Development 111, 489-496 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

Identification and expression of a regeneration-specific homeobox gene in the newt limb blastema

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Summary

Adult urodele amphibians are able to regenerate their limbs through the formation of a blastema, a growth zone of undifferentiated mesenchymal cells that arises locally at the plane of amputation. In an effort to define genes involved in pattern formation by the blastema, we isolated from a newt forelimb blastema library cDNA clones that identify a homeobox gene termed *NvHbox 2*. The amino acid sequence of the homeodomain is identical to that of the recently identified human *HOX-4f* gene (Acampora *et al.* 1989) and of the mouse *Hox-5.5* (Dolle *et al.* 1989). *NvHbox 2* is expressed in the limb blastema as a transcript of 3.4 kb that is not detectable in the normal limb. Analysis by RNAase protection demonstrates expression in limb and tail blastemas, but not in any of the adult tissues or organs tested. In the limb blastema *NvHbox 2* was expressed in mesenchymal but not epithelial tissue. When matched and normalised samples of RNA from proximal (mid-humerus) and distal forelimb (mid-radius) blastemas were compared, the level of expression of *NvHbox 2* was found to be 3- to 5-fold higher proximally. At two time points after injection of a proximalising dose of retinoic acid, the level of expression in a distal blastema was not increased in comparison to controls.

Key words: homeobox, limb regeneration, positional information, retinoic acid, axis specification.

Introduction

Limb morphogenesis is a key problem for the study of pattern formation in vertebrates. It has been most extensively investigated in avian development, and in regeneration of adult and larval urodele amphibians, but in both systems the molecular mechanisms remain largely unclear (Brockes, 1989). Limb regeneration proceeds by formation of a specialised structure called the blastema, which arises locally at the plane of amputation (Wallace, 1981). This consists of a jacket of wound epithelium surrounding mesenchymal blastemal cells that are the progenitors of the cartilage, muscle and connective tissue of the regenerate. Urodele amphibians such as the newt and axolotl are the only adult vertebrates able to regenerate their limbs. The reasons for this are not understood but it is sometimes suggested that formation of the blastema involves re-expression of genes involved in tissue patterning during development of the limb bud (Muneoka and Bryant, 1984). The identity of such genes remains unclear.

The best understood system for the genetic control of patterning is the development of the *Drosophila* embryo. Many of the genes implicated in establishing the embryonic axes (Nusslein-Volhard *et al.* 1987) and at later stages in specifying segment position and identity (Akam, 1987) are candidate transcription factors (Stanojevic *et al.* 1990; Pankratz *et al.* 1990; Dearolf *et al.* 1990). The largest group of such genes are related by a region of homology called the homeobox (Scott *et al.* 1989) and, in vertebrates, the overlapping domains of expression of homeoproteins suggests that pattern is established by co-ordinate expression of these and other transcription factors (Wright *et al.* 1989; Goulding and Gruss, 1989). For example, the 3′ members of the *Hox-2* complex in the developing mouse neural tube show progressively anterior boundaries of expression (Graham *et al.* 1989; Wilkinson *et al.* 1989). Recent studies of genes in the murine *Hox-5* complex (Dolle and Duboule, 1989) have revealed their expression in the limb bud as a nested set, where the extent of each domain is such that the genes located at the 5′ end are expressed more distally (Dolle *et al.* 1989). This progressive spatial distribution is established in conjunction with a temporal control of expression during outgrowth of the bud. These and other findings have led to the suggestion that positional information in limb morphogenesis may be encoded in part by a set of homeobox gene products (Savard *et al.* 1988; Oliver *et al.* 1988; Oliver *et al.* 1989; Dolle and

Duboule, 1989; Wedden *et al.* 1989; Robert *et al.* 1989; Hill *et al.* 1989; Dolle *et al.* 1989).

A particular focus of recent interest in limb regeneration has been the specification of pattern along the proximodistal (PD) axis running from shoulder to fingertips (Brockes, 1989). The blastemal cells arising at any level on the PD axis inherit or otherwise derive a property, sometimes referred to as positional memory, which ensures that they replace only the structures distal to their point of origin (Stocum, 1984). It is of great interest that retinoids, and in particular retinoic acid (RA), are able to re-specify this property in a dose-dependent fashion (Niazi and Saxena, 1978; Maden, 1982; Stocum and Crawford, 1987). For example, a wrist level blastema, which normally regenerates a hand, will produce an entire arm after treatment with an optimal dose of RA. The molecular basis of positional memory in the blastema and the ability of RA to respecify it are not yet understood, but it is possible to suggest that vertebrate homeoprotein genes play an important role.

In an earlier study, we identified a newt homeobox gene expressed in the normal and regenerating limbs but not in the tail (Savard *et al.* 1988). This gene, termed *NvHBox 1*, has human (Simeone *et al.* 1987), mouse (Sharpe *et al.* 1988) and *Xenopus* (Cho *et al.* 1988) homologs. It is expressed in the adult limb of the newt at comparable levels to the limb blastema, but in *Xenopus*, the homolog is expressed during limb development and not in the adult limbs, which are unable to regenerate (Savard *et al.* 1988). The present paper describes the identification of a new newt homeobox gene that is not expressed at detectable levels in the adult limb or any other adult tissue thus far examined, but is induced in blastemal mesenchyme of limbs and tails after amputation. The gene is regulated on the PD axis and we have investigated the effect of RA on its expression. The results are discussed in the context of pattern formation in the regenerating limb.

Materials and methods

Animals and treatment

Adult *Notophthalmus viridescens* were supplied by Blades Biologicals, Edenbridge, Kent. Anesthesia, amputation procedures, and the collection and storage of tissue samples were all as described earlier (Kintner and Brockes, 1985; Casimir *et al.* 1988; Savard *et al.* 1988). For RA treatment, groups of 100–200 newts received distal forelimb amputations followed 7–10 days later by a single intraperitoneal injection with 10 μl of a RA/DMSO solution (10 mg ml^{-1}) or with the DMSO vehicle alone. The first group of blastemas was harvested one week after RA treatment at the end of the delay period. The second group was taken 20 days after RA treatment, or 15 days after DMSO treatment, when they had reached the mid-bud stage. Control groups of four to six animals were allowed to regenerate for 4–8 weeks to verify that proximalisation had occurred.

For separation of blastemas into epithelial and mesenchymal fractions, blastemas were harvested and dissected as rapidly as possible in 0.1 M EDTA (pH 8). All tissue samples were stored over liquid nitrogen prior to RNA preparation.

Screening of cDNA libraries

A newt forelimb blastemal cDNA library in λgt11 (Ragsdale *et al.* 1989) was screened with a redundant oligonucleotide to the conserved homeobox sequence DRQVKIWFQNRRKEK. The probe was labelled with T4 polynucleotide kinase to a specific activity of $2–5\times10^8$ cts min^{-1} μg^{-1}. Approximately 2×10^6 plaques were screened on hybond-N+ filters at low stringency (6×SSPE, 1% SDS, 100 μg ml^{-1} each BSA and tRNA, at 37°C) with the oligonucleotide probe or at high stringency (as before but at 65°C) with the *NvHbox 1* probe N71 (Savard *et al.* 1988). Filters were washed initially for 3×15 min at room temperature in 6×SSC/1% SDS, then either in 20 mM sodium phosphate, pH 7.5, 1% SDS at 65°C (high stringency) or in 6×SSC/1% SDS at the hybridisation temperature (low stringency). Of twelve positive plaques, four reacted with both probes and the remaining 8 (oligo-positive, N71-negative) were purified and subcloned into the plasmid vector pBSM13+. The clones were initially sequenced in both orientations by double-stranded sequencing using Sequenase (US Biochemicals). Regions containing ambiguities or compressions were further subcloned into either M13 mp18 or 19 and sequenced by a combination of dITP and dGTP with Sequenase. Sequence analysis was performed using programs by Staden (1984). The EMBL Data Library Nucleotide Sequence database and SWISS-PROT Protein sequence database were searched using the programs FASTN and FASTP, respectively (Lipman and Pearson, 1985).

RNA preparation

Total RNA was prepared from newt tissue by homogenising the tissue in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarcosyl, 0.1 M beta-mercaptoethanol. The homogenate was centrifuged in a Beckman J6 centrifuge at 4200 revs min^{-1} for 40 min at 4°C and the supernatant

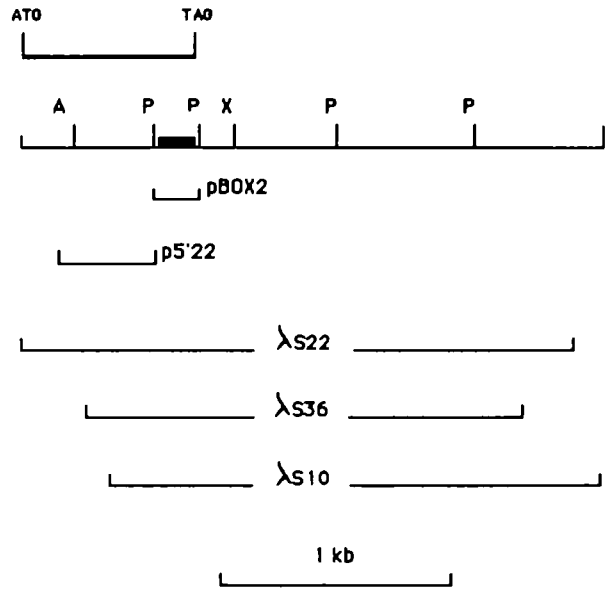

Fig. 1. Structure of *NvHbox 2* cDNA clones and probes used for RNase protection analysis. The three lambda clones were isolated as described in the text, and are indicated (λ) along with the two plasmid clones (p) containing probes used for RNAase protection. The homeobox is shown by the solid rectangle, and the open reading frame begins with the initiator ATG. A, *Ava*I; P, *Pst*I; X, *Xho*II.

precipitated with an equal volume of iso-propanol at −20°C for 1 h. Two subsequent rounds of precipitation with iso-propanol were performed before dissolving the RNA in Tris–EDTA.

RNAase protection

After subcloning the appropriate fragment into Bluescribe, antisense probes were synthesised from the plasmid template using either T3 or T7 RNA polymerase and 400 Ci mmole^{-1} ^{32}P-labelled UTP. Hybridisation was performed in 80% formamide as described previously (Casimir *et al.* 1988) at 50°C with 5 μg total RNA and 5×10^5 cts min^{-1} probe (approximately 2 ng antisense RNA). Following digestion with RNAase A and T1, the protected fragments were separated by polyacrylamide gel electrophoresis. The gel was vacuum dried and exposed for 2–3 days to Kodak XAR-5 film at −70°C. The RNA samples were normalised initially by measurement of their E_{260}. This normalisation was repeatedly checked (for example see Fig. 4B) by protection analysis with a newt satellite probe (Epstein *et al.* 1986; Epstein and Gall, 1987), or with N71 (Savard *et al.* 1988) whose expression in total RNA does not vary on the PD axis (P. Savard *et al.* manuscript in preparation), in contrast to its expression in poly(A)$^+$ RNA (Savard *et al.* 1988).

Northern blots

Poly(A)$^+$ RNA was selected by passing total RNA over a poly(A) 'Quick-kit' column (Stratagene) according to the manufacturers instructions. 5 μg of poly(A)$^+$ RNA was separated by agarose gel electrophoresis through a 1.5% agarose/0.66 M formaldehyde gel run in 1×MOPS buffer. The RNA was transferred to hybond-N+ nylon in the presence of 10×SSC for 3 h. The RNA was then crosslinked to the nylon using a UV-Stratalinker (Stratagene) under standard conditions. The filter was hybridised with radiolabelled 5′22 at 42°C in 50% formamide/6×SSC/1%SDS/100 μg ml^{-1} BSA and tRNA). High-stringency washes were performed for 2×15 min at 65°C in 15 mM sodium phosphate buffer and 1%SDS. The membrane was exposed for 8 days to Kodak XAR-5 film at −70°C with intensifying screens. The blot was stripped of probe by washing in 95% formamide/10 mM Tris, pH 7.5 and 0.1% SDS at 65°C for 15 min before being re-probed with Nor-1 (Savard *et al.* 1988) under conditions of high stringency. The blot was then exposed to XAR-5 film for 6 h at −70°C.

Results

Isolation and sequence of NvHbox 2

In order to identify novel homeobox-containing clones, a limb blastema cDNA library was screened initially both with a fully degenerate oligonucleotide probe to a region of the third helix of the *Antennapedia (Antp)* homeodomain (see Materials and methods), and with a probe from the previously identified *NvHbox 1* (Savard *et al.* 1988). Plaques that reacted with *NvHBox 1* were discarded and oligo-positive clones purified. A second round of screening at high stringency with these clones resulted in the isolation of λS36 (Fig. 1). This clone contains a homeodomain close to the C terminus of an

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                  M  T  E  F  D  G  C  T  N  G  A  T  N  M  Y  L  P  G  C  A  Y  Y  V  S  P  S  E  F  S  T  K  T  S  F  L
CGAGAAGAGGGCGACATGACCGAGTTTGATGGCTGCACCAACGGCGCCACCAACATGTATCTGCCGGGCTGCGCTTACTATGTCTCGCCATCGGAGTTCTCCACAAAAACCTCGTTCCTG
      10        20        30        40        50        60        70        80        90       100       110       120

 S  Q  G  S  S  C  P  V  T  F  P  Y  S  S  N  L  P  H  V  Q  P  V  R  E  M  A  F  R  E  Y  G  W  R  R  S  K  W  Q  Y  R
TCCCAAGGCTCTTCCTGCCCCGTGACATTCCCCTACTCGTCTAATTTACCTCACGTGCAGCCTGTGCGGGAAATGGCGTTCCGGGAGTACGGCTGGAGGCGGAGTAAGTGGCAGTACAGG
     130       140       150       160       170       180       190       200       210       220       230       240

 G  S  Y  P  S  Y  Y  P  S  E  E  V  V  A  R  D  F  I  Q  P  S  N  R  R  S  D  V  L  F  K  A  D  P  L  C  A  H  H  G  T
GGCAGCTATCCCTCCTACTACCCCTCCGAGGAGGTGGTGGCTCGGGACTTTATTCAACCGTCCAACCGGCGGTCGGACGTGCTCTTCAAAGCGGACCCACTGTGCGCCCACCACGGAACG
     250       260       270       280       290       300       310       320       330       340       350       360

 P  S  A  A  S  N  L  Y  S  T  V  G  R  N  G  V  L  P  Q  E  F  D  Q  F  Y  E  A  S  Q  P  T  S  V  P  P  E  H  V  G  S
CCCAGCGCCGCGTCCAACTTGTACAGCACGGTGGGGCGCAACGGCGTCCTGCCCCAGGAGTTCGACCAGTTCTACGAGGCCAGCCAGCCCACCTCCGTCCCGCCCGAACATGTGGGCTCT
     370       380       390       400       410       420       430       440       450       460       470       480

 L  D  K  T  G  S  K  T  Q  E  V  P  P  K  I  T  H  S  P  D  K  K  M  G  A  E  G  R  A  D  S  P  S  G  E  V  A  A  D  K
CTGGACAAGACGGGCTCCAAAACGCAGGAGGTGCCCCCTAAAATAACCCACAGTCCGGACAAGAAGATGGGCGCAGAGGGGAGAGCAGACTCGCCTTCTGGGGAAGTAGCTGCAGATAAG
     490       500       510       520       530       540       550       560       570       580       590       600

 S  N  S  S  A  T  P  Q  R  S  R  K  K  R  C  P  Y  T  K  Y  Q  I  R  E  L  E  R  E  F  F  F  N  V  Y  I  N  K  E  K  R
AGCAACAGTTCAGCCACCCCTCAGAGGTCTCGGAAGAAGAGGTGTCCCTACACCAAATACCAGATCAGAGAGCTGGAGAGGGAGTTTTTCTTCAATGTCTACATTAACAAGGAGAAGCGG
     610       620       630       640       650       660       670       680       690       700       710       720

 L  Q  L  S  R  M  L  N  L  T  D  R  Q  V  K  I  W  F  Q  N  R  R  M  K  E  K  K  L  N  R  D  R  L  Q  Y  F  T  G  N  P
CTTCAGTTGTCCCGAATGCTCAATCTCACCGACCGACAGGTCAAAATCTGGTTCCAGAACCGGAGGATGAAGGAGAAGAAACTGAACCGGGACCGGCTGCAGTATTTCACCGGCAATCCC
     730       740       750       760       770       780       790       800       810       820       830       840

 L  F  *
TTGTTTTGAGAAAGGCAGCGGGCACTTCATGTCGCGACTTGGCGGAAATAACGCGTGTAGAAGTGCGATCTGCCTTGTTGTTCTTGTGGAAGACTTTGTGTCCTCAAGGGGGGATTTCAA
     850       860       870       880       890       900       910       920       930       940       950       960

GTTCTTGGCCGGCCGGATCTTGGCACTTGGTTGTATTTTCCCAGACAGAGGGGTCCTGTCACCGCAGTATTTCCCTTCCCGATTACAGTAAATGCAAATACCGTCTATCTCTACTTAAA
     970       980       990      1000      1010      1020      1030      1040      1050      1060      1070
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Fig. 2. Nucleotide and amino acid sequences of the composite *NvHbox 2* cDNA. The boxed region is the homeobox sequence.

open reading frame encoding a protein of 67×10^3 M_r. The assignment of the initiator methionine is plausible from its neighbouring sequence (Kozak, 1987), but it must be regarded as tentative in the absence of an upstream in frame stop codon. The restriction map of this clone, and the identity of the two subclones subsequently used as probes for RNAase protection analysis are shown in Fig. 1.

The predicted amino acid sequence of the clone (Fig. 2) identifies the homeodomain as having an identical amino acid sequence to that of the *HOX-4f* gene (Acampora *et al.* 1989) and the *Hox-5.5* gene (D. Duboule, personal communication). The homeodomains of these three genes differ from the *Antp* homeobox most noticeably in the region that encodes the second helix of the domain, sharing only one residue of ten in this region. With the exception of three conservative substitutions, identity with the *Antp* sequence is restored in the third helix, this latter region of the homeodomain being implicated in binding to DNA (Otting *et al.* 1988; Hanes and Brent, 1989; Treisman *et al.* 1989). The identity with the 5.5 protein sequence extends outside the homeobox but is not sufficiently extensive to conclude that this is the newt homolog.

Tissue distribution of the NvHbox 2 *transcript*

After probing with 5′22 (Fig. 1), Northern blots revealed a single band of 3.4 kb in samples of poly(A)$^+$ RNA from the limb blastema, but no signal was detected in samples from the normal limb (Fig. 3). The level of the transcript was low, as shown by the exposure time for Fig. 3, and we have found RNAase protection analysis to be more useful for expression studies because it is quantitative and sensitive, and hence conserves the limited quantities of RNA available from the regenerating limb. The analyses reported in this paper have been performed with both p5′22 and pBOX probes (Fig. 1) with identical results. RNA samples were routinely normalised by observing their hybridisation to a ubiquitously expressed newt satellite probe (Epstein *et al.* 1986; Epstein and Gall, 1987) which gives a ladder of protected species (Casimir *et al.* 1988).

The difference in expression between normal and blastema tissues that was indicated by the Northern blot was confirmed by RNAase protection analysis of samples from normal limb and tail, and their respective blastemas (Fig. 4A). In this experiment no signal was observed from the normal samples. From other experiments we estimate the difference between normal limb and limb blastema to be at least twenty fold. The expression in the limb blastema was reproducibly 3- to 5-fold higher than in tail blastema (Fig. 4A). When a variety of adult newt tissue samples and internal organs were analysed, including one sample from a hand regenerate (digit formation complete), there was no detectable signal (Fig. 4B) indicating that adult expression of this gene is limited in extent.

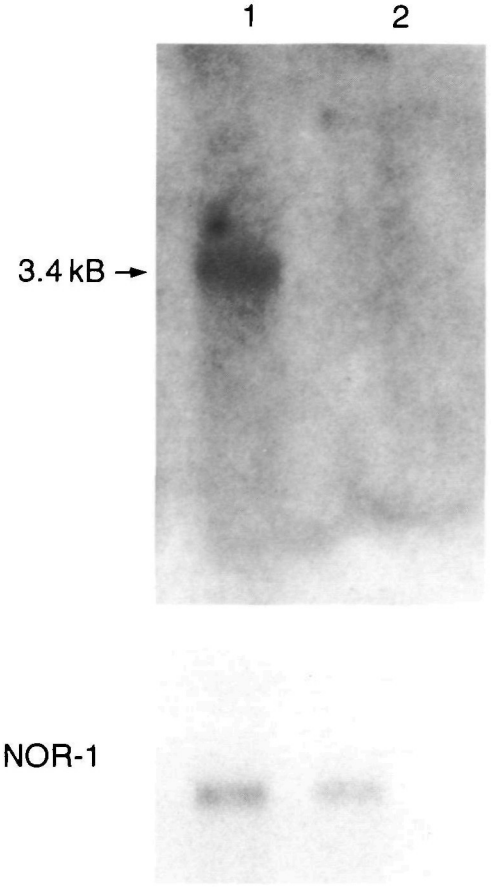

Fig. 3. Northern blot of the *NvHbox 2* transcript. Northern blot analysis was performed with 5 μg poly(A)$^+$ RNA and probed with 5′22. The nylon membrane was stripped and then probed with Nor-1 (Savard *et al.* 1988) to demonstrate RNA in both samples. The size of the transcript was estimated from the mobility of newt 28S and 18S ribosomal RNAs. Lanes: (1) proximal forelimb blastema; (2) normal adult forelimb. Note that the exposure for *NvHbox 2* was 8 days while that for Nor-1 was 6 h.

Regulation of NvHBox 2 *expression in the limb blastema*

In order to analyse the possible variation in expression along the PD axis, we obtained paired samples of proximal and distal blastemas according to the scheme of Fig. 5A. A group of newts was amputated at a distal level (mid-radius/ulna) on one side and a proximal level (mid-humerus) on the other. The RNA preparations resulting from mid-bud blastemas were normalised by optical density and by RNAase protection analysis with probes for the satellite, and also for *NvHbox 1* which does not vary in expression in total RNA preparations from proximal and distal blastemas. As shown in Fig. 5B, no protected band was observed from preparations derived from proximal and distal segments of normal limb (lanes 2 and 3), while the normalised samples from the proximal and distal blastemas showed a 5-fold difference in level (lanes 4 and 5, proximal higher). This has been confirmed by

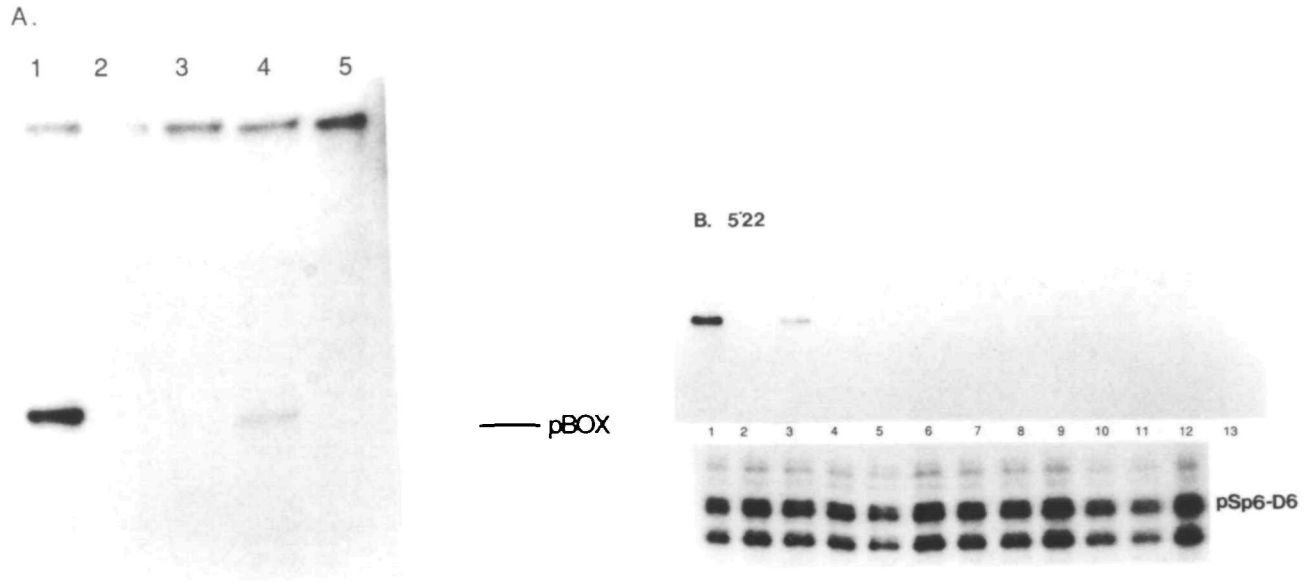

Fig. 4. Specificity of *NvHbox 2* expression for limb and tail blastemas. (A) RNAase protection analysis of expression in the limb and tail. Normalised RNA samples (5 μg) were subjected to RNAase protection analysis with pBOX as probe (see Fig. 1). Lane: (1) proximal forelimb blastema; (2) normal forelimb; (3) normal tail; (4) tail blastema; (5) tRNA (10 μg). The size of the protected fragment is 227 bp. (B) RNAase protection analysis of expression in various adult tissues. RNA samples (5 μg) were analysed with 5'22 as probe, and in parallel with the satellite probe pSp6-D6 (Casimir *et al.* 1988). Lane: (1) proximal forelimb blastema; (2) normal limb; (3) tail blastema; (4) normal tail; (5) belly mesenchyme; (6) whole torso; (7) hand regenerate; (8) jaw; (9) brain; (10) spleen; (11) heart; (12) liver; (13) tRNA (10 μg). The size of the protected fragment is 395 bp. Note that the two probes give identical results for the limb and tail and their blastemas in A and B.

multiple determinations on three independent preparations derived according to Fig. 5A.

In further experiments, a preparation of proximal blastemas was dissected into epithelial and mesenchymal fragments from which RNA was extracted. When the two normalised preparations were analysed by RNAase protection, expression was detectable only in the blastemal mesenchyme (Fig. 5B, lanes 6 and 7). By contrast, the control *NvHbox 1* gene showed somewhat higher expression in the epithelial fraction.

Effect of RA on NvHbox 2 *expression*

In view of the difference in expression between proximal and distal blastemas and the ability of RA to proximalise the blastema (see Introduction), we determined the effect of RA on the level of *NvHbox 2* RNA. Large groups of newts were amputated at the level of mid-radius/ulna and injected either with a dose of RA dissolved in DMSO that reproducibly proximalises the regenerate or with DMSO alone (Fig. 6A). The blastemas were harvested both at an early time, at the end of the delay in regeneration provoked by RA treatment (Maden, 1983), and at a later time when they had reached the mid-bud stage used for the comparison in Fig. 5B, lanes 4 and 5. Reserve animals in the group were allowed to regenerate, and all animals showed proximalisation, most commonly to the level of the mid-humerus (see Fig. 5 in Savard *et al.* 1988). RNA samples from the two sets of blastemas were normalised and analysed as before by RNAase protection. As shown in Fig. 6B, a proximalising dose of RA does not increase the level of expression of *NvHbox 2* in a distal blastema at either of the times analysed. In careful comparisons of the samples, a slight reduction of expression was sometimes observed relative to the DMSO control, but the clear result was that no increase to proximal blastemal values was observed in either the early or late populations of RA-treated distal blastemas.

Discussion

We have identified a second newt homeobox gene that is expressed in the limb regeneration blastema. Analysis of the sequence of the protein coding region shows high sequence identity with the *Hox-5.5* (Dolle *et al.* 1989) and the *HOX-4f* (Acampora *et al.* 1989) homeobox genes. The three genes contain identical homeodomains and *C* terminal amino acids. A comparison of the remainder of the newt coding sequence with *Hox-5.5* reveals extensive identity around the *N* terminus that extends for the first 60–70 amino acids. The sequences then diverge markedly until the *C* terminal homeodomains.

NvHbox 2 gene expression is not detected in the adult limb or in any other adult tissue after analysis with the sensitive RNAase protection assay. It is, however, found in the mesenchymal cells of the blastema formed after amputation. The level of expression in the blastema is low in absolute terms, and we have been

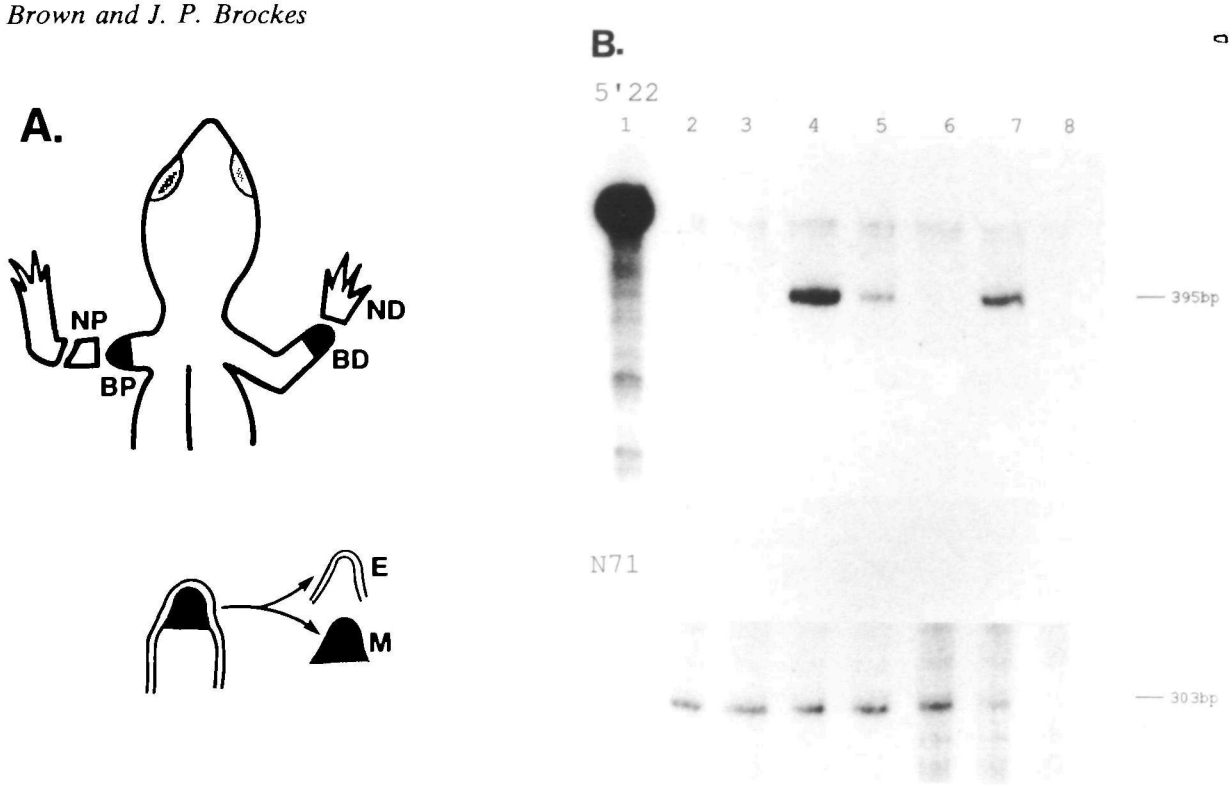

Fig. 5. Position dependence of *NvHbox 2* expression. (A) Schematic diagram of dissection to provide tissue for RNA preparations. NP, Normal limb proximal; BP, proximal limb blastema; ND, normal limb distal; BD, distal limb blastema; E, epithelium; M, mesenchyme. (B) RNAase protection analysis of *NvHbox 2* expression in tissue samples. RNA samples (5 μg) were subjected to RNAase protection analysis either with 5'22 or in parallel with N71 (see Materials and methods). Lane: (1) input 5'22 probe; (2) NP; (3) ND; (4) BP; (5) BD; (6) E; (7) M; (8) tRNA (10 μg). The size of the protected fragments is shown.

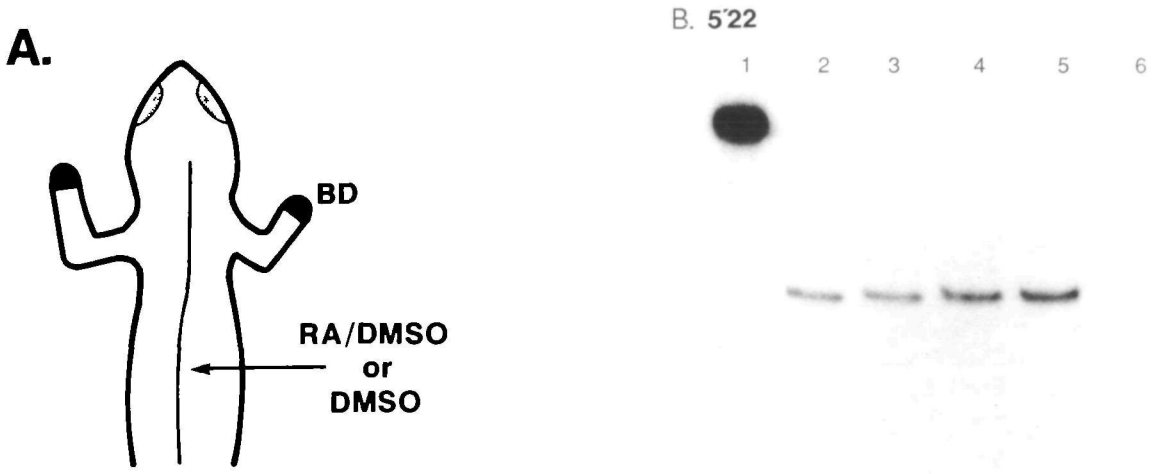

Fig. 6. Effect of RA on *NvHbox 2* expression in distal limb blastema. (A) Schematic diagram of experimental design. A group of newts with distal forelimb blastemas was injected intraperitoneally either with a proximalising dose of RA dissolved in DMSO, or with DMSO. Blastemas from RA and DMSO groups were harvested at two different times (see text) and analysed for *NvHbox 2* expression. (B) RNase protection analysis of expression in RA- and DMSO-treated tissue. RNA samples (5 μg) were normalised with the satellite probe and were analysed with 5'22 as the probe. Lane: (1) input 5'22 probe; (2) RA group, early; (3) DMSO group, early; (4) RA group, mid-bud; (5) DMSO group, mid-bud; (6) tRNA (10 μg). Note that the level of expression increases for both groups between the two times analysed.

unable to localise *NvHbox 2* mRNA by *in situ* hybridisation despite repeated attempts with both radioactive and non-radioactive probes. In the absence of cytochemical evidence, we cannot rule out the possibility that the gene is expressed in a small number of cells in the normal limb, which then proliferate to form the blastema. This hypothesis is not readily consistent with current views about the origin of the blastema (Casimir *et al.* 1988; Ferretti and Brockes, 1990) and we favour a model of gene induction over cell proliferation. These results are in contrast to those obtained with *NvHbox 1* (Savard *et al.* 1988), which is expressed in the adult forelimb of the newt, but not of the anuran *Xenopus*, which loses the ability to regenerate its limbs around the stage of metamorphosis (Dent, 1962). In view of the evidence for *Hox 5.5* expression in the mouse limb bud (Dolle *et al.* 1989), it seems likely that *NvHbox 2* is expressed in newt limb development, but this is a point that will require confirmation when suitable antibodies become available. *NvHbox 1* and 2 may exemplify two different strategies for the genetic control of regenerative events in an adult organism, these being the persistent expression of genes involved in development, and their induction after amputation.

The expression of *NvHbox 2* is significantly higher in a proximal mid-bud blastema than a distal one. It should be noted that such a blastema is composed of a mound of undifferentiated cells and that we are effectively comparing the progenitor populations arising at two different axial levels before the onset of differentiation. The locations of the blastemas (approximately mid-humerus or mid-radius) differ by no more than a half to two thirds of the total extent of the PD axis, but we observe a reproducible 3- to 5-fold difference in expression by RNAase protection analysis. As outlined in Fig. 3A, the blastemas are derived from different levels of the same group of newts, and the RNA preparations are normalised with probes for transcripts whose level does not vary on the PD axis. Models of positional information encoded in blastemas of different levels predict that a mid-bud blastema contains some complement of the positional values distal to the plane of amputation (Stocum, 1984), and hence a 3- to 5-fold difference may conceal an even greater difference between the mid-humerus and mid-radius locations than is first apparent.

In view of the difference in expression of the *NvHbox 2* RNA between a proximal and distal blastema of the same stage, we examined whether the level of expression would change in a distal blastema when exposed to a dose of RA that proximalised its positional identity. We observed no increase in RA-treated blastemas relative to control blastemas. This result was obtained both at an early time after RA treatment, when a newt keratin gene *NK2* showed a marked decrease in expression in the same RNA samples from RA treated distal blastemas (Ferretti *et al.* 1990), and at the later, mid-bud stage when the characteristic P–D difference in expression has been observed (Fig. 5B). We cannot at present rule out either that RA induces some transient change in *NvHbox 2* expression that occurs between the time points analysed here and thereby respecifies the blastema, or a still less likely possibility, that RA alters the spatial balance of transcription such that increases and decreases cancel out at the two times. Nonetheless our results argue against the hypothesis that RA resets positional value through sustained changes in transcription of *NvHbox 2*, or that the normal difference in expression between proximal and distal blastemas (Fig. 5) is obligatory for PD respecification. It is possible as noted previously in connection with *NvHbox 1* (Savard *et al.* 1988), that *NvHbox 2* expression may be regulated by RA at the level of translation, or that RA may modify the expression of genes that are downstream targets of the *NvHbox 2* protein.

Recent experiments on tissue culture cells have suggested that genes at the 5′ end of both the human *HOX-2* (Simeone *et al.* 1990) and mouse *Hox-2* complex (Papalopulu *et al.* 1990) are relatively refractory to induction by RA in comparison to their 3′ counterparts. For the murine cells it is known that this effect is predominantly post-transcriptional but is reflected in changes in the level of stable RNA. If such refractoriness is a feature of *NvHbox 2* expression in the blastema, it is no less true that a proximalising dose of RA is insufficient to change its level, and that such a change cannot be involved in mediating the morphogenetic effects. It remains an attractive possibility to identify any blastemal cDNAs that are regulated in the appropriate direction both by axial position and by RA.

We thank Dr Rob Krumlauf for helpful discussion and for the homeobox oligonucleotide, Drs D. Duboule and E. Boncinelli for communicating unpublished sequences on *Hox 5.5* and *HOX 4f* respectively, Dr Lee Crews for dissection of the blastema and Drs Cliff Ragsdale and Keith Dudley (Kings College) for critical reading of the manuscript.

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(Accepted 10 October 1990)