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Original Article

Expression of Hox A11 in the limb and the regeneration blastema of adult newt

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ABSTRACT Homeoproteins are functionally involved in pattern formation of developing systems and are potentially good candidates to regulate positional information during limb regeneration in the newt. Here we report the molecular structure of Hox A11 and its pattern of expression during the regeneration of adult newt appendages. The transcriptional unit of the gene is composed of two exons separated by an intron. Northern blots revealed two major transcripts; a size difference would result from using two different polyadenylation signals. Therefore, the gene would encode a single protein that is very homologous to other vertebrate counterparts. The pattern of expression of Hox A11 in the adult newt shows interesting findings in relation to limb regeneration. First, expression is found in both intact limb and tail, showing maintenance of expression of an important regulator of development in the appendages of the adult newt. Second, Hox A11 is expressed mainly in the muscle and the bone of intact limbs, two tissue fractions known to participate in blastemal fate determination. Third, the level of Hox A11 expression increases drastically in both limb and tail regeneration blastemas, suggesting that the population of expressing cells is preferentially recruited during blastema formation. Finally, proximal blastemas (mid-humerus) significantly express higher levels of transcript compared with distal ones (mid-radius and ulna). These features of expression suggest that Hox A11 may participate in limb pattern formation by specifying positional information to the progenitor cells of the regenerate.

KEY WORDS: *homeobox, Notophthalmus viridescens, positional information, regeneration, urodele*

Introduction

Many homeobox genes show patterns of expression during limb development that suggest their participation in pattern formation by providing cells with a molecular address that identifies their spatial identity (Oliver *et al.*, 1988; Hill *et al.*, 1989; Robert *et al.*, 1989; Davis *et al.*, 1991; Nohno *et al.*, 1991). For example, overlapping and complementary gradients of homeobox transcripts are spread along the principal axes of the limb bud, resulting in different regions of the limb expressing different combinations of homeobox genes (Dollé *et al.*, 1989; Oliver *et al.*, 1989; Izpisúa-Belmonte *et al.*, 1991). Experimental manipulations that respecify limb patterning also respecify Hox domains of expression in the limb bud, demonstrating the existence of a relationship between the code of Hox genes expressed by a cell and its developmental determination (Izpisúa-Belmonte and Duboule, 1992; Morgan *et al.*, 1992).

The spatial expression pattern in the murine and chick limb bud of genes of the Hox A cluster complies with the rule of structural colinearity whereby the spatial and temporal restriction of the expression domain of a Hox A gene is related to its position within the cluster (Dollé *et al.*, 1989; Yokouchi *et al.*, 1991; Haack and Gruss, 1993). For example, the expression domains of Hox A10, A11, and A13 suggest their involvement in the segmentation process along the proximal-distal axis of the limb, with Hox A10 expressed earlier and more proximally than Hox A11, which is expressed earlier and more proximally than Hox A13 (Yokouchi *et al.*, 1991; Haack and Gruss, 1993).

Urodeles, like the newt, can regenerate their limbs and tail in adulthood. Immediately after the amputation of a newt limb, the wound is rapidly covered by epidermal cells migrating from the stump. The epidermis responds to an induction of the underlying mesenchymal cells to thicken in the median region of its dorsal-ventral axis and form the apical ectodermal cap (Tank *et al.*, 1977). During the first few days after amputation, dedifferentiation of tissue proximal to the apical ectodermal cap occurs and the blastema forms, which is an area of growth composed of mesenchymal cells that are histologically undifferentiated. Blastemal cells divide rapidly and later undergo cytodifferentiation and morphogenesis to form the internal tissue of the regenerated limb. The apical ectodermal cap is essential to maintaining the underlying blastemal tissue undifferentiated and growing. Meanwhile, determination of the type of structure that will be reformed relies on

Abbreviations used in this paper: aa, amino acid(s); bp, base pair(s); dpc, days postcoitum; nt, nucleotide; OD, optical density; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

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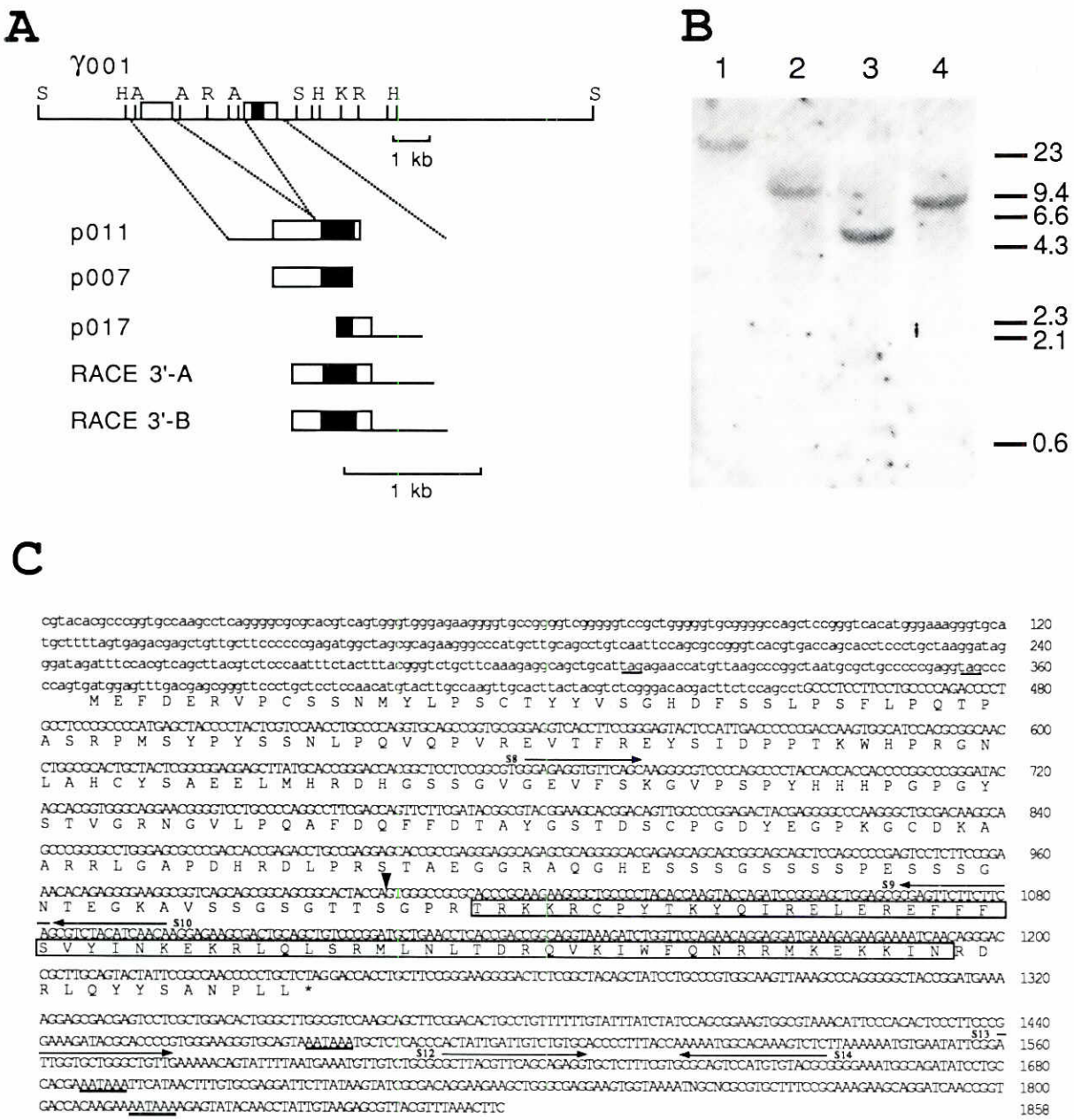

Fig. 1. Cloning and sequencing of the newt Hox A11. (A) *Schematic representation of Hox A11 genomic and cDNA clones. The phage γ001 was isolated from a newt genomic library; the 15-kb insert harbors two exons of Hox A11. p011, p007, and p017 are three overlapping cDNAs cloned from a cDNA library. RACE 3'-A and RACE 3'-B are 3'-extending cDNAs obtained by PCR amplification (see Materials and Methods). Empty boxes represent the Hox A11 coding region and black boxes represent the homeodomain. A, ApaI; H, HindIII; K, KpnI; R, EcoRI; S, SalI.* **(B)** *Southern blot analysis of newt genomic DNA hybridized with Hox A11 cDNA (p017 cDNA excised with EcoRI). Lane 1, undigested genomic DNA (25 μg); lane 2, HindIII-digested genomic DNA (25 μg); lane 3, EcoRI-digested genomic DNA (25 μg); lane 4, KpnI-digested genomic DNA (25 μg). Numbers on the right side are molecular weight markers (kb).* **(C)** *Nucleotide sequence of the newt Hox A11 and its conceptual translation product. The first 455 bp (small letters) were obtained from the genomic clone, whereas other sequences (capital letters) were obtained from cDNA clones. The location of the oligonucleotides S8, S9, S10, S12, S13, and S14 are indicated with arrows that overlie the nucleotide sequence. The arrowhead at position 1004 indicates a splicing site. The homeodomain is boxed. Finally, three putative polyadenylation signals (AATAAA) are underlined.*

the mesenchymal components of the blastema (Wallace, 1981; Stocum, 1984). The blastema is a self-organizing system that possesses a characteristic morphogenetic potential. For example, transplantation of a tail blastema to an X-irradiated limb stump leads to the formation of a tail regenerate, whereas the converse transplantation induces the growth of a limb regenerate (reviewed by Stocum, 1984). Moreover, the blastema will regenerate only the structures distal to their origin (reviewed by Stocum, 1984). There-

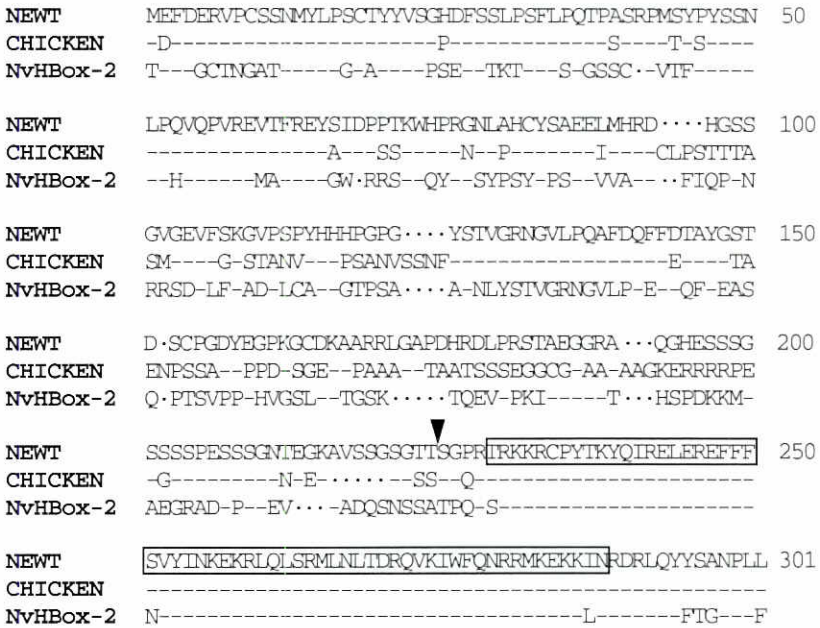

Fig. 2. Comparison of the coding region of the newt Hox A11 and related vertebrate proteins. *Amino acid sequence alignment of the newt Hox A11, the chicken Hox A11, and the newt NvHBox-2 (probably the newt Hox D11). Hyphens indicate sequence identity with the newt Hox A11 protein, whereas differences are stated. Absence of residue(s) is indicated by a dot(s). The homeodomain is boxed, and a filled arrowhead indicates a splicing site.*

fore, the blastema inherits a memory of the type of structure it has to regrow, its polarity, and its level of origin along the proximal-distal axis of the limb. The cellular and molecular mechanisms underlying specification of the limb blastema are not understood in relation to either the nature of the appendage (limb versus tail) or its axes.

Molecular evidence emphasizes that both the regeneration blastema and the developing vertebrate limb bud use a similar set of signals that regulate pattern formation (Muneoka and Sassoon, 1992). The possibility that adult newt tissues maintain, or reactivate, expression of homeobox genes to regulate pattern formation is under investigation (Savard *et al.*, 1988; Brown and Brockes, 1991; Beauchemin and Savard, 1992, 1993; Simon and Tabin, 1993). Here we report the cloning and analyses of expression of the newt Hox A11 in the adult newt limb and tail and their regeneration blastemas. Hox A11 shows differential expression along the proximal-distal axis of the limb and strong accumulation of transcripts in the blastemas. The results are discussed in relation to the putative role of Hox A11 in the determination of positional information during limb regeneration.

Results

Cloning of Hox A11

A newt tail cDNA library was screened for homeobox-containing clones. A redundant oligonucleotide probe (U-box) that showed specificity for the third helix region of the homeodomain was used to screen 2.5×10^6 plaques at moderate stringency. Clones p007, p011, and p017 were isolated and sequenced and proved to be three overlapping cDNAs of the same gene (Fig. 1A). Then we screened a genomic EMBL3 library with the p011 probe and isolated the phage γ001 (Fig. 1A). The gene harbors two exons separated by a 1.5-kb intron. Southern blot analysis of newt genomic DNA with an exon 2 probe (p017 cDNA) revealed a single band in DNA samples digested with *Hin*dIII, *Eco*RI, or *Kpn*I (Fig. 1B). The sizes of the restriction fragments generated with *Hin*dIII (9kb) and *Eco*RI (5 kb) in both the genomic DNA (Fig. 1B) and in the γ001 phage DNA (Fig. 1A) were similar. We concluded that p011 is representative of a single copy gene and that γ001 is the genomic locus.

We extended cDNA cloning in the 3' region of the gene by the PCR-RACE method (Frohman *et al.*, 1988), and isolated the RACE 3'-A and RACE 3'-B clones, which gave an additional 150 bp (Fig. 1A). Attempts to extend cDNA cloning in 5' of the gene by PCR-RACE were not successful, 5'-extensions being always truncated in a region that might represent stable secondary structures refractory to cDNA elongation; treatments with methoxymercuric acid, dimethylsulfoxide or both did not improve the yield of full-length Hox A11 cDNA.

We show the nucleotide (nt) and deduced amino acid (aa) sequences of the homeobox and adjacent regions in Fig. 1C. The sequence covers 1858 bp and harbors an ORF of 289 aa. The most 5'-sequences (446 bp) were obtained from the genomic clone; this region contains the first 30 aa of the deduced protein. A putative methionine initiation codon lies at position 367, and its sequence context fits the consensus sequence (-A/GNNATGPu-) established for other eukaryotic translation start sites (Kozak, 1987). Three polyadenylation signals were found in the 3'-untranslated region of the gene; three cDNA clones (identified as RACE-3'-A, Fig. 1A) stopped at the second termination signal, and three other cDNA clones (identified as RACE-3'-B) stopped at the third termination signal, suggesting that these two sites can be used with comparable efficiency. On the 5' side of the homeobox, an intron-exon junction was defined at position 1004 (Fig. 1C) by sequencing the genomic and cDNA clones. This junction is flanked by both donor and acceptor splicing sequences (5'-Pyr-stretch-AG/G____CCA/GTAAGT-3') that are in agreement with the consensus splice sequence characteristic of eukaryotic genes (Padgett *et al.*, 1986; Shapiro and Senapathy, 1987).

By searching for regions conserved in genes from other vertebrates, we found extensive aa conservation with the chicken Hox A11 (Rogina *et al.*, 1992) throughout the whole coding region (Fig. 2). The degree of similarity suggested that these genes are newt

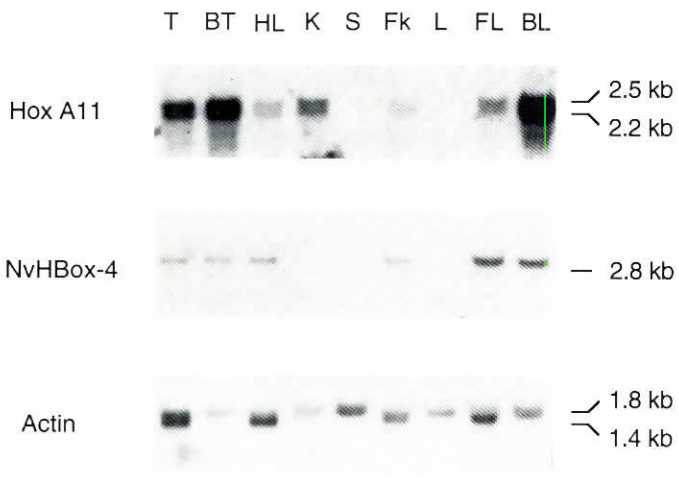

Fig. 3. Body distribution of Hox A11. *A Northern blot of poly (A)⁺ RNA (~5 μg) was serially hybridized with Hox A11, NvHBox-4, and actin. Abbreviations: T, tail; BT, tail blastema; HL, hindlimb; K, kidney; S, spleen; Fk, flank; L, liver; FL, forelimb; BL, forelimb blastema.*

and chick homologues. They have a nearly identical exon 2 (one difference in 77 aa), have a splice site located at the same position, and share long stretches of identical aa in the first exon. The newt Hox A11 also has common structural features with the human Hox D11 (Izpisúa-Belmonte *et al.*, 1991) and the newt NvHBox-2 (Brown and Brockes, 1991) (Fig. 2). Their homeodomains are 95% homologous, and the coding region ends with 12 aa that are well conserved. The first 66 aa at the amino-terminal end of Hox A11 and NvHBox-2 share 56% homology. These strong similarities suggest that Hox A11, Hox D11, and NvHBox-2 are evolutionary descendants of a common ancestral Abd-B-like homeobox gene (Izpisúa-Belmonte *et al.*, 1991).

Expression of Hox A11 in adult newt

A Northern blot of poly (A)⁺ RNA isolated from various tissues was hybridized with a Hox A11 probe (exon 1), revealing two bands of 2.5 and 2.2 kb in samples from the limb and its blastema, the hindlimb, the tail and its blastema, the flank (skin and muscles), and the kidney (Fig. 3). No signal was detectable in the spleen and liver samples.

The RNA samples were initially quantitated by reading their optical density (OD) at 260 nm. We subsequently normalized the Northern blot by probing serially with NvHBox-4 (Beauchemin and Savard, 1992) and the *Xenopus* type 8 cytoskeletal-actin (Mohun *et al.*, 1984) (Fig. 3). NvHBox-4 is a *dll*-like homeobox gene expressed ubiquitously in the skin of adult newt appendages and corresponding blastemas. The *Xenopus* actin probe revealed, at low stringency, a cytoplasmic (ß-cytoplasmic, 1.8-kb) and a muscular (α-sarcomeric, 1.4-kb) transcript. The sarcomeric transcript is present only in muscle, whereas the cytoplasmic transcript is present in the epidermis, dermis, connective tissue, and undifferentiated tissue like the mesenchyme of young blastemas. It is noteworthy that the amount of sarcomeric transcript in a muscular cell is about 20 times the level of cytoplasmic transcript in other cells; when comparing two samples like the tail and tail blastema, one must take this proportion into consideration because the tail is formed mainly with muscle, whereas the tail blastema does not contain any muscle. For example, in Fig. 3, hybridization with Hox A11 showed a strong increase in expression comparing samples of intact limb or tail with samples of limb or tail blastema, even if the ethidium bromide staining of the gel was similar for each sample. Normalization with NvHBox-4 demonstrated that the proportion of skin in samples of intact and blastemal tissue was similar. On the other hand, hybridization with actin showed that the mesenchymal content of intact and blastemal samples was quite different. The higher level of Hox A11 in the blastema (more than 10-fold) could depend on stimulation of transcription, stabilization of the mRNA, or a larger number of expressing cells. Thus, it is important to determine what cellular types are expressing Hox A11.

Tissue distribution of Hox A11

The hindlimbs of adult newts were mechanically stripped of their skin, and the muscles were dissected from the bone. Poly(A)⁺ RNA from each fraction was analyzed on Northern blot by serial hybridization with Hox A11, NvHBox-4, and actin probes (Fig. 4A). Hox A11 was detected in the muscle (HLm) and bone (HLb) fractions. No signal was revealed in the skin (HLs). However, the amount of HLs RNA loaded on gel was lower than other samples; this is demonstrated with NvHBox-4 and actin: HLP (intact proximal hindlimb) and HLs samples revealed a similar amount of skin (NvHBox-4 and cytoplasmic actin signal), whereas HLP showed at least 5 times more sarcomeric actin signal. If both samples contained the same amount of RNA, we would have expected increased levels of both NvHBox-4 and cytoplasmic actin in HLs because this tissue fraction is enriched with skin. Hybridization with actin also revealed that the bone fraction contains a large amount of sarcomeric transcript; the high level of muscle contamination in the bone fraction probably depends on the ease of extracting RNA from soft tissue compared with hard tissue like bone.

New RNA samples from each tissue fraction (HLs, HLm, and HLb) were oligo(dT) primed for reverse transcription. Then specific oligonucleotides were used to PCR-amplify Hox A11, NvHBox-4, actin, and Hox C6 (Fig. 4B). For example, the reverse transcription of 1 μg of HLs poly(A)⁺ RNA gave 200 μl of cDNA sample; 5 μl of the sample was used to PCR-amplify Hox A11, and other 5 μl samples were used to PCR-amplifiy NvHBox-4, actin, and Hox C6. The products of these reactions were loaded on gel after 21, 24, 27, and 30 cycles of amplification. Southern blots to be hybridized with a specific probe were pooled in a single bag, washed together, and exposed for the same length of time.

The signal revealed with actin demonstrated that the amounts of RNA in HLs, HLm, and HLb were quite similar. The signal revealed with Hox C6 exemplified the type of signal that is expected for a gene expressed in all tissue fractions. In contrast, NvHBox-4 expression is restricted to the skin fraction (Beauchemin and Savard, 1992); therefore, the low amount of NvHBox-4 signal detected in HLm and HLb may be representative of the level of contamination of these samples with skin. Finally, the signal revealed with Hox A11 is low in the skin and moderate in HLm and HLb. This contrasts with the Hox C6 signal, which has the same intensity in HLs and HLm. We concluded that the Hox A11 signal detected in HLs is representative of the amount of muscle contamination of the tissue fraction. Alternatively, there is no muscle contamination in HLs, and Hox A11 expression is very low in HLs.

Hox A11 expression along the proximal-distal axis of the limb

One way to gain information about the possible function of a

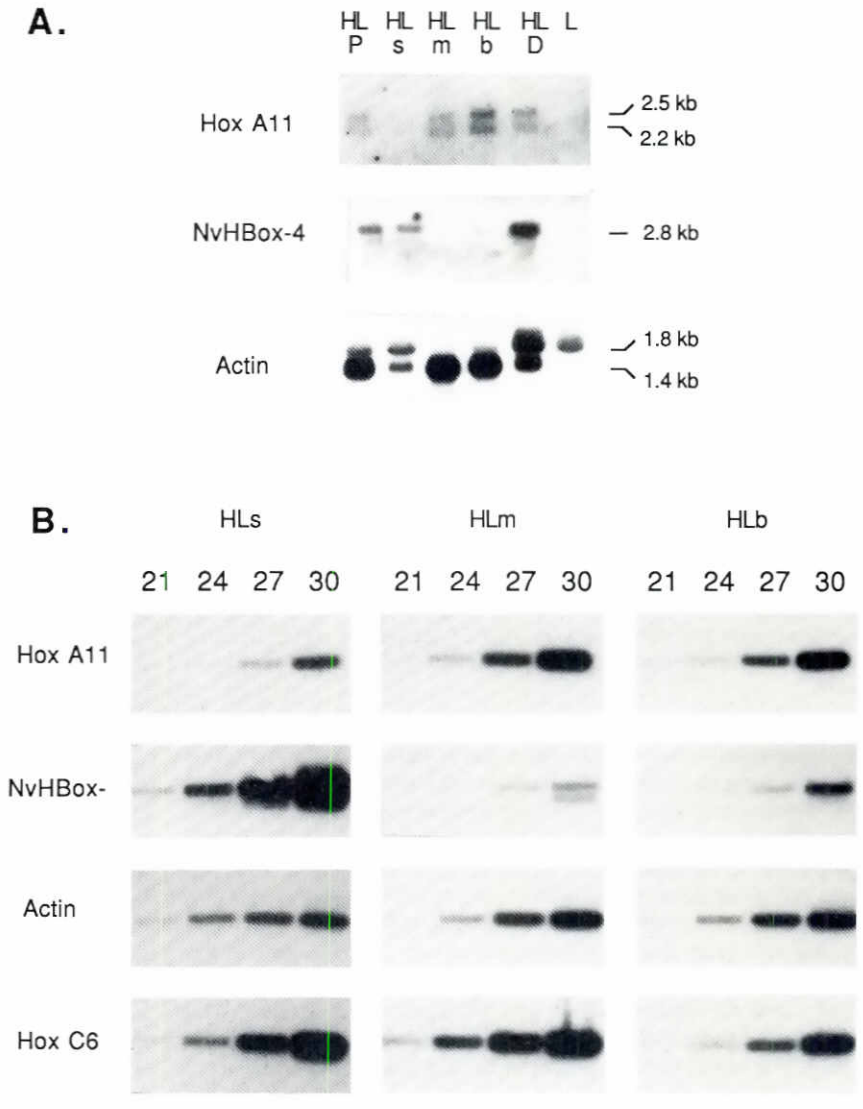

Fig. 4. Tissue distribution of Hox A11. (A) *A Northern blot of poly (A)⁺ RNA (~5 μg) was serially hybridized with Hox A11, NvHBox-4, and actin. Abbreviations: HLP, proximal hindlimb (stylopod + zeugopod); HLs, hindlimb skin; HLm, hindlimb muscle; HLb, hindlimb bone; HLD, distal hindlimb (autopod); L, liver.* **(B)** *Southern blot analysis of PCR-amplified oligo(dT)-primed cDNAs from HLs, HLm, and HLb. We used oligonucleotides specific to Hox A11, NvHBox-4, actin, and the E1-E2 transcript of Hox C6. Samples of each reaction were collected after 21, 24, 27, and 30 cycles.*

gene is to find a correlation between a special feature of its regulation and some biological process. We analyzed two RNA samples dissected from different proximal (stylopod and zeugopod) and distal (autopod) regions of the intact hindlimb (Fig. 4A). Normalization of these samples with NvHBox-4 and actin demonstrated qualitative differences in the cellular content of the samples, making it difficult to interpret quantitative differences along this axis. The signal revealed with NvHBox-4 and the ratio of cytoplasmic-to-muscular actin in each sample indicated that the proportion of skin in distal regions of the limb is higher compared with more proximal ones. It is noteworthy that both NvHBox-4 and actin levels of expression are constant along the proximal-distal axis of the limb (Beauchemin and Savard, 1992).

We also compared the level of expression of Hox A11 in blastemas of different positions of origin along the proximal-distal axis. Proximal blastemas were generated from the amputation of forelimb at the mid-humerus level, whereas distal blastemas came from amputation at the wrist level. Blastemas were allowed to develop for 15 to 20 days, at which time they will have reached the mid-bud stage (Iten and Bryant, 1973). Poly(A)⁺ RNA from these tissues were analyzed on a Northern blot along with samples of tail and tail blastema (Fig. 5). The expression of Hox A11 was threefold to fivefold higher in proximal blastema compared with the distal one; as a normalizing signal, we used the cytoplasmic actin that is representative of the amount of RNA present in each sample. The specificity of hybridization was controlled with liver poly(A)⁺ and poly(A)⁻ RNA samples.

Discussion

The newt Hox A11 transcribes two messenger RNAs of 2.5 and 2.2 kb. We cloned the entire putative coding region of the gene. The molecular structure of Hox A11 is typical for a homeobox gene; it contains two exons that code for a short protein (289 aa) having a homeodomain located at the carboxy-terminal end. The coding region is very similar to the chick Hox A11 (Rogina *et al.*, 1992);

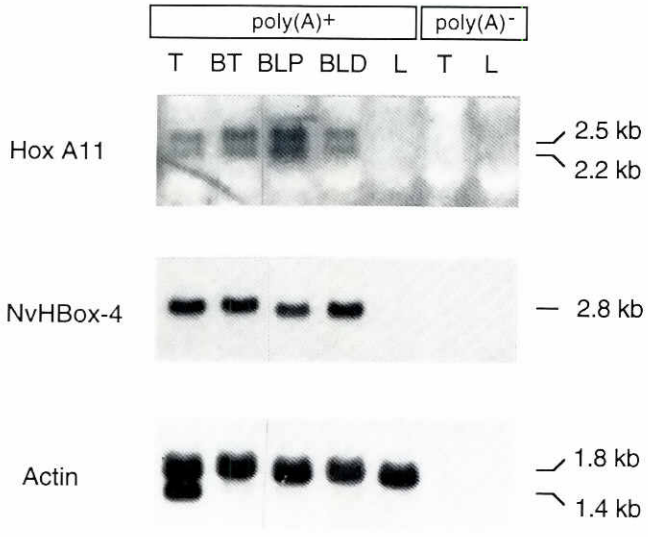

Fig. 5. Proximal-distal expression of Hox A11. *A Northern blot of poly (A)⁺ (~5 μg) or poly (A)⁻ RNA (~25 μg) was serially hybridized with Hox A11, NvHBox-4, and actin. Abbreviations: T, tail; BT, tail blastema; BLP, proximal blastema (mid-humerus); BLD, distal blastema (mid-radius and ulna); L, liver.*

both genes have two exons with a splice site located at the same position, long stretches of homology in the amino-terminal end of the protein, and identical homeodomains and carboxy-terminal ends. The degree of homology is high enough to conclude that both genes are the newt and chick versions of the same gene. The high level of conservation of Hox A11 in the phylum suggests that it has similar functions in development and regeneration.

Hox A11 also shares structural features with NvHBox-2, the newt version of the vertebrate Hox D11 (Brown and Brockes, 1991). Hox A11 and Hox D11 are two paralogous versions of a gene suspected to have duplicated during evolution (Graham *et al.*, 1989). The newt Hox A11 produces two transcripts that likely arise from utilization of different termination sites as shown by the cloning of two different 3'-extremities. The transcripts would encode the same protein. Although the difference in polyadenylation site utilization could explain the difference in length of the transcripts, it does not exclude the possibility of alternative splicing in other parts of the mRNA.

Pattern of expression of Hox A11 and regeneration

The pattern of expression of Hox A11 shows several features of interest in relation to limb regeneration. First, the genes are expressed in the tissue of the adult newt limb and tail. Most homeobox genes studied to date in both insects and vertebrates are transcribed in developing tissue and show restricted expression, if any, in the adult. This is part of the general evidence that argues for their instructive role in development. In the limb bud of vertebrates, homeobox gene expression declines with time, and expression in adult limb is not well documented. The sustained expression of homeobox genes in the appendages of the adult newt is an interesting possibility for explaining an underlying predisposition for regeneration. Knowing that the principal function of homeobox genes during development is one of positional determination, it is conceivable that their sustained expression in the mesenchymal fraction of adult tissue represents the maintenance of a positional memory that is passed on to blastemal cells during the process of dedifferentiation. The maintenance of homeobox gene expression in the appendages of the adult newt suggests that the morphogenetic potential of the cells is not completely shut off.

Several homeobox genes maintain their expression in the adult newt limb (Savard *et al.*, 1988; Tabin, 1989; Beauchemin and Savard, 1992, 1993; Simon and Tabin, 1993), whereas others are reactivated only in the regeneration blastema (Brown and Brockes, 1991; Simon and Tabin, 1993). Therefore, the persistent expression of homeobox genes in the adult newt and their induction after amputation are two different ways to use developmental genes to control regenerative events. In *Drosophila*, the functional hierarchy among homeobox genes is based on cross-regulatory interactions that modulate the expression level that is appropriate for each metamere (Jaynes and O'Farrell, 1988). Such interactions are thought to play major roles in the refinement and maintenance of the expression patterns of the homeobox genes. The same is believed to be true in vertebrate embryos (Zappavigna *et al.*, 1991). Similar cross-regulatory interactions of developmental genes may be necessary to keep active the morphogenetic capacities of the cells in the regeneration territories of the adult newt. Maintaining the expression of some homeobox genes may allow full reactivation of this or other homeobox genes under specific circumstances.

A second interesting feature of Hox A11 expression is the accumulation of transcript in blastemal tissue. A variety of possibilities may account for this increase. Expression may be low in all limb cells and then increase (either by transcriptional or post-transcriptional mechanism) in response to the amputation trauma or acquisition of the blastemal state. Alternatively, expression may be restricted to scattered cells of the limb, which would preferentially populate the blastema (by means of selective recruitment of proliferation) or would represent a higher proportion in the blastema. Analysis of Hox A11 expression at the cellular level would determine which cells are active before and after amputation.

Hox A11 is expressed mainly in the muscle and bone of intact limbs, two tissue fractions that populate the regeneration blastema during its formation. The blastema mesenchyme is known to determine pattern during regeneration, and contact of the epidermis with the mesenchyme is necessary for normal growth (Stocum and Dearlove, 1972). Therefore, Hox A11 is expressed in the correct cell types for a gene that could participate in the specification of pattern during regeneration.

A third interesting feature of Hox A11 expression is the different levels of accumulation of transcript in proximal and distal blastemas. Because of the lack of an ideal normalizing probe whose expression would be regulated like Hox A11 (the same level in each tissue fraction), it was not possible to determine whether a proximal-distal gradient of expression exists in the intact limb. Therefore, when comparing proximal and distal tissues of the limb, we limit our interpretation to blastemal tissue because it is expected to be of similar composition (same epiderm-to-mesoderm ratio) whatever its location in the limb. Under these conditions, the cytoplasmic actin transcript is an adequate normalizing signal, as it represents the overall amount of RNA isolated from blastemas without spatial restriction. The graded expression of a

homeobox gene in a morphogenetic field, like the regeneration blastema, is an interesting phenomenon in relation to the concept of positional information (Wolpert, 1969, 1989). Different combinations of homeobox genes expressed in different compartments of the limb would molecularly identify each one. Knowing the important role of homeobox genes in the patterning of the primary and secondary axes of the vertebrate embryo, we suspect that they could also be good candidates for genes involved in the control of pattern formation during regeneration. This idea is supported by the proximal-distal graded expression of other homeobox genes in the regeneration blastema (Savard *et al.*, 1988; Brown and Brockes, 1991; Simon and Tabin, 1993) and adult intact limbs (Beauchemin *et al.*, in preparation). Interestingly Hox A11, Hox C6, Hox C10, Hox D10 and Hox D11 show a proximal-to-distal gradient, whereas NvHBox-6 has a distal-to-proximal gradient.

Similarity of expression in limb development and regeneration

Early during chick limb development, Hox A11 expression is located in the mesenchyme of the presumptive zeugopodium (Yokouchi *et al.*, 1991) and in the progress zone adjacent to the apical ectodermal ridge (Rogina *et al.*, 1992). Later, expression is restricted to the mesenchyme subjacent to the epidermis of proximal peripheral regions that are differentiating into nonchondrogenic lineages, suggesting the possibility that Hox A11 is involved in mediating the antichondrogenic effect of limb ectoderm (Rogina *et al.*, 1992). The high level of expression of Hox A11 in the regeneration blastema correlates with the suggested antichondrogenic function of the gene. In addition, recent work has shown that Hox A11 is also expressed in the dermamyotome and later in the somatopleural mesoderm flanking and forming the limb bud, suggesting that Hox A11 expression is localized in the myogenic precursors that colonize the limb bud (Haack and Gruss, 1993). The expression of Hox A11 in both muscle and skin (possibly the dermis) is reminiscent of the embryonic expression in both myogenic and connective precursors.

During limb development, three genes of the Hox A cluster (Hox A10, A11, and A13) show structural and temporal colinearity of expression, that is, that the spatiotemporal restriction of expression of each gene is related to the position of the gene within its complex (Yokouchi *et al.*, 1991; Haack and Gruss, 1993). Hox A11 is the first gene on the Hox A cluster to show expression in the regeneration blastema, and it is not known whether its neighboring genes (Hox A10 and Hox A13) are also active. The demonstration that Hox A10 and Hox A13 are also expressed in the regeneration blastema would support the hypothesis that regeneration proceeds through activation of the developmental program that initially participated in elaboration of the structure.

Materials and Methods

Animals and treatment

Adult *Notophthalmus viridescens* were purchased from C.D. Sullivan Co. Inc. (Nashville, Tennessee, USA). Anaesthesia and surgical procedures were as previously reported (Savard *et al.*, 1988). Blastemas were harvested at the mid-bud stage according to the classification of Iten and Bryant (1973).

Cloning

The cDNA library construction and screening were as described in Beauchemin and Savard (1992). In brief, the library was made of tail mRNA, and the probe was a 45-bp redundant oligonucleotide (U-box) to the conserved homeobox sequence DRQVKIWFQNRRKEK (deduced by R. Krumlauf). Three overlapping cDNA clones were derived from a single mRNA, called Hox A11. The screening of a newt genomic EMBL3 library was conducted essentially as described in Savard *et al.* (1988).

The cDNA Hox A11 3'-extensions were cloned using a PCR-RACE strategy (Frohman *et al.*, 1988). In brief, 1 μg of forelimb poly(A)$^+$ RNA was oligo(dT) primed to synthesize cDNA; the mix was subsequently filtrated on a Centricon 100 spun-column and adjusted to 200 μl with water. A first series (30 rounds) of PCR amplification was done with 10 μl of the previous reaction and 150 ng of both oligonucleotides Ro-poly(T) (5'-AAGGATCCGTCGACATCGATAATACGAC(T)^{17}-3') and S13 (position 1559 to 1576 of the reported sequence in Fig. 1C) in 50 μl of PCR reaction buffer (67 mM Tris pH 8.8, 6.7 mM $MgCl_2$, 170 μg/ml BSA, 17 mM $(NH_4)_2SO_4$, 1.5 mM dNTPs, and 10% DMSO) containing 2.5 U Taq DNA polymerase (Pharmacia). The PCR program followed these conditions: a hot start (95°C for 7 min, 75°C for 2 min, addition of Taq, 45°C for 2 min, and 72°C for 40 min) and 30 regular cycles (94°C for 45 s, 50°C for 25 s, 72°C for 3 min with an extension cycle of 5 sec). A second series (30 rounds) of PCR amplification was done with 1 μl of the first amplification reaction and 150 ng of both oligonucleotides Ri (5'-GACATCGATAATACGAC-3'; poly(T) complementary sequence) and S12 (position 1611 to 1628 of the reported sequence in Fig. 1C). The final products of amplification were cloned in the PCR-1000 vector (TA-cloning kit, In Vitrogen Corporation). Positive clones were detected by colony hybridization using the oligonucleotide S14 (position 1642 to 1658 of the reported sequence in Fig. 1C) as a probe.

RNA isolation and analysis

RNA was extracted according to Chomczynski and Sacchi (1987). Poly(A)$^+$ RNA was purified on a column of oligo(dT)-cellulose following the procedure provided by the supplier (Boehringer Mannheim). Approximately 5 μg of poly(A)$^+$ RNA (evaluated with OD at 260 nm) was run onto an agarose-formaldehyde gel (Maniatis *et al.*, 1982) and transferred onto Genescreen membranes according to the supplier (New England Nuclear). The hybridization procedure has been previously described (Savard *et al.*, 1988). The Hox A11 probe used for Northern blot analyses did not contain the homeobox region. The hybridization and washing conditions for the normalizing probes (NvHBox-4 and Actin) were as previously described (Beauchemin and Savard, 1992).

Signal intensity was evaluated by densitometry; X-ray films were scanned using a RAS-1000 image analyzer (Amersham), and the data were treated with the GL-1000 software (developed by LOATS Associated). We did not use different exposure times to ensure that the signals detected on X-ray films were proportional to the amount of transcripts; therefore, the analyses remain semiquantitative.

Poly(A)$^+$ RNA samples (1 μg) were reverse transcribed for 2 h at 37°C using 200 U of Moloney reverse transcriptase (RT) (BRL) in RT buffer containing 1 mM dNTPs, 50 U RNasin (Pharmacia), and 0.5 pmol of oligo-dT primers. The mixture was diluted to 1 ml and concentrated to 200 μl onto Centricon 100 spun-columns (Amicon Division). The dilution and concentration steps were repeated. A sample of cDNA (5 μl) was then incubated with 2.5 U of Taq DNA polymerase (Pharmacia) in 50 μl of PCR reaction buffer containing 150 ng of the appropriate primers. The reactions were done in a DNA thermal cycler (Perkin Elmer Cetus) for 30 cycles under the following conditions: a hot start (95°C for 5 min, 75°C for 2 min, Taq addition, 55°C for 2 min, and 72°C for 10 min) and 30 regular cycles (94°C for 30 s, 50°C for 40 s, 72°C for 1 min with an extension cycle of 5 s). Samples (10 μl) of the reaction mixtures were taken after 21, 24, 27 and 30 cycles and were incubated 10 min at 72°C to complete DNA synthesis. Then samples were loaded on agarose gel and blotted following standard procedures. The blots from each tissue were put into a bag and hybridized with a labeled oligonucleotide specific to the gene that was PCR amplified. The three blots hybridized with any of the probes were exposed for the same length of time.

The following oligonucleotides were used during the PCR procedure: (1) Hox A11 was amplified with S8 (5'-GGAGAGGTGTTCAGC-3') and S10 (5'-

TGTTGATGTAGACGC-3'), and the product of amplification revealed with S9 (5'-CTGAAGAAGAACTCG-3'); the location of these oligonucleotides is depicted in Fig. 1C; the DNA fragment produced is 436 bp long. (2) NvHBox-4 was amplified with SAV38 (5'-ATGTGGATGTGAGTGTGT-3') and SAV39 (5'-CTCTCTTTGTGCGGTTTA-3'), and the product of amplification revealed with SAV37 (5'-GGCTTCCCGTTCACCATC-3'); the location of these oligonucleotides is depicted in Fig. 2A of Beauchemin and Savard, 1992 (position 596-613, 1040-1058, and 1536-1553); the DNA fragment produced is 957 bp long. (3) Actin was amplified with BAR18 (5'-TTTGAGACCTTCAACACCCC-3') and BAR19 (5'-CACCAGACAACAC TGTGTTG-3'), and the product of amplification revealed with BAR20 (5'-GAGCAACATAGCACAGCTTC-3'); the location of these oligonucleotides is depicted in Fig. 2 of Alonso *et al.*, 1986 (position 292-312, 564-583, and 807-826); the DNA fragment produced is 534 bp long. (4) The transcript E1-E2 of Hox C6 was amplified with SAV27 (5'-AGTGTCCAGGAGCAGAAG-3') and SAV3 (5'-ATCCATATTCATTGTGCCAGT-3') and the product of amplification revealed with Op158 (5'-ACTCCAGGGTCTGGTAG CGCGAGT-3'); the location of these oligonucleotides is depicted in Fig. 2 of Savard *et al.*, 1988 (position 550-568, 659-683, and 978-998); the DNA fragment produced is 448 bp long.

DNA sequence analysis

The nt sequence was determined using a chain termination kit and method provided by the United States Biochemical Corporation or Pharmacia. Double-strand DNA from bluescribe was denatured for 30 min at 37°C in 0.2 M NaOH and 0.2 mM EDTA before ethanol precipitation. Six micrograms of double-stranded or single-stranded DNA was used per sequencing reaction.

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