Regulation of HoxA expression in developing and regenerating axolotl limbs

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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 Hox 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 Capecechi, 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 spawn at either UCI or the Axolotl Colony, Indiana University. 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 32P-labeled probes in 50% formamide, 5x SSPE, 5x 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 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) and the RNA-containing supernatant was phenol-chloroform extracted and precipitated with 1/10 volume 5 N NaCl and 2 volumes ethanol.

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 8×10^5 independent clones and the distal-blastema library contained 2×10^6 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(T,C,T)T(G,T)(A,G)ACCA(A,G)-AT(C,T)TT] that are complementary to all possible variations of the DNA sequence encoding the conserved amino acid sequence KWFQ(K/N)RR. 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(T,C,T)T(G,T)-(A,G)ACCA] 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 treated with proteinase K (20 μg/ml sonicated salmon sperm DNA at 42°C for 48 hours. Filters were washed at 65°C in 0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) and the RNA-containing supernatant was phenol-chloroform extracted and precipitated with 1/10 volume 5 N NaCl and 2 volumes ethanol.

Preparation of digoxigenin-labeled RNA probes

Digoxigenin-labeled RNA probes for whole-mount in situ hybridization were synthesized as described to the manufacturer’s protocol (Boehringer). The 5’ HoxA13 probes were transcribed from the 430-bp EcoRI 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 EcoRI-Xhol 3’ UTR fragment (Fig. 1A). The HoxA9 probe was transcribed from the 620-bp EcoRI-BglII fragment from clone Hpi4 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 MgSO4, 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 protease 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, 5x SSC, 1 mg/ml yeast RNA, 100 μg/ml heparin, 1x 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 2x SSC (20 minutes each) and twice with 0.2x 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 1:4000 in blocking solution in 2% sheep serum 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 MgCl2). 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 stored in methanol 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 MgCl2). 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 stored in methanol at 4°C.

**Results**

Isolation and identification of axolotl homebox-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 (40% 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

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

<table>
<thead>
<tr>
<th>Deduced homeobox AA sequence</th>
<th>Probable homolog</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>KFRR Contractor TRQQVLEKEFHFNYLRTRRRIEIAHLMCLSERQKV1WFQNRMKWKKDHD</td>
<td><em>HoxA4</em></td>
<td>1</td>
</tr>
<tr>
<td>GKRARTA YQTRQLEKEFHFNYLR <em>K</em></td>
<td><em>HoxA5</em></td>
<td>3</td>
</tr>
<tr>
<td>RKRGRQTYTRQLEKEFHFNYLRTRRRIEIAHLMCLSERQKV1WFQNRMKWKKHEN</td>
<td><em>HoxA7</em></td>
<td>4</td>
</tr>
<tr>
<td>TRKKRCPTYKHTQLEKEFHFNYLDRRYEVARLNLTERQKV1WFQNRMKWKKKIN</td>
<td><em>HoxA9</em></td>
<td>12</td>
</tr>
<tr>
<td>GGRKKRCPTYKHTQLEKEFHFNYLTERRELEISRS</td>
<td><em>HoxA10</em></td>
<td>3</td>
</tr>
<tr>
<td>TRKKRCPTYKQIESFSEIYIKEKRLQSRLNLTDVRQKV1WFQNRMKWKKKIN</td>
<td><em>HoxA11</em></td>
<td>1</td>
</tr>
<tr>
<td>GKRKPVYTRKQVLEKEEREYATNKF1TDRRRI3ATTNLSERQVT1WFQNRVKEKVKI</td>
<td><em>HoxA13</em></td>
<td>–</td>
</tr>
<tr>
<td>SKRATAYTSAQLVEKEFHFNYLICPRRVMANMLNLTERQID1WFQNRMKYKKDQ</td>
<td><em>HoxB3</em></td>
<td>5</td>
</tr>
<tr>
<td>GRGRR8QYTRQLEKEFHFNYLRTRRRIEIAHLMCLSERQKV1WFQNRMKWKKKEN</td>
<td><em>HoxB6</em></td>
<td>1</td>
</tr>
<tr>
<td>DRRKRVYQSKGQLEKEYA3SKF1TDRRRQIAATNLSERQI1WFQNRVREKVF</td>
<td><em>HoxC13</em></td>
<td>–</td>
</tr>
<tr>
<td>RRRGRRQYTRQFOPLTELKEFHFNYLTERREIEVSHAGLTERQKV1WFQNRMKWKKKEN</td>
<td><em>HoxD8</em></td>
<td>3</td>
</tr>
<tr>
<td>GKRKRPYTRQHTQLEKEFHFNYLTERRELEISKSVESSLTDVRQKV1WFQNRMKLKKMS</td>
<td><em>HoxD10</em></td>
<td>8</td>
</tr>
<tr>
<td>SRRKRCPTYKQRERFFNV1IEKRLQSRLNLTDVRQKV1WFQNRNRMEKLN</td>
<td><em>HoxD11</em></td>
<td>3</td>
</tr>
<tr>
<td>NKRPPTTFCTTSQALERKFKQGLSIAERAEFSNSLALTEQTV1WFQNRKAAKRLQ</td>
<td><em>Msx2</em></td>
<td>4</td>
</tr>
<tr>
<td>IKRHPITYSSQLAQLQRKFGCALPERAEIVAQLQGLTQTQVK1WFQNRSKFKLY</td>
<td><em>Dlx3</em></td>
<td>–</td>
</tr>
<tr>
<td>KRRKRSARVFSNLQRKLEKRFQKYVTKPDRLQ</td>
<td><em>Hlx</em></td>
<td>1</td>
</tr>
<tr>
<td>RARPRTKFSTEQLSERFSQEQRIGVAEKRLARENLSELRIKTWFQNRMKFNGSE</td>
<td><em>en/Msx</em></td>
<td>1</td>
</tr>
</tbody>
</table>

**Region**

*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 EcoRI site and the vector EcoRI 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 remaining 11 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 cells.
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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...
Table 3. Pattern duplicating activity of retinol palmitate on axolotl distal forelimb medium bud blastemas

<table>
<thead>
<tr>
<th>Treatment duration</th>
<th>n</th>
<th>No. of normal</th>
<th>No. of duplicated</th>
<th>Duplication index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>2.4</td>
</tr>
<tr>
<td>2 day</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>3.25</td>
</tr>
<tr>
<td>3 day</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>4.25</td>
</tr>
</tbody>
</table>

*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 \textit{HoxA13} expression further by whole-mount in situ hybridization as described below.

Northern hybridization analysis of axolotl \textit{HoxA9} expression

Axolotl \textit{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 \textit{HoxA9} expression is similar to \textit{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, \textit{HoxA9} is expressed by mesenchymal cells but not epidermal cells (Fig. 2E). We analyzed the response of \textit{HoxA9} to retinoids by whole-mount in situ hybridization as described below.

In situ hybridization analysis of \textit{HoxA} expression in developing limb buds

We used whole-mount in situ hybridization to analyze the patterns of \textit{HoxA13} and \textit{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 \textit{Ambystoma punctatum} (Harrison, 1969). \textit{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. \textit{HoxA9} transcripts were localized with a 5' probe (Fig. 1C).

\textit{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 \textit{HoxA9} (Fig. 3C). At later stages, \textit{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 \textit{HoxA9} expression extends from the distal third of the humerus through the lower arm and hand (Fig. 3F).

\textit{HoxA13} expression is first detected later and in a more distally restricted population of limb bud cells as compared to \textit{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 \textit{HoxA13} and \textit{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) \textit{HoxA9} expression; (G-L) \textit{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.
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(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 early stage (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.

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

Reexpression of both *HoxA9* and *HoxA13* is initiated at a very early stage (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.

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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 4DJ, 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. 4EJ), 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
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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.
HoxA begins soon after amputation and is considered to be critical that forms distally (see Wallace, 1981). Dedifferentiation process referred to as 'dedifferentiation'. This term refers to and HoxA13 induction of associated with the initiation of the regeneration cascade; dis-tinguish limb regeneration from limb development are those discussed elsewhere (Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992), the key developmental processes that dis- counted 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 reexpression of 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.

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).

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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

**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
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