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Expression of *Hoxb13* and *Hoxc10* in Developing and Regenerating Axolotl Limbs and Tails

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The expression of Hox complex genes in correct spatial and temporal order is critical to patterning of the body axis and limbs during embryonic development. In order to understand the role such genes play in appendage regeneration, we have compared the expression of two 5′ Hox complex genes: Hoxb13 and Hoxc10 during development and regeneration of the body axis and the limbs of axolotls. In contrast to higher vertebrates, Hoxb13 is expressed not only in the tip of the developing tail, but also in the distal mesenchyme of developing hind limbs, and at low levels in developing forelimbs. Hoxc10 is expressed as two transcripts during both development and regeneration. The short transcript (Hoxc10S) is expressed in the tip of the developing tail, in developing hind limbs, and at low levels in developing forelimbs. The long transcript (Hoxc10L) is expressed in a similar pattern, with the exception that no expression in developing forelimbs could be detected. Hoxb13 and both transcripts of Hoxc10 are expressed at high levels in the regenerating spinal cord during tail regeneration, and in both regenerating hind limbs and forelimbs. The up-regulation of expression of these genes during forelimb regeneration, relative to the very low levels of expression during forelimb development, suggests that they play a critical and perhaps unique role in regeneration. This is particularly true for Hoxc10L, which is not expressed during forelimb development, but is expressed during forelimb regeneration; thus making it the first truly "regeneration-specific" gene transcript identified to date.

Key Words: **amphibian; axolotl; urodele; limb; tail; regeneration; development; homeobox; Hox.**

INTRODUCTION

The means by which urodele salamanders achieve perfect limb regeneration remains one of the most fascinating puzzles in developmental biology. Recent work suggests that regeneration takes place in two phases. The first phase (preparation phase) involves unique processes that promote the transition from mature limb tissue to a population of undifferentiated, proliferating blastemal cells. The second phase involves the control of growth and pattern formation within the blastema, similar to that of limb development (Gardiner *et al.*, 1999). Since regeneration involves signals controlling these events, it is of considerable interest to identify the genes that are expressed in response to amputation, particularly those genes involved in the first phase involving the transition to a blastema. Identification of the signals that induce expression of these genes will lead to studies of how those signals are in turn regulated. The ultimate goal of such studies is to stimulate regeneration in animals that normally are unable to regenerate their limbs, such as humans.

Genes of the *Hox* complexes have been implicated in pattern formation and growth control during both limb development (Nelson *et al.*, 1996; Shubin *et al.*, 1997) and limb regeneration (Gardiner *et al.*, 1998; Brockes, 1997). The onset of *HoxA* gene expression is an early indication of activation of the genetic cascade controlling limb regeneration (Gardiner *et al.*, 1999). As regeneration progresses, overlapping patterns of expression of the *HoxA* and *HoxD* genes are established that correspond to the morphological patterns of the limb in a fashion comparable to what occurs during limb development (Gardiner *et al.*, 1998). These conserved patterns of *Hox* gene expression are referred to as *Hox* codes (Kessel, 1991), and it is the establishment of these *Hox* codes during limb development and regeneration

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that controls the specification of the limb pattern (Dollé *et al.*, 1989; Gardiner *et al.*, 1998; Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991).

For genes of the *HoxA* and *HoxD* complexes, functional studies involving gene disruption or misexpression have demonstrated a role in controlling the patterning and growth of specific limb segments, both in forelimbs and in hind limbs (Shubin *et al.*, 1997). Genes of the *HoxB* and *HoxC* complexes are expressed in distinct spatial domains along the rostral–caudal axis and are expressed in either fore limbs or hind limbs in a manner corresponding to their anterior axial boundaries (Charité *et al.*, 1994; Nelson *et al.*, 1996; Schughart *et al.*, 1991; Zeltser *et al.*, 1996). Functional studies support the hypothesis that the *HoxB* and *HoxC* complex genes are involved in the specification of forelimb identity as distinct from hind limb identity (Charité *et al.*, 1994; Papenbrock *et al.*, 2000; Stratford *et al.*, 1997).

Studies of *Hox* gene expression during limb regeneration have not involved functional analysis; however, the patterns of expression indicate that their functions are conserved between development and regeneration (Gardiner *et al.*, 1998). Genes of the *HoxD* complex are expressed during regeneration with the same temporal and spatial patterns as in development (Torok *et al.*, 1998). Although expression of *HoxA* genes during the initial, preparation phase of regeneration is different than during development, expression during the later phase of growth and pattern formation is equivalent, which is consistent with the function being conserved also (Gardiner *et al.*, 1995, 1998). *HoxC* genes are expressed in restricted domains corresponding to the primary body axis, although the correspondence between restriction to either forelimb or hind limb during development compared to regeneration is unresolved (Khan *et al.*, 1999; Savard *et al.*, 1988; Savard and Tremblay, 1995).

In this paper, we report the results from whole-mount *in situ* hybridization and RT-PCR analyses of the expression patterns of *Hoxb13* and *Hoxc10* in developing and regenerating limbs and tails of axolotls. Although we have found some differences in the spatial patterns of expression of these genes during axolotl development, their expression is generally comparable to that reported for other developing vertebrates. In contrast, expression during regeneration differs in remarkable ways compared to development, suggesting that these genes may have unique functions during regeneration.

MATERIALS AND METHODS

Animals. Experiments were performed on albino or white axolotls (*Ambystoma mexicanum*) spawned at either UCI or the Axolotl Colony, Indiana University. For isolation of RNA, blastemas were generated on animals measuring 10–15 cm, snout to tail tip. Animals measuring 4 to 5 cm were used to generate blastemas for whole-mount *in situ* hybridization. Animals were anesthetized in a 0.1% solution of MS222 (Sigma), and limbs or tails were amputated to initiate regeneration. For tails, amputations of either 1/3 (distal amps) or 2/3 (proximal amps) of the length from the tip of the tail to its base were performed. For limbs, amputations were either proximal (midhumerus or femur) or distal (midradius/ulna or tibia/fibula). Pre limb bud embryos were staged according to the normal tables of Bordzilovskaya *et al.* (1989) and are referred to as stage B. Embryos at limb bud stages were based on stages for *Ambystoma punctatum* described by Harrison (1969) and referred to as stage H.

Some animals were treated with retinol palmitate (Sigma) following amputation. Animals were immersed in 10 I.U./ml of retinol palmitate in 40% Holtfreters solution for either 5 or 7 days. Treated animals and controls were kept in the dark during treatment.

Northern hybridization. Total RNA (7 μg) from regenerating tail and limb tissues was separated by electrophoresis in 1.0% agarose–0.66 M formaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham). The filters were hybridized with [^{32}P]-labeled probe in 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 50% formamide, 20 μg/ml sonicated salmon sperm DNA at 42°C for 24–48 h, and then washed in 0.1× SSPE/0.1% SDS at 65°C. Autoradiography was performed at −70°C with intensifying screens for several hours to 7 days.

Cloning of Hoxc10 and Hoxb13. A partial clone of axolotl *Hoxc10* was isolated by RT-PCR using the following degenerate primers: (a) 5′-GGGATCCCATHAARGCNGARAAYACNACNGG; (b) 5′-GGGATCCCGTRAARTTRAARTTNGANGTNA: (c) 5′-CKNCKRTTYTKRAACCARATYYTT. First-strand cDNA was synthesized from 10 μg total RNA from hind limb blastemas using $(dT)_{12–16}$ as a primer. The cDNA was purified through a MicroSpin S-400 HR column (Pharmacia) to remove primer. The first PCR reaction was performed with the primer pair a and b; reaction product of the appropriate size (282 bp) was purified by gel electrophoresis and subjected to the second PCR with primer pair a and c. The final PCR product (211 bp) was labeled with [^{32}P] and used to screen a regenerating axolotl tail cDNA library. *Hoxb13* was isolated in a previous screen and at that time was identified as *Hoxc13* (Gardiner *et al.*, 1995).

Detection of Hoxb13 and Hoxc10 with nested RT-PCR. Messenger RNA was isolated and purified using the dynal bead isolation kit according to the manufacturer's protocol (Dynal). RT reactions were performed using superscript enzyme according to the manufacturer's protocol (Gibco). For the first round of nested RT-PCR, 10% of the RT reaction product was used, and for the second round, 1 μl of 1:20 diluted PCR reaction from the first round of PCR was used.

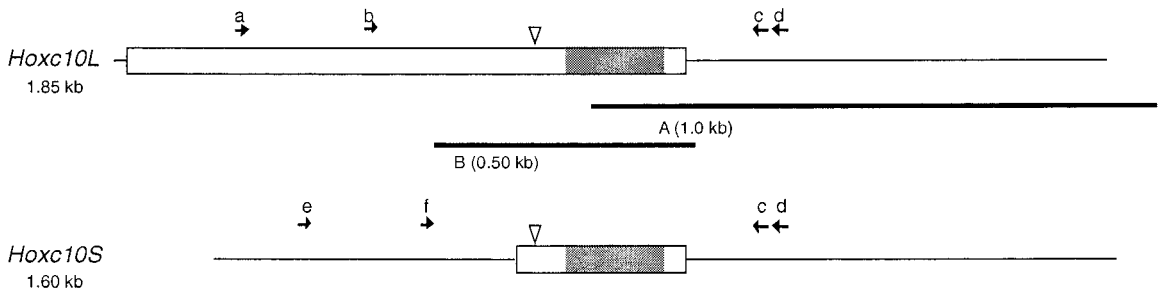
Transcripts of *Hoxb13* were amplified by the following primers (illustrated in Fig. 1D): (a) 5′-CGAGAAGTACACGGACA; (b) 5′-GTGGAGGCTGGAATAAC; (c) 5′-GGGCTTCATTTTGGAGA; (d) 5′-TATGTGGTGGTGGAGCT. For the first round, PCR was carried out for 35 cycles using the following conditions: 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, [MgCl] = 1.5 mM. For the second round, PCR was carried out for 25 cycles using the following conditions: 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, [MgCl] = 2 mM.

Transcripts of *Hoxc10* were amplified by the following primers (illustrated in Fig. 1B): (a) 5′-AGCTGGACAATTGGTGC; (b) 5′-GTTACTACAGACCCAGC; (c) 5′-CACCCAGAGAACTTGCA; (d) 5′-TTGCTCCACACGGCAT; (e) 5′-GTTGTTGGACGTGACTG; (f) 5′-CAGAGAGTTAGTGGAGG. For all the primer combinations used to detect the two *Hoxc10* transcripts, the PCR conditions were the same. Each round of PCR was carried out for 30 cycles using the following conditions: 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, [MgCl] = 1.5 mM.

A

Axolotl	C10	GRKKRCPYTKHQTLELEKEFLFNMYLTRERRLEISKSINLTDRQVKIWFQNRRMKLKKMN	
Newt	C10	.....	100%
Mouse	C10	.....T.....	98.3%
Mouse	A10	.....R.VH...S.....S.....	91.6%
Human	A10	.....R.VH.....	95.0%
Axolotl	A10	.....R.	
Mouse	D10	..E.....V.....S	95.0%
Axolotl	D10	.....V.....S	96.6%
Chick	D10	.....V.....S	96.6%

B



C

Axolotl	B13	DRKKRVPYSKGQLRELEKEYASSKFITKDRRRQIATATNLSERQITIWFQNRRVKEKKVF	
Human	B13	G....I.....R...AN.....K..K.SA..S.....L	81.7%
Mouse	B13	G....I.....R...AN.....K..K.SA..S.....L	81.7%
Human	C13	G.....T.V..K.....A.....EK..R.SAT.....V.....V	78.3%
Mouse	C13	G.....T.V..K.....A.....EK..R.SAT.....V.....V	78.3%
Axolotl	A13	G.....T.V..K...R...TN.....K..R.SAT.....V.....I	76.7%
Human	A13	G.....T.V..K...R...TN.....K..R.SAT.....V.....I	76.7%
Human	D13	G.....T.L..K...N...IN...N..K..R.SA.....V.....D..IV	73%
Mouse	D13	G.....T.L..K...N...IN...N..K..R.SA.....V.....D..IV	73%
Chick	D13	G.....T.L..K...N...IN...N..K..R.SA.....V.....D..IV	73%

D

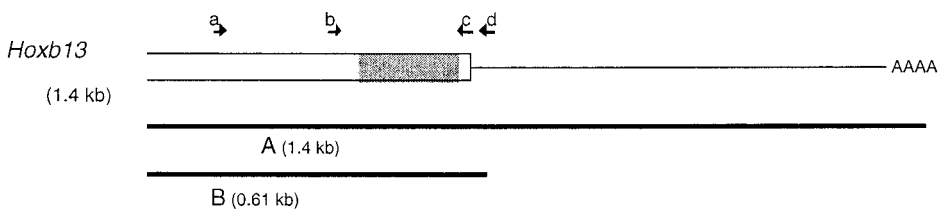


FIG. 1. Homeodomain sequence comparisons and schematic maps of *Hoxc10* and *Hoxb13*. (A) Comparison of the deduced amino acid sequence for the axolotl *Hoxc10* homeodomain with *Hox10* paralogs from various species. Degree of sequence identity within the homeodomain for each gene compared to axolotl *Hoxc10* is shown on the right. (B) Schematic maps for the two transcripts of axolotl *Hoxc10*. The boxed areas indicate the open reading frame and the shaded boxes within the ORF indicate the homeodomain regions. The open arrowheads indicate the splice site 5′ of which the nucleotide sequence for these two clones are not identical, whereas the nucleotide sequences 3′ of the splice site are identical. Probes used to detect these two transcripts in Northern hybridization (probe A) or in whole-mount *in situ* hybridization (probe B) are shown as heavy black lines. Primers used for nested RT-PCR analysis are shown as small dark, lettered arrows. (C) Comparison of the deduced amino acid sequence for the axolotl *Hoxb13* homeodomain with *Hox13* paralogs from various species. The degree of sequence identity within the homeodomain for each gene relative to axolotl *Hoxb13* is shown on the right. (D) Schematic map for the transcript of axolotl *Hoxb13*. The boxed area indicates the open reading frame and shaded box within the ORF indicates the homeodomain region. Probes used to detect this transcript in Northern hybridization (probe A) or in whole-mount *in situ* hybridization (probe B) are shown as heavy black lines. Primers used for nested RT-PCR analysis are shown as small dark arrows.

Transcripts of EF-1α were amplified by the following primers: 5′-AACATCGTGGTCATCGGCCAT and 5′-GGAGGTGCCAGTGATCATGTT. PCR was carried out for 30 cycles on 10% of the RT reaction using the following conditions: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, [MgCl] = 1.5 mM.

Whole-mount in situ hybridization. Whole-mount *in situ* hybridization was performed as described previously (Gardiner *et al.*, 1995) with the following modifications. Conditions for proteinase K treatment were adjusted for each tissue: embryos, 10 μg/ml at room temperature for 10–15 min; limb buds, 10 μg/ml at room temperature for 25 min; limb blastemas, 30 μg/ml at 37°C for 30 min; tail blastemas, 10 μg/ml at room temperature for 10 min. Probes for both *Hoxb13* and *Hoxc10* were hybridized at 65°C. Following hybridization, tissues were washed at 70°C in 2× SSC three times for 20 min each, then in 0.2× SSC twice for 30 min each. Staining of all specimens was done with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in alkaline phosphatase buffer, with the exception of limb regenerates which were stained with BCIP and 4-nitro blue tetrazolium chloride (NBT) as in the original protocol. After staining, specimens were postfixed and stored in neutral-buffered formalin, transferred into Bouin's fixative to counterstain for 45 min, dehydrated in methanol, and finally cleared in methyl salicylate for photography.

Most tissues were collected from animals lacking pigmentation; however, some of the tail regenerates were collected from pigmented animals. In those cases, the fixed tissues were bleached in 6% hydrogen peroxide in maleic acid buffer for 24 h to remove enough pigment to allow visualization of the reaction product. Some specimens were sectioned after whole-mount *in situ* hybridization. For sectioning, samples were rehydrated in phosphate buffered saline, frozen in OCT compound, and cryosectioned at a thickness of 40 μm.

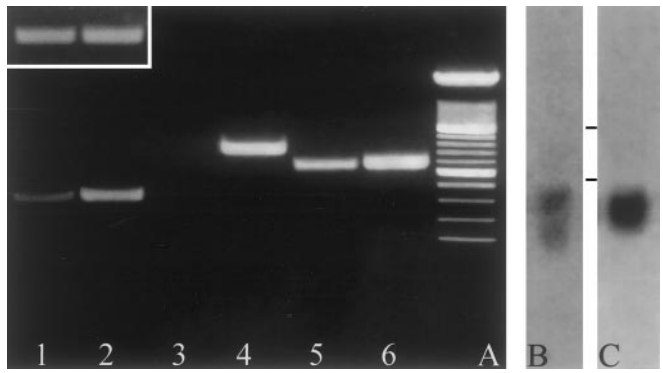


FIG. 2. RT-PCR and Northern hybridization analysis of *Hoxb13* and *Hoxc10* expression in developing and regenerating axolotl limbs. (A) *Hoxb13* expression is detected as a faint band in developing forelimbs (lane 1) and at higher levels in regenerating forelimbs (lane 2); *Hoxc10L* expression is not detected in developing forelimbs (lane 3) but is detected at high levels in regenerating forelimbs (lane 4); *Hoxc10S* expression is detected in developing forelimbs (lane 5) and at higher levels in regenerating forelimbs (lane 6). Lane 7 is 100 base pair ladder, and the inset (upper left corner) illustrates the expression level of EF-1 transcripts for the RT reactions used for developing (left) and regenerating (right) forelimbs. (B) Northern hybridization of *Hoxc10*. (C) Northern hybridization of *Hoxb13*. For B and C, the location of the 18s and 28s rRNA bands are indicated by the hash marks between the two.

RESULTS

Cloning and characterization of axolotl Hoxc10 and Hoxb13. We used RT-PCR to isolate a 211 base pair fragment of axolotl cDNA with a predicted amino acid sequence that was identical to the corresponding region of mouse and newt homologues for *Hoxc10*. We used this fragment to screen an axolotl tail blastema cDNA library to obtain full-length clones. The longest cDNA clone isolated with an in-frame translation start site is 1.85 kb in length and contains an ORF of 343 amino acids (Accession # AF298185). A second, shorter clone, also with a translation start site, has a length of 1.6 kb and an ORF of 100 amino acids (Fig. 1B; Accession # AF298186). Both clones have identical nucleotide sequences 3′ to a splice site that is also present in *Hoxc10* in newt and mouse (Peterson *et al.*, 1992; Simon and Tabin, 1993; Fig. 1B, open arrowheads). The homeobox is contained in this region of sequence identity which extends 15 amino acids 3′ to the homeodomain to an in-frame stop codon. Both clones have an identical 3′ UTR of about 800 bp. The conceptual amino acid translation of the homeodomain is 100% identical to that reported for the newt homolog and 98% identical to the mouse homolog (Fig. 1A). Northern hybridization analysis of axolotl *Hoxc10* with a probe that would detect both clones (probe A in Fig. 1B) detected the presence of two transcripts of approximately 1.95 and 1.6 kb in length (Fig. 2B).

Two clones isolated from an earlier screen for homeobox-containing genes (Gardiner *et al.*, 1995) have identical nucleotide sequences with a deduced amino acid sequence of the homeodomain that has a high degree of identity to members of paralogous group 13 of vertebrate *Hox* genes (Fig. 1C; Accession # AF298184). Neither clone is complete at the 5′ end. Axolotl *Hoxa13* has already been identified (Gardiner *et al.*, 1995), and of the remaining members of the paralogous group, the unknown axolotl paralog has a higher degree of identity to mouse *Hoxb13* (82%) and human *Hoxb13* (82%) than to human (42.0%), mouse (42.0%), or chicken *HoxD13* (39.4%). When the axolotl gene was first cloned, *Hoxb13* had not been reported in any species, and at that time it was tentatively identified as the axolotl homolog of *Hoxc13* with 78% amino acid identity within the homeodomain (Gardiner *et al.*, 1995). Most recently, it has been determined that the renamed axolotl *Hoxb13* maps between the thyroid hormone receptor alpha gene (*Thra*) and distalless-3 genes (*Dlx-3*; Randal Voss, personal communication). In humans, *Thra*, *HoxB*-complex genes and *Dlx3* are syntenic, and *Thra* and *HoxB*-complex genes are syntenic in the mouse (Nakamura *et al.*, 1996; also see the human-to-mouse homology map available at NCBI: www.ncbi.nlm.nih.gov/Omim/Homology/human17.html). Northern hybridization analysis of axolotl *Hoxb13* detected the presence of one transcript of approximately 1.8 kb in length (Fig 2C).

In situ hybridization analysis of Hoxb13 expression in developing and regenerating tails. A probe that includes the homeodomain and 412 bp 5′ to that was used for whole-mount *in situ* hybridization to regenerating and developing axolotl tails and limbs (probe B in Fig. 1D).

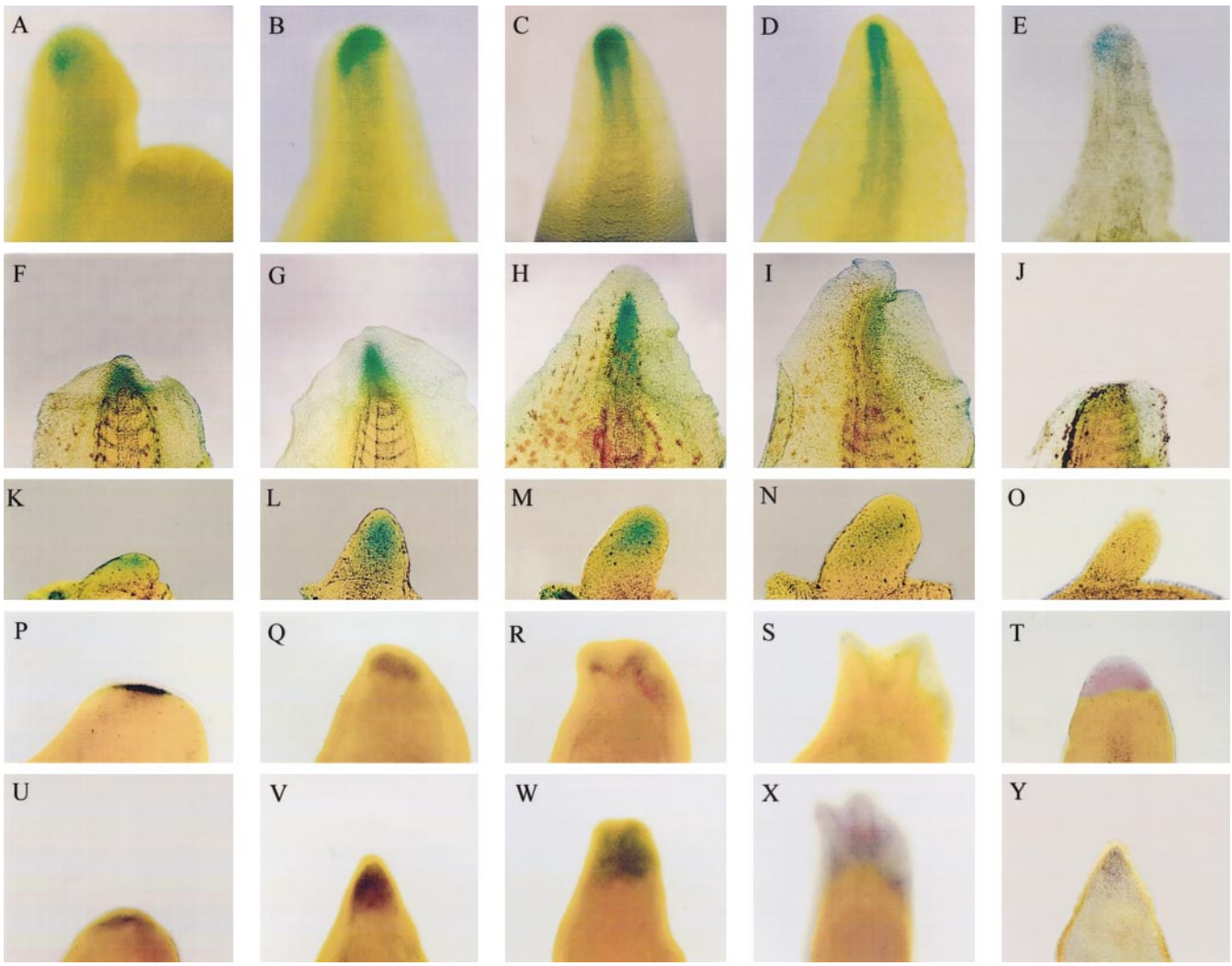


FIG. 3. Whole-mount *in situ* hybridization analysis of *Hoxb13*. (A–E) Expression in tails at embryonic stages B34 (A); B35 (B); B38 (C); and B41 (D); (E) section of whole-mount *in situ* hybridization of a stage B35 tail. Dorsal is on the left in each figure. (F–J) Expression in regenerating tails from proximal level amputations. (F) 4 days postamputation; (G) 7 d; (I) 10 d; (J) 11 d; (K) expression in an amputated tail treated with retinol palmitate for 7 days post amputation. Dorsal is on the left in each figure. (K–N) Expression in developing hind limbs at stages H36 (K); H38 (L); H39 (M); H40 (N); developing forelimb at stage H39 (O). Anterior is on the left in each figure. (P–S) Expression in regenerating forelimbs from distal level amputations at stages of early bud (P); medium bud (Q), palette (R), and early digits (S). (T) Expression in regenerating hind limb at early bud stage. (U–Y) Expression in regenerating forelimbs from proximal level amputation at stages of dedifferentiation (U); medium bud (V); palette (W), and early digits (X). (Y) Section of whole-mount *in situ* hybridization of proximal medium bud forelimb regenerate. Anterior is on the left in each figure.

During tail development, expression of *Hoxb13* is confined to the unsegmented cap of mesenchyme and the terminal region of the neural tube at the tip of the tail bud (Figs. 3A–3E). Expression is first detected at stage B34 in the dorsal mesenchyme at the tip of the tail (Fig. 3A) and continues through stages B35, B38, and B41 (Figs. 3B, 3C, and 3D). In tails that have been sectioned after whole-mount *in situ* hybridization, it is evident that expression is confined to the terminal mesenchyme and the tip of the neural tube (Fig. 3E). At stage B35 and later, expression is detected in the neural tube, dorsal mesenchyme, and ventral mesenchyme, but not in the notochord. As a consequence, expression appears to extend rostrally as two stripes with a nonexpressing region in between (Figs. 3C and 3D). Expression appears to be most intense around stage B38 (Fig. 3C) and is not detected after stage B41 (data not shown).

Hoxb13 is not detected in mature tails, or in the differentiated tissues adjacent to regenerating tail blastemas; however, it is expressed in the mesenchyme of tail blast-

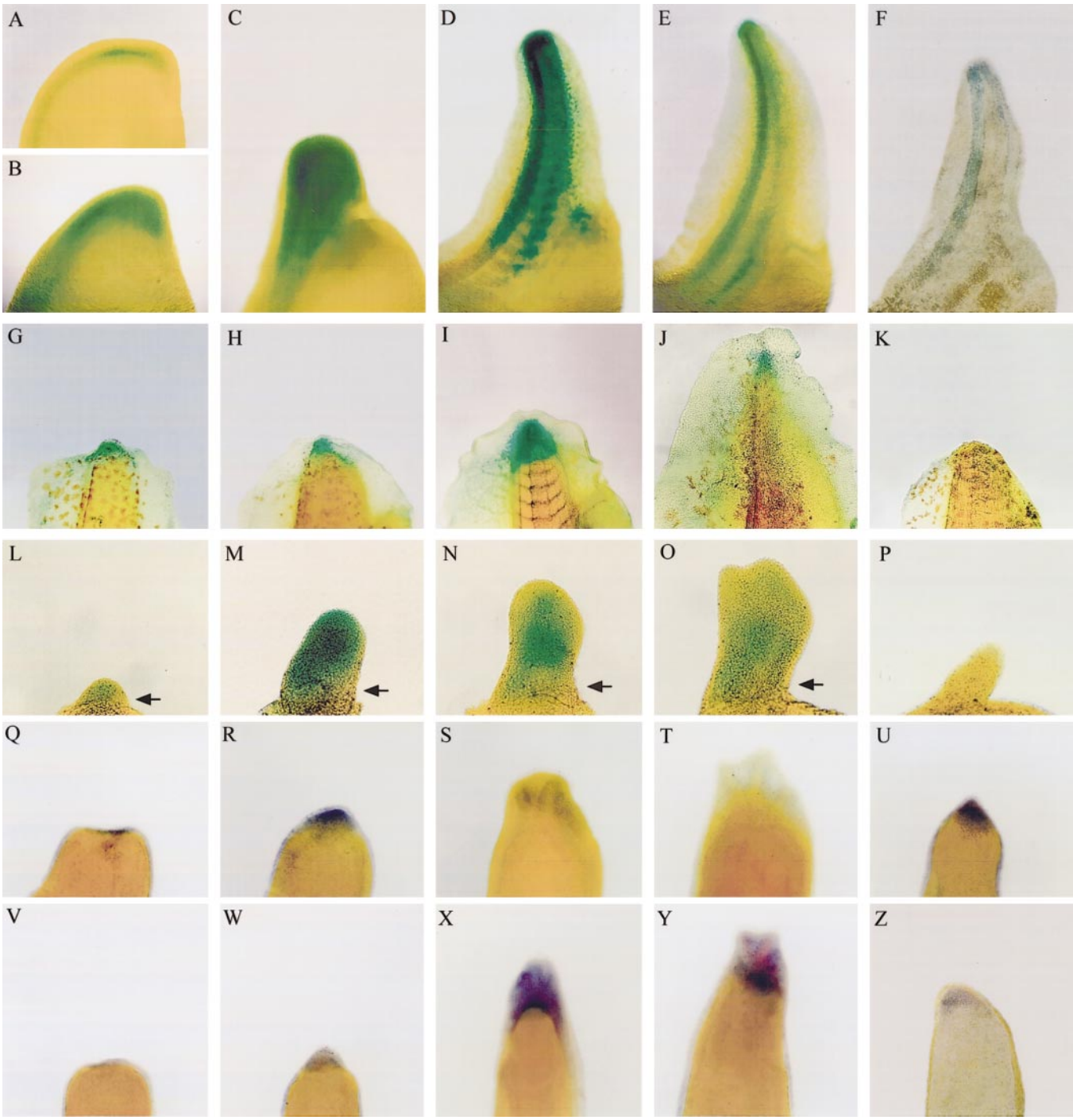


FIG. 4. Whole-mount *in situ* hybridization analysis of *Hoxc10*. (A–F) Expression in tails at embryonic stages B28 (A); B29 (B); B32 (C); B35 (D); B38 (E). (F) Sectioned whole-mount *in situ* hybridization of a stage B35 tail. Dorsal is on the left in each figure. (G–K) Expression in regenerating tails from proximal level amputations at (G) 3 days post amputation; (H) 4 d; (I) 5 d; (J) 12 d. (K) Expression in an amputated tail treated with retinol palmitate for 7 days post amputation. Dorsal is on the left in each figure. (L–O) Expression in developing hind limbs at stages H36 (L); H39 (M); H40 (N); H42 (O). (P) Expression in a developing forelimb bud at stage H39. Anterior is on the left in each figure. (Q–T) Expression in regenerating forelimbs from distal level amputation at stages of dedifferentiation (Q); early bud (R); palette (S); early digits (T). (U) Expression in regenerating hind limb at medium bud. (V–Y) Expression in regenerating forelimbs from proximal level of amputation at stages of dedifferentiation (V); early bud (W); palette (X); early digits (Y). (Z) Sectioned whole-mount *in situ* hybridization of a proximal medium bud forelimb regenerate. Anterior is on the left in each figure.

emas (Figs. 3F–3I). Expression is first detected 3 days after amputation, at a stage corresponding to late stage II of newt tail regeneration (Iten and Bryant, 1976b), or a stage roughly equivalent to medium bud or late bud blastema for limb regenerates. The onset of expression was the same whether tails were amputated at proximal levels or distal levels (data not shown). Expression appears to be maximal by 5 to 7 days post amputation, at which time it is detected throughout the tail blastema in both the mesenchymal tissues and the terminal vesicle of the regenerating spinal cord. As the tail tissues begin to differentiate at the base of the blastema (Stage III according to Iten and Bryant, 1976b; Fig. 3H), the level of expression begins to decrease, and thus *Hoxb13* expression is higher in the more distal, less-differentiated tissues. Expression is progressively down-regulated as differentiation progresses at later stages of regeneration (Fig. 3I) and is not detected in either proximal or distal blastemas by day 11–12 postamputation.

Treatment of regenerating tails with retinoids inhibits tail regeneration in some amphibians (Niazi, 1979; Pietsch, 1987) and induces the formation of legs in others (Maden, 1993; Mohanty-Hejmadi *et al.*, 1992), depending on the dose and timing of exposure and stage of development of the animal. Treatment of regenerating axolotl tails with retinol palmitate in this study inhibited tail regeneration and also inhibited the expression of *Hoxb13* (Fig. 3J).

In situ hybridization analysis of Hoxb13 expression in developing and regenerating limbs. *Hoxb13* expression was detected in the distal region of developing hind limb buds (Figs. 3K–3N), but not in developing forelimbs (Fig. 3O). Expression was detected in the distal mesenchyme between stages H36 and H40 (Figs. 3K–3N) and was down-regulated at later stages, coincident with the onset of differentiation. Consistent with the results from whole-mount *in situ* hybridization, RT-PCR analysis detected the expression of *Hoxb13* in developing hind limb buds (data not shown). In addition, a very low level of expression was detected in developing forelimb buds (Fig. 2A, lane 1). Presumably this level of expression is below the detection limit for whole-mount *in situ* hybridization.

Hoxb13 expression was detected in the distal region of regenerating forelimbs and hind limbs (Figs. 3P–3Y). Expression appears to be restricted to mesenchymal cells in whole-mount preparations, an observation that is confirmed in sections of whole-mount stained blastemas (Fig. 3Y). The patterns of *Hoxb13* expression during forelimb regeneration are illustrated in Fig. 3 and are comparable to the expression patterns observed during hind limb regeneration. Expression was first detectable 5 to 6 days post amputation, when blastema cells first begin to accumulate at the amputation plane (Figs. 3P and 3U) and remains detectable until differentiation is almost complete (Figs. 3S and 3X). The highest levels of expression were detected at the medium and late bud stages of regeneration from proximal (mid stylopod) level amputations (Figs. 3V and 3W). Overall, in blastemas from a proximal level amputation, expression appeared to be at a higher level and to persist for a longer period of time as compared to distal level amputations (distal zeugopod) of the contralateral limb of the same animal (compare Figs. 3Q, 3R, and 3S with Figs. 3V, 3W, and 3X).

Because expression of *Hoxb13* was not detected by whole-mount *in situ* hybridization in developing forelimb buds, we did not anticipate that we would detect expression in regenerating forelimbs. We used RT-PCR to verify the differences in the level of expression during forelimb regeneration as compared to forelimb development (Fig. 2A). Whereas *Hoxb13* expression is only detectable at very low levels in developing forelimb buds (Fig. 2A, lane 1), it is strongly up-regulated in forelimb blastemas (Fig. 2A, lane 2).

In situ hybridization analysis of Hoxc10 expression in developing and regenerating tails. A 463-bp probe from the 3′ half of the coding region containing the homeodomain and 283 bp 5′ to it was used for whole-mount *in situ* hybridization. This probe would be expected to detect both the long and short transcripts of *Hoxc10* (probe B, Fig. 1B).

During the embryonic stages of tail development, *Hoxc10* is expressed in the posterior trunk as well as the tail (Figs. 4C–4F). Expression was detected as early as stage B28 (Fig. 4A) and increased as the tail bud developed through stages B29, B32, and B35 (Figs. 4B–4D), reaching peak expression at stage B35, with declining levels of expression at stage B38, and no detectable expression by stage B42 (not shown). At the stages of peak expression, the anterior expression boundary was at somite 16 and covered the last 4 or 5 trunk somites and the entire tail (Fig. 4D). The trunk domain corresponds to the location of the hindlimb field (Peterson *et al.*, 1992). A longitudinal section through a stage B36 embryo showed that *Hoxc10* is expressed in the neural tube and in the surrounding mesenchyme, but not in the notochord or fin fold (Fig. 4F).

Hoxc10 expression is not detectable in mature tails, but is present at high levels in regenerating tail blastemas (Figs. 4G–4J). Expression is first detected at a stage equivalent to late stage II tail blastemas (Iten and Bryant, 1976b), while dedifferentiation is occurring (Fig. 4G). Expression is most intense by 5–7 days post amputation (Fig. 4I), at a stage corresponding to late stage III when differentiation is beginning in the proximal region of the blastema. In sections (not shown), it is evident that *Hoxc10* is expressed in the ependymal tube and the surrounding mesenchyme. Expression decreases at later stages of regeneration (Fig. 4J) and is no longer detectable by day 11–12 postamputation, when regeneration was complete. Expression of *Hoxc10* was not detected in tails that were exposed to retinol palmitate, which also inhibits tail regeneration (Fig. 4K).

In situ hybridization analysis of Hoxc10 expression in developing and regenerating limbs. *Hoxc10* expression was detected with whole-mount *in situ* hybridization in developing hind limbs (Figs. 4L–4O), but not in developing forelimbs (Fig. 4P). In hind limbs, expression was first detected at early stages (H36), where it was seen at low levels throughout the mesenchyme, except for a region of proximal–posterior cells within which expression was not detected (arrow, Fig. 4L). At later stages, the level of

expression was more intense, but was not detected in the proximal–posterior region of the limb bud (Figs. 4M–4O). At later stages when chondrogenesis begins (H40 and H42), *Hoxc10* expression begins to decrease, particularly in distal regions associated with differentiation of the autopod (Fig. 4O). RT-PCR analysis confirmed that both transcripts of *Hoxc10* are expressed in developing hind limbs (data not shown). Expression of the long transcript (*Hoxc10L*) was not detected in developing forelimb buds (Fig. 2A, lane 3), which is consistent with the whole-mount *in situ* results. In contrast, expression of the short transcript (*Hoxc10S*) is detectable in developing forelimbs (Fig. 2A, lane5). Presumably the level of expression of this transcript is below the detection limit for our whole-mount *in situ* hybridization probe, possibly because only about half of the probe would detect this transcript (Fig. 1B).

Hoxc10 expression was not detected by whole-mount *in situ* hybridization in either mature forelimbs or mature hind limbs; however, a high level of expression was detected in both regenerating forelimbs (Figs. 4Q–4T, 4V–4Y) and hind limbs (Fig. 4U). In sections of whole-mount-stained, regenerating forelimbs it is evident that *Hoxc10* is expressed in the mesenchyme but not the epidermis (Fig. 4Z). The patterns of *Hoxc10* expression during forelimb regeneration are illustrated in Fig. 4 and are comparable to the expression patterns observed during hind limb regeneration. Expression was first detectable 5 to 6 days post amputation, when blastema cells first begin to accumulate at the amputation plane (Figs. 4Q and 4V), and remains detectable until differentiation is almost complete (Figs. 4T and 4Y). The highest levels of expression were detected at the medium and late bud stages of regeneration from amputations at both proximal (Figs. 4X and 4Y) and distal level amputations (Fig. 4R). Overall, expression in blastemas from proximal level amputations appeared to be at a higher level and persisted for a longer period of time as compared to distal level amputations (distal zeugopod) of the contralateral limb of the same animal (compare Figs. 4X and 4Y with Figs. 4S and 4T).

Because expression of *Hoxc10* was not detected by whole-mount *in situ* hybridization in developing forelimb buds, we did not anticipate that we would detect expression in regenerating forelimbs. In response to this finding, we used RT-PCR to analyze expression of each of the two *Hoxc10* transcripts. As reported above, expression of the *Hoxc10S* transcript can be detected in developing forelimbs (Fig. 2A, lane 5) and is up-regulated in regenerating forelimbs (Fig. 2A, lane 6). Consistent with the results from whole-mount *in situ* hybridization, *Hoxc10L* is not expressed in developing forelimbs (Fig. 2A, lane 3), but is expressed at high levels in regenerating forelimbs (Fig. 2A, lane 4).

DISCUSSION

Expression patterns of Hoxb13 and Hoxc10. In this study, we have examined the expression of two 5′ *Hox* genes, *Hoxb13* and *Hoxc10*, during axolotl axis and limb development, and during limb and tail regeneration. As in other vertebrates (Peterson *et al.*, 1992, 1994; Zeltser *et al.*, 1996), axial expression in embryos is restricted to posterior regions of the body, encompassing the hind limb field and the entire tail in the case of *Hoxc10*, and the tail tip in the case of *Hoxb13*. Expression of *Hoxb13* has been reported in a similar location in the mouse tail bud (Zeltser *et al.*, 1996). Similarly, *Hoxc10* has an anterior border in the mouse trunk that includes the hind limb region (Peterson *et al.*, 1992); as we describe here for the axolotl, and in both animals, expression is very strong in the neural tube.

Since the embryonic expression of these genes suggests a role in tail formation, we investigated their expression during tail regeneration and found that both are re-expressed with a similar pattern, being detected in the ependymal vesicle (regenerating neural tube) and surrounding mesenchyme of the regenerating tail blastema. The peak of expression intensity for each gene was slightly different, with *Hoxc10* showing peak expression at an earlier stage than *Hoxb13*. The treatment of regenerating tails with retinoids can, in some species of amphibians, lead to the transformation of tails into legs (Maden, 1993; Mohanty-Hejmadi *et al.*, 1992). In other species, including axolotls, retinoid treatment inhibits tail regeneration, but does not promote leg development (Niazi, 1979; Pietsch, 1987). Neither *Hoxb13* nor *Hoxc10* were expressed in regenerates exposed to retinoids, indicating that these genes are not merely activated by wound healing or trauma, but are part of a regeneration pathway that is sensitive to retinoid exposure.

The axial expression domain of *Hoxb13* in embryos does not extend anteriorly into the trunk region; nevertheless, *Hoxb13* is expressed in the distal mesenchyme of developing hind limbs, but not forelimbs. In this characteristic, expression of *Hoxb13* resembles that of 5′ *HoxA* and *HoxD* genes, but is dissimilar to that of *HoxC* genes. In the latter, expression of a particular *HoxC* gene is only seen in those limbs that arise within the axial expression domain of a particular *HoxC* gene. In the case of *Hoxb13* expression in the developing hind limb, expression is not continuous with and is more anterior than the axial expression domain. In the only other study to look for *Hoxb13* expression in limb development, it was not detected in either developing forelimbs or hind limbs (Zeltser *et al.*, 1996). This result is not inconsistent with the findings in axolotls, because at the relatively advanced stages of mouse limb development examined, equivalent limb bud stages in axolotls were also negative for *Hoxb13* expression.

The axial expression domain of *Hoxc10* includes the hind limb region, and as expected, *Hoxc10* expression was detected in developing hind limb, but not in forelimb buds. Previous studies have described *Hoxc10* expression in developing hind limbs of chick (Nelson *et al.*, 1996) and mouse (Peterson *et al.*, 1992, 1994), and regenerating hind limbs of the newt (Simon and Tabin, 1993). Unlike most *Hox* genes expressed in limbs, which show conserved

expression patterns, the patterns of expression of *Hoxc10* in chick and mice are not very similar to one another. In mice, expression is found in almost the entire bud except the distal tip. In chicks, it is only expressed in a proximal–anterior domain. Yet a third pattern is present in axolotls, where expression is found throughout the early bud except for the proximal posterior. Later, expression is downregulated distally in the developing hand plate. This expression pattern is similar to that observed for *HoxC6* in chick, *Xenopus*, and mouse forelimbs (Nelson *et al.*, 1996; Oliver *et al.*, 1988).

Hoxc10 and Hoxb13 have a unique expression pattern in regenerating forelimbs. Studies of limb regeneration have emphasized the similarities between limb regeneration and development (Muneoka and Bryant, 1982, 1984) as well as the differences (Scadding and Maden, 1986a,b). As more has been learned about the molecular events of regeneration, it has become clear that there are two phases of regeneration; an early one that differs from development and a later one that is similar (Gardiner *et al.*, 1999). Little is known about tail regeneration, despite the fact that it involves complete regeneration of the spinal cord and could potentially provide clues for future treatments of spinal cord injury in humans.

The first, early phase is unique to regeneration and involves the steps required to develop a blastema from the differentiated cells of the stump. It is during this phase that regeneration is expected to differ most from development, since there is no comparable stage of differentiated tissues in developing embryos. One characteristic of the early phase is that patterns of gene expression are not predictable based on their expression during limb development. The expression of *Hoxb13* and *Hoxc10* in regenerating forelimbs are particularly dramatic examples of how gene expression is regulated differently during the early stages of regeneration. The function of these genes during early regeneration is unknown, but does not appear to be involved in wound healing, which is completed prior to the onset of their expression. The expression in forelimbs reveals a function in regeneration that coincides temporally with the initial accumulation of dedifferentiated blastema cells. The role of these genes in controlling the transition from differentiated connective tissue cells to undifferentiated blastema cells will be testable with the development of vectors for somatic cell transgenesis in urodeles (Gardiner *et al.*, 1999).

In the second phase, regeneration and development appear to employ common mechanisms of growth control and pattern formation to form a limb (Gardiner *et al.*, 1999) and thus are likely to be more similar than different. Grafting studies of the interaction between cells of developing limb buds and regenerating limb blastemas have led to the conclusion that the patterning mechanisms of limb development and regeneration are identical during this later phase (Muneoka and Bryant, 1982). The functional significance, if any, of the persistent expression of *Hoxb13* and *Hoxc10* during the later stages of forelimb regeneration will remain unclear until functional studies can be performed. However, this persistent expression in regenerating forelimbs is the first evidence suggesting that there are developmental events occurring during the later stages of regeneration that do not occur during limb development. As discussed below, these genes may have a role in the intercalary interactions between cells of the blastema. A reinvestigation of the interactions between cells from developing and regenerating tissues utilizing molecular markers would be valuable in better understanding the process of intercalary growth and pattern formation during both limb development and limb regeneration (Bryant *et al.*, 1981).

The expression of the *Hoxc10L* transcript in regenerating forelimbs is particularly significant in the context of understanding the molecular control of regeneration. Axolotl *Hoxc10L* corresponds to the transcript that has been described in other vertebrate organisms (Peterson *et al.*, 1992; Simon and Tabin, 1993); there are no reports in the literature of a second transcript corresponding to axolotl *Hoxc10S*. Expression of *Hoxc10L* is not detectable in developing forelimbs, even by highly sensitive RT-PCR. In contrast, *Hoxc10L* is expressed in regenerating forelimbs. Although the spatial and temporal patterns of expression of genes expressed during regeneration do not always correspond exactly to their respective patterns of expression during development; each gene studied to date has always been expressed during limb development as well as during regeneration. *Hoxc10L* is unique in being the first gene identified to date whose expression is truly regeneration-specific. The expression of *Hoxc10L* in regenerating forelimbs indicates the presence of at least one unique factor that controls gene expression during regeneration. Studies to isolate this and other regeneration-specific factors are in progress.

The expression of a transcript that is homologous to axolotl *Hoxc10S* has not been reported previously, and its function in axolotl limb development and regeneration is unclear. Since it encodes a protein that would be a truncated version of *Hoxc10L*, it has the potential to modulate the function of *Hoxc10L*, possibly through a mechanism of dominant-negative regulation. A previous report based on Northern hybridization analysis did not detect *Hoxc10* expression in regenerating forelimbs in a species of newt (Simon and Tabin, 1993). Although there may be a difference in gene expression between these two species of salamanders, it seems more likely that *Hoxc10* expression was below the level of detection by the methods employed in that study.

The expression of *Hoxc10* and *Hoxb13* during limb regeneration persists for a longer time in blastemas from proximal-level amputations as compared to distal-level amputations. It is possible that the persistent expression in proximal regenerates is related to the intercalation of the pattern between the distal tip of the limb and the proximal stump that appears to be a property of regeneration (Gardiner *et al.*, 1999). *Hoxa13*, which functions in the control of hand/foot development (Fromental-Ramain *et al.*, 1996; Mortlock *et al.*, 1996; Muragaki *et al.*, 1996), is

expressed very early in limb regeneration, suggesting that the distal part of the pattern is specified first (Gardiner *et al.*, 1995). Specification of the distal tip of the limb pattern is proposed to stimulate blastema cell proliferation that results in the intercalation of missing limb segments between the distal tip and the proximal stump. The process of intercalation during regeneration has been well established by tissue-grafting experimentation in the limb (Iten and Bryant, 1975; Pescitelli and Stocum, 1980) and tail (Iten and Bryant, 1976a). The prolonged expression of *Hoxb13* and *Hoxc10* could be functionally related to the additional growth needed to intercalate more of the missing pattern from a proximal-level than a distal-level amputation. These genes may therefore play a role in this unique aspect of regeneration, namely the intercalary growth and patterning that are a result of interactions between the newly specified cells of the distal tip and the dedifferentiating cells of the proximal stump.

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