DEVELOPMENTAL BIOLOGY **200,** 225–233 (1998)
ARTICLE NO. DB988956

Expression of *HoxD* Genes in Developing and Regenerating Axolotl Limbs

Maureen A. Torok,*,[1] David M. Gardiner,* Neil H. Shubin,† and Susan V. Bryant*,[2]

**Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, Irvine, California 92612; and †Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

***Hox* genes play a critical role in the development of the vertebrate axis and limbs, and previous studies have implicated them in the specification of positional identity, the control of growth, and the timing of differentiation. Axolotl limbs offer an opportunity to distinguish these alternatives because the sequence of skeletal differentiation is reversed along the anterior–posterior axis relative to that of other tetrapods. We report that during early limb development, expression patterns of *HoxD* genes in axolotls resemble those in amniotes and anuran amphibians. At later stages, the anterior boundary of *Hoxd-11* expression is conserved with respect to morphological landmarks, but there is no anterior–distal expansion of the posterior domain of *Hoxd-11* expression similar to that observed in mice and chicks. Since axolotls do not form an expanded paddle-like handplate prior to digit differentiation, we suggest that anterior expansion of expression in higher vertebrates is linked to the formation of the handplate, but is clearly not necessary for digit differentiation. We also show that the 5′ *HoxD* genes are reexpressed during limb regeneration. The change in the expression pattern of *Hoxd-11* during the course of regeneration is consistent with the hypothesis that the distal tip of the regenerate is specified first, followed by intercalation of intermediate levels of the pattern. Both *Hoxd-8* and *Hoxd-10* are expressed in non-regenerating wounds, but *Hoxd-11* is specific for regeneration. It is also expressed in the posterior half of nerve-induced supernumerary outgrowths.** © 1998 Academic Press

***Key Words: Hoxd-8; Hoxd-10; Hoxd-11;* limb development; limb regeneration; handplate; digit; digit sequence.**

INTRODUCTION

The homeobox-containing transcription factors, particularly the *Hox* complex genes, have been shown to play critical regulatory functions in axial and appendicular patterning among vertebrates. The patterns of expression of genes of the *HoxA* and *HoxD* complexes in amniotes show three distinct phases (Nelson *et al.*, 1996; Shubin *et al.*, 1997). Phase I, involving expression of the 3′ members of the limb *Hox* genes, is considered to be relevant to specification of the stylopod (Fromental-Ramain *et al.*, 1996a; Nelson *et al.*, 1996; Shubin *et al.*, 1997). Phase II, where *HoxA* and *HoxD* genes are expressed with spatial and temporal colinearity along the proximal–distal (PD) (*HoxA*) and anterior–posterior (AP) (*HoxD*) axes, has been linked to specification of the zeugopod (Davis and Capecchi, 1994; Davis *et al.*, 1995; Favier, 1995; Favier *et al.*, 1996; Nelson *et al.*, 1996; Shubin *et al.*, 1997). The final phase, III, in amniotes involves expansion of expression across the handplate concomitant with an inversion of the relative anterior boundaries of the 5′ most *HoxD* genes that had been posteriorly restricted in phase II. The posterior to anterior nested expression in phase II and the posterior to anterior distal expansion in phase III are correlated with the posterior to anterior differentiation of the zeugopo-

GenBank accession numbers: Accession numbers for *Hoxd-8*, *Hoxd-10*, and *Hoxd-11* are AF031246, AF031245, and AF031481, respectively.

[1] Present address: Department of Molecular Genetics, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030

[2] To whom correspondence should be addressed at Developmental Biology Center and Department of Developmental and Cell Biology, 4205 BioSci II, University of California, Irvine, Irvine, CA 92697-2275 Fax: (949) 824 5385. E-mail: svbryant@uci.edu.

0012-1606/98 $25.00

dium, expansion of the distal region into a handplate, and posterior to anterior differentiation of the digits (Sordino *et al.,* 1995; Nelson *et al.,* 1996; Shubin *et al.,* 1997). Gene disruption and targeted misexpression analyses (Morgan *et al.,* 1992; Dollé *et al.,* 1993; Small and Potter, 1993; Davis and Capecchi, 1994; Davis *et al.,* 1995; Favier, 1995; Yokouchi *et al.,* 1995; Beckers *et al.,* 1996; Davis and Capecchi, 1996; Fromental-Ramain *et al.,* 1996a,b; Gerard *et al.,* 1996; Herault *et al.,* 1996; Mortlock *et al.,* 1996; Muragaki *et al.,* 1996; Zakany and Duboule, 1996; Goff, 1997; Zakany *et al.,* 1997), as well as patterns of expression (Dollé and Duboule, 1989; Dollé *et al.,* 1989; Nohno *et al.,* 1991; Yokouchi *et al.,* 1991; Izpisúa-Belmonte *et al.,* 1992a,b; Haack and Gruss, 1993; Gardiner *et al.,* 1995; Nelson *et al.,* 1996) of individual *HoxA* or *HoxD* genes, or of combinations of genes lend support to the view that *HoxA* and *HoxD* genes operate together in the formation of the limb segments and that different members of the complexes are critically involved in patterning each of the major limb segments.

Tetrapod limbs provide a classic example of adaptation around a conserved structural theme, such that unity of the plan of the limb skeleton underlies extensive functional diversity. Not surprisingly, key features of the development of limbs are highly conserved (see review by Shubin and Alberch, 1986). Hence, the three major segments of the limb (stylopod, zeugopod, and autopod) generally differentiate in a proximal to distal sequence. The pattern of elements across the AP axis has also been well conserved, and an anatomical relationship between the skeletal elements distal to the forearm, known as Gregory's Pyramid, has long been recognized as reflecting a very ancient feature of tetrapod appendages (Westoll, 1943). It is surprising therefore to discover that despite anatomical conservation, the sequence of differentiation across the AP axis is less conserved (Shubin and Alberch, 1986; Blanco and Alberch, 1992). Whereas in most tetrapods skeletal elements in the zeugopod and autopod appear in a posterior to anterior sequence, urodele amphibians are unique because the sequence of skeletal differentiation is from anterior to posterior in both the zeugopod and the autopod (Shubin and Alberch, 1986).

Recent studies of the expression of *HoxA* and *HoxD* genes in developing zebrafish fins have led to the hypothesis that the autopod is an evolutionary innovation in tetrapods and is formed by an overproliferation of posterior mesenchymal cells, accompanied by a posterior to anterior expansion of the expression domain of the 5′ *HoxD* genes (Sordino *et al.,* 1995; Sordino and Duboule, 1996). This hypothesis proposes a causal relationship between the anterior–distal expansion of *HoxD* gene expression, the control of cellular proliferation, and the posterior origin of digits. We are able to provide new information about the proposed relationship between *HoxD* expression and the sequence of digit differentiation by studying axolotls in which digits differentiate in the reverse sequence in comparison to other tetrapods.

Another unique feature of urodeles is that, alone among tetrapods, they are capable of limb regeneration as adults. Recent studies suggest that many of the same molecules that function during development are reexpressed during limb regeneration. Interestingly, in contrast to their expression in developing limbs, *Hoxa-9* and *Hoxa-13* are reexpressed simultaneously in the stump mesenchyme within 1 to 2 days of amputation and the spatial pattern of expression typical of development is only established at late stages of regeneration (Gardiner *et al.,* 1995). The early expression of *Hoxa-13* suggests that during regeneration the distal tip of the limb is reestablished first. In other studies, *Hoxd-11* and *Hoxd-10,* believed to function in limb patterning during development, have been shown to be reexpressed during regeneration (Brown and Brockes, 1991; Simon and Tabin, 1993; Gardiner *et al.,* 1995), but the timing of their upregulation and their spatial expression have not been reported. In this paper, we have analyzed the expression patterns of three *HoxD* genes during limb regeneration, in non-regenerating wounds, and in induced supernumerary limbs.

MATERIALS AND METHODS

***Isolation of Hoxd-8, Hoxd-10,* and *Hoxd-11* clones.** As previously described (Gardiner *et al.,* 1995), several cDNA clones of *Hoxd-8, Hoxd-10,* and *Hoxd-11* were isolated from axolotl regenerating medium bud blastema cDNA libraries screened with a degenerate oligonucleotide probe corresponding to the conserved third helix of the homeodomain KIWF(Q/K)NRR. Isolated clones, which were inserted within the *Eco*RI and *Xho*I restriction sites of pBluescript II SK (Stratagene), were sequenced using primers for the T7 and T3 promoters within the vector. Additionally, primers were designed from sequenced regions of each clone such that internal sequence information could be obtained that would overlap and yield the entire sequence of each clone.

To obtain a full-length clone of *Hoxd-11,* we performed a second cDNA library screen. A proximal blastema library was screened (Gardiner *et al.,* 1995) with a probe corresponding to the largest *Hoxd-11* cDNA clone from the original screen. Several positive plaques were purified and the inserts were excised as subclones into the *Eco*RI and *Xho*I restriction sites of the pBluescript II SK phagemid vector (Stratagene). Clones were sequenced, as above, and a single full-length clone was found to share identity to the previously described transcriptional start site of *Hoxd-11* from other organisms.

Animal procedures. Axolotls (*Ambystoma mexicanum*) were spawned at either the Indiana University Axolotl Colony or at UCI. Embryos were hatched and maintained at 20–22°C in 40% Holtfreter's solution. Animals were anesthetized in 0.1% MS222 solution for surgical procedures and for collection of staged limb buds (Harrison, 1969). For Alcian and Victoria blue staining, samples were fixed overnight with agitation in Bouin's fixative and then processed as in Wanek *et al.* (1989) and Bryant and Iten (1974), respectively. For whole-mount *in situ* analysis animals

were fixed overnight at room temperature with gentle agitation in freshly prepared MEMFA (0.1M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde). Fixed samples were washed once for 5–10 min in methanol (MeOH) and then stored in fresh MeOH at −20°C.

Preparation of labeled RNA probes. Digoxigenin-labeled RNA probes were *in vitro* transcribed from linearized subcloned plasmid templates according to the manufacturer's protocol (Boehringer-Mannheim) using either T3 (*Hoxd-8*) or T7 (*Hoxd-10* and *Hoxd-11*) RNA polymerase. For *Hoxd-8* the *Eco*RI–*Cla*I fragment containing approximately 850 bp was used. The *Xho*I–*Xho*I fragment corresponding to the 3′ untranslated region of *Hoxd-10* was removed to leave only the coding region for the transcription reaction. For *Hoxd-11* the *Pvu*II–*Hin*dIII fragment was utilized which contains the entire homeodomain and 3′ coding and noncoding regions. In all cases linearized templates were phenol: CIAA extracted after digestion, precipitated, and resuspended. Reaction mixtures were treated with DNAseI prior to precipitation with 4 M LiCl, 200 mM EDTA, glycogen, and ethanol at −20°C. The digoxigenin-labeled RNA was diluted to 1 mg ml^{-1} in hybridization solution (50% formamide, 5× SSC, 1 mg ml^{-1} yeast RNA, 100 mg ml^{-1} heparin, 1× Denhart's solution, 0.1% Tween 20, 0.1% Chaps, 5 mM EDTA) and stored at −20°C. Probes were denatured by heating to 80°C for 2 min prior to hybridization.

Whole-mount in situ hybridization. The procedure is as in Gardiner *et al.* (1995) with the following changes. Samples were rehydrated to PTw and then treated with 20 μg ml^{-1} proteinase K for 30 min on ice followed by either 15 min (embryos) or 30 min (regenerating limbs) at 37°C. The samples were then acetylated in 0.1 M triethanolamine and refixed in 4% formaldehyde in PTw. Embryos were prehybridized for 4 to 6 h, and then probes were added overnight at 50°C for *Hoxd-8* and *Hoxd-10* or 57°C for *Hoxd-11*. Regenerates were prehybridized 5 h to overnight and hybridized with digoxigenin–UTP-labeled probes for 12 h to 2 days. Samples were washed with hybridization solution for 10 min at 55–60°C and then 3 times in 2× SSC and 2 times in 0.2× SSC. Embryos and regenerates were washed with MAB and then blocked with MABB (MAB plus 0.1% BSA) for 1 h. Both embryos and regenerates were blocked with MABBS (MABB plus 10% heat-inactivated sheep serum) for 4 h to overnight at 4°C. Anti-digoxigenin conjugated to alkaline phosphatase antibody was blocked in MABBS with axolotl powder and diluted 1:1000 in MABBS before addition to samples overnight at 4°C. Samples were washed in MAB 10 times for 25 min each and then twice in AP buffer before development in BM purple (Boehringer-Mannheim) for 3 to 10 h. Postfixation was done in formalin prior to analysis. This was followed by 5 min in Bouin's for regenerating limbs which were later dehydrated and cleared in methyl salicylate for photography.

Surgical procedures. To obtain regenerates, limbs were removed at different proximal–distal levels from animals that were 50 to 60 mm stout to tail tip. Non-regenerating wounds were created by removing a cuff of skin from the zeugopod. To induce lateral outgrowths, a square of skin (2–3 mm) from the posterior–dorsal stylopod of the host limb was replaced by a similar-sized graft from the anterior–ventral side of the stylopod. A brachial nerve was transected at the elbow and deviated to the wound site. To secure the skin graft the proximal and distal edges were tucked under the wound edges.

RESULTS

***Characterization of axolotl homologues of HoxD* genes.** Hoxd-11, Hoxd-10, and *Hoxd-8* were cloned from axolotl regenerating blastema cDNA libraries with a degenerate probe for the conserved third helix of the homeodomain (Gardiner *et al.*, 1995). Axolotl *Hoxd-8* has 100, 98, and 97% amino acid identity within the homeodomain to reported sequences for chick, human, and mice, respectively, and contains the residues outside the homeodomain that are characteristic of the *Hox8* paralogs (Sharkey *et al.*, 1997). Deduced amino acid sequence analysis of axolotl *Hoxd-10* indicates that it is 100% identical to chick, mice, and human within the homeodomain and for the characteristic amino acids of the *Hox10* paralogs (Sharkey *et al.*, 1997). The library was rescreened with one of the isolated *Hoxd-11* clones and a full-length clone was identified which has a translational start site in a similar position to that reported for chick (Izpisúa-Belmonte *et al.*, 1991; Rogina *et al.*, 1992) and newt (Brown and Brockes, 1991). The deduced amino acid sequence of *Hoxd-11* is 98% identical within the homeodomain to newt, frog, mouse, human, and chick. There is also a region of high identity (83–90%) between axolotl and other species at the N-terminus and 100% identity to conserved residues of the *Hox11* family outside the homeodomain (Sharkey *et al.*, 1997). When newt and axolotl amino acid sequences are compared, they show 83% identity overall.

HoxD expression in forelimb development analyzed by whole-mount in situ hybridization. Expression of *Hoxd-8* is first detectable in the flank mesenchyme in the position of the future limb bud (Fig. 1A). As described in Izpisúa-Belmonte *et al.* (1990) for higher vertebrates, *Hoxd-8* transcripts are also detectable in the mesonephros adjacent to the prelimb mesenchymal disc (Figs. 1A and 1B). As the limb buds emerge at stage H36 (Harrison, 1969), *Hoxd-8* is detected uniformly in the limb bud mesenchyme (Fig. 1B). By H38, *Hoxd-8* expression is more intense distally (Fig. 1C). As digits form, expression is weak proximally where the humerus is condensing and stronger distally, especially at the tips of digits 1 and 2 (Fig. 1D). After formation of the first two digits, expression of *Hoxd-8* is significantly reduced (Fig. 1E).

Like *Hoxd-8*, expression of *Hoxd-10* is first detectable in the mesenchyme of the prelimb bud flank (Fig. 1F). As the limb bud emerges, *Hoxd-10* is expressed in the mesenchyme and in the adjacent mesonephros (Fig. 1G). As in other vertebrates, expression is not detected in anterior–proximal cells of the limb bud (Figs. 1G and 1H). Over time expression becomes reduced proximally (Fig. 1I) and by H40 *Hoxd-10* expression is no longer detectable in the proximal cells of the condensing humerus (Figs. 1J and 1K). At the onset of digit differentiation, expression is most intense in the condensing radius and ulna (Fig. 1J). Later, *Hoxd-10* expression is seen in association with the condensing

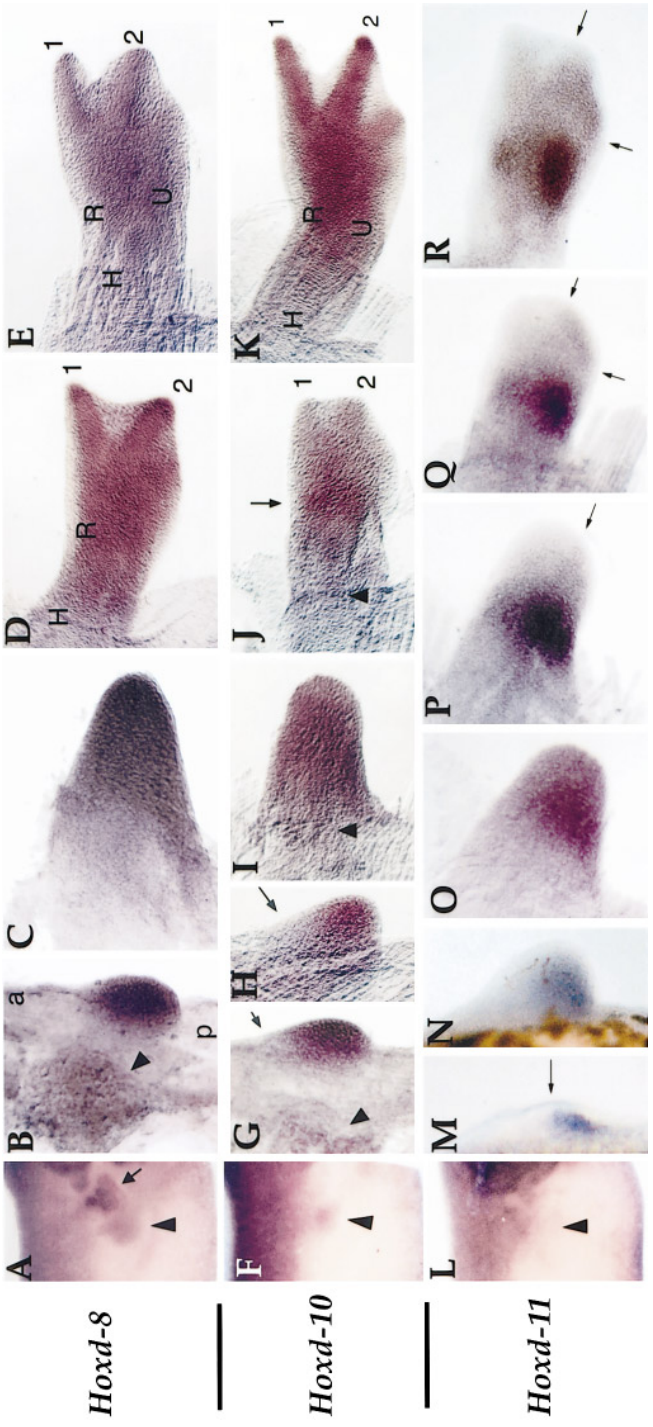
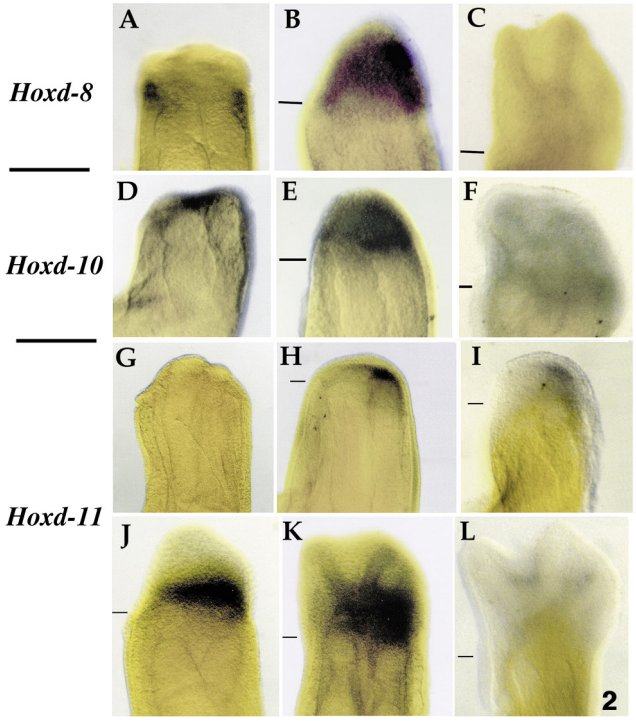


FIG. 1. Whole-mount *in situ* hybridization analysis of *Hoxd-8*, *Hoxd-10*, and *Hoxd-11* expression in developing limb buds. (A–E) *Hoxd-8*: A (H34); B (H36); C (H38); D (H42); E (H44). (F–K) *Hoxd-10:* G (H36); H (H37); I (H38); J (H41); K (H45). (L–R) *Hoxd-11:* M (H36); N (H37); O (H38); P (H39); Q (H40); R (H41). Arrowhead (A, F, L): prelimb bud region; arrow (A) and arrowhead (B, G): mesonephros; arrow (G and H): reduced expression in the proximal–anterior limb bud; arrowhead (I and J): condensing cartilage of the humerus; arrow (J): position of radius and ulna. Arrow (M, P): anterior extent of *Hoxd-11* expression. Arrows (Q, R): extent of the distal–posterior domain. A, F, L: Rostral to right and dorsal at top. All rest: distal to the right, anterior (a) at the top, posterior (p) at bottom. Digits are indicated by numbers. H, humerus; R, radius; U, ulna.



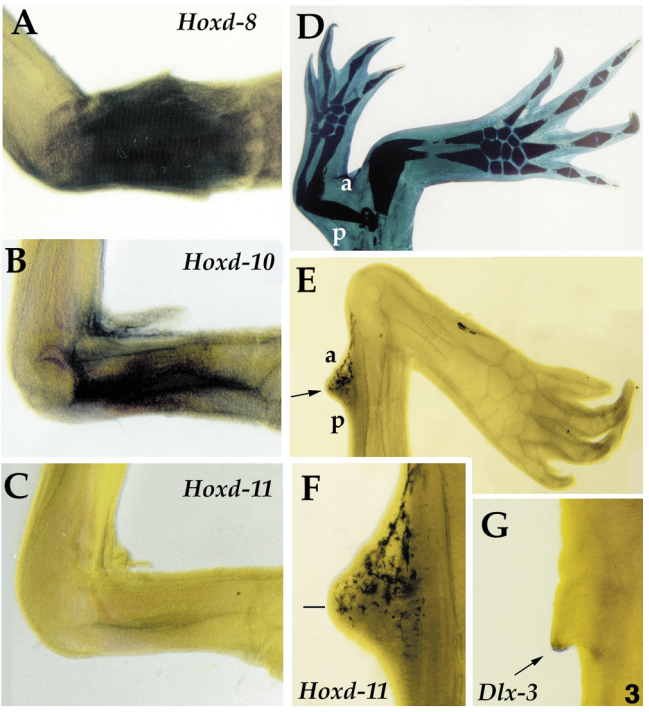


FIG. 2. Expression of *Hoxd-8*, *Hoxd-10*, and *Hoxd-11* in regenerating limbs. (A–C) *Hoxd-8:* A (24 h); B (medium bud); C (palette). (D–F) *Hoxd-10:* D (24 h); E (medium bud); C (palette). (G–L) *Hoxd-11:* G (24 h); H (early bud); I (medium bud); J (late bud); K (palette); L (early digits). All panels are forelimbs amputated distally, with anterior to the left and distal at the top. Hash marks identify the level of amputation.
FIG. 3. HoxD gene expression in superficial wounds (A–C) and induced regenerates (E–G). (A–C) 24 h: A (*Hoxd-8*); B (*Hoxd-10*); C (*Hoxd-11*). Proximal to left. (D) Skeletal preparation of induced supernumerary limb (at left); (E, F) expression of *Hoxd-11* in early induced outgrowth (arrow, E). Hash mark (F): approximate boundary between graft-derived (pigmented) and host-derived cells. (G) *Dlx-3* expression in distal epidermis of an early induced outgrowth.

cartilages of the zeugopod and autopod and at the tips of the digits (Fig. 1K).

Hoxd-11 expression is first detectable later than *Hoxd-8* and *Hoxd-10* and is not expressed in prelimb bud flank cells (Fig. 1L). Expression is detected in the early limb bud (H36) where it is confined to the posterior half of the bud, and expression remains posterior through stage H37 (Figs. 1M and 1N). As the limb bud grows, *Hoxd-11* expression becomes strongest in a band across the AP axis (Figs. 1O–1R). This proximal band is most intense on the posterior side of the limb, and expression is absent from the most anterior cells. As the proximal band intensifies a weak distal domain remains and is restricted to the posterior half of the limb bud (Fig. 1P). This posterior–distal domain is later separated from the proximal band (Fig. 1Q) leaving an area without expression in the region of the future wrist. The posterior–distal domain includes cells that will form digits 2 through 4 (Fig. 1R). Expression in the distal region is not observed anterior to digit 2 (Fig. 1R). By stage H42, the proximal band of *Hoxd-11* expression has separated into two domains associated with the forming radius and ulna (Fig. 1R). The expression of *Hoxd-11* in the proximal band is at all times stronger on the posterior half of the limb bud, the location of the forming ulna. Both the distal and proximal expression domains fade once the elements of the pattern have been established.

HoxD gene expression in regenerating limbs. Within 24 h of amputation, *Hoxd-8* (Fig. 2A) and *Hoxd-10* (Fig. 2D) transcripts are detectable in the stump mesenchyme, especially in the dermis. Weak expression of both genes can be detected in some limbs as early as 12 h after amputation (data not shown). As the stump cells migrate under the wound epidermis to form the blastema, expression of *Hoxd-10* and *Hoxd-8* becomes restricted to the blastema (Figs. 2B and 2E). When digits begin to redifferentiate, the level of expression of both genes (Figs. 2C and 2F) is greatly reduced.

In contrast, *Hoxd-11* is not expressed at 24 h after amputation (Fig. 2G), but first becomes detectable on the posterior–distal part of an early bud blastema (Fig. 1H). Hence, the reexpression of *Hoxd-11* is delayed by 3–4 days relative to that of *Hoxd-8* and *Hoxd-10*. In an early

bud blastema (Fig. 2H) *Hoxd-11* is expressed on the posterior side of the blastema, and at medium bud (Fig. 2I) expression is strongest in the posterior–distal mesenchyme and less intense in the more proximal–posterior part of the blastema. At late bud, *Hoxd-11* is no longer expressed distally, and a new expression domain forms a strong band across the AP axis adjacent to the stump. Expression is strongest in the posterior of this band and is absent from a small group of the most anterior cells (Figs. 2J and 2K). At this stage and beyond, the distal expression domain is downregulated. As digits begin to differentiate on the anterior, *Hoxd-11* transcripts remain abundant proximally as a band across the reforming zeugopod and wrist (Fig. 2K). After the first digits form, *Hoxd-11* expression decreases in intensity (Fig. 2L).

***Expression of HoxD* genes in non-regenerating wounds.** As described above, *Hoxd-8* and *Hoxd-10* transcripts are rapidly upregulated after amputation (Figs. 2A and 2D). *Hoxd-11,* however, is not detectable 24 h after amputation (Fig. 2G), but is first detectable several days after amputation when a blastema is present (Fig. 2H). To test whether expression of *Hoxd-8, Hoxd-10,* or *Hoxd-11* is unique to regeneration or is activated in response to wounding, we made wounds in the zeugopodium (forearm) by removing a cuff of skin. Such wounds heal without forming outgrowths or regenerates. Within 24 h of surgery, both *Hoxd-8* and *Hoxd-10* transcripts are expressed in the mesenchyme under the healed wound epidermis of the zeugopod (Figs. 3A and 3B, respectively), as they are in amputated limbs. The expression of *Hoxd-8* is more intense than that of *Hoxd-10* at this time. Transcripts of *Hoxd-11* were not detected at the site of lateral wounds either at 24 h (Fig. 3C) or at 6 days after wounding (data not shown) when expression in the regenerate is strong (Fig. 2H).

Expression of HoxD genes in nerve-induced supernumerary limbs. Lateral wounds can be induced to form supernumerary outgrowths (Maden and Holder, 1984) by grafting anterior–ventral skin from a donor limb onto a posterior–dorsal location of the stylopod and deviating a transected nerve to the site of the wound. For skeletal analysis, as well as analysis of *Hoxd-11* expression, pigmented donor animals and white or albino hosts were used to establish the approximate location of graft cells within the outgrowths. Analysis of limbs fixed for Victoria blue cartilage staining after 6 weeks revealed that 30% ($n = 10$) of the limbs developed outgrowths with identifiable skeletal elements. The best-developed outgrowth, a complete limb, is shown in Fig. 3D. Other supernumeraries were less complete and consisted of spikes and finger-like segmented skeletal cartilage elements, as described previously (Lheureux, 1977; Maden and Holder, 1984).

Operated limbs were fixed at early outgrowth stages for whole-mount *in situ* hybridization. *Hoxd-11* transcripts are visible in the mesenchyme on the posterior half of the outgrowth, as determined by the location of pigment cells from the anterior donor skin (Figs. 3E and 3F). As in regenerating limbs (Mullen *et al.*, 1996), *Dlx-3* transcripts are detected in the apical epidermis of the supernumerary outgrowth (Fig. 3G).

DISCUSSION

***Expression of HoxD* genes in developing axolotl limb buds.** We have examined the expression of *Hoxd-8, Hoxd-10,* and *Hoxd-11* genes in axolotl limb buds and find many similarities between the patterns of expression in axolotls and those described for other vertebrates. Similarities are most pronounced in phases I and II (Nelson *et al.,* 1996; Shubin *et al.,* 1997) and include spatial and temporal colinearity and equivalence of expression domains. The most obvious differences between axolotls and higher vertebrates become evident in phase III and concern the expression of *Hoxd-11.* First, although the zeugopodial domain of *Hoxd-11* is strong, the distal–posterior domain is much weaker than in other tetrapods, and it does not show the anterior expansion characteristic of all other vertebrates except fish (Sordino *et al.*, 1995). It is not unreasonable to consider that the failure of the posterior–distal domain to exhibit an anterior expansion is related to the reversal in the sequence of digit differentiation in urodeles relative to that of other tetrapods. Rather than form a handplate prior to differentiation, in axolotls the distal tip of the limb bud remains unexpanded as differentiation of the most anterior digit begins. Digit 1 forms from the anterior half of the limb bud, from cells that are not expressing *Hoxd-11.* The remaining digits are formed from *Hoxd-11*-expressing cells. Hence, the anterior border of the weak distal domain is in a conserved position relative to limb anatomy, such that in all tetrapods this border falls between digits 1 and 2. Based on these observations, we suggest that the anterior expansion of *Hoxd-11* and other 5′ *Hox D* genes across the distal limb bud is not indispensable for hand and foot development. Similar conclusions can be drawn from studies of mutant mice (Davis *et al.*, 1995). Rather, our studies suggest that the expansion could be related to the formation of a handplate prior to digit differentiation and may be a necessary feature of limbs in which the sequence of digit formation is from posterior to anterior.

Reexpression of HoxD genes during regeneration. Given the conserved expression of *HoxD* genes in development, it would be expected that they would also be reexpressed during regeneration, as shown for *HoxA* genes by Gardiner *et al.* (1995). We have examined reexpression of *HoxD* genes during regeneration and find that the temporal pattern of reexpression is similar to that in development, with *Hoxd-8* and *Hoxd-10* being expressed earlier in the process than *Hoxd-11.* This is in contrast to the findings with *HoxA* genes, where both 3′ and 5′ members of the

complex are expressed simultaneously (Gardiner *et al.*, 1995). *Hoxd-11* is not expressed until a blastema has formed, and the earliest expression is confined to the posterior half of the early bud blastema. At medium bud, expression is very strong in a limited region of the posterior–distal tip of the blastema and is weak in more proximal regions. It is possible to interpret the expression of *Hoxd-11* in early regenerates as a modified version of the pattern seen in early development. However, in light of previous findings that make it very likely that the order in which limb parts are specified in regeneration is not the same as in development, another interpretation is possible. The data suggest that it is the distal tip of the new pattern that is specified first, followed later by intercalation of forearm regions between the newly respecified tip and the stump at the base of the regenerate (Gardiner *et al.*, 1995). Hence, the early distal posterior expression of *Hoxd-11* would occur at a time when only the hand region is represented in the blastema. At later times when *HoxA* expression patterns indicate the emergence of the zeugopod, expression that extends across the proximal base of the regenerate develops, and the distal expression domain is downregulated. A more thorough understanding of the relationship between *Hox* expression patterns and the order in which parts are specified might be possible by combining cell lineage and gene expression data.

The rapid upregulation of *Hoxd-8* and *Hoxd-10* in regenerates suggests that these genes are induced in response to wounding of the limb and might therefore be expressed in non-regenerating wounds. We found that both of these genes are rapidly induced (within 24 h) in non-regenerating wounds, as they are in amputated limbs. However, *Hoxd-11*, which is not detected until a blastema has formed, is not expressed in non-regenerating wounds. It is possible that the early upregulation of *Hoxd-8* and *Hoxd-10*, as part of wound healing, may be a prerequisite for the establishment of the signal to form a regeneration blastema. It will be interesting to find out whether activation of any *Hox* genes occurs during wound healing in non-regenerating animals and, if not, whether loss of expression correlates with decline in regenerative ability during development.

The later time of *Hoxd-11* induction during regeneration relative to other *Hox* genes and the absence of expression in response to wounding suggest that a secondary signal is necessary to upregulate *Hoxd-11* expression. To test the relationship between *Hoxd-11* expression and limb patterning, we analyzed induced lateral supernumerary limbs for expression of the transcripts. Our data show that *Hoxd-11* transcripts are associated with cells on the posterior half of limbs, whether in supernumerary limb outgrowths, blastemas, or limb buds in normal development. Since we have shown that superficial wounding is insufficient to induce *Hoxd-11* expression, the induction of expression in lateral supernumerary limbs is correlated with pattern formation events.

Comparing the limited data available so far for axolotls with that for other tetrapods, we suggest that patterns of *Hox* gene expression are not consistent with a direct and conserved role in either the timing or the sequence of differentiation of limb elements. On the other hand, the expression domains are consistent with a role in the formation of the conserved features of limb anatomy. For example, in both the forearm and the hand, where the order of skeletal differentiation is AP-reversed relative to that of other tetrapods, *Hoxd-11* expression is nevertheless restricted to the same posterior expression domain relative to the skeletal elements themselves. Additional evidence from the expression patterns of *HoxA* genes in developing and regenerating axolotl limbs (Gardiner *et al.*, 1995) also suggests a conserved role relative to morphology, but lends no support for a conserved role in either the timing or sequence of differentiation. Hence, in development, *Hoxa-13*, expressed in the hand region, is the last gene to be activated in the *HoxA* complex and the hand is the last part of the pattern to differentiate. In contrast, *Hoxa-13* is expressed precociously during regeneration such that the hand region is established prior to that of more proximal domains. However, the distal part of the pattern still differentiates last.

Despite the forgoing, there remains an obvious relationship between pattern formation and growth control (French *et al.*, 1976; Bryant *et al.*, 1981), and it is likely that there is at some level a functional interaction between *Hox* gene expression, pattern formation, and the control of growth and differentiation (Duboule, 1994; Morgan and Tabin, 1994). One possibility is that the patterns of growth and timing of differentiation are downstream consequences of the activities of *Hox* genes in the specification of morphological identity. This would allow for variation in the response to expression of *Hox* genes whose expression with respect to morphology is conserved. Such variation could result in changes in the rate and timing of growth and differentiation and thus generate the heterochrony upon which much evolutionary change has relied (Gould, 1977; McKinney and McNamara, 1991).

We conclude that the underlying patterning mechanisms of all vertebrate limbs are homologous, despite differences in the sequence of developmental events. The AP asymmetry in structural design that is a major feature of both tetrapod limbs and sarcopterygian fins is underlain by a conserved pattern of asymmetry in gene expression, as exemplified by the expression of *Hoxd-10*, *Hoxd-11*, and *shh* (Imokawa *et al.*, 1997; Torok *et al.*, submitted for publication).

ACKNOWLEDGMENTS

We thank Bruce Blumberg for his participation in the initial isolation and identification of axolotl homeobox genes and Mandy Tran for her assistance with skeletal preparations. Research was supported by PHS Grant HD 33465 (to S.V.B. and D.M.G.); an allocation of computer resources by the University of California, Irvine; and the Indiana University Axolotl Colony (Bloomington, IN).

REFERENCES

Beckers, J., Gerard, M., and Duboule, D. (1996). Transgenic analysis of a potential *Hoxd-11* limb regulatory element present in tetrapods and fish. *Dev. Biol.* **180,** 543–553.

Blanco, M. J., and Alberch, P. (1992). Caenogenesis, developmental variability, and evolution in the carpus and tarsus of the marbled newt *Triturus marmoratus*. *Evolution* **46,** 677–687.

Brown, R., and Brockes, J. P. (1991). Identification and expression of a regeneration-specific homeobox gene in the newt limb blastema. *Development* **111,** 489–496.

Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration and symmetry. *Science* **212,** 993–1002.

Bryant and Iten (1974). The regulative ability of the limb regeneration blastema of *Notophthalmus viridescens:* Experiments *in situ*. *Wilhelm Roux's Arch.* **174,** 90–101.

Davis, A. P., and Capecchi, M. R. (1994). Axial homeosis and appendicular skeleton defects in mice with a targeted disruption of *hoxd-11*. *Development* **120,** 2187–2198.

Davis, A. P., and Capecchi, M. R. (1996). A mutational analysis of the 5′ *HoxD* genes: Dissection of genetic interactions during limb development in the mouse. *Development* **122,** 1175–1185.

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.

Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbaur, B., Chambon, P., and Duboule, D. (1993). Disruption of *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. *Cell* **75,** 431–441.

Dollé, P., and Duboule, D. (1989). Two gene members of the murine *HOX-5* complex show regional and cell-type specific expression in developing limbs and gonads. *EMBO J.* **8,** 1507–1515.

Dollé, P., Izpisúa-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.

Duboule, D. (1994). Temporal colinearity and the phylotypic progression: A basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Development Suppl.* 135–142.

Favier, B., LeMeur, M., Chambon, P., and Dolle, P. (1995). Axial skeleton homeosis and forelimb malformations in *Hoxd-11* mutant mice. *Proc .Natl. Acad. Sci. USA* **92,** 310–314.

Favier, B., Rijli, F. M., Fromental-Ramain, C., Fraulob, V., Chambon, P., and Dolle, P. (1996). Functional cooperation between the non-paralogous genes *Hoxa-10* and *Hoxd-11* in the developing forelimb and axial skeleton. *Development* **122,** 449–460.

French, V., Bryant, P. J., and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193,** 969–981.

Fromental-Ramain, C., Warot, X., Lakkaraju, S., Favier, B., Haack, H., Birling, C., Dierich, A., Dolle, P., and Chambon, P. (1996a). Specific and redundant functions of the paralogous *Hoxa-9* and *Hoxd-9* genes in forelimb and axial skeletal patterning. *Development* **122,** 461–472.

Fromental-Ramain, C., Warot, X., Messadecq, N., LeMeur, M., Dolle, P., and Chambon, P. (1996b). *Hoxa-13* and *Hoxd-13* play a crucial role in the patterning of the limb autopod. *Development* **122,** 2997–3011.

Gardiner, D. M., Blumberg, B., Komine, Y., and Bryant, S. V. (1995). Regulation of *HoxA* expression in developing and regenerating axolotl limbs. *Development* **121,** 1731–1741.

Gerard, M., Chen, J. Y., Gronemeyer, H., Chambon, P., Duboule, D., and Zakany, J. (1996). *In vivo* targeted mutagenesis of a regulatory element required for positioning the *Hoxd-11* and *Hoxd-10* expression boundaries. *Genes Dev.* **10,** 2326–2334.

Goff, D. J., and Tabin, C. J. (1997). Analysis of *Hoxd-13* and *Hoxd-11* misexpression in chick limb buds reveals that *Hox* genes affect both bone condensation and growth. *Development* **124,** 627–636.

Gould, S. J. (1977). ''Ontogeny and Phylogeny.'' Harvard Univ. Press, Cambridge.

Haack, H., and Gruss, P. (1993). The establishment of murine *Hox-1* expression domains during patterning of the limb. *Dev. Biol.* **157,** 410–422.

Harrison, R. G. (1969). ''Organization and Development of the Embryo.'' Yale Univ. Press., New Haven, CT.

Herault, Y., Hraba-Renevey, S., van der Hoeven, F., and Duboule, D. (1996). Function of the *Evx-2* gene in the morphogenesis of vertebrate limbs. *EMBO J.* **15,** 6727–6738.

Imokawa, Y., and Yoshizato, K. (1997). Expression of Sonic hedgehog gene in regenerating newt blastemas recapitulates that in developing limb buds. *Proc. Natl. Acad. Sci. USA* **94,** 9159–9164.

Izpisúa-Belmonte, J.-C., Brown, J. M., Crawley, A., Duboule, D., and Tickle, C. (1992a). *Hox-4* gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. *Development* **115,** 553–560.

Izpisúa-Belmonte, J.-C., Brown, J. M., Duboule, D., and Tickle, C. (1992b). Expression of *Hox-4* genes in the chick wing links pattern formation to the epithelial–mesenchymal interactions that mediate growth. *EMBO J.* **11,** 1451–1457.

Izpisúa-Belmonte, J.-C., Dollé, 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.

Izpisúa-Belmonte, J.-C., Tickle, C., Dollé, P., Wolpert, L., and Duboule, D. (1991). Expression of the homeobox *Hox-4* genes and the specification of position in chick wing development. *Nature* **350,** 585–589.

Lheureux, E. (1977). Importance of limb tissue associations in the development of nerve-induced supernumerary limbs in the newt *Pleurodeles waltlii* Michah. *JEEM* **38,** 151–173.

Maden, M., and Holder, N. (1984). Axial characteristics of nerve induced supernumerary limbs in the axolotl. *Roux's Arch. Dev. Biol.* **193,** 394–401.

McKinney, M. L., and McNamara, K. J. (1991). ''Heterochrony: The Evolution of Ontogeny.'' Plenum Press, New York.

Morgan, B. A., Izpisúa-Belmonte, J.-C., Duboule, D., and Tabin, C. J. (1992). Targeted misexpression of *Hox-4.6* in the avian limb bud causes apparent homeotic transformations. *Nature* **358,** 236–239.

Morgan, B. A., and Tabin, C. (1994). Hox genes and growth: Early and late roles in limb bud morphogenesis. *Development Suppl.,* 181–186.

Mortlock, D. P., Post, L. C., and Innis, J. W. (1996). The molecular basis of hypodactyly (*Hd*): A deletion in *Hoxa-13* leads to arrest of digital arch formation. *Nature Genet.* **13,** 284–289.

Mullen, L., Torok, M. A., Bryant, S. V., and Gardiner, D. M. (1996). Nerve dependency of regeneration: Role of *Dlx* and FGF signaling in amphibian limb regeneration. *Development* **122,** 3487–3497.

Muragaki, Y., Mundlos, S., Upton, J., and Olsen, B. R. (1996). Altered growth and branching patterns in synpolydactyly caused by mutations in *HOXD13*. *Science* **272,** 548–551.

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.

Nohno, T., Noji, S., Koyama, E., Ohyama, K., Myokai, F., Kuroiwa, A., Saito, T., and Taniguchi, S. (1991). Involvement of the *Chox-4* chicken homeobox genes in determination of anteroposterior axial polarity during limb development. *Cell* **64,** 1197–1205.

Rogina, B., Coelho, C. N. D., Kosher, R. A., and Upholt, W. B. (1992). The pattern of expression of the chicken homolog of *HOX1I* in the developing limb suggests a possible role in the ectodermal inhibition of chondrogenesis. *Dev. Dynam.* **193,** 92–101.

Sharkey, M., Graba, Y., and Scott, M. P. (1997). Hox genes in evolution: Protein surfaces and paralog groups. *TIG* **13,** 145–151.

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

Shubin, N. H., and Alberch, P. (1986). A morphogenetic approach to the origin and basic organization of the tetrapod limb. *Evol. Biol.* **20,** 319–387.

Simon, H.-G., and Tabin, C. J. (1993). Analysis of *Hox-4.5* and *Hox-3.6* expression during newt limb regeneration: Differential regulation of paralagous *Hox* genes suggests different roles for members of different *Hox* clusters. *Development* **117,** 1397–1407.

Small, K. M., and Potter, S. S. (1993). Homeotic transformations and limb defects in Hox A11 mutant mice. *Genes Dev.* **7,** 2318–2328.

Sordino, P., and Duboule, D. (1996). A molecular approach to the evolution of vertebrate paired appendages. *TIEE* **11,** 114–119.

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

Torok, M. A., Gardiner, D. M., Izpisua-Belmonte, J.-C., and Bryant, S. V. Analysis of *Sonic hedgehog* expression in developing and regenerating axolotl limbs. Submitted for publication.

Wanek, N., Muneoka, K., Holler-Dinsmore, G., Burton, R., and Bryant, S. V. (1989). A staging system for mouse limb development. *J. Exp. Zool.* **249,** 41–49.

Westoll, T. (1943). The origin of the primitive tetrapod limb. *Proc. R. Soc. London B* **131,** 373–393.

Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto, Y., Kameda, T., Iba, H., and Kuroiwa, A. (1995). Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev.* **9,** 2509–2522.

Yokouchi, Y., Sasaki, H., and Kuroiwa, A. (1991). Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* **353,** 443–445.

Zakany, J., and Duboule, D. (1996). Synpolydactyly in mice with a targeted deficiency in the *HoxD* complex. *Nature* **384,** 69–71.

Zakany, J., Gerard, M., Favier, B., and Duboule, D. (1997). Deletion of a HoxD enhancer induces transcriptional heterochrony leading to transposition of the sacrum. *EMBO J.* **16,** 4393–4402.

Received for publication January 28, 1998
Revised May 11, 1998
Accepted May 11, 1998