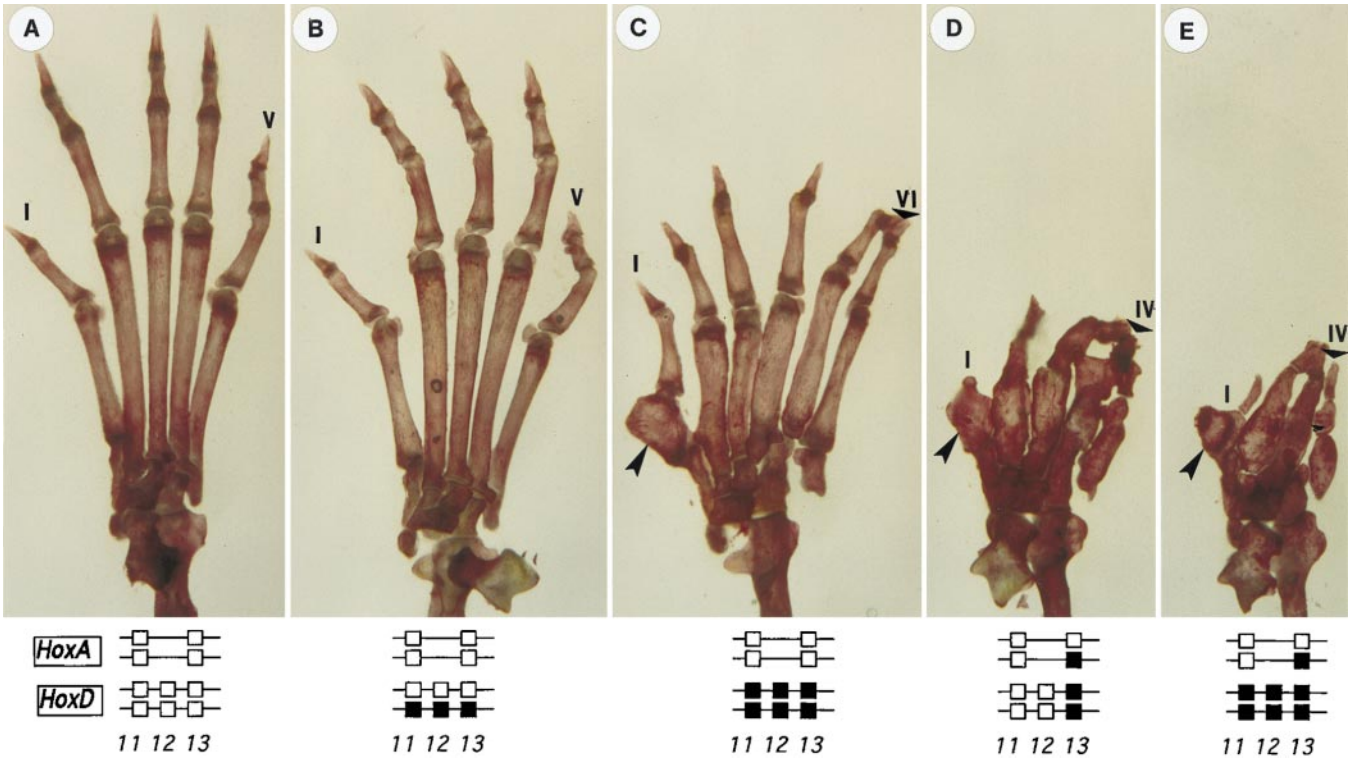

FIG. 2. Dose-dependent variation of the dactyly, as illustrated by the feet of mice of various genotypes. The posterior parts of both complexes are schematized below with the genes whose functions were removed in black. (*A*) Wild type. (*B*) *HoxD*$^{Del/+}$. (*C*) *HoxD*$^{Del/Del}$. (*D*) *Hoxd-13*$^{-/-}$;*Hoxa-13*$^{+/-}$. (*E*) *HoxD*$^{Del/Del}$;*Hoxa-13*$^{+/-}$. The progressive ectrodactyly goes together with a transition from pentadactyly (*A* and *B*) to polydactyly (*C*) to oligodactyly (*E*). Digits I (anterior to the left) and V are indicated as well as the most posterior digit IV or VI (small arrowheads in *C* and *D*). The large arrowheads in *C* and *D* point to a digit I specific alteration produced in absence of *Hoxd-13* (2), which identifies this digit. Thus, although the phalanx pattern does not allow homologies with other digits, the extra digit belongs to the II-V domain.

embryonic day 14 (not shown), more than one digit was missing, indicating that the defect could not be solely ascribed to the absence of digit I.

The loss of *Hoxa-11* function did not significantly modify the dose balance required for proper digit development. Mice of the *HoxD*$^{Del/Del}$;*Hoxa-11*$^{-/-}$ genotype were indeed polydactylous in their hindlimbs, with a reduced incidence of polydactyly in the forelimbs, and their digits were only marginally shorter than those of *HoxD*^{Del} homozygous mice (not shown). Oligodactyly was not observed in this configuration. Even though both the *Hoxa-11* as well as the *Evx-2* (located near *Hoxd-13*) genes were shown to contribute to digit development (22, 23), their combined function was insufficient to rescue digit growth in *HoxD*$^{Del/Del}$;*Hoxa-13*$^{-/-}$ mice, suggesting that the four wild-type alleles of *Hoxa-11* and *Evx-2* together contribute less than two "*Hox* dose units," as measured in the crosses above.

In summary, it appears that both the size and number of digits are dependent on *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, and *Hoxa-13*, making these four genes the major *Hox* determinants of digit morphogenesis. Step-wise reduction of *Hox* dosage induced not only an increasing severity in digit size defects, i.e., ectrodactyly, but also led to a transition from pentadactyly to polydactyly, whereas further reduction generated oligodactyly and finally adactyly, i.e., complete digit loss. This result suggests that a common *Hox* dose-dependent mechanism may control both the number and the size of digits. Although the effect on digit number may reflect the early role of these genes in the formation of prechondrogenic condensations, the effect on digit growth also might involve the control of chondroblast cell proliferation and maturation (4, 11). Hence, these two important parameters may reflect the two levels at which *Hox* genes previously were proposed to be required during limb development (2).

Size of Baculum. As in many rodent and in some other mammalian species, male mice have a bone in their penis. This baculum (os priapi) originates from cells strongly expressing posterior *HoxD* genes during development, which led to the proposal that limb and genital buds had similar developmental strategies in which posterior *Hox* genes were essential components (24). Furthermore, this small bone was slightly altered, because of a cellular deficit, in mice lacking *Hoxd-13* function (3). We therefore looked for further baculum size reduction in these various compound genotypes.

The functional cooperation of the same four genes was clearly observed (Fig. 3). Whereas the inactivation of *Hoxd-13* led to a minor localized alteration of the baculum, the simultaneous inactivation of *Hoxd-11*, *Hoxd-12*, and *Hoxd-13* (*HoxD*$^{Del/Del}$) resulted in an overall size reduction of the bone and indeed of the entire organ. The functional input from *Hoxa-13* was best evidenced in *HoxD*$^{Del/+}$;*Hoxa-13*$^{+/-}$ males, and an almost complete agenesis of the baculum was seen in *HoxD*$^{Del/Del}$;*Hoxa-13*$^{+/-}$ (Fig. 3*A*). The remarkably similar functional cooperation of the same four genes during both digit and genital eminence development reflects the coexpression of *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, and *Hoxa-13* in overlapping domains of both developing autopods and external genitalia. It suggests that these apparently different structures share important developmental mechanisms, perhaps as a consequence of a common phylogenetic history (18).

Autoregulation and Crossregulation. The existence of autoregulatory and/or crossregulatory interactions between vertebrate *Hox* genes and their products (e.g., refs. 25 and 26) has introduced an uncertainty in the interpretation of loss-of-function phenotypes, in particular when multiple genes are involved. This uncertainty may be of particular concern when evaluating additive gene doses. For instance, inactivating one gene could in turn double the functional output of another by relieving a repressive effect. Consequently, we looked at *HoxD*

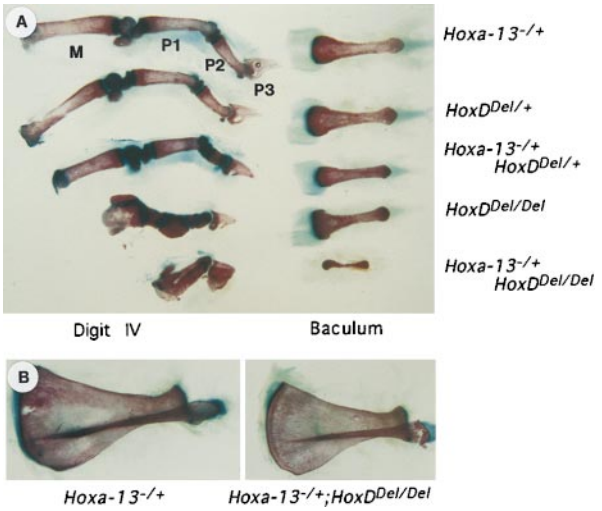

FIG. 3. Relationship between the sizes of the penian bone, the baculum, and digit IV in *HoxD* and *HoxA* mutant juvenile male mice. (*A*) Forelimb digit IV (*Left*) and the penis (*Right*) of 11-day-old juvenile sibling males were isolated, cleared, and compared. The genotypes are indicated on the right. An extreme reduction of the baculum was observed in mice of the $HoxD^{Del/Del}$;$Hoxa\text{-}13^{+/-}$ genotypes, i.e., mice with the shortest digits. In such specimen, the baculum was ill-formed and barely half of the expected length and thickness. (*B*) As control for the overall size of the mice, the scapula of the same two genotypes are shown. Although a noticeable reduction of the scapula in $HoxD^{Del/Del}$;$Hoxa\text{-}13^{+/-}$ animals indicate their general shorter statures, this reduction was much less drastic than that observed for the penian bone, which was almost absent.

gene activation in selected compound genotypes leading either to the absence of digits, or allowing very limited digit development.

We isolated $HoxD^{Del/+}$ and $HoxD^{Del/Del}$ fetuses which, in addition, lacked *Hoxa-13* function (Fig. 4). In the latter case, the extent of skeletal condensations was expected to be even less developed than in $Hoxd\text{-}13^{-/-}$;$Hoxa\text{-}13^{-/-}$ mice, which did not show any genuine digit (3). These animals were used to asses the activity of the *Hoxd-11/lacZ* maker gene present at the 5′ end of the $HoxD^{Del}$ locus (4). $HoxD^{Del/Del}$ and $HoxD^{Del/Del}$;$Hoxa\text{-}13^{-/-}$ forelimbs and hindlimbs showed comparable levels of *Hoxd* marker gene expression (Fig. 4 *B* and *D*), similarly to $HoxD^{Del/+}$ and $HoxD^{Del/+}$;$Hoxa\text{-}13^{-/-}$ (Fig. 4 *A* and *C*). Activation of the *Hoxd* marker in $HoxD^{Del/Del}$;$Hoxa\text{-}11^{-/-}$ mice also was unaltered as shown by the blue staining in both forearm and digits (Fig. 4*E*). These results illustrated that neither the *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, *Hoxa-13* combination, nor the *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, *Hoxa-11* combination was required for *Hoxd* gene activity. These data strongly suggested that activation of *Hoxd* genes in digit primordia was independent of both *Hoxa-13* and *Hoxa-11*, as well as of autoregulatory mechanisms. Activation of the *Hoxd* reporter in the genital bud and genital eminence mirrored the situation in digits, supporting a previous suggestion that a common regulatory mechanism is responsible for posterior *Hoxd* gene expression in the primordia of these two structures (18).

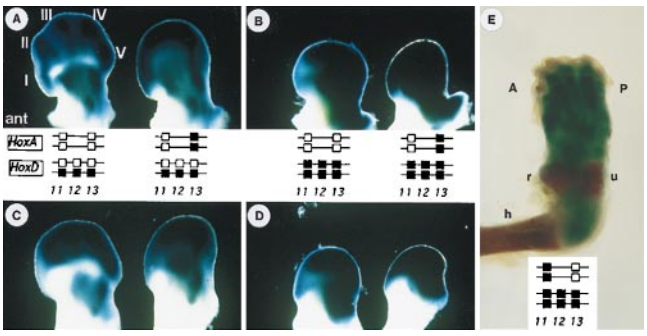

FIG. 4. Absence of crossregulatory and autoregulatory interferences as judged by a *HoxD* reporter gene in *HoxA* and *HoxD* compound mutant mice. *Hoxd-11/lacZ* reporter gene expression (4) in pairs of forelimbs (*A* and *B*) and hindlimbs (*C* and *D*) derived from mice of four different genotypes schematized in between as in Fig. 2. The presumptive digits are labeled from I (anterior to the left) to V. Although the morphology of these developing limbs vary with the genotypes, they all strongly expressed the *HoxD* reporter gene in both the distal and proximal domains. This expression indicated that neither the HOXD, nor the HOXA13 proteins are necessary for the function of the *Hoxd* transgene in limbs. (*E*) *Hoxd-11/lacZ* reporter gene expression in a forelimb of $HoxD^{Del/Del}$;$Hoxa\text{-}11^{-/-}$ mouse 5 days after birth. Expression of the *Hoxd-11/lacZ* reporter gene was detected in both the autopod (digits) and around the zeugopod (radius and ulna), indicating that HOXA11 is not required for the activation of *HoxD* genes in the developing limbs. The forearm of the limb shown under *E* is truncated because of the absence of both *Hoxd-11* and *Hoxa-11* functions (7). A, anterior; P, posterior; r, radius; u, ulna; h, humerus.

CONCLUSIONS

From these and previous results (6), it appears that, similar to vertebral specification (e.g., refs. 27 and 28), digit size and number are fixed as a quantitative function of *Hox* protein dose, rather than by a qualitative *Hox* code. However, various *Hox* complexes seem to have particular roles in digit patterning, as in these mutant stocks we were not able to generate a polydactylous mouse without affecting the *HoxD* complex, whereas oligodactylous mice could be obtained only by perturbing *HoxA*. This observation may be relevant in an evolutionary context. It has been established, that at least four *Hox* complexes were present at the time of the emergence of digits, including all the loci described in this work (29, 30). We therefore may speculate that, in the course of this important remodeling of distal limb structures, the two complexes became involved independently and at different times through the design of complex-specific regulatory mechanisms (Fig. 5). In this model, incipient limiting doses of *Hox* proteins, initially

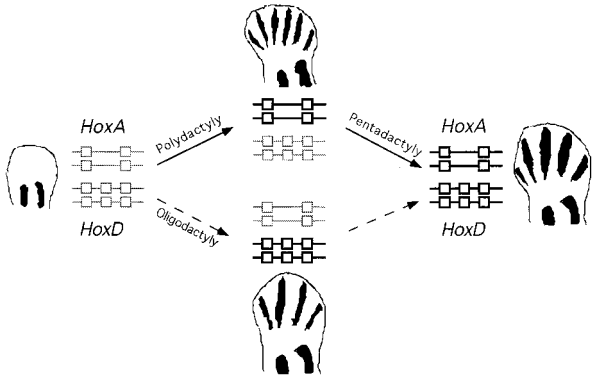

FIG. 5. Scheme showing the relationships between the function of the *HoxA* and *HoxD* complexes in distal limbs and the dactyly. *Hox* complexes are shown either in light gray, when not functional, or bold boxes when functional. In the absence of *HoxA* and *HoxD* expression, a complete adactyly is observed. This situation may reflect an ancestral step in which neither complexes had been recruited in distal limbs. Activation of the *HoxA* complex genes in distal limb, in absence of *HoxD* function (pathway on the top) coincides with the appearance of a series of truncated digit-like bony elements with a clear polydactyly. The subsequent recruitment of *Hoxd* genes in the digit domain could lengthen all the digits while reducing their number to the pentadactyl formula. Alternatively, activation of the *Hoxd* genes first (pathway in the bottom) would have generated an oligodactyl limb, with potentially long digits, and the subsequent activation of the *Hoxa* genes would have shortened digits and extended their number to five. Paleontological and developmental evidence support the first pathway (see the text).

provided by *Hoxa* genes (16, 17) could have been involved in distal appendages of the polydactylous short-digited stem tetrapod forms (14, 15), or even before, as suggested by distal *Hoxa-13* expression in developing teleost paired fins (30). The occurrence of polydactyly in the *Hoxa-11/HoxD*^{Del} compound homozygous mice, a situation where digit development almost exclusively relied on the function of *Hoxa-13*, is consistent with this view. It therefore is reasonable to propose that the emergence of a novel distal expression domain for posterior *Hoxd* genes may have contributed to abbreviation of serial iterative generation of digits and provided for an intensive and elongated growth phase of the endoskeleton necessary for long-digit formation (17), possibly through the elaboration of a shared-digit activation unit involving at least *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, and *Evx-2* (18). Whenever a threshold *Hox* dose was surpassed, digits could have become longer and the pentadactyl pattern stabilized. The dual role of the *Hox* dose in digit number and size determination was deduced from mouse mutants and may not directly apply to anamniotes, which, according to fossil evidence, reached pentadactyly independently (15). It is also likely that in different species, the output of the *HoxD* complex may have varied independently in the early limb bud, thereby influencing digit number and, subsequently, in digital condensations, contributing to the final phalanx length. In this context, important parameters could be determined by the specific times of recruitment of the *HoxA* and *HoxD* complexes in distal appendages, followed by gene-specific expression patterns, to build up locally effective *Hox* protein doses to determine cellular growth rates.

The potential successive activation of these two *Hox* complexes in the course of limb evolution is supported by both developmental and phylogenetic arguments (17, 31–33). During limb development, distal expression of *Hoxa-13* is observed before that of *Hoxd-13* and appears to be generated through a different dynamic, which does not necessarily involve the apparent anterior expansion of *Hoxd* expression domains (32, 34–36). And, *Hoxa-13* is expressed during teleost fin development in a way similar to what is seen in developing limbs (30), in contrast to *Hoxd* genes that show fundamental differences (16), suggesting that *Hoxa-13* was already functional in distal appendages at the time of the origin of teleosts whereas *Hoxd* genes became involved secondarily.

We thank S. S. Potter and S. Small for the gift of mice lacking *Hoxa-11* function and P. Dollé for his early involvement in this work. We also thank M. Kumar for her suggestions and all of the members of the laboratory for discussions and sharing reagents. This work was supported by funds from the Canton de Genève, the Swiss National Research Fund, the Human Frontier Programme, and the Claraz and Latsis foundations.

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