The EMBO Journal vol.7 no.13 pp.4275-4282, 1988

Position dependent expression of a homeobox gene transcript in relation to amphibian limb regeneration

Pierre Savard, Phillip B.Gates and Jeremy P.Brockes

MRC Cell Biophysics Unit, King's College London, 26-29 Drury Lane, London WC2B 5RL and [1]The Ludwig Institute for Cancer Research, Courtauld Building, 91 Riding House Street, London W1P 8BT, UK

[1]Present address

Communicated by L.Wolpert

Adult urodele amphibians such as the newt *Notophthalmus viridescens* are capable of regenerating their limbs and tail by formation of a blastema, a growth zone of mesenchymal progenitor cells. In an attempt to identify genes implicated in specification of the regenerate, we screened a newt forelimb blastema cDNA library with homeobox probes, and isolated and sequenced clones that identify a 1.8 kb polyadenylated transcript containing a homeobox. The transcript is derived from a single gene called NvHbox 1, the newt homologue of XlHbox 1 (*Xenopus*), HHO.c8 (human) and Hox-6.1 (mouse). The cDNA for the 1.8 kb transcript has two exons as determined by isolation and partial sequencing of a genomic clone. The expression of the transcript shows several interesting features in relation to limb regeneration: (i) Hybridization of Northern blots of poly(A)$^+$ RNA from limb and tail and their respective blastemas shows that the transcript in limb tissues has exons 1 and 2, whereas a 1.8 kb transcript in tail tissues has only exon 2. (ii) The transcript is expressed in limbs of adult newt but not of adult *Xenopus*, raising the possibility that this contributes to an explanation of the loss of regenerative ability with maturation in adult anurans. (iii) The transcript is expressed at a higher level in a proximal (mid-humerus) blastema than in a distal one (mid-radius). When distal blastemas were proximalized by treatment with retinoic acid, no change in the level of the transcript was detected by Northern analysis at a single time point after amputation. Study of NvHbox 1 may shed light on the general problem of limb specification, as well as the particular mechanisms involved in regeneration of adult appendages.

Key words: alternative splicing/homeobox/limb regeneration/*Notophthalmus viridescens*/urodele

Introduction

The limbs and tail of urodele amphibians are encompassed by epimorphic fields (French *et al.*, 1976) that can be activated repeatedly by amputation, resulting in regeneration of the missing structures. This ability is absent in most other vertebrates, and even in anuran amphibians, such as *Xenopus*, it is lost at about the time of metamorphosis (Dent, 1962). Regeneration in urodeles proceeds by local formation of a blastema, a region containing mesenchymal progenitor cells that proliferate under control of the peripheral nervous system (Brockes, 1984). The blastemal cells divide and undergo morphogenesis to produce the internal tissues of the regenerate (reviewed by Wallace, 1981).

The blastema is a self-organizing system that can express its morphogenetic potential when transplanted to a new location. Thus the transplantation of a tail blastema to an X-irradiated limb stump leads to formation of a tail regenerate while the converse transplantation of limb blastema to tail induces the formation of a limb regenerate (reviewed by Stocum, 1984). Moreover the blastema derives from its parent limb or tail cells a memory of its level of origin along the proximo-distal axis. This positional memory specifies the proximal boundary of the regenerate such that blastemal cells do not form structures proximal to their origin (Stocum, 1984). It is of great interest that retinoic acid is the sole compound to date that is able to re-specify the limb blastema to form proximal structures (Niazi and Saxema, 1978; Maden, 1983, 1985). The cellular and molecular mechanisms underlying specification of the limb blastema are not understood, either in relation to the nature of the appendage (limb versus tail) or its axes.

Candidates for developmental genes controlling the body plan were first identified as homeotic mutations in *Drosophila* (Bridges and Morgan, 1923). Homeotic mutations transform certain appendages, or body segments, thereby changing the architecture of the organism. Thus genes of the bithorax complex specify the posterior thoracic and abdominal segments (Lewis, 1978), whereas a separate cluster of homeotic genes, the *Antennapedia* complex, controls the anterior thoracic and the head segments (Kaufman, 1983).The structural analysis of the *Antennapedia* (*Antp*) gene led to the discovery of the homeobox, a small DNA segment of ~180 bp, that is characteristic of homeotic genes (McGinnis *et al.*, 1984a; Scott and Weiner, 1984). The homeobox sequence codes for a peptide of 60 amino acids that is well conserved from the fly to the human (Carrasco *et al.*, 1984; Levine *et al.*, 1984; McGinnis *et al.*, 1984b). Certain proteins harbouring a homeobox domain have been shown to be DNA-binding proteins suggesting that they regulate segment identity by acting at the level of transcription (Laughon and Scott, 1984; Desplan *et al.*, 1985; Fainsod *et al.*, 1986). Screening with the homeobox sequence could be an approach to identify the genes that control the architecture of body structure during regeneration in urodeles.

We have screened a forelimb blastema cDNA library of the newt *Notophthalmus* with a variety of *Drosophila* and *Xenopus* homeobox probes. These have identified a transcript of the newt homologue (referred to as NvHbox 1) of the human homeobox gene HHO.c8 (Simeone *et al.*, 1987) and XlHbox 1 in *Xenopus* (Cho *et al.*, 1988). The pattern of expression of this transcript shows several features of interest in relation to amphibian limb regeneration.

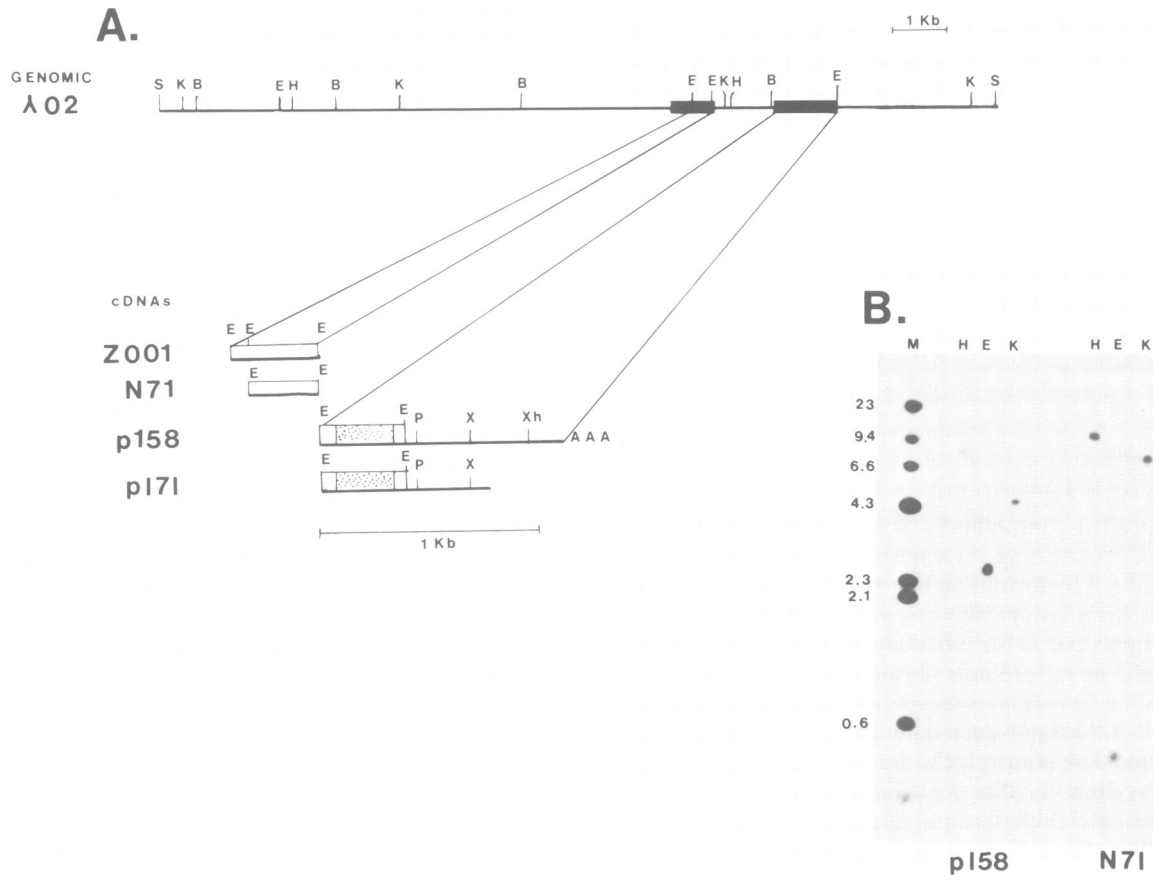

Fig. 1. Structure of the NvHbox 1 locus and summary of the cDNAs cloned. (**A**) The solid rectangles represent the two exons identified in the genomic clone. The open rectangles represent the predicted coding region in the cDNA clones. Included within this region is the homeobox sequence (hatched area). E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I; B, *Bgl*II; Xh, *Xho*I. (**B**) Analysis of newt genomic DNA (25 μg per lane) digested with H, E and K. The fragment sizes of lambda DNA digested with H are indicated on the left in kilobase pairs. The Southern blots were hybridized to the probes indicated below the lanes. These probes are shown in (A).

Results

Isolation of NvHbox 1 genomic and cDNA clones

We screened a newt forelimb blastema cDNA library in λgt10 at low stringency with a mixture of the homeobox probes *Ant, fushi tarazu* (*ftz*) and *Xenopus* XlHbox 1 and isolated two cDNA clones that harbour a homeobox sequence. The clone p158 is a polyadenylated cDNA of 1.1 kb while the clone p171 is a shorter version lacking the 3′ end of p158. The clone N71 (0.3 kb) does not have a homeobox but hybridizes at high stringency with a segment of XlHbox 1 that is located 5′ to the box. After screening another blastema cDNA library in λZAP at a high stringency with N71, we isolated clone Z001 (0.45 kb) which is a 5′ extension of N71 and provides the complete coding portion of the gene.

In order to isolate the corresponding genomic clone we screened an amplified *Notophthalmus* library in EMBL3 with the p158 and N71 probes. These identified a genomic clone (λ02) with a 15 kb insert that hybridizes with both probes (Figure 1). The restriction map of λ02 is equivalent to that determined by Southern analysis of newt genomic DNA (Figure 1). The gene contains two exons that are separated by a 1.2 kb intron, with the sequence of N71 being contained in the first exon and that of p158 in the second (with the exception of 14 bp in the first).

Nucleotide sequence of the NvHbox 1 cDNA

The nucleotide and deduced amino acid sequences of the NvHbox 1 homeobox and adjacent regions are shown in Figure 2. The sequence of the 5′ untranslated portion was obtained from the genomic clone. It shows extensive conservation with the corresponding region in XlHbox 1 which was present in mature mRNA as demonstrated by RNase protection analysis (Cho *et al.*, 1988). The polyadenylated cDNA has an open reading frame (ORF) of 234 amino acid residues and a 3′ untranslated portion of 780 bp. On the 5′ side of the homeobox an intron–exon junction has been defined at position 618 by sequencing of the genomic and cDNA clones. This junction is flanked by both donor and acceptor splicing sequences that are in agreement with the consensus splice sequence characteristic of eukaryotic genes (Shapiro and Senapathy, 1987). The 3′ end of the cDNA N71 and the 5′ end of p158 end at the *Eco*RI restriction site in position 604 of the sequence shown in Figure 2. The first 14 nucleotides at the 5′ end of p158 are found in the genomic clone λ02 adjacent to the N71 sequence at the 3′ end of the first exon. This observation strongly indicates that the cDNA clones N71 and p158 are derived from the same gene.

We have searched for regions conserved in genes from other vertebrates and have found extensive amino acid conservation of NvHbox 1 with XlHbox 1 (Cho *et al.*,

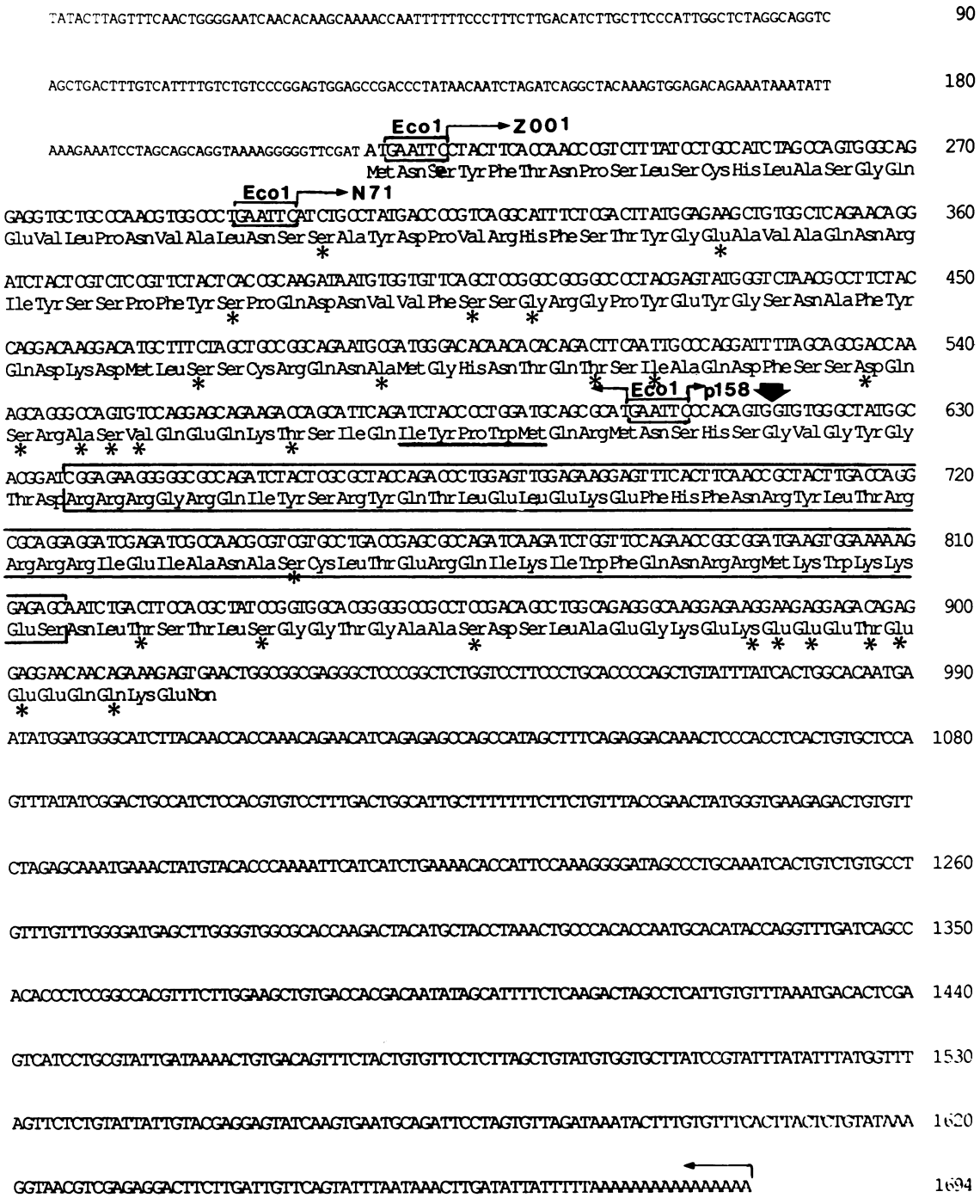

Fig. 2. Nucleotide and amino acid sequences of the NvHbox 1 cDNA. The complete nucleotide sequence compiled from genomic (small letters) and cDNA sequences (big letters) is shown. The boxed region is the homeobox sequence, and the position of the splice site is indicated with a filled arrow. A conserved pentapeptide just before the homeodomain is underlined. The *Eco*RI restriction sites delimiting the probes N71 and p158 are boxed and indicated *Eco*I. The non-conserved amino acid residues of NvHbox 1 when compared with the PRII-XlHbox 1 (Cho *et al.*, 1988) protein are starred.

1988), Hox-6.1 (Sharpe *et al.*, 1988), and HHO.c8 (Simeone *et al.*, 1987) throughout the whole coding region (Table I).The degree of similarity of these genes suggests that these are the newt, frog, mouse and human homologues. They all have a near-identical homeobox (one amino acid change out of 60) and an intron−exon boundary at position 618 (Figure 2). We also found extensive nucleotide conservations (88%) in the 5′ untranslated region of NvHbox 1 and XlHbox 1, immediately preceding the initiator AUG, while the 3′ untranslated region shows no sequence conservation (Table I). This striking sequence homology in the 5′ untranslated region is not without precedent among homeotic genes and has been reported for the genes XlHbox 2 and HHO.c1, Xhox-36 and Hox-1.1, XlHbox 2 and Hox-1.1,

Table I. Homology of the NvHbox 1 gene with its human (HHO.c8) and *Xenopus* (XlHbox 1) homologues

	NvHbox 1/HHO.c8	NvHbox 1/XlHbox 1
5′ untranslated DNA sequence (n.a.)	—	161/183 (88%)
Translated DNA sequence (n.a.)	331/528 (63%)	554/724 (77%)
3′ untranslated DNA sequence (n.a.)	<20%	<20%
Protein homology (a.a.)	114/134 (85%)	192/213 (90%)

n.a., nucleic acid residues; a.a., amino acid residues. Analysis was performed using MacGene software (Applied Genetic Technology Inc.).

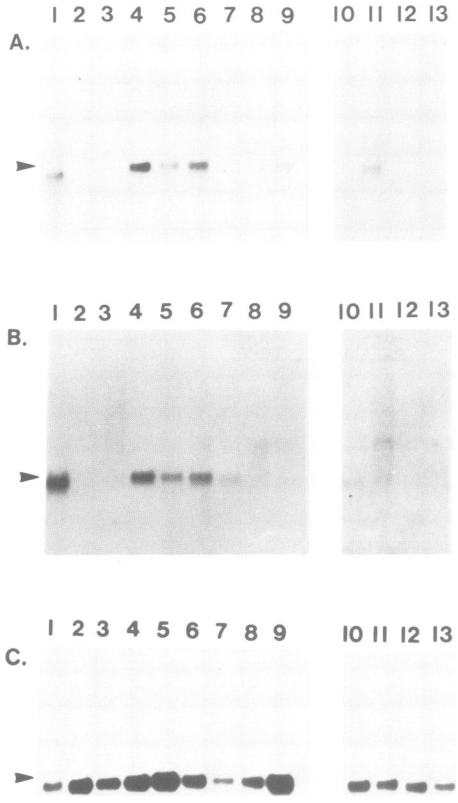

Fig. 3. Tissue distribution of the NVB1 gene transcript. Northern blot analysis was performed with poly(A)$^+$ RNA extracted from different tissues (5 μg in lanes 1−11 and 2 μg in lanes 12 and 13), and hybridized sequentially with (**A**) p158; (**B**) N71; (**C**) NOR1. Lane: (**1**) *Pleurodeles* embryo (stage 35−37); (**2**) liver; (**3**) kidney; (**4**) forelimb; (**5**) hindlimb; (**6**) proximal forelimb blastema; (**7**) distal forelimb blastema; (**8**) tail blastema; (**9**) tail; (**10**) liver; (**11**) cultured cells; (**12**) heart; (**13**) spleen.

Hox-2.1 and Hox-1.3. The possible significance of such a conservation has been discussed by Burglin *et al.* (1987), and Kessel and Gruss (1988).

The putative NvHbox 1 protein shares structural features with many other mammalian homeodomain proteins. The protein consists of 234 amino acids residues and has a calculated mol. wt of 28 kd. The homeobox is preceded by a conserved pentapeptide which has been identified in all homeodomain proteins analysed to date (Mavilio *et al.*, 1986; Kessel *et al.*, 1987; Krumlauf *et al.*, 1987). At the carboxy terminus, the NvHbox 1 sequence has a region rich in glutamic acid residues (nine out of 34 residues). Similar polyglutamic acid stretches have been observed in murine, human and *Xenopus* homeotic proteins (Kessel *et al.*, 1988).

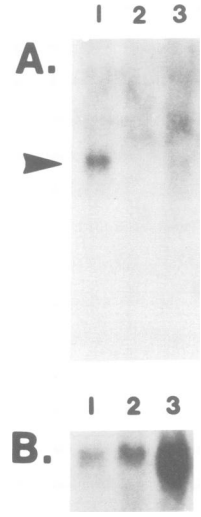

Fig. 4. Tissue distribution of the XlHbox 1 gene in *Xenopus*. (**A**) Tissue distribution of the XlHbox 1 gene in *Xenopus*. Lane (**1**) embryonic tissue 5 μg; (**2**) adult liver 25 μg; (**3**) adult forelimb mesenchyme 25 μg (**B**) Normalization of the RNA samples with cytoplasmic actin probe X1-424.

Tissue distribution of the NvHbox 1 transcript

We have analysed the tissue distribution of the NvHbox 1 transcript by Northern analysis of newt RNA. Poly(A)$^+$ and poly(A)$^-$ RNAs were isolated from adult tissues of *Notophthalmus* and from embryonic tissue of the ribbed newt *Pleurodeles waltl* (stage 35−37; see Gallien and Durocher, 1957). Northern blots were hybridized at high stringency with radioactive probes N71 and p158 which detect the first and the second exon respectively of the NvHbox 1 gene (see Figure 1).

A single rare transcript of ~1.8 kb was detected with p158 in poly(A)$^+$ RNA from whole embryos, kidney, forelimb, forelimb blastema, hindlimb, tail, tail blastema and cultured mesenchymal cells (Ferretti and Brockes, 1988) from hindlimb muscle (Figure 3A). We did not detect a signal in samples from liver, heart and spleen, or various poly(A)$^-$ RNAs. The blot was washed and re-hybridized with the first exon probe N71 (Figure 3B). This detected a 1.8 kb transcript in poly(A)$^+$ RNA from embryos, forelimb, forelimb blastema and hindlimb, but we did not detect a signal from tail, tail blastema, liver, heart, spleen and cultured cells. While the RNA samples were initially quantitated by reading their optical density, we subsequently normalized the Northern blots by probing with NOR1 cDNA (Figure 3C) which detects a very abundant poly(A)$^+$ transcript of ~1.6 kb that is present in all tissue samples that we have analysed (see Materials and methods).

We conclude that the transcript expressed in limb blastema harbours both the first and second exons, whereas the

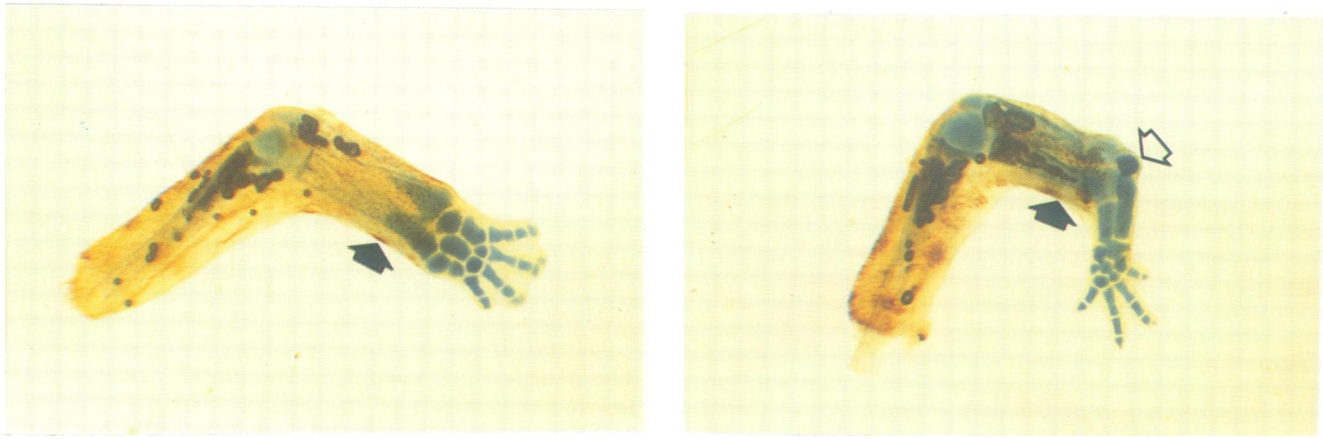

Fig. 5. Whole mounts of *Notophthalmus* limbs stained with Alcian green to show the cartilage elements. (**A**) Regeneration after distal forelimb amputation followed by DMSO injection. (**B**) Regeneration after distal forelimb amputation followed by a RA−DMSO injection. The filled arrow shows the plane of amputation. Note the extra elbow (open arrow) in (**B**).

transcript expressed in tail and tail blastema has the second but not the first, even though the size is 1.8 kb in both tissues. The observations have been repeated on four independent preparations of tail and limb, and their blastemas. An attractive model to explain these results is the insertion of an alternative exon 1 in the tail-derived tissues (see Discussion).

A second feature of interest in Figure 3 is that the 1.8 kb transcript is expressed at ~3-fold higher level (after normalization) in a proximal forelimb blastema (mid-humerus) as compared to a distal forelimb (mid-radius and ulna). This difference is reproducible but its cellular basis requires further clarification (see Discussion). The level is also 3-fold higher in forelimb as compared to hindlimb. In subsequent experiments (see below) we have further investigated the significance of the proximal−distal difference in the blastema by proximalizing a forelimb distal blastema with retinoic acid.

A third aspect of the distribution of the transcript is that while it is expressed in the limb of adult *Notophthalmus*, the XlHbox 1 homologue is not detectable in the normal limb mesenchyme of adult *Xenopus*. In the experiment of Figure 4, we hybridized Northern blots of embryonic and adult *Xenopus* limb mesenchyme poly(A)$^+$ RNA with an XlHbox 1 probe. The 1.8 kb transcript was not detected in the RNA from adult tissues, whereas it was clearly possible to see a band in 5 times less RNA from *Xenopus* embryos. The *Xenopus* blots were normalized with a probe for the 3′ untranslated region of cytoskeletal actin (Mohun *et al.*, 1984).

Expression of the NvHbox 1 transcript following retinoic acid treatment

We have used retinoic acid (RA) to proximalize the positional memory of a forelimb distal blastema (Niazi and Saxema, 1978; Maden, 1985), and have determined if the expression of the homeobox transcript is reset to a value characteristic of a more proximal blastema. Newts were amputated distally such that the resulting forelimb blastemas arose at approximately the level of mid-radius and ulna. The tails of this group were also amputated distally as an internal control, since RA does not affect a tail regenerate. The newts

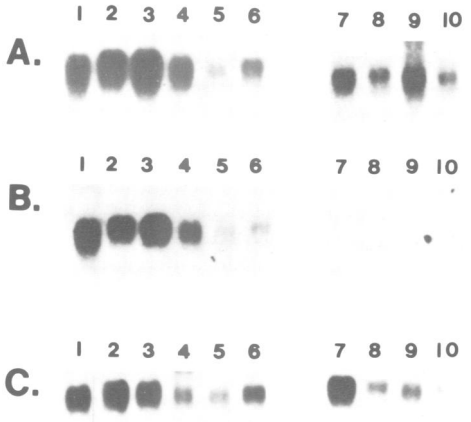

Fig. 6. Effect of retinoic acid treatment on the level of expression of the NvHbox 1 transcript. A Northern blot of poly(A)$^+$ RNA from various tissues was hybridized successively with probes p158 (**A**), N71 (**B**) and NOR1 (**C**). The densitometric evaluation of each loading and the number of cycles of purification on oligo(dT) cellulose were: (**1**) proximal limb 5 μg (2 cycles); (**2**) distal limb 5 μg (2 cycles); (**3**) proximal limb blastema 5 μg (2 cycles); (**4**) distal limb blastema 5 μg (2 cycles); (**5**) DMSO-treated distal limb blastema 2.5 μg (1 cycle); (**6**) RA-treated distal limb blastema 4.0 μg (1 cycle); (**7**) tail 5 μg (2 cycles); (**8**) tail blastema 5 μg (2 cycles); (**9**) RA-treated tail blastema 4 μg (1 cycle); (**10**) DMSO-treated tail blastema 4 μg (1 cycle).

were injected intraperitoneally at 4 and 10 days after amputation either with RA dissolved in dimethylsulphoxide (DMSO), or with DMSO alone folowing the procedures of Crawford and Stocum (1988). The blastemas were allowed to proceed to the mid-bud stage, ~20 days after the last injection, and then harvested for poly(A)$^+$ RNA extraction. A small group was allowed to regenerate for another 30 days in order to establish that the group injected with RA−DMSO showed duplication from the level of mid-humerus, whereas the DMSO group showed a normal pattern (Figure 5).

The poly(A)$^+$ RNA samples from the limb and tail blastemas were Northern blotted and hybridized sequentially with p158, N71 and NOR1 (Figure 6). When the signal from the DMSO group (which had fewer animals than the RA group) was corrected for the difference in load, there was

no discernible effect of RA on the level of the NvHbox 1 transcript in the distal limb blastema, while samples of RNA from proximal and distal blastema from another group of untreated newts showed a clear difference on the same blot (Figure 6A). The level of the NvHbox 1 transcript in tail tissues was less than in the forelimb (Figure 6A and see also Figure 3), and as before there was no detectable signal with the N71 first exon probe (Figure 6B). The treatment with RA did not change the level of the tail transcript as detected with the p158 second exon probe.

Discussion

The NvHbox 1 gene and its transcripts

There is adequate evidence to conclude that the two exons of N71 and p158 belong to the same gene. Both probes detect a transcript of the same size on Northern blots of poly(A)$^+$ RNA. We have detected a single locus on genomic Southerns probed with both N71 and p158. The locus has been cloned and the two exons are found to be separated by an intron of 1.2 kb flanked by a donor and an acceptor splice site. Finally we have sequenced 14 bp at the 5′ end of the p158 cDNA (second exon) that are also found at the 3′ end of the first exon in the genomic locus. Both *Xenopus* (Cho *et al.*, 1988) and human (Simeone *et al.*, 1987) homologues to NvHbox 1 are known to contain the same two exons.

The NvHbox 1 transcript is ~1.8 kb long including an ORF of 234 amino acids that is conserved to 88% when compared to the XlHbox 1 PRII-protein of *Xenopus* (Cho *et al.*, 1988). On Northern blots of poly(A)$^+$ RNA from various tissues we see reproducibly a single band that contrasts with the two transcripts of 1.8 and 2.2 kb that are detected in both *Xenopus* and human tissues. The 2.2 kb band has recently been shown to encode an alternative form of the protein, and involves the utilization of an alternative promoter and upstream exon (Cho *et al.*, 1988). In the present case while some preparations of poly(A)$^+$ RNA give a weak signal at ~2.2 kb, the intensity of the band is greater in the poly(A)$^-$ RNA sample suggesting that it could be a ribosomal signal. Therefore we have failed to detect convincingly a 2.2 kb transcript and this could be due either to the level of transcription being too low for our detection, or simply because there is a single transcript in adult *Notophthalmus*. The amino terminal sequence of the proposed NvHbox 1 protein (Met-Asn-Ser-Tyr-Phe-Thr-Asn) is identical to the amino terminal sequence of XlHbox 1 and similar to many other homeodomain-containing proteins (Met-Ser-Ser-Tyr-Phe-X-Asn). The high level of conservation of the protein from urodeles to human (>85%) suggests that it plays a similar role in limb regeneration and development in vertebrates.

Differential expression of the NvHbox 1 gene in tail and forelimb tissues

Although exon two is expressed in cells of both limb and tail and their respective blastemas, only limb cells have a transcript hybridizing to exon 1. Interestingly, the size of the transcript detected with the exon 2 probe is similar in both limb and tail tissues. Although we do not yet know the sequence of the tail transcript, it is simplest to propose that both limb and tail transcripts share the second exon, while their first exons are different. The exon shared by limb and tail harbours the DNA binding domain, and it is therefore possible that this portion of the NvHbox 1 protein is necessary to bind specific genomic sequences while the portion coded by the first exon is involved in the regulation of genetic events implicated in the determination of limb and tail.

Examples of such differential splicing occur in the homologues XlHbox 1 and HHO.c8 (Simeone *et al.*, 1987; Cho *et al.*, 1988). In both cases the gene express two types of transcript: a short one of 1.8 kb that is similar to the NvHbox 1 transcript we are reporting, and a long one of 2.2 kb that possess an extra exon 1 located 9 kb in upstream. The splicing event joining the new first exon (exon 1a) to the first exon of the 1.8 kb transcript (exon 1b) occurs at position 271 of the sequence we report (middle of the exon 1b, see Figure 2). The alternative splicing event that is discussed above for limb and tail would replace the entire exon 1 by insertion at position 608. Therefore the coding portion of the amino terminus of the protein would be different, as opposed to being shorter in *Xenopus* (Cho *et al.*, 1988). Examples of alternative splicing in homeobox genes have been noted in the *Drosophila Ultrabithorax* gene (Lipshitz *et al.*, 1987), in *Xenopus* XlHbox 2 gene (Wright *et al.*, 1987) and in the mouse Hox-1.6 gene (Baron *et al.*, 1987). In addition it has recently been shown that the first exon of the 2.2 kb transcript of HHO.c8 is also found on messages for two other homeobox genes in the same cluster (Simeone *et al.*, 1988). In vertebrates the gene NvHbox 1 might be the first example where alternative splicing patterns give distinct homeobox proteins in related body structures.

Expression of the NvHbox 1 gene and regeneration

Most homeobox genes studied to date in both insects and vertebrates are transcribed in developing tissues and show restricted expression, if any, in the adult. This is part of the general evidence that argues for their instructive role in development. As far as we know, the NvHbox 1 gene is unusual for a homeobox gene in showing expression in the appendages of an adult, in addition to the regeneration blastema. Is there a relation between the expression of this gene and the regenerative ability of urodeles?

The homologues XlHbox 1 and HHO.C8 are known to be expressed in the developing limb bud (Carrasco and Malacinski, 1987; Simeone *et al.*, 1987) but there is no report of their expression in the adult limb. It is perhaps significant that we failed to detect XlHbox 1 transcripts in the forelimb of adult *Xenopus*. During development, *Xenopus* can regenerate their forelimbs and hindlimbs but this ability is progressively lost between stages 50 and 55 (Dent, 1962). It would be of future interest to compare the time course of loss of the XlHbox 1 transcript with the loss of regenerative ability, and possibly even to maintain expression in the adult limb through appropriate manipulations.

The dorsal body surface of adult urodeles is divided into territories which allow regeneration of a particular appendage (forelimb, hindlimb, tail, dorsal crest). The limits of these territories have been delineated by a combination of experiments involving deviation of major nerve branches and transplantation of neutral tissue (Guyenot *et al.*, 1948; Kiortsis, 1953). It would be worthwhile to compare a map of these territories with a map of the distribution of NvHbox 1 expression in adult *Notophthalmus*. A similar map

would provide strong circumstantial evidence that the expression of NvHbox 1 is related to regenerative potential. We are currently producing several antibodies to the NvHbox 1 protein (see Figure 2) in order to facilitate such mapping experiments.

Proximal–distal differences in expression of the NvHbox 1 transcript

Our results of analysing RNA samples from proximal and distal blastemas have provided the first evidence for a difference in expression along this axis. Although the difference between a mid-humerus blastema and one at mid-radius and ulna is only ~3-fold, it has been supported by preliminary results with *in situ* hybridization (D.M.Fekete, personal communication). It will be important to determine with cytochemical assays if the NvHbox 1 RNA and protein vary continuously along the limb, or if there is differential representation of distinct cell types in the blastema at different levels.

Although RA is able to 'convert' a distal blastema into a proximal one, we did not detect an increase in the expression of the NvHbox 1 transcript to a level characteristic of a more proximal blastema. One reservation about this result is that a single time point was analysed for the control and experimental groups, since a large number of animals were required to obtain an adequate quantity of poly(A)$^+$ RNA. It is desirable to extend the analysis to a series of time points, preferably using a cytochemical assay to look at expression in mesenchymal and epithelial cells of the limb blastema. Our result raises the possibility that RA does not proximalize a blastema by altering NvHbox 1 expression. This in turn could be due to the fact that NvHbox 1 has nothing to do with specification on the proximal–distal axis, or alternatively that RA may act at a 'downstream' level from NvHbox 1 expression. This serves to underline how little we understand about the mechanism of action of RA on patterning in the limb. The recent identification of genes for nuclear receptors of RA (Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988) should help this.

Materials and methods

Animals and treatment

N. viridescens were supplied by Xenopus Ltd and Blades Biologicals while adult *Xenopus* were from Xenopus Ltd. *Pleurodeles* embryos were obtained as described in Fekete and Brockes (1987, 1988). *Xenopus* forelimb mesenchyme was obtained form adult limbs after removal of the epidermis. Newts were anaesthetized by immersion in 0.1% tricaine (methane sulphonate) in sterile distilled water. Forelimbs were amputated by cutting the limb either just above the elbow (proximal) or the wrist (distal), pushing the soft tissue down the bone, cutting the bone and then trimming the soft tissue to produce a flat amputation surface (Kintner and Brockes, 1985). Tails were amputated by cutting off the distal 30–50% followed by pinching the wound for a few seconds to minimize the loss of blood. Amputated newts were allowed to recover overnight in 1% sulphamerazine before returning them to the aquarium. We cut off the bud of blastemal tissue (stage mid-bud according to the classification of Iten and Bryant, 1973) at 13–16 days post-amputation. The newts generally grew another bud of blastemal tissue in <12 days and this second bud with a subsequent one were harvested. Blastemas and other tissues were routinely stored over liquid nitrogen prior to RNA extraction.

In order to proximalize the positional memory of blastemal cells we injected intraperitoneally 20 μl of a RA–DMSO solution (15 mg/ml) 4 days post-amputation. We repeated the injection 6 days later to maximize proximalization of pattern in regenerating limbs. A control group of animals were injected in parallel with DMSO. The blastemas reached the mid-bud stage ~20 days following the last injection of RA.

Genomic and cDNA libraries

Total cytoplasmic RNA was isolated by homogenization in 4 M guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion (Maniatis *et al.*, 1982). Poly(A)$^+$ RNA was isolated by two cycles of oligo(dT)-cellulose chromatography (Maniatis *et al.*, 1982). Ten micrograms of forelimb blastemal mRNA were sent to Clontech Inc. who produced a cDNA library in the *Eco*RI site of bacteriophage λgt10 of 1.5×10^6 plaques containing inserts. In addition we produced a cDNA library in the *Eco*RI site of bacteriophage λZAP of 5×10^4 plaques containing inserts.

Screening at low stringency was carried out essentially as described by McGinnis *et al.* (1984a,b). The probes were a mixture of a 600 bp DNA fragment (*Bam*HI–*Pvu*II) of the *Antennapedia* cDNA clone p903G (McGinnis *et al.*, 1984a,c), a 1040 bp DNA fragment (*Pvu*II) of the genomic clone *fushi tarazu* pFS2 (McGinnis *et al.*, 1984a,c), and a 1700 bp DNA fragment (*Pst*I) of the genomic clone AC1 (Carrasco *et al.*, 1984). Positive plaques were picked, purified and analysed by hybridization. The cDNA insert was excised and subcloned into bluescribe (M13$^+$) vector for subsequent analysis.

A genomic library for a single individual of *N. viridescens* was prepared using a partial *Sau*3A digest of liver DNA and insertion of size selected fragments into *Eco*RI/*Bam*H1 digested EMBL3 phage vector (Kaiser and Murray, 1985). The initial size of the library was 8×10^6 plaques. We screened at high-stringency 6×10^6 plaques of the amplified EMBL3 library with a mixture of the probes N71 and p158. Positive plaques were picked, purified and analysed by restriction enzyme mapping. Fragments of interest were subcloned into the bluescribe vector (M13$^+$) prior to sequence analysis.

Southern and Northern blot analysis

For Southern blot analysis 25 μg of newt liver DNA was digested with the appropriate restriction enzyme and electrophoresed on 1.0% agarose gel. The DNA was transferred to a Gene-screen filter and hybridized according to the protocol of the supplier (New England Nuclear). For Northern blot analysis ~5 μg of poly(A)$^+$ RNA per lane was run on an agarose–formaldehyde gel (Maniatis *et al.*, 1982) and transferred onto Gene-screen membranes according to the supplier (New England Nuclear). After hybridization, Northern and Southern blots were washed 3 × 20 min in 0.1 SSC/1% SDS/60°C, and exposed to X-ray film in the presence of intensifying screens, generally for 4–7 days at −70°C, except for NOR1 probed filters which were exposed for 4 h. Autoradiographic intensity was quantitated by use of an LKB Ultroscan laser densitometer, followed by weighing of selected peaks from the resulting trace. The signals were normalized with reference to the weight of the NOR1 peak for the appropriate sample.

Gel purified fragments were either nick-translated or randomly primed for use as probes. N71 is derived from a cDNA clone identifying the first exon of the gene NvHbox 1; p158 is from a cDNA clone identifying the second exon of the gene NvHbox 1; X1-424 is a 210 bp cDNA of the 3′ untranslated region of the *Xenopus laevis* type 5 cytoskeletal actin gene; NOR1 is derived from a 700 bp cDNA clone present at high frequency in the newt λgt10 library (DNA sequence analysis of NOR1 and search of the EMBL library have failed to identify a known homologue to this polyadenylated message).

DNA sequencing analysis

The nucleotide sequence of cDNA and genomic subclones was determined using two chain-termination methods: (i) the transcript sequencing method as described by Stratagene Corporation (Bluescribe vector kit); (ii) the Sequenase DNA sequencing kit and method provided by United States Biochemical Corporation (Cambridge Bioscience). The DNA sequence was determined along both strands with exception of 200 nucleotides at the 3′ end of the clone p158 where only one strand was sequenced due to the poly(A) tail.

Acknowledgements

We thank Drs Malcolm Maden and Robb Krumlauf for stimulating discussion. We are also grateful to Drs W.Gehring, D.Melton and T.Mohun for the generous gifts of homeobox and *Xenopus* clones. We are indebted to Dr Maden for providing Figure 5, to Dr D.Fekete for *Pleurodeles* embryos and to Dr P.Ferretti for cultured cells. Finally we thank Drs Donna Fekete, Patrizia Ferretti, Colin Casimir, Robin Brown and Clifton Ragsdale

for many helpful discussions and encouragements. PS is a postdoctoral fellow from the Medical Research Council of Canada.

References

Baron,A., Featherstone,M.S., Hill,R.E., Hall,A., Galliot,B. and Duboule,D. (1987) *EMBO J.*, **6**, 2977–2986.
Benbrook,D., Lernhardt,E. and Pfahl,M. (1988) *Nature (London)*, **333**, 669–672.
Brand,N., Petkovich,M., Krust,A., Chambon,P., De The,H., Marchio,A., Tiollais,P. and Dejean,A. (1988) *Nature (London)*, **332**, 850–853.
Bridges,C.B. and Morgan,T.H. (1923) Carnegie Institute of Washington, publication, **327**, 137.
Brockes,J.P. (1984) *Science*, **225**, 1280–1287.
Burglin,T.R., Wright,C.V.E. and De Robertis,E.M. (1987) *Nature (London)*, **330**, 701–702.
Carrasco,A.E. and Malacinski,G.M. (1987) *Dev. Biol.*, **121**, 69–81.
Carrasco,A.E., McGinnis,W., Gehring,W.J. and De Robertis,E.M. (1984) *Cell*, **37**, 409–414.
Cho,K.W.Y., Goetz,J., Wright,C.V.E., Fritz,A., Hardwicke,J. and De Robertis,E.M. (1988) *EMBO J.*, **7**, 2139–2149.
Crawford,K. and Stocum,D.L. (1988) *Development*, **102**, 687–698.
Dent,J.N. (1962) *J. Morphol.*, **110**, 61–77.
Desplan,C., Theis,J. and O'Farrell,P.H. (1985) *Nature (London)*, **318**, 630–635.
Fainsod,A., Bogarad,L.D., Ruusala,T., Lubin,M., Crothers,D.M. and Ruddle,F.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9532–9536.
Fekete,D.M. and Brockes,J.P. (1987) *Development*, **99**, 589–602.
Fekete,D.M. and Brockes,J.P. (1988) *Development*, **103**, 567–573.
Ferretti,P. and Brockes,J.P. (1988) *J. Exp. Zool.*, **247**, 77–91.
French,V., Bryant,P.J. and Bryant,S.V. (1976) *Science*, **193**, 969–981.
Gallien,L. and Durocher,M. (1957) *Bull. biol. Fr. Belg., fasc*, **2**, 97–114.
Giguere,V., Ong,E.S., Segui,P. and Evans,R.M. (1987) *Nature (London)*, **330**, 624–629.
Guyenot,E., Dinichert-Favarger,J. and Galland,M. (1948) *Rev. Suisse Zool.*, **55**, 1–120.
Iten,L.E. and Bryant,S.V. (1973) *Wilhelm Roux' Arch. EntwMech. Org.*, **173**, 263–282.
Kaiser,K. and Murray,N.E. (1985) In Glover,D.M. (ed.), *DNA Cloning. A Practical Approach.* IRL Press, Oxford, Vol. 1, pp. 1–48.
Kaufman,T.C. (1983) *Time, Space and Pattern in Embryonic Development.* Allan R.Liss, New York, pp. 365–383.
Kessel,M. and Gruss,P. (1988) *Nature (London)*, **332**, 117–118.
Kessel,M., Schulze,F., Fibi,M. and Gruss,P. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5306–5310.
Kessel,M., Fibi,M. and Gruss,P. (1988) In Sato,G.H. and Harris,S.E. (eds), *Cellular Factors in Development and Differentiation.* Alan R.Liss, New York, in press.
Kintner,C.R. and Brockes,J.P. (1985) *J. Embryol. Exp. Morph.*, **89**, 37–55.
Kim,W.S. and Stocum,D.L. (1986) *Dev. Biol.*, **114**, 170–179.
Kiortsis,V. (1953) *Rev. Suisse Zool.*, **60**, 303–410.
Krumlauf,R., Holland,P.W.H., Mevey,J.H. and Hogan,B.L. (1987) *Development*, **90**, 603–617.
Laughon,A. and Scott,M.P. (1984) *Nature (London)*, **310**, 25–31.
Levine,M., Rubin,G.M. and Tjian,R. (1984) *Cell*, **38**, 667–675.
Lewis,E.B. (1978) *Nature (London)*, **276**, 565.
Lipshitz,H.D., Peattie,D.A. and Hogness,D.S. (1987) *Genes Dev.*, **1**, 307–322.
Maden,M. (1983) *J. Embryol. Exp. Morphol.*, **77**, 273–295.
Maden,M. (1985) *Trends Genet.*, **1**, 103–107.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mavilio,F., Simeone,A., Giampaolo,A., Faiella,A., Zappavigna,V., Acampora,D., Poiana,G., Russo,G., Peschle,C. and Boncinelli,E. (1986) *Nature*, **324**, 664–668.
McGinnis,W., Levine,M.S., Hafen,E., Kuroiwa,A. and Gehring,W.J. (1984a) *Nature (London)*, **308**, 428–433.
McGinnis,W., Hart,C.P., Gehring,W.J. and Ruddle,F.H. (1984b) *Cell*, **38**, 675–685.
McGinnis,W., Garber,R.L., Wirz,J., Kuroiwa,A. and Gehring,W.J. (1984c) *Cell*, **37**, 403–408.
Mohun,T.J., Brennan,S., Dathan,N., Fairman,S. and Gurdon,J.B. (1984) *Nature (London)*, **311**, 716–721.
Niazi,I. and Saxema,S. (1978) *Folia Biol. (Krakow)*, **26**, 3–11.
Petkovich,M., Brand,N.J., Krust,A. and Chambon,P. (1987) *Nature (London)*, **330**, 444–450.
Scott,M.P. and Weiner,A.J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4115–4125.
Sharpe,P.T., Miller,J.R., Evans,E.P., Burtenshaw,M.D. and Gaunt,S.J. (1988) *Development*, **102**, 397–407.
Shapiro,M.B. and Senapathy,P. (1987) *Nucleic Acids Res.*, **15**, 7155–7174.
Simeone,A., Mavilio,F., Acampora,D., Giampaolo,A., Faiella,A., Zappavigna,V., D'Esposito,M., Pannese,M., Russo,G., Boncinelli,E. and Peschle,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4914–4918.
Simeone,A., Pannese,M., Acampora,D., D'Esposito,M. and Boncinelli,E. (1988) *Nucleic Acids Res.*, **16**, 5379–5390.
Stocum,D.L. (1984) *Differentiation*, **27**, 13–28.
Wallace,H. (ed.) (1981) *Vertebrate Limb Regeneration.* John Wiley and Sons, Chichester.
Wright,C.V.E., Cho,K.W.Y., Fritz,A., Burglin,T.R. and De Robertis,E.M. (1987) *EMBO J.*, **6**, 4083–4094.

Received on July 27, 1988; revised on September 12, 1988