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Authors
Gardiner, David M
Blumberg, Bruce
Komine, Yuriko
et al.

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Regulation of *HoxA* expression in developing and regenerating axolotl limbs

David M. Gardiner[1,*], Bruce Blumberg[2], Yuriko Komine[1] and Susan V. Bryant[1]

[1]Developmental Biology Center and Department of Developmental and Cell Biology, University of California Irvine, Irvine, CA 92717, USA
[2]The Salk Institute for Biological Studies, La Jolla, CA 92037, USA

*Author for correspondence

SUMMARY

Homeobox genes are important in the regulation of outgrowth and pattern formation during limb development. It is likely that homeobox genes play an equally important role during limb regeneration. We have isolated and identified 17 different homeobox-containing genes expressed by cells of regenerating axolotl limbs. Of these, nearly half of the clones represent genes belonging to the *HoxA* complex, which are thought to be involved in pattern formation along the proximal-distal limb axis. In this paper we report on the expression patterns of two 5′ members of this complex, *HoxA13* and *HoxA9*. These genes are expressed in cells of developing limb buds and regenerating blastemas. The pattern of expression in developing axolotl limb buds is comparable to that in mouse and chick limb buds; the expression domain of *HoxA13* is more distally restricted than that of *HoxA9*. As in developing mouse and chick limbs, *HoxA13* likely functions in the specification of distal limb structures, and *HoxA9* in the specification of more proximal structures. In contrast, during regeneration, *HoxA13* and *HoxA9* do not follow the rule of spatial colinearity observed in developing limbs. Instead, both genes are initially expressed in the same population of stump cells, giving them a distal *H*ox code regardless of the level of amputation. In addition, both are reexpressed within 24 hours after amputation, suggesting that reexpression may be synchronous rather than temporally colinear. Treatment with retinoic acid alters this *Hox* code to that of a more proximal region by the rapid and differential downregulation of *HoxA13*, at the same time that expression of *HoxA9* is unaffected. *HoxA* reexpression occurs prior to blastema formation, 24-48 hours after amputation, and is an early molecular marker for dedifferentiation.

Key words: homeobox, *HoxA13*, *HoxA9*, urodele, axolotl, limb development, pattern formation, limb regeneration, retinoic acid, dedifferentiation

INTRODUCTION

Several lines of evidence support the hypothesis that genes of the *Hox* complexes are involved in pattern formation of vertebrate embryos. Among this evidence is the colinearity between the position along the rostrocaudal axis at which a particular *Hox* gene is expressed and the physical location of that *Hox* gene within the complex (Duboule and Dollé, 1989; Graham et al., 1989). Hence, 3′ genes are expressed rostrally and early, whereas more 5′ genes are expressed more caudally and later. The domains of *Hox* gene expression overlap, leading to characteristic combinations of *Hox* gene products in particular segments of the body (Kessel and Gruss, 1991). Support for the idea that combinations of *Hox* genes specify positional identity comes from experiments in transgenic mice. When *Hox* gene expression is forced in ectopic locations or eliminated by gene knockout, mice often develop predictable transformations in segment identity (see Krumlauf, 1993, 1994).

Hox genes are also involved in pattern formation in developing limbs. Expression patterns suggest an involvement of 5′ *HoxA* genes in specification of the proximal-distal axis (Haack and Gruss, 1993; Yokouchi et al., 1991) and 5′ *HoxD* genes in patterning of the anterior-posterior limb axis (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; Yokouchi et al., 1991). Overexpression of *HoxD11* in chick leg buds results in digit one sometimes developing with the morphology of digit 2, presumably because of a change in the *Hox* code (Morgan et al., 1992). Misexpression of *HoxB8* in mice, under the control of an *RARβ* promoter, leads to major limb duplications (Charité et al., 1994). Although loss of function experiments have yielded less dramatic phenotypes, possibly due to redundancy in function between paralogs, the location of the defects have been consistent with their domains of expression. Hence, in limbs lacking *HoxA11* (Small and Potter, 1993) or *HoxD11* (Davis and Capecchi, 1994) function, defects are observed in the forearm and wrist (and for *HoxA11*, equivalent regions of the hind limb), which corresponds to the region between the proximal border of normal expression and the proximal boundary of the next most 5′ gene. In *HoxD13* knockout mice, defects are observed in the hand/foot regions where this gene is normally expressed (Dollé et al., 1993).

Retinoids, retinoic acid (RA) in particular, are powerful experimental tools for studying the mechanisms of pattern

formation. When developing embryos are treated with RA, dramatic alterations in pattern of the main body axis, as well as the limbs, are induced (see Bryant and Gardiner, 1992). In some cases it has been shown that RA treatment leads to changes in *Hox* gene expression, further evidence of the relationship between *Hox* genes and pattern formation. For example, when mouse embryos are treated with RA, changes in segment identity are accompanied by changes in the combination of *Hox* genes expressed (Kessel and Gruss, 1991). Equivalent evidence from developing limbs has been reported; when anterior chick limb bud cells are exposed to RA released from an implanted bead, ectopic expression of posterior-distal *Hox* genes accompany changes in the limb pattern (Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; Hayamizu and Bryant, 1994).

We are interested in the remarkable ability of urodele amphibians to regenerate perfect replacement limbs after amputation. Among the many questions regarding how regeneration occurs, the role of *Hox* genes is of particular importance considering the role of these genes in pattern formation during embryogenesis and limb development. In addition, because retinoids have such dramatic effects on pattern formation during regeneration, it is important to understand the relationship between pattern alterations induced by retinoids and the corresponding changes in *Hox* gene expression. In this paper we report on the isolation and identification of a large number of homeobox genes expressed in regeneration blastemas. We also have characterized the expression of two 5′ members of the *HoxA* complex, *HoxA13* and *HoxA9*. We report that both genes are reexpressed early in the regeneration cascade and are among the earliest molecular markers for dedifferentiation of limb stump cells. Their initial expression does not conform to the usual pattern of temporal and spatial colinearity. It is not until later blastemal stages that they show differential expression patterns along the proximal-distal axis. We also report that the two genes differ in their response to retinoid treatment.

MATERIALS AND METHODS

Preparation of blastemas

Experiments were performed on 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 4-5 cm were used to generate blastemas for whole-mount in situ hybridization. To initiate regeneration, we anesthetized animals in a 0.1% solution of MS222 (Sigma) and amputated limbs either at proximal (mid-humerus), middle (mid-radius/ulna) or distal (carpals) levels. The amputation surface was trimmed flat.

RNA isolation

Total RNA was extracted using urea/LiCl (Auffray and Rougeon, 1980). Tissues were homogenized in 3 M LiCl/6 M urea/0.1% SDS and stored at 4°C for 2-5 days to precipitate RNA. Insoluble materials were collected by centrifugation at 10,000 ***g*** and rinsed twice with 3 M LiCl to remove contaminants. The pellet was solubilized in 10 mM Tris-HCl (pH 8.0)/0.5% SDS, centrifuged at 10,000 ***g*** and the RNA-containing supernatant was phenol-chloroform extracted and precipitated with 1/10 volume 5 N NaCl and 2 volumes ethanol.

Construction of cDNA libraries

Blastema cDNA libraries were constructed in λZAPII (Stratagene) as detailed in Blumberg et al. (1991), with the following variations. Total RNA was isolated from the mesenchymal component of medium bud forelimb blastemas after the wound epidermis was removed manually. Each library was constructed from 2 μg of poly(A)$^+$ RNA selected by standard oligo(dT) chromatography. The proximal-blastema library contained 8×10^7 independent clones and the distal-blastema library contained 2×10^8 independent clones.

Isolation and sequencing of axolotl *Hox* genes

We screened 5×10^5 unamplified clones from the distal-blastema library and 10^6 unamplified clones from the proximal-blastema library with a mixture of 1024 oligonucleotides [C(G,T)(A,C,G,T)C(G,T)-(A,G)TT(C,T)T(G,T)(A,G)AACCA(A,G)-AT(C,T)TT] that are complementary to all possible variations of the DNA sequence encoding the conserved amino acid sequence KIWF(Q/K)NRR. We used the tetramethylammonium chloride method for degenerate oligonucleotide screening (Burglin et al., 1989) , and plaque-purified the clones. The inserts were excised as subclones in the Bluescript SK$^-$ phagemid vector according to the manufacturer's protocol (Stratagene). We sequenced plasmid DNA with the use of a degenerate oligonucleotide [C(G,T)(A,C,G,T)C(G,T)(A,G)TT(C,T)T(G,T)-(A,G)AACCA] corresponding to the amino acid sequence WF(Q/K)NRR as a primer. In addition we used a T3 primer to obtain sequence information from the 5′ end of the directionally cloned inserts. Sequence data were analyzed using the GCG Sequence Analysis Software Package; similarity searches were performed using the Blast Programs, NCBI.

Northern hybridization analysis

For RNA-blot analysis, total RNA (5 μg, 10 μg or 20 μg) from limbs at various stages of regeneration were separated by electrophoresis in 1% agarose-0.66 M formaldehyde gels, and transferred to nylon membranes (Hybond-N, Amersham) according to the manufacturer's protocol. The amount of RNA loaded was quantitated spectrophotometrically. To check that equal amounts of RNA were loaded, we visualized the 18S and 28S ribosomal RNA bands by either UV shadowing or by ethidium bromide staining of the gels. Blots were hybridized with ^{32}P-labeled probes in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS and 20 μg/ml sonicated salmon sperm DNA at 42°C for 48 hours. Filters were washed at 65°C in 0.1 × SSPE, 0.1% SDS and autoradiographed at −70°C with intensifying screens. Autoradiographs were digitized (HP Scanjet IICX) and quantitated using Scan Analysis software (Biosoft).

Preparation of digoxigenin-labeled RNA probes

Digoxigenin-labeled RNA probes for whole-mount in situ hybridization were synthesized according to the manufacturer's protocol (Boehringer). The 5′ *HoxA13* probes were transcribed from the 430-bp *Eco*RI fragment that contains 320 bp of coding region (including the entire homeobox) and 110 bp of 3′ untranslated region (UTR) (Fig. 1A). The 3′ probe was transcribed from the 1100-bp *Eco*RI-*Xho*I 3′ UTR fragment (Fig. 1A). The *HoxA9* probe was transcribed from the 620-bp *Eco*RI-*Bgl*II fragment from clone Hp14 that contains 140 bp of the homeobox and 580 bp of coding region 5′ to the homeobox (Fig. 1C). The probes were not hydrolyzed.

Whole-mount in situ hybridization

Our procedure for whole-mount in situ hybridization to axolotl blastemas and limb buds is based largely on the protocol of Harland (1991) with the modifications reported in Lamb et al. (1993).

Tissues were fixed overnight at room temperature in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) with gentle agitation and then stored at −20°C in 100% methanol. Tissues were rehydrated to PTw (PBS with 0.1% Tween-20) and treated with proteinase K (20 μg/ml) at 37°C for 30 minutes for blastemas or 15 minutes for limb buds. Tissues were acetylated with 0.5% acetic anhydride in 0.1M triethanolamine (pH 7.8) for 10

minutes, rinsed with PTw, refixed in 4% formaldehyde in PTw for 20 minutes and rinsed in PTw.

Tissues were prehybridized overnight in hybridization solution (50% formamide, 5× SSC, 1 mg/ml yeast RNA, 100 μg/ml heparin, 1× Denhardt's solution, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA) at 50°C. The digoxigenin-labeled probe (10 μg/ml in hybridization solution) was heated to 95-98°C for 30 minutes, diluted to 1 μg/ml in hybridization solution and added to samples for hybridization at 50°C for 48-72 hours. Following hybridization the tissues were washed once with hybridization solution without probe, three times with 2× SSC (20 minutes each) and twice with 0.2× SSC (30 minutes each); all these washes were done at 60°C.

Tissues were rinsed twice with maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, pH 7.5), and then rinsed with MAB-B (MAB with 2 mg/ml BSA). Tissues were treated with antibody-blocking solution (20% heat-inactivated sheep serum in MAB-B) overnight at 4°C. At the same time, the alkaline-phosphatase (AP) conjugated anti-digoxigenin antibody (Boehringer) was diluted 1:400 in blocking solution and preabsorbed overnight at 4°C with 10 mg/ml axolotl powder (acetone extracted limb and blastema tissues). The next day, the antibody solution was diluted to 1:1000 with blocking solution and added to the samples. After an overnight incubation at 4°C, the tissues were rinsed 10 times with MAB, and twice with AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Tissues were then incubated in AP substrate: 340 μg/ml NBT and 175 μg/ml BCIP (Boehringer) in AP buffer with 1 mM levamisol (Sigma). After the chromogenic reaction was complete (1 to 3 hours), tissues were postfixed overnight in Bouin's fixative, rinsed in 70% ethanol, and stored in methanol at –20°C. Tissues were cleared in methyl salicylate for photography.

Retinoid treatment

For systemic retinoid treatment, 4-6 cm animals that had regenerated limbs to an early to medium bud stage (7 days post amputation) were treated by adding retinol palmitate (all trans type VII, SIGMA) at a concentration of 50 IU/ml to the aquarium water for 1 to 5 days. Blastema tissues were treated locally with RA by implanting 200 μm beads (AG1-X2, Bio-Rad) that had been soaked in a 1 mg/ml solution of RA (all *trans*, Sigma) in DMSO for 20 minutes and rinsed twice, briefly, in PBS. Beads were implanted into the anterior distal-most region of blastemas, adjacent to the wound epithelium by making a tunnel with a 27G hypodermic needle and inserting a single bead using watchmaker forceps. Animals that had retained the bead were fixed for analysis by whole-mount in situ hybridization.

RESULTS

Isolation and identification of axolotl homeobox-containing cDNAs

We used a degenerate oligonucleotide complementary to the conserved third helix of the homeobox [KIWF(Q/K)NRR] to screen axolotl blastema cDNA libraries for homeobox-containing clones. From that screen we isolated and determined the nucleotide sequence of 105 clones, of which 80 had an open reading frame with a high degree of deduced amino acid identity to known vertebrate homeobox genes. These 80 homeobox containing clones represent the axolotl homologs of 17 different homeobox genes (Table 1). Twenty five clones contained nucleotide sequences complementary to the probe, but did not contain a long open reading frame and were not very similar to any sequences in the data bases.

The two most abundant axolotl homeobox genes isolated were *HoxA9* and *HoxD10*, which together accounted for one third of all the homeobox-containing clones. The *HoxA* complex was the most complete of the four vertebrate *Hox* complexes both in terms of total number of clones (49% of the total) and the number of members (7 of 11 total, and 7 of the 8 most 5′ members of the complex). Only one clone (*en/Msx*) did not have a very high degree of amino acid identity with any single homeobox gene. It encodes an homeobox-containing protein that is equally similar to both engrailed proteins and Msx proteins. We are investigating the possibility that this

Table 1. Identification of homeobox-containing genes isolated from axolotl blastema cDNA libraries

Deduced homeobox AA sequence	Probable homolog	No. of clones		
		Prox	Dist	Total
PKRSRTAYTRQQVLELEKEFHFNRYLTRRRRIEIAHTLCLSERQVKIWFQNRRMKWKKDH	*HoxA4*	1	1	2
GKRARTAYTRYQTLELEKEFHFNRYLTRR**********IKIWFQNRRMKWKKDN	*HoxA5*	3	–	3
RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLSERQIKIWFQNRRMKWKKEH	*HoxA7*	4	–	4
TRKKRCPYTKHQTLELEKEFLFNMYLTRDRRYEVARLLNLTERQVKIWFQNRRMKMKKIN	*HoxA9*	12	4	16
GRKKRCPYTKHQTLELEKEFLFNMYLTRERRLEISRS**********	*HoxA10*	3	4	7
TRKKRCPYAKYQIRELEREFFFSIYINKEKRLQLSRMLNLTDRQVKIWFQNRRMKEKKIN	*HoxA11*	1	4	5
GRKKRVPYTKVQLKELEREYATNKFITKDKRRRISATTNLSERQVTIWFQNRRVKEKKVI	*HoxA13*	–	2	2
SKRARTAYTSAQLVELEKEFHFNRYLCRPRRVEMANLLNLTERQIDIWFQNRRMKYKKDQ	*HoxB3*	5	2	7
GRRGRQTYTRYQTLELEKEFHYNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN	*HoxB6*	1	2	3
DRKKRVPYSKGQLRELEKEYASSKFITKDRRRQIATATNLSERQITIWFQNRRVKEKKVF	*HoxC13*	–	2	2
RRRGRQTYSRFQTLELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQNRRMKWKKEN	*HoxD8*	3	–	3
GRKKRCPYTKHQTLELEKEFLFNMYLTRERRLEISKSVNLTDRQVKIWFQNRRMKLKKMS	*HoxD10*	8	3	11
SRKKRCPYTKYQIRELEREFFFNVYINKEKRLQLSRMLNLSDRQVKIWFQNRRMREKKLN	*HoxD11*	3	2	5
NRKPRTPFTTSQLLALERKFRQKQYLSIAERAEFSNSLALTETQVKIWFQNRRAKAKRLQ	*Msx2*	4	3	7
IRKPRTIYSSYQLAALQRRFQKAQYLALPERAELAAQLGLTQTQVKIWFQNRRSKFKKLY	*Dlx3*	–	1	1
KRKRSWSRAVFSNLQRKGLEKRFEIQKYVTKPDRKQLA**********	*Hlx*	1	–	1
RARPRTKFSTEQLQELERSFQEQRYIGVAEKRRLARELNLSELRIKTWFQNRRMKFNGSE	*en/Msx*	1	–	1

****Regions of incomplete sequence information.

novel homeobox gene may be specific to regenerating blastemas.

Identification of the axolotl homologs of *HoxA13* and *HoxA9*

Two clones had identical nucleotide sequences between an internal *Eco*RI site and the vector *Eco*RI site at the 5′ end of the inserts (Fig. 1A,B), and were identified as the axolotl homolog of *HoxA13* (Table 2). Comparison of the deduced amino acid sequence within the homeodomain with sequence data from other vertebrate species (Table 2) indicates that axolotl *HoxA13* is completely identical to both mouse and human sequences (see Gehring et al., 1994). Sequence data have not been reported for regions outside the homeobox for the mouse homolog. Data for the human homolog include only the homeodomain and five amino acids 3′ to the homeodomain. The axolotl and human genes are identical in this short 3′ region also (data not shown). There is an in-frame stop codon six amino acids 3′ to the homeodomain and a 3′ UTR of approximately 1200 nucleotides.

Sixteen clones had identical nucleotide sequences within the homeobox and in regions of overlap 5′ to the homeobox (Fig. 1C,D) and were identified as the axolotl homolog of *HoxA9*. Comparison of the deduced amino acid sequence within the homeodomain with sequence data from other vertebrate species (Table 2) indicates that axolotl *HoxA9* is completely identical to mouse, human, chick, guinea pig and frog sequences (see Gehring et al., 1994). This conservation of sequence extends beyond the homeobox; 20 of the next 21 more 5′ amino acids and 10 of the 11 more 3′ amino acids are identical to mouse and guinea pig sequences (data not shown). There is an in-frame stop codon 11 amino acids 3′ to the homeodomain and a 3′ UTR of approximately 900 nucleotides.

Northern hybridization analysis of *HoxA13* expression

Axolotl *HoxA13* expression is detected as a single transcript of approximately 2.3 kb on northern blots of total RNA from regenerating blastemas (Fig. 2A-D). Probes from both the 5′ region of the transcript, which includes the homeobox, and the 3′ region (Fig. 1A) detect the same size transcript and reveal the same expression pattern. The blots illustrated in Fig. 2A-D have been probed with the 3′ probe.

HoxA13 is expressed in developing limb buds (Fig. 2B), is not detected in mature limbs (Fig. 2A), and is reexpressed (or dramatically upregulated) during regeneration of forelimbs, hind limbs, and tails (Fig. 2B). Low levels of *HoxA13* transcripts are first detected within a few days post-amputation, coincident with the period of dedifferentiation (Fig. 2C). Higher levels of transcription are detected several days after amputation (early bud stages) when blastemal cells are first present as an observable accumulation of undifferentiated

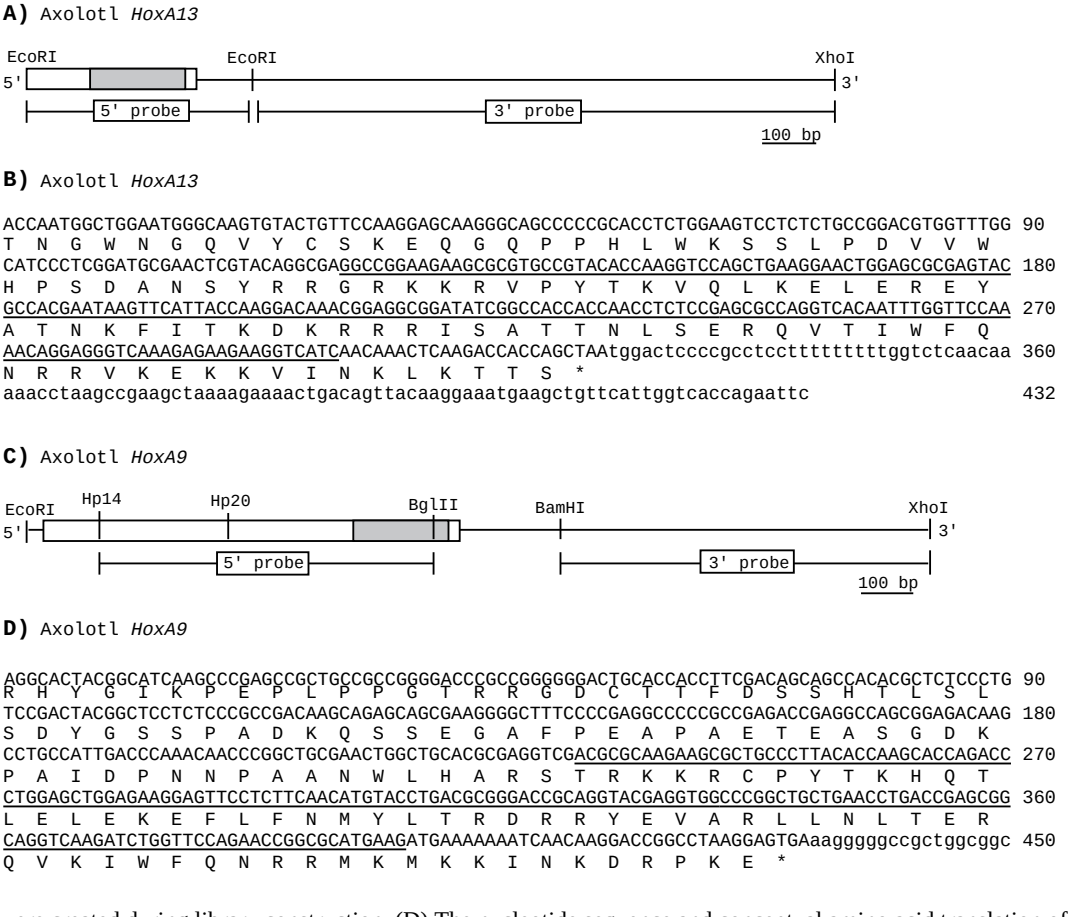

Fig. 1. Schematic map and sequence of the axolotl *HoxA13* and *HoxA9* cDNAs. (A) Schematic map indicating the coding region (box) and homeodomain (hatched box) of *HoxA13*. Two fragments obtained by digestion with *Eco*RI and *Xho*I were used as probes. The 5′ *Eco*RI and 3′ *Xho*I sites were created during library construction and are the sites at which the insert was cloned into Bluescript SK$^-$. (B) The nucleotide sequence and conceptual amino acid translation of the axolotl *HoxA13* cDNA from the 5′ *Eco*RI site to the internal *Eco*RI site located in the 3′UTR. The homeodomain is underlined. (C) Schematic map of *HoxA9* indicating the position of the two fragments used as probes. The 5′ probe contains nucleotides from the coding region (box) and extends from the 5′ end of clone Hp14 to the *Bgl*II site within the homeobox (hatched box). The *Eco*RI and *Xho*I sites were created during library construction. (D) The nucleotide sequence and conceptual amino acid translation of the axolotl *HoxA9* cDNA from the 5′ end of clone Hp20 to the end of the coding region. The homeodomain is underlined. Genbank accession numbers: *HoxA9*, U20941; *HoxA13*, U20942.

Table 2. Comparison of deduced amino acid sequence for the homeodomain of axolotl *HoxA9* and *HoxA13* to *Drosophila* Abd-B and to homologs from other species

Animal	Gene	Amino acid sequence
Drosophila	Abd-B	VRKKRKPYSKFQTLELEKEFLFNAYVSKQKRWELARNLQLTERQVKIWFQNRRMKNKKNS
Axolotl	*HoxA9*	...T----C--T-H-----------M-LTRDR-Y-V--L-N---------------M--IN
Human	*HoxA9*	T----C--T-H-----------M-LTRDR-Y-V--L-N---------------M--IN
Mouse	*HoxA9*	T----C--T-H-----------M-LTRDR-Y-V--L-N---------------M--IN
Chick	*HoxA9*	T----C--T-H-----------M-LTRDR-Y-V--L-N---------------M--IN
Guinea pig	*HoxA9*	T----C--T-H-----------M-LTRDR-Y-V--L-N---------------M--IN
Xenopus b.	*HoxA9*	T----C--T-H-----------M-LTRDR-Y-V--L-N---------------M--IN
Axolotl	*HoxA13*	G----V--T-V-LK---R-YAT-KFIT-D--RRISATTN-S----T-------V-E--VI
Human	*HoxA13*	G----V--T-V-LK---R-YAT-KFIT-D--RRISATTN-S----T-------V-E--VI
Mouse	*HoxA13*	G----V--T-V-LK---R-YAT-KFIT-D--RRISATTN-S----T-------V-E--VI

Dashes (---) represent amino acids that are identical to the *Drosophila* Abd-B sequence at that position.

cells. Expression remains high over the next several days as the blastema increases in size, then decreases as differentiation begins (early digit stage, Fig. 2C), and eventually is undetectable when the limb is fully regenerated (as in Fig. 2A).

HoxA13 is not uniformly expressed in blastemas. Expression is restricted to the mesenchymal tissues; transcripts are not detected in RNA samples from epidermal tissues separated from the underlying mesenchyme (Fig. 2A). Expression is differentially regulated along the proximal-distal axis; expression is about 30% greater in middle level blastemas as compared to proximal blastemas (Fig. 2B). The reason for this difference in level of expression is apparent from analysis of the whole-mount in situ hybridization results reported below.

Expression of *HoxA13* decreases in response to treatment with retinoids that cause pattern duplications along the proximal-distal limb axis (Table 3). Regenerating forelimbs treated for as little as 1 day with retinol palmitate at the medium bud stage of regeneration exhibit some degree of proximal-distal duplication. The proportion and degree of duplicated limbs increases after 2 days of treatment, and all limbs are duplicated after 3 days of treatment. The level of *HoxA13* expression in blastemas collected after 3 days of

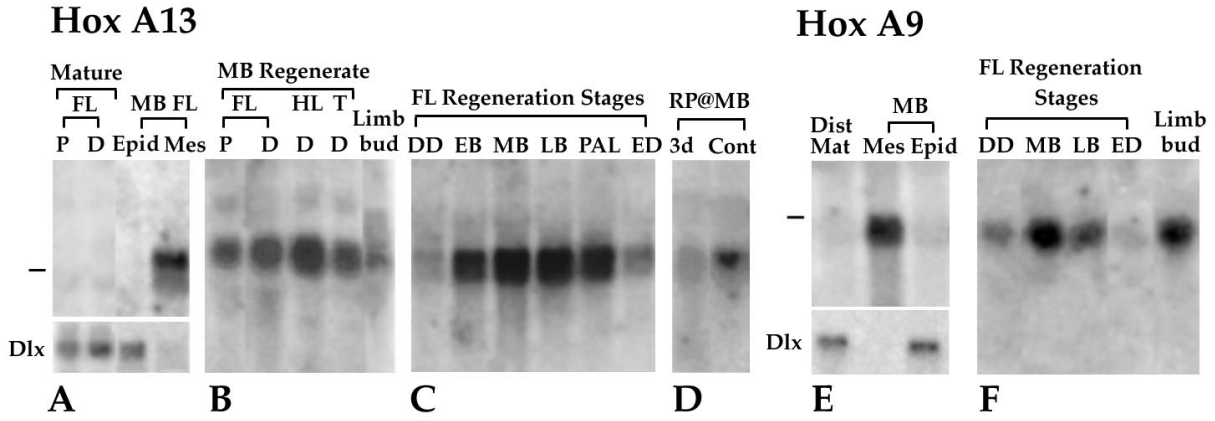

Fig. 2. Expression of *HoxA13* and *HoxA9* in developing, regenerating and mature axolotl limbs as visualized by northern blot analysis. The sizes of the transcripts were determined relative to the mobility of axolotl 28S rRNA (3.9 kb) and 18S rRNA (1.95 kb). The position of the 18S rRNA band is indicated by the hash mark on the left. Equal amounts of total RNA were loaded for each blot as determined spectrophotometrically and verified by either UV shadowing or ethidium bromide staining of the gels. The blots in A and E were also hybridized with a probe for axolotl *Dlx3* (inserts) to confirm that transcripts could be detected in those lanes that are negative for *HoxA13* and *HoxA9*. (A) Expression of *HoxA13* in mature limbs (Mature) and medium bud forelimb blastemas (MB FL). Tissues were from either humerus levels (P) or mid radius/ulna levels (D). In the MB FL samples, blastemas were separated into epidermal fractions (Epid) and mesenchymal fractions (Mes) prior to RNA extraction. 10 μg total RNA/lane. (B) Expression of *HoxA13* in medium bud blastemas (MB Regenerate) from forelimbs (FL), hindlimbs (HL), and tails (T) and in developing forelimb buds. 5 μg total RNA/lane. (C) Expression of *HoxA13* at different stages of regeneration from radius/ulna level amputations of forelimbs (FL). Stages are dedifferentiation (DD), early bud (EB), medium bud (MB), late bud (LB), palette (PAL) and early digits (ED). 20 μg total RNA/lane. (D) The effect of retinol palmitate (RP) treatment on *HoxA13* expression. RNA was extracted from control medium bud (MB) blastemas (Cont) that had not been treated with retinoids and medium bud blastemas from animals that had been treated with retinol palmitate for 3 days (3d). 5 μg total RNA/lane. (E) Expression of *HoxA9* in mature distal limbs (Dist Mat) and in epidermal (Epid) and mesenchymal fractions (Mes) of medium bud forelimb blastemas (MB). 10 μg total RNA/lane. (F) Expression of *HoxA9* in developing forelimb buds and at different stages of regeneration from humerus level amputations of forelimbs (FL). Stages are as in C. 5 μg total RNA/lane.

Table 3. Pattern duplicating activity of retinol palmitate on axolotl distal forelimb medium bud blastemas

Treatment duration	*n*	No. of normal	No. of duplicated	Duplication index*
1 day	5	1	4	2.4
2 day	4	1	3	3.25
3 day	4	0	4	4.25

*Duplication index is from Maden (1983). The maximum proximal-distal duplication in which an entire limb,including a pectoral girdle forms from a distal amputation stump has a score of 5; the index is the mean score for all limbs.

treatment is less than half (45%) the level in equivalent staged blastemas not treated with retinoids (Fig. 2D). We have analyzed retinoid-induced downregulation of *HoxA13* expression further by whole-mount in situ hybridization as described below.

Northern hybridization analysis of axolotl *HoxA9* expression

Axolotl *HoxA9* expression is detected as a single transcript of approximately 2 kb on northern blots of total RNA from regenerating blastemas (Fig. 2E-F). Probes from both the 5′ region of the transcript, which includes most of the homeobox, and the 3′ UTR (Fig. 1C) detect the same size transcript and reveal the same expression patterns. The blot illustrated in Fig. 2E has been probed with the 5′ probe and that in Fig. 2F with the 3′ probe.

The pattern of *HoxA9* expression is similar to *HoxA13*. It is expressed in developing limb buds (Fig. 2F), is not detected in mature limbs (Fig. 2E), and is reexpressed during regeneration (Fig. 2F). The maximal level of expression occurs during the blastemal stages, then decreases during redifferentiation (Fig. 2F). Within the blastema, *HoxA9* is expressed by mesenchymal cells but not epidermal cells (Fig. 2E). We analyzed the response of *HoxA9* to retinoids by whole-mount in situ hybridization as described below.

In situ hybridization analysis of *HoxA* expression in developing limb buds

We used whole-mount in situ hybridization to analyze the patterns of *HoxA13* and *HoxA9* expression in limb buds ranging from the earliest stage of forelimb outgrowth (stage 36) to later stages when digits one and two have formed (stages 41/42) (Fig. 3). Stages are based on the comparable limb bud morphologies for *Ambystoma punctatum* (Harrison, 1969). *HoxA13* probes from both the 5′ and 3′ regions of the transcript (Fig. 1A) revealed the same expression pattern; the 5′ probe was used because it resulted in a more intense signal. *HoxA9* transcripts were localized with a 5′ probe (Fig. 1C).

HoxA9 is expressed early in limb development (stage 36) throughout the limb bud mesenchyme and in the adjacent flank region (Fig. 3A). At stage 37, the intensity of staining increases, but the pattern is the same (Fig. 3B). At stage 38, expression is no longer detected in the flank and there is a small region of cells at the base of the limb bud that does not express *HoxA9* (Fig. 3C). At later stages, *HoxA9* continues to be expressed throughout the limb bud except in the proximal-most region (Fig. 3C,D). There are no fate maps for axolotl limb buds to compare with these expression patterns; however, expression is still evident during differentiation of skeletal elements, at which time *HoxA9* expression extends from the distal third of the humerus through the lower arm and hand (Fig. 3F).

HoxA13 expression is first detected later and in a more distally restricted population of limb bud cells as compared to *HoxA9*. Transcripts are not detected at stage 36 (Fig. 3G), and staining is limited to the distal region of stage 37 limb buds

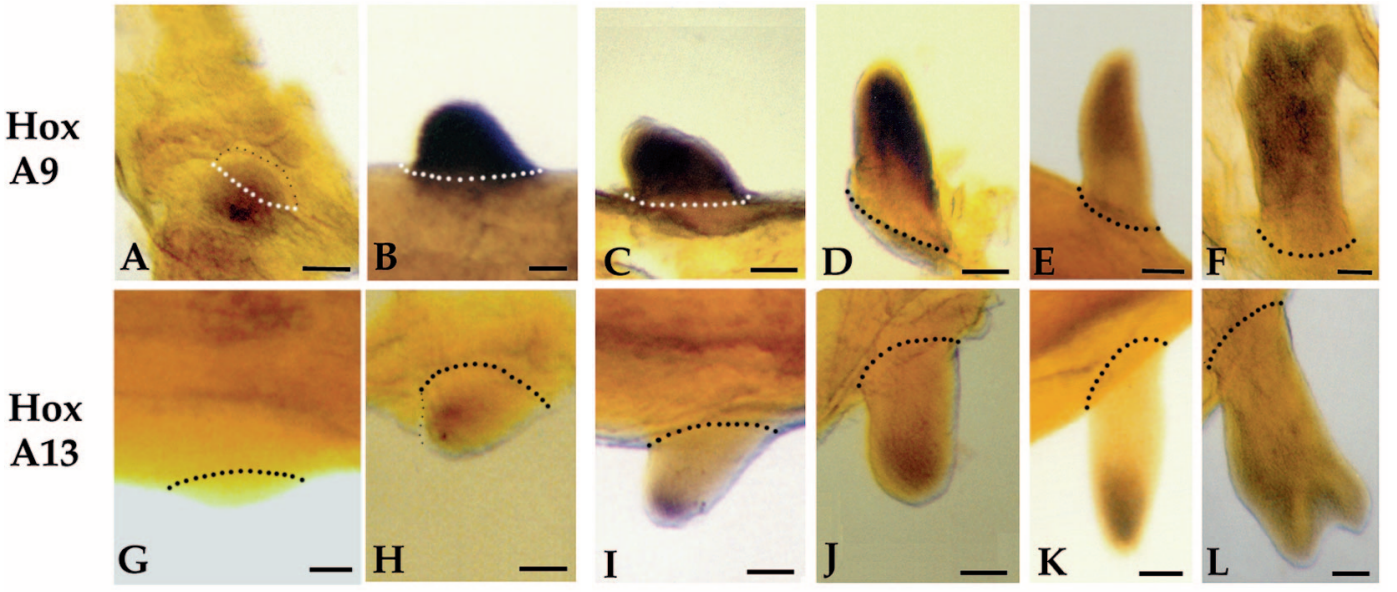

Fig. 3. Expression of *HoxA13* and *HoxA9* in developing axolotl forelimbs as visualized by whole-mount in situ hybridization. The yellow coloration of the limbs is due to the Bouin's postfixative, which we do not completely remove so as to provide enhanced contrast with the blue reaction product. Anterior is to the right in all limb buds. (A-F) *Hox A9* expression; (G-L) *HoxA13* expression; (A,G) stage 36; (B,H) stage 37; (C,I) stage 38; (D,J) stage 39; (E,K) stage 40; (F, L) stage 41/42 (stages after Harrison, 1969). Large dots indicate the base of the bud; small dots in A and H outline the edge of the bud where it overlaps the body. Scale bar, 100 μm.

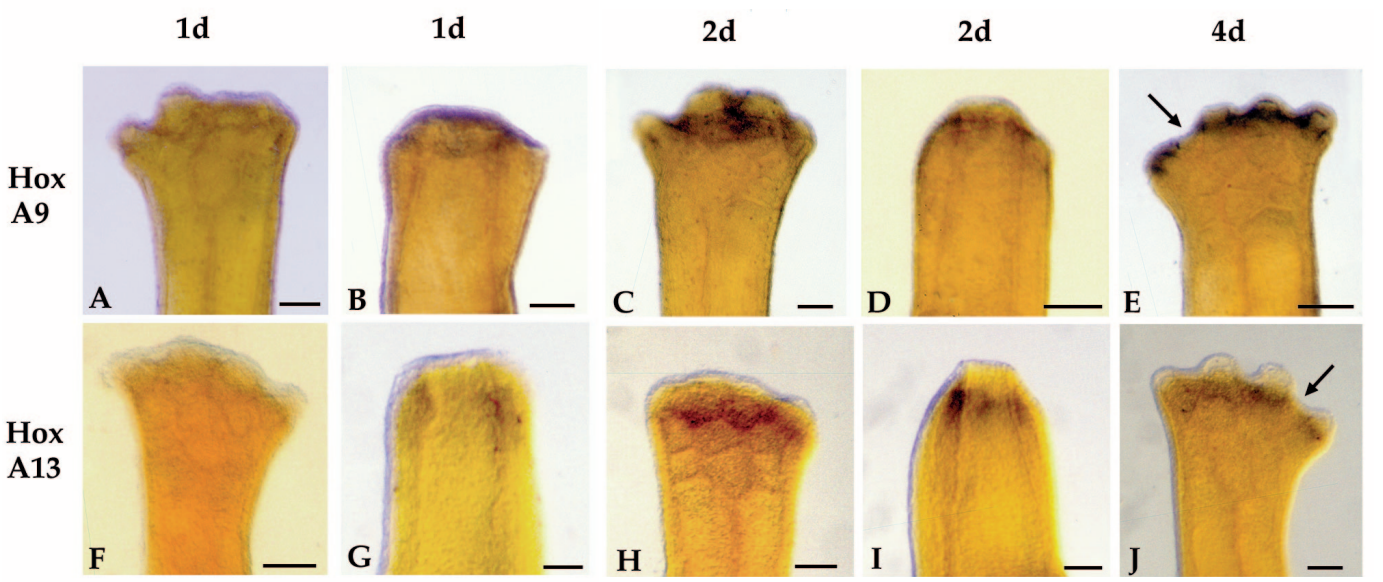

Fig. 4. Expression of *HoxA13* and *HoxA9* during early stages of axolotl limb regeneration as visualized by whole-mount in situ hybridization. All samples are forelimbs amputated at either the level of the humerus (B,D,G and I) or the level of the carpals (A,C,E,F,H and J). The limbs illustrated were collected 1 day (1d), 2 days (2d) or 4 days (4d) after amputation. Limbs are viewed from the dorsal side and are matched left limbs (*HoxA9*, anterior to the right) and right limbs (*HoxA13*, anterior to the left) from the same animal. Arrows in E and J indicate regions of stump tissues not expressing *HoxA9* or *HoxA13*. Scale bar, 200 μm.

(Fig. 3H). *HoxA13* expression remains distally restricted at later stages (Fig. 3I-K), and is localized to the region in which hand structures differentiate (Fig. 3L).

In situ hybridization analysis of *HoxA* expression during limb regeneration

Reexpression of both *HoxA9* and *HoxA13* is initiated at a very

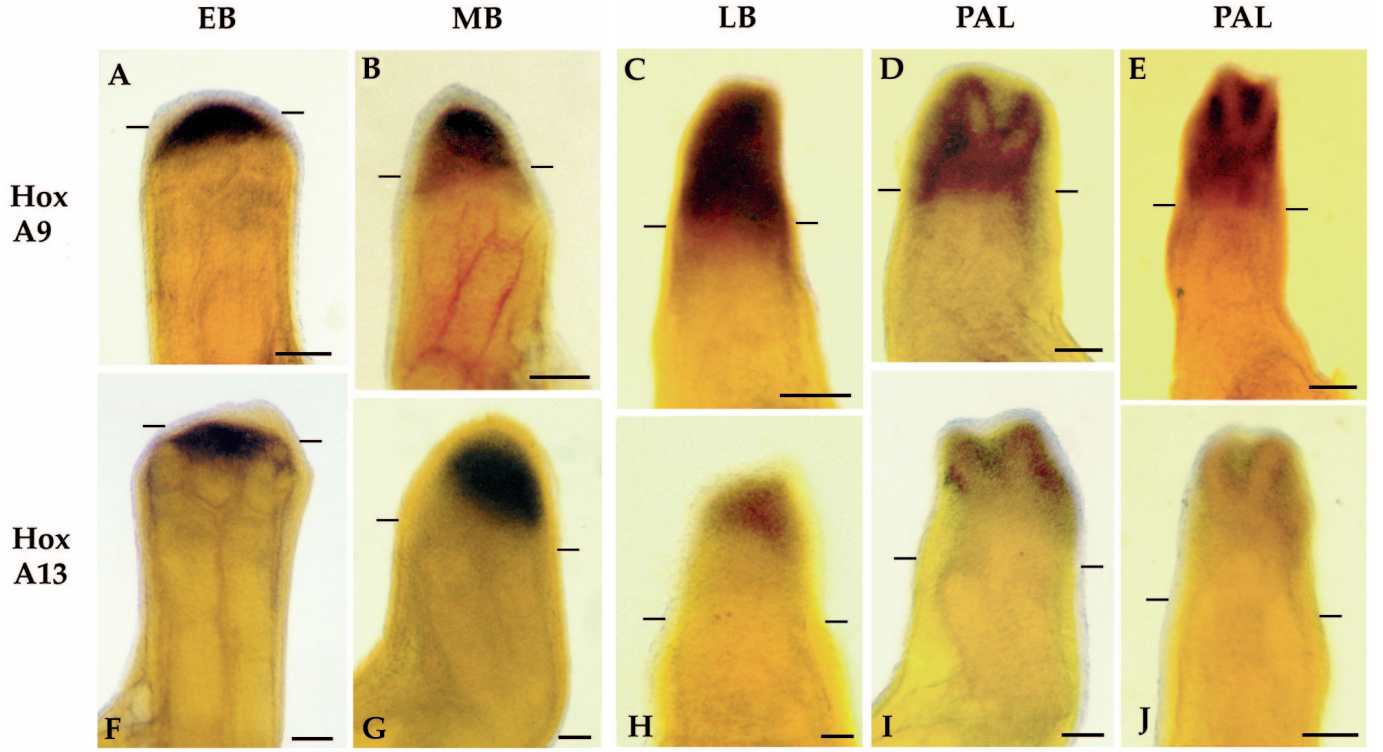

Fig. 5. Expression of *HoxA13* and *HoxA9* during blastema stages of axolotl limb regeneration as visualized by whole-mount in situ hybridization. All samples are forelimbs amputated at either the level of the humerus (C,E,H and J) or the level of the radius/ulna (A,B,D,F,G and I). The amputation plane is indicated by hash marks. All limbs are viewed from the dorsal side and are either left limbs with anterior to the right (*HoxA9*) or right limbs with anterior to the left (*HoxA13*). The limbs illustrated represent progressively later stages of regeneration; early bud (EB), medium bud (MB), late bud (LB) and palette (PAL). Scale bar, 200 μm.

early stage of regeneration in internal tissues of the stump, but not in epidermal cells (Fig. 4). Activation of this early expression domain precedes the accumulation of blastema cells, and is associated with the stump tissues that will dedifferentiate to form the blastema. We observed a similar pattern of early expression at each of the three levels of amputation along the proximal-distal limb axis (mid-stylopod, mid-zeugopod and distal-carpals). Expression of both genes is detectable 24 hours post-amputation in most limbs (Fig. 4A,B,F,G). Expression increases such that it is readily detectable in all limbs by 48 hours (Fig. 4C,D,H,I). In comparing the expression of *HoxA9* and *HoxA13* in left and right limbs of the same animal, we have not detected a difference in the time of onset of expression. Expression increases over the next few days in a stripe 100-150 μm wide, localized immediately proximal to the amputation surface (Figs 4D,J, 5A,F). Activation is localized to regions adjacent to the wound epidermis. In some limbs the amputation plane occurred distal to a digit base (Fig. 4E,J), and *HoxA* expression is not activated in the region covered by mature, interdigital epidermis (arrows).

At later stages of regeneration, when a blastema is present distal to the amputation plane, mesenchymal cells of the blastema express both *HoxA9* and *HoxA13* (Fig. 5); expression is not detected in epidermal cells. Dedifferentiating stump cells (proximal to the amputation plane) continue to express *HoxA9* as the blastema elongates (Fig. 5A-C); however, expression becomes restricted to the blastema at later stages when dedifferentiation has ceased (Fig. 5D,E). In contrast, *HoxA13* is not expressed in dedifferentiating stump cells beyond the early bud stage of blastema formation (Fig. 5F-J).

In contrast to the preblastema stages in which *HoxA9* and *HoxA13* transcripts are colocalized in both proximal and distal stump cells, the expression domains of these two genes within the blastema become spatially distinct as growth progresses (Fig. 5). *HoxA9* is expressed throughout both proximal and distal blastemas at all stages, and at late stages is expressed in the distal third of the redifferentiating humerus, the lower arm, and hand (Fig. 5E). *HoxA13* expression is detected throughout the early bud blastema (Fig. 5F), but subsequently becomes restricted to the distal region of the blastema (Fig. 5G-J). Thus the *HoxA13* expression domain becomes nested within the *HoxA9* expression domain. At late stages of regeneration, the distal domain of *HoxA13* expression corresponds to the differentiating digits (Fig. 5J); *HoxA13* is not expressed in more proximal regions of the regenerate, such as the distal radius and ulna reformed from a distal amputation. Work is in progress to develop blastema fate maps, which will allow for a more precise correlation between expression domains and structures regenerated.

The size of the *HoxA13* expression domain relative to the total size of the blastema differs between distal and proximal blastemas. In distal blastemas (Fig. 5G), cells throughout most of the blastema express *HoxA13*; only a narrow zone of cells at the base and in the anterior-proximal region of the blastema do not express *HoxA13*. In proximal blastemas (Fig. 5H) the zone of non-expressing cells at the base of the blastema is much larger than in distal blastemas; thus the distal expression domain represents a smaller proportion of the blastema. This difference is probably a consequence of the fact that only distal structures are regenerated from a distal amputation, but both proximal and distal structures are regenerated from a proximal amputation. This difference accounts for the results from northern analysis indicating a higher level of *HoxA13* expression in distal blastemas (greater proportion of expressing cells) as compared to proximal blastemas (lesser proportion of expressing cells; Fig. 2B).

The expression domain of *HoxA13* is asymmetric with respect to the anterior-posterior axis of the limb. The expression domain extends further proximally in the posterior region and is more distally restricted in the anterior region. This pattern is observed in both distal (Fig. 5G) and proximal (Fig. 5H) blastemas. A similar anterior-posterior asymmetry has been observed in *HoxA13* expression during limb development in the chick and mouse (Haack and Gruss, 1993; Yokouchi et al., 1991). *HoxA9* expression does not exhibit such an asymmetrical pattern, and both genes are expressed uniformly with respect to the dorsal-ventral limb axis. A dorsal-ventral asymmetry in *HoxA13* expression has been observed in developing mouse limbs (Haack and Gruss, 1993), but not in developing chick limbs (Yokouchi et al., 1991).

HoxA13 and *HoxA9* respond differently to retinoids

The expression of *HoxA13* during regeneration is downregulated in response to exposure to retinoids. After one day of systemic treatment with retinol palmitate, *HoxA13* expression is decreased at the distal tip of the blastema in some but not all samples (e.g. Fig. 6A). By 3 days of treatment, the level of *HoxA13* expression ranged from not detectable (not illustrated) to noticeably downregulated and uniformly expressed with respect to the anterior-posterior limb axis (Fig. 6B). Corresponding to this inhibition of *HoxA13* expression, the frequency and degree of pattern duplication increased. After 1 day of treatment, a few of the limbs exhibited pattern alterations; whereas, after 3 days of treatment all limbs exhibited some degree of pattern alteration (Table 3).

HoxA13 expression is downregulated in less than 24 hours when blastema cells are treated directly with RA released from beads implanted into the blastema (Fig. 6D). The level of expression is lowest adjacent to the bead (implanted into the anterior-distal region) and is higher in the posterior-proximal region away from the RA bead. As with systemic treatment of regenerating limbs, RA bead implants into blastemas result in pattern duplications at high frequency (Sessions, Wanek and Bryant, unpublished observations).

In contrast to *HoxA13*, the expression of *HoxA9* is not noticeably altered in response to RA released from beads. Expression in both proximal and distal RA-treated blastemas does not appear to differ from control blastemas at either 17 hours or 24 hours after bead implantation (Fig. 6E).

DISCUSSION

Regulation of homeobox gene expression during limb regeneration is complex

As a result of a screen for homeobox genes expressed in regenerating limb blastemas, we have identified a total of 17 different axolotl genes with homology to known homeobox genes. Previous screens of cDNA libraries from regenerating newt limbs have resulted in the identification of nine homeobox genes expressed during limb regeneration: *HoxA11* and *Hox B3* (Beauchemin and Savard, 1993); *HoxC6* (Savard

et al., 1988; Tabin, 1989); *HoxC10* and *HoxD10* (Simon and Tabin, 1993); *HoxD11* (Brown and Brockes, 1991) ; *Dlx1*, *Dlx3* and *Emx2* (Beauchemin and Savard, 1993). We isolated the axolotl homologs of five of these newt genes in our screen (*HoxA11*, *HoxB3*, *HoxD10*, *HoxD11*, and *Dlx3*). We have subsequently isolated the axolotl homolog of *HoxC10* from axolotl limb blastema RNA by RT-PCR (Komine, Gardiner and Bryant, unpublished data). Thus, to date, a total of 21 different homeobox genes are known to be expressed in regenerating limb blastemas. It is now apparent that there is not a single, or even just a few, but many homeobox genes that might be involved in regulating growth and pattern formation during limb regeneration. In addition, several of these genes are expressed as multiple transcripts with spatially distinct expression patterns (Beauchemin and Savard, 1993; Savard et al., 1988; Torok, Gardiner and Bryant, unpublished data), indicating an even more complex role in regeneration.

Almost all of the homeobox genes expressed in regenerating blastemas are also expressed in developing limb buds of other vertebrates (see Izpisúa-Belmonte and Duboule, 1992). The broad overlap in the homeobox genes expressed during limb development and regeneration is supportive of the view that these two developmental processes involve common mechanisms of growth regulation and pattern formation (see Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992). Although most, perhaps even all, of the same homeobox genes are expressed, the ways in which their expression is regulated differs (as discussed below), and the events involved in the initiation of outgrowth are different (see Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992). It may prove to be the case that some homeobox genes are uniquely expressed in either developing or regenerating limbs. We note that the novel axolotl *en/Msx* gene, though clearly a homeobox gene (50% amino acid identity to both engrailed and msh proteins within the homeodomain), may be unique to regeneration.

A striking result of our screen is the relative abundance and complexity of members of the *HoxA* complex expressed during limb regeneration. Studies of developing mouse and chick limbs indicate that the 5′ *HoxA* genes function in specifying the proximal-distal limb pattern. Since regeneration is essentially reforming the proximal-distal axis, it is perhaps not surprising that *HoxA* genes are so abundantly expressed.

HoxA genes are expressed in a colinear sequence in axolotl limb buds

The expression of *HoxA13* and *HoxA9* during axolotl limb development is consistent with the principle of temporal and spatial colinearity as described for other vertebrate limbs (Izpisúa-Belmonte and Duboule, 1992). The more 3′ gene, *HoxA9*, is first expressed at an earlier stage of limb development than is the more 5′ gene, *HoxA13*. Similarly, *HoxA9* has a more extensive expression domain with a more proximal boundary than *HoxA13*, which has a more distally restricted expression domain that is nested within the *HoxA9* domain. Thus *HoxA* gene expression in developing axolotl limbs appears to be regulated the same as in developing limbs of other vertebrates.

Although presently there are no fate maps for developing axolotl limbs, it is possible to correlate expression domains with structures that differentiate during the later stages of limb development. At these stages, *HoxA13* is expressed in the region that forms the hand, as in chick and mouse limbs (Haack and Gruss, 1993; Yokouchi et al., 1991). This domain is nested within the *HoxA9* domain, which at early stages includes the entire limb bud as well as the adjacent flank. As the limb grows, the proximal border of expression moves out onto the bud, and staining remains intense from there to the tip of the bud. At later stages, *HoxA9* is expressed throughout the region that will form the hand, the forearm and the distal third of the humerus. *HoxA9* expression does not appear to be less intense distally in regions of overlap with *HoxA13* expression, as has been reported for *HoxA11* in mouse and chick (Haack and Gruss, 1993; Yokouchi et al., 1991).

Reexpression of *HoxA* genes is not colinear during initiation of regeneration

The pattern of *HoxA* gene reexpression during regeneration departs markedly from the spatial and temporal colinearity characteristic of developing limbs of axolotls and other vertebrates. Neither gene is expressed in mature limbs, but both can be detected within 1-2 days after amputation in a stripe of mesenchymal cells immediately beneath the wound epidermis. Expression is similar, regardless of the proximal-distal level of the amputation. The exact time of onset of expression is somewhat variable within the first 24-48 hours, with some but not all limbs showing clear expression at 24 hours. However, in matched contralateral limbs, whenever *HoxA9* was detected in one limb, *HoxA13* was detected in the other. While these results suggest that the reexpression of *HoxA9* and *HoxA13* is synchronous rather than colinear, further experiments are necessary to rule out a very rapid activation via the canonical *HoxA9 – HoxA10 – HoxA11 – HoxA13* sequence.

Both *HoxA9* and *HoxA13* continue to be colocalized during the early stages of blastema formation, at both proximal and distal limb levels. Spatially distinct domains of expression emerge during growth of the blastema as *HoxA13* expression becomes confined to a distal subset of the cells that also express *HoxA9*. During these later stages of regeneration, the relationship of *HoxA* expression patterns is the same as in developing limbs, with *HoxA13* expression correlated with regeneration of the hand, and *HoxA9* with regeneration of the hand, lower arm and distal humerus.

According to the hypothesis that segmental identity is based on combinatory expression of *Hox* genes (Kessel and Gruss, 1991), the most distal part of the limb pattern (hand) would be characterized by the overlapping expression of *HoxA9* and *HoxA13*, whereas more proximal regions would express *HoxA9* but not *HoxA13*. The *HoxA* expression patterns in developing limbs and blastema stage regenerating limbs are consistent with this hypothesis. Given that the coexpression of *HoxA13* and *HoxA9* is characteristic of the distal limb pattern, then the distal part of the limb pattern is the first to be respecified during regeneration, regardless of the level of amputation. The regions that are intermediate between the stump and the newly formed distal tip appear to arise later, during growth of the blastema, as the domains of *HoxA9* and *HoxA13* become spatially distinct. Whether the cells for this intermediate zone originate from the distally specified cells of the early blastema, or from the stump, remains to be determined. It is generally thought that the pattern of both developing and regenerating limbs is specified in a strict proximal to distal sequence. We conclude that for regeneration at least, this is not the case.

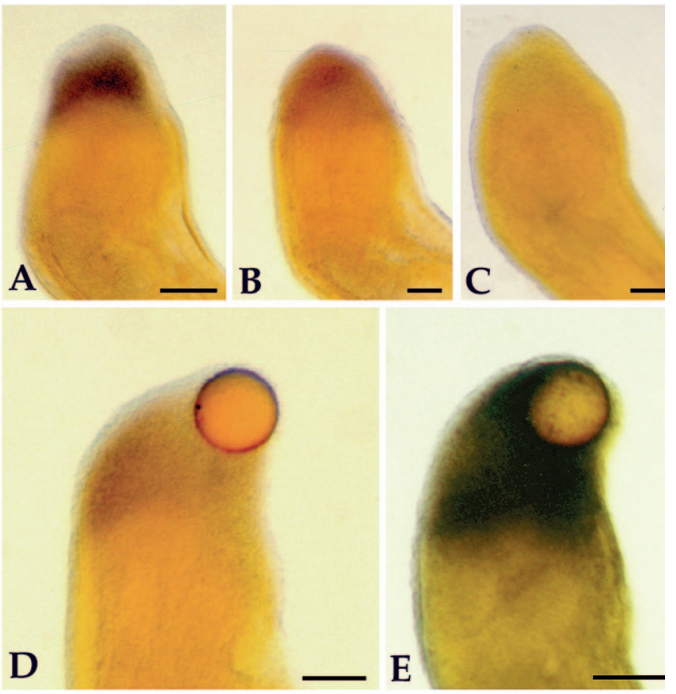

Fig. 6. Effects of retinoid treatment on the expression of *HoxA13* and *HoxA9* as visualized by whole-mount in situ hybridization. (A) *HoxA13* expression in a medium bud blastema on an animal treated with retinol palmitate for 1 day. (B) *HoxA13* expression in a medium bud blastema on an animal treated with retinol palmitate for 3 days. (C) A medium bud blastema that was hybridized with the sense probe from the 5′ region of *HoxA13*. (D) *HoxA13* expression in a medium bud blastema into which a retinoic acid-containing bead was implanted 23 hours earlier. (E) *HoxA9* expression in a medium bud blastema into which a retinoic acid-containing bead was implanted 23 hours earlier. Scale bar, 200 μm

In addition to providing insights as to how the proximal-distal limb axis is reestablished during regeneration, *HoxA9* and *HoxA13* expression are early molecular markers for the process referred to as 'dedifferentiation'. This term refers to events occurring in the transition zone between mature stump tissues proximal to the amputation surface and the blastema that forms distally (see Wallace, 1981). Dedifferentiation begins soon after amputation and is considered to be critical for the initiation of regeneration. Considering how rapidly *HoxA* reexpression is induced in the stump, it must be situated close to the beginning of the regeneration cascade. As discussed elsewhere (Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992), the key developmental processes that distinguish limb regeneration from limb development are those associated with the initiation of the regeneration cascade; induction of *HoxA* reexpression during dedifferentiation appears to be one such process. The possibility that regeneration is initiated with a generalized activation of gene expression, of which the *HoxA* genes described here are only a part, will become clear as more of the genes expressed in regeneration are studied.

Hox A13 and *HoxA9* respond differently to retinoid treatments that cause proximalization of blastemas

The effects of retinoids on developing and regenerating limbs have been well characterized at the level of cell biology; whereas, effects on gene expression have been less well characterized, especially for regenerating limbs. Retinoids cause a distal blastema to regenerate as if it had been transformed to a proximal blastema, leading to the formation of duplicated pattern along the proximal-distal axis (Maden, 1982). It is presumed that the changes in positional information are a consequence of retinoid-induced changes in gene expression.

The finding that proximalization of the blastema by retinoid treatment is associated with an early downregulation of *HoxA13*, but not of *HoxA9* expression, is consistent with the idea that the *Hox* code of the treated cells is changed to that of a more proximal region. However, as discussed above, blastemas initially express a distal *Hox* code, and thus a retinoid-treated blastema, with a proximal *Hox* code, is not the same as a blastema arising at a proximal limb level. The steps leading from a retinoid-proximalized blastema to the final duplicated limb pattern have not yet been studied. Despite this gap in our knowledge, the coincidence between altered *HoxA13* expression and altered pattern, provides further evidence of the importance of *HoxA* genes in limb pattern formation. In addition, we note that this is the second report of retinoid-responsive *Hox* gene expression during regeneration (see Simon and Tabin, 1993). Taken together with our finding that stump cells initially acquire a distal *Hox* code regardless of their position along the proximal-distal axis, regenerating amphibian limbs no longer provide support of the hypothesis that *Hox* gene expression patterns become permanently imprinted and cannot be modified (Mavilio, 1993).

The response of *HoxA9* and *HoxA13* to retinoid treatment of regenerating limbs in vivo is consistent with that of teratocarcinoma cells in vitro (Simeone et al., 1991). Genes at the 3′ end of the *Hox* complexes are activated by RA, genes located in the middle of the complex do not react strongly to RA, and genes at the 5′ end are either not affected, inhibited or strongly downregulated. In blastemas, *HoxA9*, which is in the middle of the complex is relatively unresponsive to retinoids; whereas, *HoxA13*, at the 5′ most end of the complex, is inhibited. In addition, another 5′ *Hox* gene, *HoxD13*, is inhibited by RA in vivo during chick limb development (Hayamizu and Bryant, 1994). The coordinated upregulation of 3′ *Hox* genes and downregulation of 5′ *Hox* genes could cause positional identity to be shifted to a more rostral position along the rostrocaudal axis, and a more proximal position along the limb axis.

An intriguing feature of limb regeneration is that the regenerate is always appropriate to the level of amputation and precisely matches the stump. This implies that mature limb cells retain a memory of their identity along the proximal-distal limb axis. This memory is not simply that of upper arm versus lower arm or hand, but of a precise position within a particular limb segment. Discovering how limb cells store and reaccess this information is one of the challenges of regeneration research that is of importance to understanding both limb regeneration and limb development. Were it not for animals that can regenerate so flawlessly, we would not appreciate that positional information is so fine-grained, that this information is stored by differentiated cells, and that it can be accessed. The observations that *HoxA* genes are expressed in specific patterns along the proximal-distal limb axis, and that alterations in these patterns coincide with changes in the positional identity of blastema cells, suggest that understanding how

HoxA genes are regulated is an important step in meeting this challenge.

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