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Expression of HoxD Genes in Developing and Regenerating Axolotl Limbs

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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-
sequence of digit differentiation by studying axolotls in a hypo-
thesis proposes a causal relationship between the digits. We are able to provide new information about the control of cellular proliferation, and the posterior origin of expansion of the expression domain of the 5 mesenchymal cells, accompanied by a posterior to anterior tetrapods and is formed by an overproliferation of posterior genes in developing zebrafish fins have led to the hypoth-
ersis 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 pattern-
ing 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 previ-
ously 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 KWIIFQ/KNNR. Isolated clones, which were inserted within the EcoRI and XhoI 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 EcoRI and XhoI 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, 1989). 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 MgSO₄, 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 EcoRI–ClaI fragment containing approximately 850 bp was used. The XhoI–Xhol 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 PvuII–HindIII 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 DNAsel 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× Denhardt’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 (MAB plus 0.1% BSA) for 1 h. Both embryos and regenerates were blocked with MABBS (MAB 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 Brookes, 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

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**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.
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). 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
As described above, wrist (Fig. 2K). After the first digits form, proximally as a band across the reforming zeugopod and on the anterior, expression is downregulated. As digits begin to differentiate, the distal expression is more intense than that of 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), 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 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), 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 Hox-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 spines and finger-like segmented cartilage elements, as described previously (Lheurieux, 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 foregoing, 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).

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REFERENCES


Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration of the regenerative ability of the limb regenera-

Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration of the limb regenera-

Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration of the limb regenera-

Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration of the limb regenera-


Tak, 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.


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