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Analysis of *Hox-4.5* and *Hox-3.6* expression during newt limb regeneration: differential regulation of paralogous *Hox* genes suggest different roles for members of different *Hox* clusters

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SUMMARY

Adult urodele amphibians can regenerate their limbs and tail. Based on their roles in other developing systems, *Hox* genes are strong candidates for genes that play a role in regulating pattern formation during regeneration. There are four homologous clusters of *Hox* genes in vertebrate genomes. We isolated cDNA clones of two newt homeobox genes from homologous positions within two *Hox* clusters; *Hox-4.5* and *Hox-3.6*. We used RNase protection on nonamputated (normal) and regenerating newt appendages and tissue to compare their transcriptional patterns. Both genes show increased expression upon amputation with similar kinetics. *Hox-4.5* and *Hox-3.6* transcription is limited to the mesenchymal cells in the regenerates and is not found in the epithelial tissue. In addition to regenerating appendages, both genes are transcriptionally active in adult kidney of the newt. Striking differences were found in the regulation of *Hox-4.5* and *Hox-3.6* when they were compared in unamputated limbs and in regenerating forelimbs versus regenerating hindlimbs. *Hox-4.5* is expressed in the blastema of regenerating fore- and hindlimbs, but *Hox-4.5* transcripts are not detectable in normal limbs. In contrast, *Hox-3.6* transcripts are found exclusively in posterior appendages, but are present in normal as well as regenerating hindlimbs and tails. *Hox-4.5* is also expressed at a higher level in proximal (mid-humerus) regenerates than in distal ones (mid-radius). When we proximalized the positional memory of a distal blastema with retinoic acid, we find that the early expression level of *Hox-4.5* is also proximalized. When the expression of these genes is compared to the expression of two previously reported newt *Hox* genes, a consistent pattern emerges, which can be interpreted in terms of differential roles for the different *Hox* clusters in determining regenerative limb morphology.

Key words: *Hox-3.6*, *Hox-4.5*, paralogues, homeobox genes, limb regeneration, positional information, retinoic acid, axis specification

INTRODUCTION

Following amputation, adult newts are able to regenerate complete limbs. In many respects, the mechanisms underlying control of regenerative pattern are believed to be the same as those controlling normal limb development. One major difference between the two processes is the origin of the mesenchymal cells that give rise to the limb. The embryonic limb bud is derived from undifferentiated lateral plate mesoderm, with a contribution from myogenic precursors migrating from the myotome of the somite. In the case of limb regeneration, the new limb comes from differentiated adult cells at the cut surface. These are thought to undergo a process of dedifferentiation to form a mass of cells called a blastema. These mesenchymal progenitor cells then proliferate, redifferentiate and give rise to that part of the limb that was lost (Wallace, 1981).

One way to conceptualize the patterning of the limb is in terms of positional information. Applying this framework to regeneration, there is a continuum of positional values extending along the length of the proximodistal axis from the shoulder to the tips of the fingers, distal values being lost in amputation. The ability to regenerate precisely the structures that have been amputated then depends on a 'positional memory' of the cells at the amputation plane giving them the property of initializing the regenerates at the proper value (Stocum, 1984).

Regeneration can be thought of as generating new positional values distal to the cut surface. It is of great interest that retinoic acid (RA) has the unique ability to change the positional values in the blastema (Niazi and Saxena, 1978; Maden, 1982; Stocum and Crawford, 1987). For example, if a wrist level blastema is treated with an optimal dose of RA, the positional values are altered to those at the level of the shoulder. The regenerate formed is no longer just a hand but a complete new limb. The molecular basis of positional memory in the blastema and the ability of RA to respecify it remain largely unclear.

Urodele amphibians such as the newt and axolotl are the only adult vertebrates able to regenerate their limbs. The

cellular and molecular basis for this unique ability is not understood (Brockes, 1989). One critical step for the formation of the blastema is the reactivation of genes controlling the formation of pattern during development of the limb bud (Muneoka and Bryant, 1984).

One class of molecules that are candidates for playing a role in both providing the capacity for regeneration and for carrying positional information are the products of the *Hox* genes. Studies on *Drosophila* and *Xenopus* homeodomain proteins indicate that these transcription factors control key developmental events (Akam, 1987; Nusslein-Volhard et al., 1987; Wright et al., 1989). Mammalian homeodomain proteins may also regulate differentiation events since they display temporally and spatially restricted expression patterns during embryogenesis (Goulding and Gruss, 1989). In *Drosophila*, homeobox genes are genetically linked to two clusters, termed the *Antennapedia* complex (*Antp-C*) and the *Bithorax* complex (*BX-C*), which together are known as the Homeotic complex *HOM-C* (Akam, 1989). Vertebrates have four homologous sets of clustered genes related to ancestral *HOM-C*, termed *Hox-1*, *Hox-2*, *Hox-3* and *Hox-4* (Duboule and Dolle, 1989; Graham et al., 1989; Kappen et al., 1989; Schughart et al., 1989), which most probably arose during evolution by amplification and duplication events. As a consequence, specific genes in each of the different complexes are evolutionary related to each other, forming a subfamily or 'paralogous' group (Boncinelli et al., 1989). Fig. 1 illustrates the homology relationship between the vertebrate *Hox* complexes and the *Drosophila HOM-C* complex. In both vertebrates and insects, the gene clusters are organized in a physical order colinear with the anterioposterior axis of the developing organism such that the 3′-most genes are expressed in the most anterior domains (Duboule and Dolle, 1989; Gaunt et al., 1988; Gaunt, 1991; Graham et al., 1989). The *Hox* genes that have the most intriguing pattern described thus far in the developing limb are at the 5′ end of the *Hox-4* cluster (*Hox-4.4*, *-4.5*, *-4.6*, *-4.7* and *-4.8*), and the *Hox*-1 cluster (*Hox-1.7*, *-1.8*, *-1.9*, and *-1.10*), respectively. The genes in the *Hox-4* cluster are expressed coordinately along the anterioposterior and proximodistal axis forming a nested set, with the genes located in a more 5′-position expressed in progressively more posteriodistal areas (Dolle et al., 1989). Homeobox genes in the *Hox -1* cluster have expression patterns forming domains along the proximodistal axis (Yokouchi et al., 1991). The genes in these two clusters are thus candidates to be involved in the determination of the anterioposterior, and proximodistal fates.

Like other vertebrates, the genomes of newts contain clusters of *Hox* genes (Belleville et al., 1992). In earlier studies, we and others have described a newt homolog to the *Hox-3.3* gene that is expressed in normal and regenerating newt limbs. Its expression is restricted to the mesenchyme of the forelimb bud, although it is found in the ectoderm of both the forelimb and hindlimb buds (Savard et al., 1988; Tabin, 1989). In addition, a newt homolog to the *Hox-4.6* gene has been described that is expressed in the mesenchymal tissue of regenerating limbs and tails, but not differentiated limbs; it has been termed 'regeneration-specific' (Brown and Brockes, 1991). These two *Hox* genes belong to different homeobox clusters, and also occupy different positions within each cluster. We wondered whether the difference in their expression patterns is a consequence of their belonging to the *Hox-3* and *Hox-4* clusters, respectively, or whether it is due to the different position they each occupy within their cluster. We therefore decided to examine the expression pattern of two additional homeobox genes, one from each of these clusters, but from an equivalent paralogous position within the clusters.

In this paper, we present two new paralogous newt homeobox genes, the homologues of *Hox-3.6* and *Hox-4.5*. We compare the transcript patterns of these two cognate genes in unamputated (normal) and regenerating tissue, and find that the regulation of *Hox-3.6* is similar to that of *Hox-3.3* while the regulation of *Hox-4.5* is similar to that of *Hox-4.6*. This has important implications for the roles of these *Hox* clusters in specifying regenerative limb territories and positional values in axis formation during limb regeneration.

MATERIALS AND METHODS

Animals and treatment

Adult newts (*Notophthalmus viridescens*) were supplied by M. Tolley, Donelson, Tennessee. Anesthesia, amputation procedures, and the collection and storage of tissue samples were all as described earlier (Tabin, 1989). For RA treatment, groups of about 100 newts were amputated distally at the level of the mid-forearm (mid-radius/ulna). 5 days later the animals received a single intraperitoneal injection with 10 μl of either a RA/DMSO solution (10 mg/ml) or DMSO alone. Blastemas were harvested 7 days after RA treatment, at the end of the delay period in regeneration

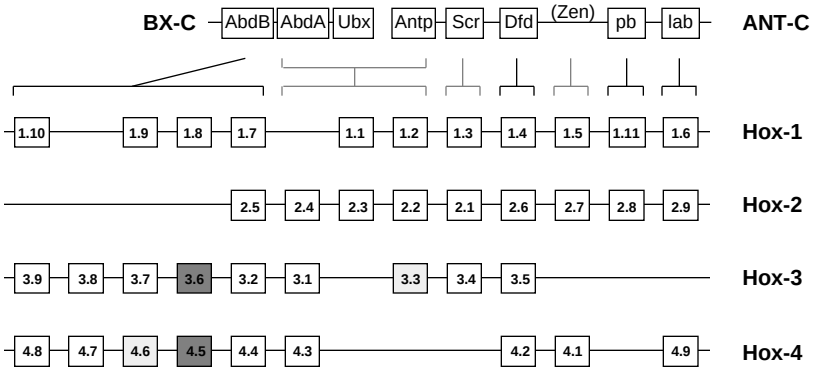

Fig. 1. Organization of the four vertebrate Hox complexes and the *Drosophila BX-C* and *ANT-C* homeotic (HOM) complexes. The two cognate genes *Hox-3.6* and *Hox-4.5* (dark shaded) represent together with *Hox-1.8* one paralogous group. The two newt homeobox genes described before (*Hox-3.3*, Savard et al., 1988; Tabin, 1989, and *Hox-4.6*, Brown and Brockes, 1991) belong to different paralogous groups (light shaded). The scheme has been adapted from Krumlauf (1992).

provoked by RA (Maden, 1983), and when they had reached the mid-bud stage: 20 days after RA treatment, or 15 days after DMSO treatment. Control groups were allowed to regenerate completely to verify that proximalization had occurred. For separation of blastemas into epithelial and mesenchymal fractions, blastemas were harvested and treated with 0.1 M EDTA (pH 8) for 2 minutes on ice. The epidermis was then manually separated from the mesenchymal tissue. All tissue samples were quick frozen in liquid nitrogen and stored at −80°C prior to RNA preparation.

Isolation and analysis of cDNA clones

Individual cDNA libraries were made from newt forelimb and hindlimb mid-bud-stage blastema RNA applying the cloning strategy described by Aruffo and Seed (1987). In brief, a plasmid vector pBluescript KS(+) (Stratagene) was modified to contain two identical *Bst*XI sites placed in inverted orientation with respect to each other and separated by a short replaceable segment of DNA. Non-self-complementary *Bst*XI adaptors were ligated to the cDNA, size-selected molecules were ligated into the compatible *Bst*XI site of the vector, and the ligation mixture was used to transform *E. coli* XL1-Blue (Stratagene) by electroporation (Dower et al., 1988). Aliquots of the forelimb and hindlimb blastema cDNA libraries were mixed and screened with a completely degenerate oligonucleotide probe to the most conserved 8-amino acid motif (K/Q-I/V/M-W-F-Q/S-N-R-R) in the recognition helix of the homeodomain (Burglin et al., 1989). Hybridization and washing conditions were as described by Burglin et al. (1989). Positive clones were initially sequenced using the degenerate oligonucleotides used for screening to verifiy their homology to the homeobox gene family. Further sequencing was performed using vector-derived primers and internal cDNA-specific primers. The clones were sequenced in both orientations by double-stranded sequencing using Sequenase (US Biochemicals). Sequence analysis was performed using the sequence analysis software package (version 7.1), Genetics Computer Group, Inc. (Devereux et al., 1984).

RNA preparation

Total RNA was isolated from frozen tissue by homogenizing the tissue in 4 M guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion (Sambrook et al., 1989). Poly $(A)^+$ RNA was purified using the Poly(AT) tract System III (Promega) following the instructions of the manufacturer.

Northern blot analysis

5 μg of poly$(A)^+$ RNA was separated by electrophoresis through a 1% agarose-0.66 M formaldehyde gel run in 1× MOPS buffer. The RNA was transferred to Genescreen membrane using a pressure blotter (Stratagene) and then crosslinked to the nylon membrane using an UV-Stratalinker (Stratagene) under standard conditions. For use as probes, gel-purified DNA fragments were randomly primed using the Multiprime DNA labelling system (Amersham). The filter was hybridized with 1×10^6 cts/minute/ml of the radiolabelled probes at 42°C in 50% formamide; 5× SSPE; 0.5% SDS; 5× Denhardt's solution; 100 mg/ml salmon sperm DNA. High stringency washes were carried out in 0.2× SSC; 0.5% SDS at 65°C for 3× 20 minutes. The filter was exposed to Kodak XAR-5 film for 7 days at −70°C with intensifying screens after hybridization to homeobox probes, or for 1 hour at room temperature after hybridization to a newt EF-1α probe isolated by homology to a *Xenopus* EF-1α clone provided by D. Melton (Krieg et al., 1989). Before being reprobed, the blot was stripped of probe by washing in boiling water for 20 minutes.

RNase protection

RNase protection was done according to a modified procedure described by Melton et al. (1984). Appropriate fragments of the cDNAs were derived by PCR and cloned into Bluescript KS+. Antisense RNA probes were synthesized from the linearized plasmid template using either T3 or T7 RNA polymerase incorporating [α-^{32}P]UTP. 2×10^6 cts/min probe (approximately 800 pg radiolabelled transcript) was hybridized to 5 μg of each total RNA sample in a final volume of 40 μl of hybridization solution (50% formamide; 40 mM Pipes, pH 6.7; 400 mM NaCl; 1 mM EDTA) at 60°C overnight. 300 μl of 0°C RNase solution (10 mM Tris, pH 7.5; 300 mM NaCl; 1 mM EDTA; 40 μg/ml of RNaseA; 2 μg/ml of $RNaseT_1$) was added to the hybridization reactions and the reaction mixtures were incubated at 30°C for 30 minutes. 50 μl of proteinase K solution (4 % SDS; 1 mg/ml proteinase K; 0.4 mg/ml tRNA) was added to each and the samples were incubated for an additional 15 minutes at 37°C. The solutions were extracted with phenol, precipitated with ethanol and resuspended in 90% formamide dye. The protected fragments were separated on standard sequencing gels. The gels were vacuum dried and exposed to Kodak XAR-5 film for 2-3 days at −70°C with intensifying screens in RNase protections with homeobox probes, or for 15 hours at room temperature in RNase protections with an EF-1α probe. All RNA samples were initially normalized by measurement of their absorbance at 260 nm. The autoradiographic intensity of the protected fragments was quantitated by use of a Molecular Dynamics densitometer. The signals were normalized with reference to the signals of an EF-1α probe for each sample.

RESULTS

Isolation and sequence of the newt *Hox-4.5* and *Hox-3.6* cDNAs

In order to identify homeobox-containing genes in the newt, we screened a mixed forelimb and hindlimb blastema cDNA library with a fully degenerate oligonucleotide probe to the most conserved motif in helix 3 of the homeodomain (see Materials and methods). Southern analysis and sequence analysis of the resulting hybridizing colonies identified four distinct groups of cDNAs containing a homeobox. Two of these cDNA groups were chosen for this study. The predicted amino acid sequence of the homeodomain of one of these cDNA groups was found to have the highest degree of homology to those of the mouse *Hox-4.5* and the human *HOX 4D* genes (98.3% and 100% identical, respectively at the amino acid level) as shown in Fig. 2. Three overlapping *Hox-4.5* cDNAs were isolated. Two of them (1.62 kb and 0.82 kb) lack a poly(A) tail whereas the third clone (0.84 kb) represents a polyadenylated cDNA. The composite newt *Hox-4.5* cDNA has an open reading frame of 356 amino acids and a 3′ untranslated portion of 581 bp (Fig. 3). A potential initiator methionine is located 36 nt downstream of the 5′ end of the cDNA. Its sequence context fits the consensus sequence (-$^{A}/_{G}$NNATGPu-) established for many eukaryotic translation start sites (Kozak, 1987). This suggested protein start site is in perfect agreement with the one found for the cognate gene in the mouse (Renucci et al., 1992). The second group of cDNA isolates is represented by two overlapping clones. The analysis of their homeodomain reveals the highest degree of homology to the homeodomain of mouse *Hox-3.6* and human *HOX 3I* genes (98.3% and 96.6% identical respectively on the amino acid level) as shown in Fig. 2. Both clones (1.4 kb and 1 kb) span the homeobox region,

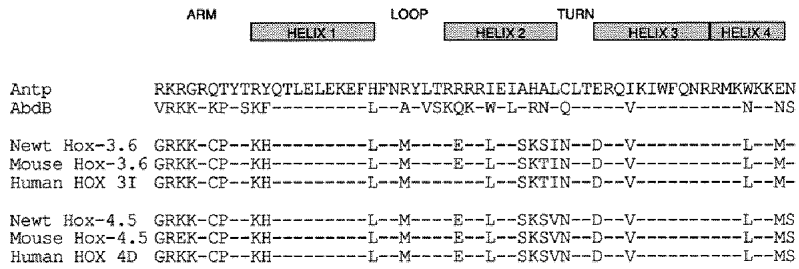

Fig. 2. Comparison of the *Drosophila Antp* and *AbdB* homeodomains to the newt, mouse and human *Hox-3.6* and *Hox-4.5* homeodomains. The amino acid sequence of the *Drosophila Antp* homeodomain is given in the one letter code. In case of the other homeodomains only the residues differing from the *Antp* sequence are shown, identical amino acids are represented by a dash. Sequences are from: *Antp* (McGinnis et al., 1984), *AbdB* (Regulski et al., 1985), newt *Hox-3.6* and *Hox-4.5* (this study), mouse *Hox-3.6* (Peterson et al., 1992), human *HOX* 3I (Boncinelli et al., 1991), mouse *Hox-4.5* (Duboule and Dolle, 1989), and human *HOX* 4D (Zappavigna et al., 1991). On top the overall structure of the homeodomain is indicated (Otting et al., 1990).

but lack a 3′ poly(A) tail and do not cover the complete *Hox-3.6* protein as recently reported in the mouse system (Peterson et al., 1992). Thus, for the newt *Hox-3.6* only partial cDNA clones were obtained (Fig. 4).

Hox-3.6 and *Hox-4.5* are paralogous Hox genes, meaning they are evolutionary homologues in the *Hox-3* and *Hox-4* gene clusters. When we compared the predicted amino acid sequence of the newt *Hox-4.5* and *Hox-3.6*, we found their homeodomains to be highly conserved (58 out of 60 amino acids and 96.6%, respectively) as shown in Fig. 2. Further evidence for this relationship can be found in a conserved tryptophan (W) residue at position −6 from the N-terminal end of the homeodomain. Like most members of the *AbdB*-like subfamily, the mouse, human and newt *Hox-4.5* and *Hox-3.6* isolates have this amino acid (Izpisua-Belmonte et al., 1991).

The composite sequence of the newt *Hox-4.5* gene appears to represent the complete coding region (Fig. 3). The alignment of the entire newt *Hox-4.5* and its mouse cognate gene revealed a high identity of the protein around the aminoterminus that extends for the first 60-70 amino acids, and across the carboxyterminal homeodomain down to the termination codon (16 amino acids 3′ of the homeodomain), which is further extended 5′ of the homeobox for about 35 amino acids. Although we find a Pro/Tyr motif in the newt gene at a similar position upstream of the homeodomain as reported for its mouse *Hox-4.5* homolog and other *AbdB* genes (Izpisua-Belmonte et al., 1991; Renucci et al., 1992), their sequences diverge markedly between the aminoterminus and the homeodomain. Besides a different amino acid composition, we found deletions and insertions of amino acid residues in the newt gene; most striking is an insertion of 7 glutamines (Q) at position −67 with respect to the homeodomain. The comparison of the newt *Hox-3.6* and its mouse cognate gene is not as complete because the cDNA clones that we obtained do not represent the entire

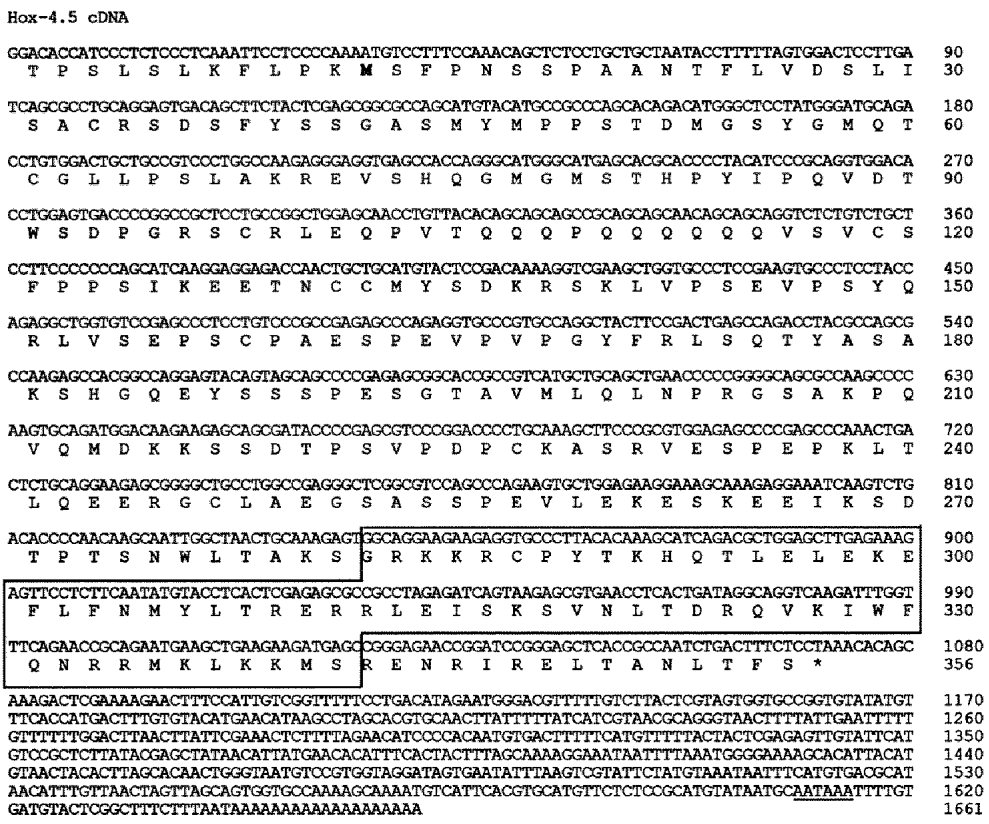

Fig. 3. Structure of the newt *Hox-4.5* cDNA. Nucleotide and predicted amino acid sequence of the composite newt *Hox-4.5* cDNA. The potential initiator methionine (M) is shown in bold print. The homeobox domain is boxed. The location of the polyadenylation signal (AATAAA) is underlined. The sequence data are available from the EMBL data library, accession number X68976.

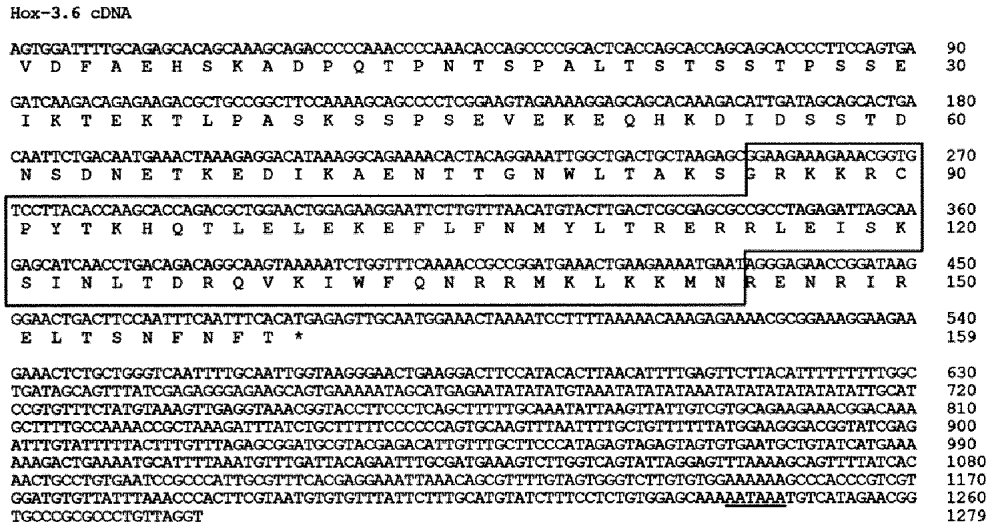

Fig. 4. Structure of the newt *Hox-3.6* cDNAs. Nucleotide and predicted amino acid sequence of the newt *Hox-3.6* cDNA. The homeobox domain is boxed. The location of a potential polyadenylation signal (AATAAA) is underlined. The sequence data are available from the EMBL data library, accession number X68975.

coding region (Fig. 4). Nonetheless, the conservation observed between the newt and mouse *Hox-3.6* homeodomains is extended on the carboxyterminal portion of the protein flanking the homeodomain down to the termination codon (16 amino acids 3′ of the homedomain), and on the aminoterminal side of the homeodomain for about 23 amino acids. Although the predicted newt and mouse proteins are virtually identical at their carboxyterminus, the peptide sequence 5′ of the homeodomain appears to be very diverged.

Tissue distribution of the *Hox-4.5* transcript and the *Hox-3.6* transcript

To obtain an initial indication of the transcriptional regulation of the *Hox-4.5* and *Hox-3.6* genes, northern analysis was performed using poly(A)$^+$ RNA from unamputated (normal) forelimbs, blastema forelimbs, unamputated (normal) hindlimbs, blastema hindlimbs and liver. After hybridization with a *Hox-4.5* probe (C8.2), a single band of ~1.8 kb was visible in RNA from forelimb and hindlimb blastemas, but no signal was detected in RNA from unamputated forelimbs, unamputated hindlimbs and liver (Fig. 5A). Thus, like *Hox-4.6*, *Hox-4.5* appears to be regeneration specific. A *Hox-3.6* probe (A6.1) revealed a band of ~2.3 kb in RNA from unamputated hindlimbs and blastema hindlimbs. No transcripts were found in RNA from unamputated forelimbs, blastema forelimbs, or liver. Thus, like *Hox-3.3*, *Hox-3.6* is expressed in unamputated and regenerating tissue but is specific to one limb territory (the hindlimb in the case of *Hox-3.6*). The RNA samples were normalized by reference to transcripts detected with a newt translation elongation factor 1-α (EF-1α) probe. EF-1α was found to be equally expressed in all newt tissues tested (H.-G. Simon, unpublished data).

To analyse further the expression of *Hox-4.5* and *Hox-3.6* in a variety of organs, we performed RNase protection experiments. The RNase protections were done with preparations of total RNA. The individual samples were normalized by optical density and by RNase protection analysis with a probe for the newt EF-1α. As shown in Fig. 5B, we could not detect protected fragments of the *Hox-4.5* and *Hox-3.6* probes in any organ except kidney. While the expression of these homeobox genes in adult tissues is very limited, it is striking that the genes are expressed at comparable levels in the kidney.

Temporal expression pattern of *Hox-4.5* and of *Hox-3.6*

Because it is quantitative and more sensitive than northern analysis, we used RNase protection to evaluate the expression profile of *Hox-4.5* and *Hox-3.6* during regeneration (Figs 6A, 7A). The differences in expression of *Hox-4.5* and *Hox-3.6* in unamputated and blastema tissues from forelimbs and hindlimbs was confirmed by the RNase protection analysis. With a *Hox-4.5* probe (Fig. 6B), we did not detect a protected band in unamputated forelimbs. After amputating forelimbs at a proximal level (mid humerus), we found a strong induction of the message when the regenerates reached mid-bud stage blastemas (BFL(P)) (when pattern is believed to be specified). In early digit stage regenerates of forelimbs (DFL(P)), we saw a much weaker expression and, in fully regenerated forelimbs (RFL(P)), no *Hox-4.5* message was detectable. When we examined the expression profile of *Hox-3.6* (Fig. 7B), a protected band was found in unamputated hindlimbs. After proximal amputation (mid femur) of the hindlimbs, an increased level of the *Hox-3.6* message was visible in mid-bud stage blastemas (BHL(P)). In digit stage regenerates (DHL(P)) and fully regenerated hindlimbs (RHL(P)), the level of expression was found to be significantly reduced and equivalent to the expression level detected in unamputated hindlimbs.

Spatial expression pattern of *Hox-4.5* and of *Hox-3.6*

In order to analyze the spatial expression of *Hox-4.5* and *Hox-3.6* in newt appendages, we obtained tissue samples following amputation at various proximodistal levels (Fig. 8A for *Hox-4.5* and Fig. 9A for *Hox-3.6*). Fig. 8B shows the RNase protection analysis with the *Hox-4.5* RNA probe. No protected fragment was observed in total RNA preparations derived from distal (NFL(D)) and proximal (NFL(P)) segments of unamputated (normal) forelimbs,

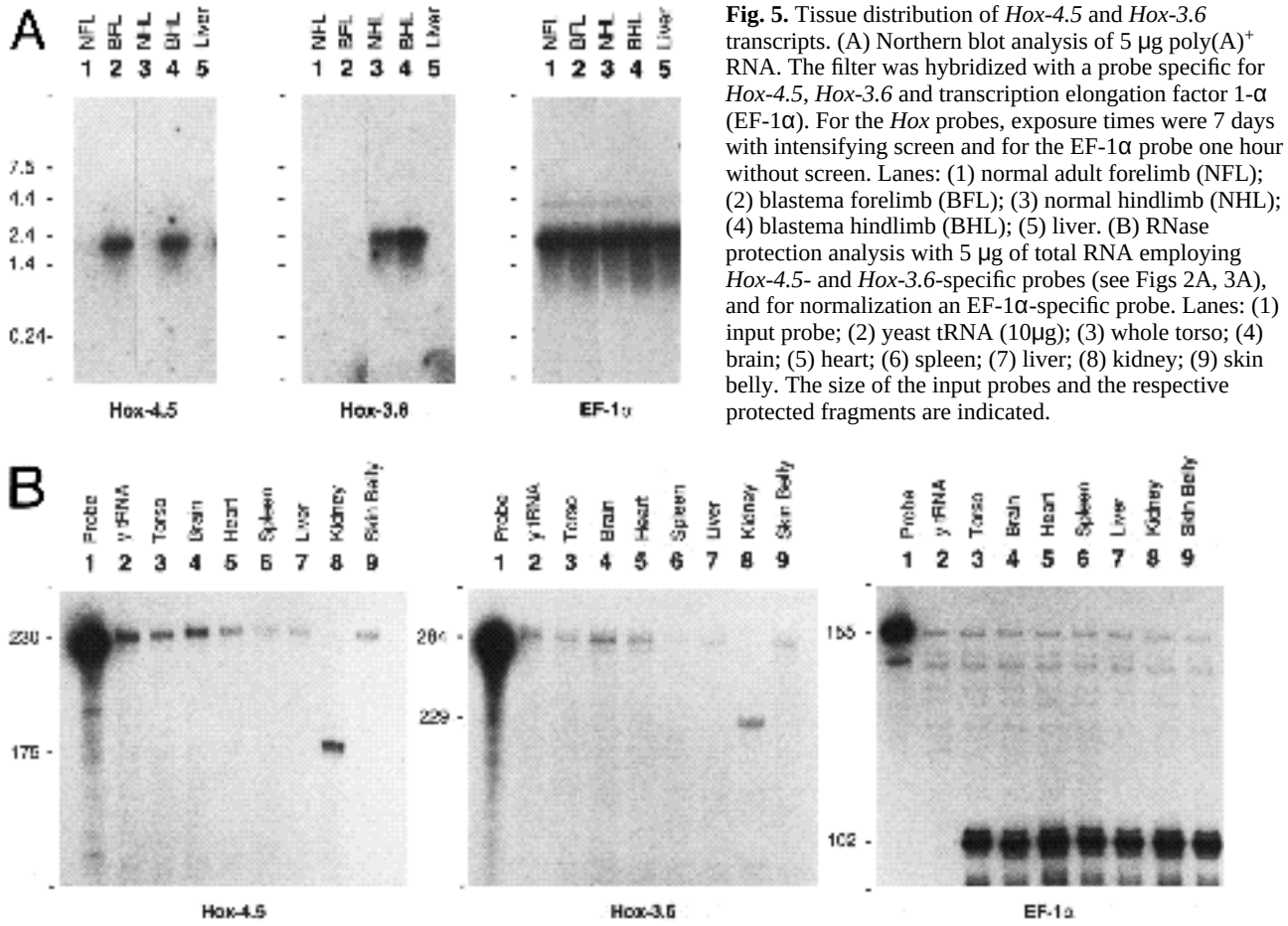

Fig. 5. Tissue distribution of *Hox-4.5* and *Hox-3.6* transcripts. (A) Northern blot analysis of 5 μg poly(A)$^+$ RNA. The filter was hybridized with a probe specific for *Hox-4.5*, *Hox-3.6* and transcription elongation factor 1-α (EF-1α). For the *Hox* probes, exposure times were 7 days with intensifying screen and for the EF-1α probe one hour without screen. Lanes: (1) normal adult forelimb (NFL); (2) blastema forelimb (BFL); (3) normal hindlimb (NHL); (4) blastema hindlimb (BHL); (5) liver. (B) RNase protection analysis with 5 μg of total RNA employing *Hox-4.5*- and *Hox-3.6*-specific probes (see Figs 2A, 3A), and for normalization an EF-1α-specific probe. Lanes: (1) input probe; (2) yeast tRNA (10μg); (3) whole torso; (4) brain; (5) heart; (6) spleen; (7) liver; (8) kidney; (9) skin belly. The size of the input probes and the respective protected fragments are indicated.

although after long exposures a faint signal was detectable in the proximal segments of unamputated forelimbs. When we compared the *Hox-4.5* expression in distal (BFL(D)) and proximal (BFL(P)) blastemas, we found in repeated experiments a 4- to 5-fold higher level in the proximal blastemas. In normal tail, we could not detect a protected band and, in tail blastemas, only after long exposures was a faint signal visible. It has been shown by transplantation experiments that the information for producing limb pattern resides in the mesodermally derived 'blastema' cells rather than in the wound epidermis that covers them (Stocum and Dearlove, 1972). It was therefore important to ascertain whether *Hox-4.5* is expressed in the mesenchymal blastema cells. Proximal hindlimb mid-bud blastemas (BHL(P)) were dissected into epithelial (Epi) and mesenchymal (Mes) fragments. When the two preparations were analyzed by RNase protection, expression of *Hox-4.5* was observed only in the blastema mesenchyme.

Fig. 9B shows the RNase protection analysis with the *Hox-3.6* RNA probe. In unamputated (normal) hindlimbs, we observed a higher level of *Hox-3.6* expression in proximal than distal limb segments. RNA from proximal (BHL(P)) and distal (BHL(D)) blastemas revealed a protected fragment similar in intensity. *Hox-3.6* is expressed in normal tail and in regenerating tail as well, and the level of expression in the tail blastema seemed to be slightly higher than in the normal tail. As with *Hox-4.5*, we analyzed the expression of *Hox-3.6* in dissected epithelial and mesenchymal fractions of proximal hindlimb blastemas. In the hindlimb blastema, *Hox-3.6* is expressed exclusively in the mesenchymal but not in the epithelial tissue. The northern analysis indicated a restricted expression of *Hox-3.6* in the hindlimb territory of the newt. To test how far the expression of *Hox-3.6* extends anteriorly along the flank, we included surgically dissected flank tissue into the spatial expression analysis. No protected fragment was detected in anterior or posterior flank tissue. The very faint signals for *Hox-4.5* and *Hox-3.6* observed in the epithelial tissue is most likely due to slight contamination of mesenchymal tissue in the preparation of the two tissue fractions.

Effect of RA on *Hox-4.5* expression

When we analyzed the expression of *Hox-4.5* along the proximodistal axis of the regenerating limb, a reproducibly 4- to 5-fold higher level of expression was observed in RNA from proximal blastemas. This raised the possibility that *Hox-4.5* might be involved in specifying proximodistal positional information to a regenerating limb. RA is said to 'proximalize' the blastema's positional values because a limb treated with exogenous RA will regenerate as if it had been amputated at a more proximal level. We therefore examined the effect of RA on the level of *Hox-4.5* RNA. Newts were amputated distally at the level of the mid forearm (mid-radius/ulna) (Fig. 10A). 5 days later the animals were injected with a proximalizing dose of RA dissolved in DMSO or with DMSO alone. The blastemas were har-

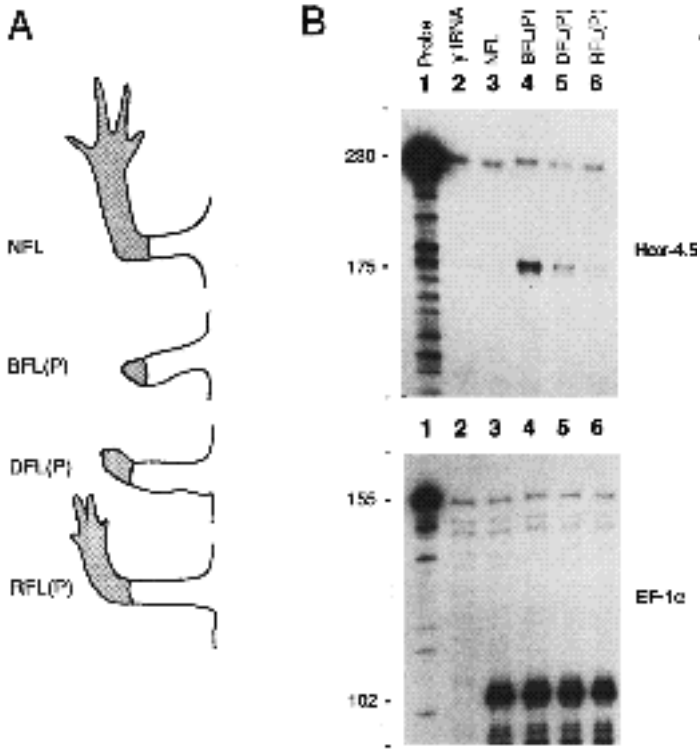

Fig. 6. Temporal expression pattern of *Hox-4.5*. (A) Schematic diagram representing the tissue (shaded) that has been used for RNA preparation. (B) RNase protection analysis with a *Hox-4.5*-specific probe and in parallel with the normalizing EF-1α probe (see Materials and methods). Lanes: (1) input probe; (2) yeast tRNA; (3) normal forelimb (NFL); (4) proximal blastema forelimb (BFL(P)); (5) proximal digit-stage forelimb (DFL(P)); (6) proximal regenerated forelimb (RFL(P)). The size of the input probes and the protected fragments are shown.

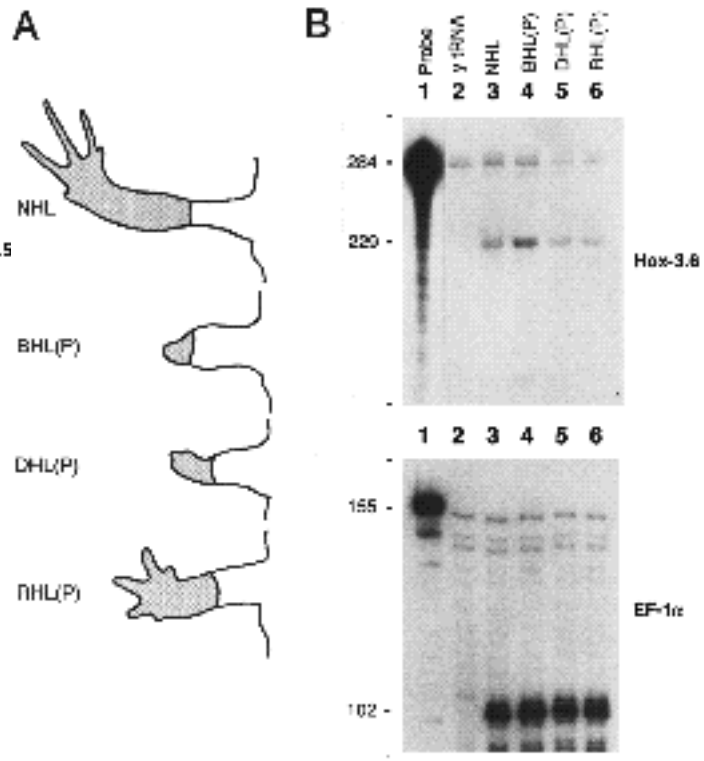

Fig. 7. Temporal expression pattern of *Hox-3.6*. (A) Schematic diagram representing the tissue (shaded) that has been used for RNA preparation. (B) RNase protection analysis with a *Hox-3.6*-specific probe and in parallel with the normalizing EF-1α probe (see Materials and methods). Lanes: (1) input probe; (2) yeast tRNA; (3) normal hindlimb (NHL); (4) proximal blastema hindlimb (BHL(P)); (5) proximal digit-stage hindlimb (DHL(P)); (6) proximal regenerated hindlimb (RHL(P)). The size of the input probes and the protected fragments are shown.

vested at an early time (day 7), and at a later time when they had reached the mid-bud stage (day 20/day 15). As shown in Fig. 10B, a 2- to 3-fold increased level of expression is observed in the early blastemas of RA-treated animals compared to the DMSO-treated control animals. RA had no effect on the expression level of *Hox-4.5* in the mid-bud stage blastemas. For comparison, the expression level of *Hox-4.5* in proximal and distal mid-bud blastemas of untreated animals is shown. Thus, RA proximalizes the expression of *Hox-4.5* at an early time point in regeneration, when blastema cells are recruited from underlying stump tissue and start proliferation.

This result is in contrast to the reported expression of *Hox-4.6*, whose expression was found not to be affected by RA treatment (Brown and Brockes, 1991). Since both are adjacent genes in the same *Hox* cluster, and considering the low level of effect that we observed with *Hox-4.5* (2- to 3-fold), we wanted to verify this difference when compared under identical experimental conditions. Identical RNA samples from each time point were used in RNase protections with a *Hox-4.6* probe (5′22) obtained from R. Brown and J. Brockes. No differences were observed between RA-treated and DMSO controls at any time point (data not shown) consistent with earlier reports.

DISCUSSION

We have used the newt *Hox-4.5* and *Hox-3.6* genes as a model system for studying the expression of paralogous homeobox genes during limb regeneration. Upon amputation, expression of *Hox-4.5* is reactivated and *Hox-3.6* is upregulated in the regenerating limb. The expression of both is highest in the undifferentiated mid-bud blastema, the time when the pattern is thought to be respecified. Both newt homeobox genes described in this paper are expressed in the mesenchymal blastema cells, which are known to determine pattern during regeneration (Stocum and Dearlove, 1972), and not in the overlying ectoderm. Thus, *Hox-4.5* and *Hox-3.6* are expressed in the correct cell type and in a time window to be expected of a gene involved in the specification of pattern during regeneration. Besides these common expression features, there are significant differences in the regulation of the patterns of *Hox-4.5* and *Hox-3.6* expression in the regenerating newt.

Our expression data support the existence of a posteriorly restricted expression domain along the longitudinal body axis for *Hox-3.6* similar to that shown in the mouse embryo (Peterson et al., 1992). Because the level of expression of *Hox-3.6* and *Hox-4.5* in the newt blastema is

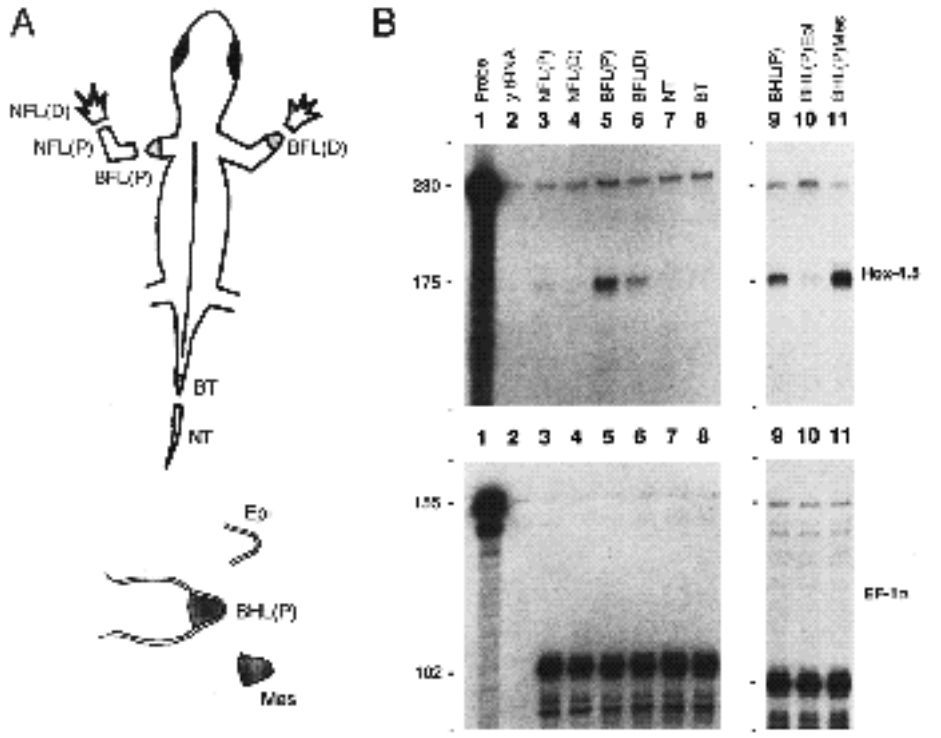

Fig. 8. Spatial expression pattern of *Hox-4.5*. (A) Schematic diagram of dissection of tissue for RNA preparation. (B) RNase protection analysis of *Hox-4.5* expression in respective tissue samples. RNA samples were analysed with a *Hox-4.5*-specific probe and normalized with the EF-1α probe (see Materials and methods). Lanes: (1) input probe; (2) yeast tRNA; (3) proximal normal forelimb (NFL(P)); (4) distal normal forelimb (NFL(D)); (5) proximal blastema forelimb (BFL(P)); (6) distal blastema forelimb (BFL(D)); (7) normal tail (NT); (8) blastema tail (BT); (9) proximal blastema hindlimb (BHL(P)); (10) proximal blastema hindlimb - epidermis (BHL(P)Epi); (11) proximal blastema hindlimb - mesenchyme (BHL(P)Mes). The size of the input probes and the protected fragments are indicated.

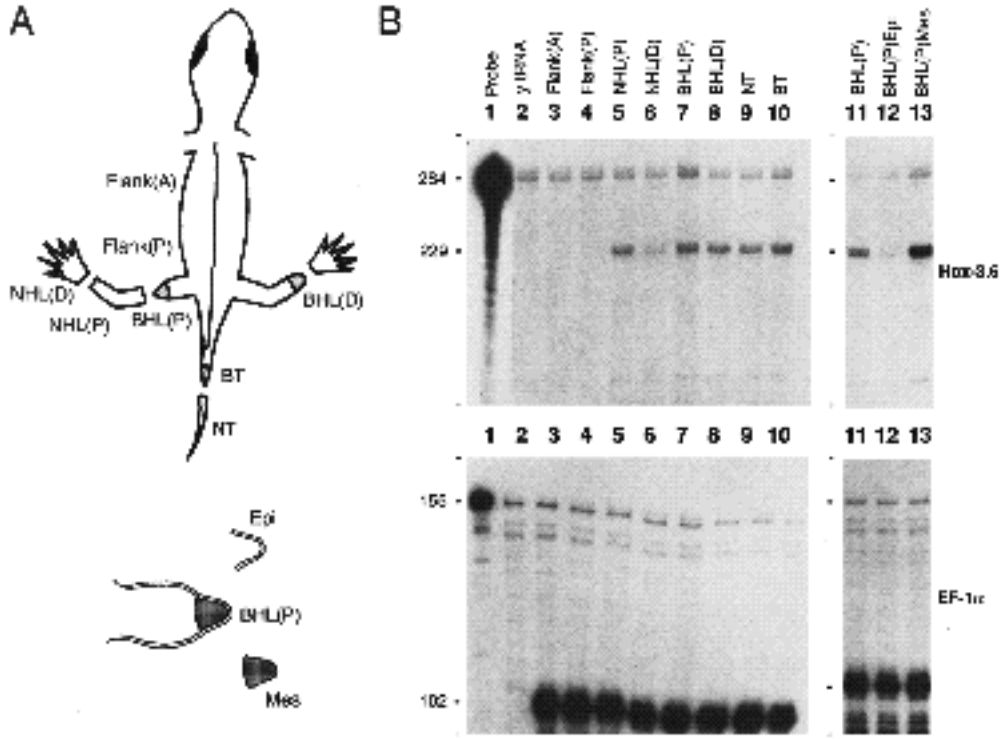

Fig. 9. Spatial expression pattern of *Hox-3.6*. (A) Schematic diagram of dissection of tissue for RNA preparation. (B) RNase protection analysis of *Hox-3.6* expression in respective tissue samples. RNA samples were analysed with a *Hox-3.6*-specific probe and normalized with the EF-1α probe (see Materials and methods). Lanes: (1) input probe; (2) yeast tRNA; (3) anterior flank tissue (Flank (A)); (4) posterior flank tissue (Flank (P)); (5) proximal normal hindlimb (NHL(P)); (6) distal normal hindlimb (NHL(D)); (7) proximal blastema hindlimb (BHL(P)); (8) distal blastema hindlimb (BHL(D)); (9) normal tail (NT); (10) blastema tail (BT); (11) proximal blastema hindlimb (BHL(P)); (12) proximal blastema hindlimb - epidermis (BHL(P)Epi); (13) proximal blastema hindlimb - mesenchyme (BHL(P)Mes). The size of the input probes and the protected fragments are indicated.

extremely low, we have not been able to localize their mRNA by in situ hybridization. Based on RNase protection, *Hox-3.6* is expressed in the adult hindlimb and tail of the newt, in addition to the regenerating blastema. A similar persistent expression in adult newt tissue has been described for *Hox-3.3* ((NvHbox1) Savard et al., 1988; (FH-2) Tabin, 1989), and it has been speculated that the expression of this gene in adult tissue might be related to the

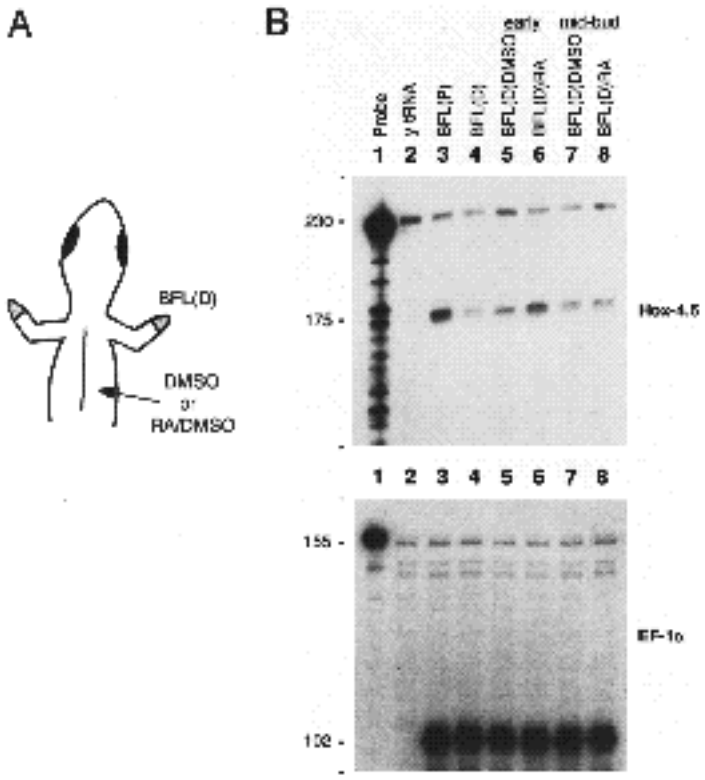

Fig. 10. Expression of *Hox-4.5* after retinoic acid treatment. (A) Schematic diagram of experimental design. Groups of newts with distal forelimb amputations were injected intraperitoneally either with a proximalizing dose of RA dissolved in DMSO or with DMSO alone. Blastemas from RA- and DMSO-treated animals were harvested at two different time points for RNA preparation (see Materials and methods). (B) RNase protection analysis of respective RNA samples with a *Hox-4.5* probe. For normalization the RNA samples were hybridized to the EF-1α probe. Lanes: (1) input probe; (2) yeast tRNA: (3) proximal blastema forelimb (BFL(P)); (4) distal blastema forelimb (BFL(D)); distal forelimb blastemas (5) DMSO group, early; (6) RA group, early; (7) DMSO group, mid-bud; (8) RA group, mid-bud. For reference samples (3) and (4) see also Fig. 8. The size of the input probes and the protected fragments are indicated.

regenerative ability of urodele amphibians since the *Hox* genes are not expressed in other non-regenerating adult vertebrates (Savard et al., 1988). The data presented here might suggest that the continued expression may be a general property of genes of the *Hox-3* cluster in adults capable of regeneration.

In contrast to *Hox-3.6*, the *Hox-4.5* gene is not expressed in unamputated appendages of the newt. After amputation, *Hox-4.5* gene expression is detected in regenerating fore- and hindlimbs. The expression of *Hox-4.5* is significantly higher in a proximal mid-bud blastema than a distal one. A very similar expression pattern in limb regeneration has been reported for *Hox-4.6*, the adjacent gene in the *Hox-4* cluster (Brown and Brockes, 1991). Like *Hox-4.5*, *Hox-4.6* is expressed in the limb blastema, but not in the normal limb. In addition *Hox-4.6* is clearly expressed in the tail blastema; in contrast, we did not find evidence of *Hox-4.5* expression in regenerating tail. Thus, the A/P domain of expression of *Hox-4.6* seems to be posterior to *Hox-4.5*. Since *Hox-4.5* is 3′ of *Hox-4.6* within the *Hox-4* cluster, this is in agreement with the general finding of a colinear relationship between the structural order of *Hox* genes within their respective cluster and the order of anterior expression limits along the longitudinal body axis (Gaunt et al., 1988; Duboule and Dolle, 1989).

According to a model on how positional information is encoded in blastemas, cells along the adult limb maintain positional values that were set up during embryonic development (Stocum, 1984). In regeneration new positional values are generated distal to the cut surface, which means that the structures of the regenerate depend on the inherent positional values at the amputation plane. Since there is a difference in *Hox-4.5* expression between proximal and distal blastemas, *Hox-4.5* expression could play a role of providing positional information. To test this, we examined whether the level of expression in a distal blastema could be altered by a dose of RA that proximalized the blastema's positional identity. In several previous experiments, RA was not found to alter *Hox* gene expression during regeneration. However, some of these analyzed expression levels at a single time point, when the blastemas had reached a mid-bud stage (Savard et al., 1988; Tabin, 1989). In this study, we in addition analyzed the effect of RA on distal blastemas very early in regeneration, and were indeed able to detect an increased *Hox-4.5* transcript level in RA-treated blastemas relative to control blastemas at this early time point but not at the later one (mid-bud-stage blastemas). This result is striking because it is the first time that any blastemal cDNA has been isolated for a gene whose expression is regulated by RA in a direction consistent with it providing axial positional information. When Brown and Brockes (1991) examined the effect of RA on the newt *Hox-4.6* gene in a very similar experimental design, they could not detect any change of expression level. The difference in responsiveness of the two *Hox* genes is unexpected since they are directly adjacent in the same *Hox-4* cluster. It was possible though, that the contrary results were related to minor differences in the experimental protocols. When *Hox-4.6* expression was analyzed under identical conditions, we saw no evidence for regulation by RA, confirming Brown and Brockes' results. They speculated on a possible transient change of *Hox-4.6* provoked by RA, which they might have missed in their analysis. We in fact provide evidence for a very early transient effect of RA on *Hox-4.5* gene expression in a defined time window, when blastema cells are still being recruited by dedifferentiation and just start

proliferation. A slightly different time window might reveal RA regulation of *Hox-4.6*. The finding that RA proximalizes the expression of *Hox-4.5*, at least transiently, suggests that endogenous RA levels might play a role in specification of pattern along the proximodistal (PD) axis. Regardless of whether or not this turns out to be correct, the fact that the pharmacological RA treatment of regenerating limbs results in concurrent shifts in *Hox-4.5* expression and regenerative pattern supports the model that *Hox-4.5* plays a role in the specification of limb axis.

The comparison of the expression patterns of the two paralogous genes *Hox-3.6* and *Hox-4.5* in limb regeneration demonstrates that the regulation of *Hox-3.6* is similar to that of *Hox-3.3* while the regulation of *Hox-4.5* is similar to that of *Hox-4.6*. In view of this, and considering the current information on the expression of *Hox-3* and *Hox-4* genes in limb development, it seems likely that different *Hox* clusters address different functional roles in development. The genes from different parts of the *Hox-3* cluster (*AbdB*-like and *Antp*-like genes) might be spatially regulated to specify the morphogenesis of specific body parts, whereas the 5′ *Hox-4* genes (*AbdB*-like genes) appear to be involved in the generation of positional values in axis formation. The fact that the expression of *Hox-3* genes is maintained in adult newt limbs suggests that the positional memory they provide, differentiating forelimbs from hindlimbs, must be retained to initiate the proper regeneration of appendages. Conversely, the fact that *Hox-4* genes are only reactivated upon amputation implies that the positional values they specify in both limbs can be autonomously established within the regenerating limb field.

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REFERENCES

Akam, M. E. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development* **101**, 1-22.

Akam, M. E. (1989). Hox and HOM: homologous gene clusters in insects and vertebrates. *Cell* **57**, 347- 349.

Aruffo, A. and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. natn. Acad. Sci. USA* **84**, 8573-8577.

Belleville, S., Beauchemin, M., Tremblay, M., Noiseux, N. and Savard, P. (1992). Homeobox-containing genes in the newt are organized in clusters similar to other vertebrates. *Gene* **114**, 179-186.

Boncinelli, E., Acampora, D., Pannese, M., D'Esposito, M., Somma, R., Gaudino, G., Stornaiuolo, A., Cafiero, M., Faiella, A. and Simeone, A. (1989). Organization of human class I homeobox genes. *Genome* **31**, 745-756.

Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F. (1991). HOX gene activation by retinoic acid. *Trends Genet.* **7**, 329-334.

Brockes, J. P. (1989). Retionoids, homeobox genes, and limb morphogenesis. *Neuron* **2**, 1285-1294.

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

Burglin, T. R., Finney, M., Coulson, A. and Ruvkun, G. (1989). *Caenorhabditis elegans* has scores of homeobox-containing genes. *Nature* **341**, 239-243.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acid Res.* **12,** 387-395.

Dolle, P., Izpisua-Belmonte, J. C., Falkenstein, H., Renucci, A. and Duboule, D. (1989). Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature* **342**, 767-772.

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucl. Acids Res.* **16**, 6127-6145.

Duboule, D. and Dolle, P. (1989). The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. *EMBO J.* **8**, 1497-1505.

Gaunt, S. J., Sharpe, P. T. and Duboule, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. In *Mechanisms of Segmentation* (ed. V. French, P. Ingham, J. Cooke and J. Smith) *Development* **104**, 169-179.

Gaunt, S. J. (1991). Expression patterns of mouse *Hox* genes: Clues to an understanding of developmental and evolutionary strategies. *BioEssays*. **13**, 505-513.

Goulding, M. D. and Gruss, P. (1989). The homeobox in vertebrate development. *Current Opinion in Cell Biology* **1**, 1088-1093.

Graham, A., Papalopulu, N. and Krumlauf, R. (1989). The murine and *Drosophila* homeobox clusters have common features of organization and expression. *Cell* **57**, 367-378.

Izpisua-Belmonte, J. C., Falkenstein, H., Dolle, P., Renucci., A. and Duboule, D. (1991). Murine genes related to the Drosophila AbdB homeotic gene are sequentially expressed during development of the posterior part of the body. *EMBO J.* **10**, 2279-2289.

Kappen, C., Schughart, K. and Ruddle, F. (1989). Two steps in the evolution of antennapedia-class vertebrate homeobox genes. *Proc. Natn. Acad. Sci. USA.* **86**, 5459-5463.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucl. Acids Res.* **15**, 8125-8131.

Krieg, P. A., Varnum, S. M., Wormington, W. M. and Melton, D. A. (1989). The mRNA encoding elongation factor 1-α (EF-1α) is a major transcript at the mid blastula transition in *Xenopus. Dev. Biol.* **133**, 93-100.

Krumlauf, R. (1992). Evolution of the Vertebrate Hox Homeobox genes. *BioEssays* **14**, 245-252.

Maden, M. (1982). Vitamin A and pattern formation in the limb. *Nature* **295**, 672-675.

Maden, M. (1983). The effect of vitamin A on the regenerating axolotl limb. *J. Embryol. Exp. Morph.* **77**, 273-295.

McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A. and Gehring, W. J. (1984). A conserved DNA sequence in homeotic genes of the *Drosophila Antennapedia* and *Bithorax* complexes. *Nature* **308**, 428-433.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**, 7035-7056.

Muneoka, K. and Bryant, S. V. (1984). Cellular contribution to supernumerary limbs resulting from the interaction between developing and regenerating tissues in the axolotl. *Dev. Biol.* **105**, 179-187.

Niazi, I. A. and Saxena, S. (1978). Abnormal hind limb regeneration in tadpoles of the toad, *Bufo andersoni*, exposed to excess vitamin A. *Folia biol*. (Krakow) **26**, 3-11.

Nusslein-Volhardt, C., Frohnhofer, H. G. and Lehman, R. (1987). Determination of anterioposterior polarity in the *Drosophila* embryo. *Science* **238**, 1675-1681.

Otting, G., Qian, Y. Q., Billeter, M. Muller, M., Affolter, M., Gehring, W. J. and Wuthrich, K. (1990). Protein-DNA contacts in the structure of a homeodomain-DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J.* **9**, 3085-3092.

Peterson, R. L., Jacobs, D. F. and Awgulewitsch, A. (1992). Hox-3.6: isolation and characterization of a new murine homeobox gene located in the 5′ region of the Hox-3 cluster. *Mechan. Dev.* **37**, 151-166.

Regulski, M. Harding, K., Kostriken, R., Karch, F., Levine, M. and McGinnis, W. (1985). Homeo box genes of the Antennapedia and Bithorax complexes of *Drosophila. Cell* **43**, 71-80.

Renucci, A., Zappavigna, V., Zakany, J., Izpisua-Belmonte, J. C., Burki, K. and Duboule, D. (1992). Comparison of mouse and human HOX-4 complexes defines conserved sequences involved in the regulation of Hox-4.4. *EMBO J.* **11**, 1459-1468.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Savard, P., Gates, P. B. and Brockes, J. P. (1988). Position dependent expression of a homeobox gene transcript in relation to amphibian limb regeneration. *EMBO J.* **7**, 4275-4282.

Schughart, K., Kappen, C. and Ruddle. F. (1989). Duplication of large genomic regions during the evolution of vertebrate homeobox genes. *Proc. Natn. Acad. Sci. USA.* **86**, 7067-7071.

Stocum, D. L. and Dearlove, G. E. (1972). Epidermal-mesodermal interaction during morphogenesis of the limb regeneration blastema in larval salamanders. *J. Exp. Zool.* **181**, 49-61.

Stocum, D. L. (1984). The urodele limb regeneration blastema. Determination and organization of the morphogenetic field. *Differentiation* **27**, 13-28.

Stocum, D. L. and Crawford, K. (1987). Use of retinoids to analyse the cellular basis of positional memory in regenerating amphibian limbs. *Biochem. Cell Biol.* **65**, 750-761.

Tabin, C. J. (1989). Isolation of potential vertebrate limb-identity genes. *Development* **105**, 813-820.

Wallace, H. (1981). *Vertebrate Limb Regeneration.* Chichester: John Wiley and Sons.

Wright, C. V. E., Cho, K. Y. W., Oliver, G. and DeRobertis, E. M. (1989). Vertebrate homeodomain proteins: families of region-specific transcription factors. *Trends Biochem. Sci.* **14**, 52-56.

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

Zappavigna, V., Renucci, A., Izpisua-Belmonte, J. C., Urier, G., Peschle, C. and Duboule, D. (1991). HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. *EMBO J.* **10**, 4177-4187.

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