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A newt type II keratin restricted to normal and regenerating limbs and tails is responsive to retinoic acid

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Summary

In order to understand the molecular mechanisms underlying the regenerative ability of the urodele limb, it is important to identify regeneration-associated proteins and to study their regulation. We have recently shown that the anti-cytokeratin monoclonal antibody LP1K reacts strongly with newt blastemal cells, while its reactivity is restricted in normal limbs. By screening a cDNA expression library from the newt blastema with LP1K, we have identified cDNA clones coding for a type II keratin (NvKII) expressed both in the mesenchyme and the specialized wound epithelium of the blastema. While the rod domain of the protein is highly conserved, the homology between NvKII and mammalian type II keratins drops markedly at the N- and C-terminal regions. The expression of this keratin was analysed by Northern blotting and RNAase protection analysis of various newt tissues, and appears to be organ specific, since it is restricted to normal and regenerating limbs and tails. In particular, we have investigated the expression of this keratin mRNA in normal and regenerating limbs. The transcript is barely detectable in the proximal portion of the normal limb, but its level is high in the distal one. After amputation, NvKII mRNA is expressed both in proximal and distal blastemas, although at higher levels distally, indicating that this keratin is regeneration associated. The NvKII transcript is detectable both in mesenchyme and in the wound epithelium of the regenerate, while no transcript is detectable in normal epidermis. The level of NvKII mRNA is markedly down-regulated both in normal and regenerating limbs following intraperitoneal injection with retinoic acid, a putative endogenous morphogen in limb regeneration.

Key words: keratin, limb, newt, regeneration, retinoic acid.

Introduction

Adult urodeles such as the newt and axolotl are the only vertebrates that can regenerate their limbs and tails, although all vertebrates can repair tissues such as epithelia, muscle, bone and liver (for reviews see Thornton, 1968; Goss, 1969; Wallace, 1981; Sicard, 1986). The first event that occurs after limb amputation is migration of basal cells at the cut edge of the epidermis, and within a few hours the amputation plane is covered by an epithelial sheet whose thickness subsequently increases to form a specialized wound epithelium. This epithelium is distinct from the wound epidermis that forms during normal healing in that it lacks a basement membrane, and therefore maintains direct contact with the underlying mesenchyme. Undifferentiated mesenchymal cells (blastemal cells) are generated from the stump mesoderm in a way that is not fully understood (Ferretti and Brockes, 1991), and accumulate beneath the wound epithelium. The blastemal cells begin to divide within 4–5 days after amputation to form a mound of undifferentiated cells called the blastema. The blastema subsequently undergoes differentiation, which occurs in a proximal–to-distal sequence, and morphogenesis leading to reconstruction of the limb tissues in a spatially organized structure. The nerve supply and the wound epithelium control cell proliferation, and the wound epithelium may also maintain a gradient of differentiation in the underlying mesenchyme and control the direction of outgrowth (Thornton, 1968; Wallace, 1981).

Keratins represent the more complex subclasses of the intermediate filament gene family, since both a type I and a type II keratin gene need to be expressed in order to have filament assembly (Moll *et al.* 1982; Weiss *et al.* 1984; Cooper and Sun, 1986; Steinert and Roop, 1988). The expression of specific keratin pairs has been regarded as a marker for different epithelial cell types and for epidermal differentiation (Lane *et al.* 1985; Osborn and Weber, 1986; Fuchs *et al.* 1987). More recently, however, some instances of non-epithelial expression of keratins have emerged, although in some

cases the levels of expression are rather low (Franko *et al.* 1987; Jahn *et al.* 1987; Bader *et al.* 1988; Jahn and Franke, 1989; Achstaetter *et al.* 1989). We have recently shown, by using a panel of monoclonal antibodies, that the undifferentiated mesenchymal cells of the regenerating newt limb express high levels of the keratin pair 8 and 18 both *in vivo* and *in vitro* (Ferretti *et al.* 1989). One of the monoclonal antibodies used, LP1K (Lane *et al.* 1985), reacted on Western blots of blastemal cytoskeletal fractions with a 52000 M_r protein, the putative newt keratin 8, and with another cytoskeletal protein of 57000 M_r. By immunocytochemistry, reactivity was observed in the majority of blastemal cells and in scattered cells of the wound epithelium, but the normal epidermis was negative. Since these keratins appear to be expressed at a high level in the blastema, we became interested in the possible correlation between the expression of such an atypical mesenchymal phenotype and the regenerative capacity of the urodele limb.

The idea that intermediate filaments are not simply 'housekeeping' constituents of the cell, but that their expression is probably related to the specific function of a certain cell type, has gained increasing popularity in the last few years (Franke *et al.* 1987; Pondel and King, 1988). Furthermore, the expression of specific keratins is regulated by retinoic acid (RA), a potent modulator of epithelial differentiation (Pitt, 1971; Fuchs and Green, 1981; Stellmach and Fuchs, 1989). There is currently great interest in the effects of RA on limb regeneration, where its most striking effect is to provoke duplication of structures along the proximodistal axis by changing the positional value of the blastema (Niazi and Saxena, 1978; Maden, 1982; Stocum, 1984; Brockes, 1989). For example, after RA treatment a wrist blastema will give rise not only to a hand, but also to more proximal structures (Stocum and Crawford, 1987). It is not yet understood how these effects are mediated and which cell populations are the main target of RA action. We therefore became interested in analyzing the expression and regulation of blastemal keratins in order to study molecular and cellular events characteristic of regeneration and to identify cell populations responsive to retinoic acid.

We report here that the 57000 M_r protein recognized by the monoclonal antibody LP1K is a type II keratin that appears to be organ-specific, since its expression is restricted to normal and regenerating limb and tail. This keratin is expressed both in the mesenchyme and in the wound epithelium, but not in the epidermis, and is regulated by RA.

Materials and methods

Animals

All the experiments were carried out on regeneration blastemas and cultured cells derived from the limb of the newt *Notophthalmus viridescens* (Lee's Newt Farm, Tennessee, USA). The animals were maintained in the laboratory at 23°C and fed shredded bovine heart on alternate days. Regeneration blastemas were obtained by anesthetizing the newts in 0.1% tricaine (3-aminobenzoic acid ethylester methanesulphate salt, Sigma) and amputating their limbs bilaterally either at the level of the mid-humerus (proximal amputation), or of the mid-radius/ulna (distal amputation). For comparison of proximal and distal blastemas, newts were amputated proximally on one side, and distally on the other as described previously (Brown and Brockes, 1991). The animals were allowed to recover in 0.5% sulfamerazine (Sigma) for 18–24 h before being returned to the tank. Long-term newt cultures were started from cells that had migrated from small explants of normal limb and grown and passaged as previously described (Ferretti and Brockes, 1988). *Pleurodeles waltlii* embryos, stage 32–35 (Gallien and Durocher, 1957) were supplied by Blades Biologicals Ltd.

Retinoic acid (RA, Sigma) treatment was performed by injecting intraperitoneally (i.p.) 10 μl of a RA-DMSO solution (20 or 30 mg ml^{-1}) as described previously (Savard *et al.* 1988). Control animals were injected i.p. with 10 μl of DMSO. Newts were injected either with RA or DMSO 12 days after distal amputation, and both controls and RA-treated blastemas were harvested one week later. A few animals were allowed to regenerate in order to verify that retinoic acid had effectively proximalized the regenerating limbs. The effect of RA was also tested in unamputated limbs 4 days after injection.

Tissues for RNA extraction were dissected, pooled and stored in liquid nitrogen. Skin or epidermis from normal limbs and the torsos was mechanically separated from the underlying tissues; the tissues from which underlying mesenchyme was carefully removed are defined 'epidermis', while the word 'skin' is used to indicate the presence of subepidermal tissues including some muscle contamination. The blastemal mesenchyme and the wound epithelium were mechanically separated after brief immersion in 0.1 M EDTA (pH 8).

Screening of blastema λgt11 library

The forelimb blastema cDNA library in λgt11 (7.7×10^6 plaque forming units, pfu) described by Savard *et al.* (1988) was screened for cytokeratin proteins recognized by the monoclonal antibody LP1K. 2.5×10^6 pfu of the amplified library were plated onto *E. coli* strain Y1090rk$^-$ and after 3–4 h incubation at 37°C nitrocellulose filters, previously soaked in 10 mM IPTG and dried, were laid over the plates. The filters were removed after 12–16 h incubation at 37°C, rinsed three times in 100 mM Tris-buffered saline pH 8 (TBS), and incubated in 1% low-fat milk protein (Marvel) in TBS for 1 h. After three washes in TBS containing 0.5% Tween 20 (TBS–Tween), the filters were incubated for 1 h in 5% rabbit serum in TBS–Tween to block false positives, and then overnight at 4°C with LP1K hybridoma supernatant diluted 1:2 with TBS–Tween. Extensive washes in TBS–Tween were followed by 4 h incubation with affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse IgG (AP-Rba-MIgG, Miles) in 1% rabbit serum in TBS–Tween. The filters were thoroughly washed and alkaline phosphatase activity was detected by using bromochloroindolyl-phosphate as a substrate.

Analysis of cDNA clones

Selected LP1K positive plaques were purified, cloned into the bluescribe vector (Stratagene Corporation) and analyzed for their size, restriction map and nucleotide composition. The dideoxy sequencing reactions were performed using the Sequenase DNA sequencing kit (United States Biochemical Corporation).

Northern blot and RNAase protection

RNA was extracted from newt tissues by the guanidine isothiocyanate procedure as described by Brown and Brockes (1991). 5 μg of poly(A)$^+$ RNA were used for Northern blot analysis, which was performed essentially according to Casimir *et al.* (1988). Hybridization was performed in 50% formamide at 42°C, and the filters were washed twice for 15 min at 55°C with 0.2 M SSC. A 140 bp *Pst*I–*Pst*I fragment, which encodes part of the α-helix 1B, was used to probe Northern blots (see Fig. 1). The same fragment was transcribed to give a riboprobe for the RNAase protection experiments (performed essentially as described by Casimir *et al.* 1988) and was hybridized with 5 μg of total RNA. In some experiments, a 231 bp *Sac*I–*Fok*I fragment, which encodes part of the C terminus, was used as a template (Fig. 1). The protected fragments were separated on 6% polyacrylamide–urea gels. The satellite 2 DNA probe pSP6D6 (Epstein and Gall, 1987) was used to standardize all RNA samples, in conjunction with measurments of the OD at 260 nm.

Immunochemistry

Detection of LP1K immunoreactivity either on nitrocellulose by Western blotting of cytoskeletal fractions, or on 10 μm cryostat sections of different stages of regeneration blastemas, was performed as previously described (Ferretti *et al.* 1989)

Results

Isolation and analysis of newt keratin clones

As previously observed, the LP1K mAb reacted with a protein of apparent relative molecular mass of 52 000 in cytoskeletal fractions of cultured limb cells, while in blastemal cytoskeletal fractions it reacted in addition with a 57 000 M_r protein (Fig. 1A). When LP1K was used to screen an expression cDNA library from forelimb blastema, reactivity was observed with about 65 plaques of the 2.5×10^6 screened. Of the ten clones purified, seven represented keratin cDNAs, while the other three coded for non-keratin proteins, which presumably express the antigenic determinant recognized by LP1K. The seven keratin clones had the same *Pst*I and *Sac*I restriction map, and three of them (3B3, 1B3 and 4D3) were chosen for further analysis on the basis of their length and 5′ extent. These were overlapping clones coding for the same polypeptide of the type II (basic) cytokeratin family, which we named NvKII (newt keratin type II). 3B3 (~1950 bp) and 1B3 (1554 bp) extended into the 3′ untranslated region, while the 4D3 clone (1334 bp) was contained within the coding region, but extended further towards the 5′ end than the other two (Fig. 1B).

When poly(A)$^+$ RNA from limb blastema and various other tissues was analyzed by Northern blotting using the *Pst*I–*Pst*I fragment as a probe, a single mRNA transcript of ~2.9 kb was detected (Fig. 2).

Nucleotide and amino acid sequence

The nucleotide and deduced amino acid sequences of NvKII determined by the analysis of clones 1B3, 3B3 and 4D3 are shown in Fig. 3. None of the clones analyzed coded for the complete protein. The predicted amino acid sequence contained part of the N-terminal domain, the complete rod domain characteristic of type II cytokeratins, which is rich in basic and neutral amino acids and consists of α-helical tracts 1A, 1B and 2 connected by non-helical linkers, and the complete C-terminal region. The overlapping sequences of 3B3, 1B3 and 4D3 were identical except for four nucleotides in the helical tract 2 of clone 1B3, which identified 4 amino acid substitutions. This probably represents a naturally occurring polymorphism, since these four amino acids are found in many mammalian type II keratins.

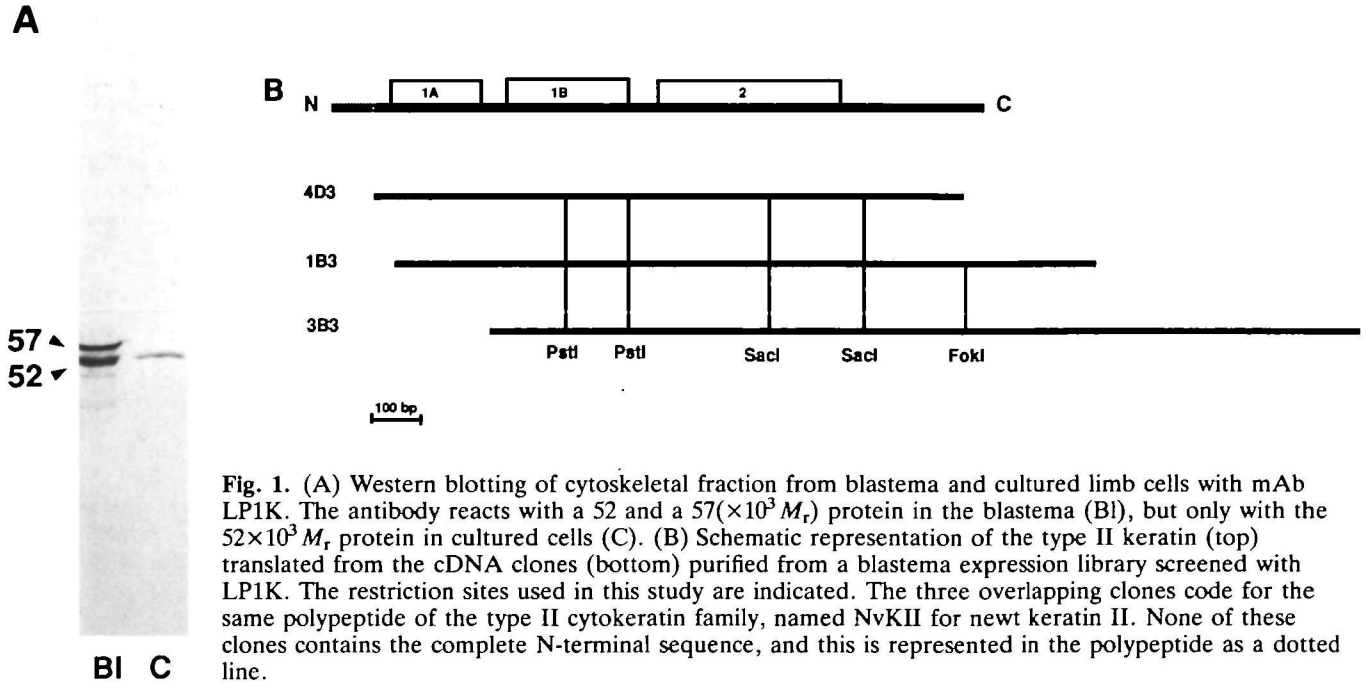

Fig. 1. (A) Western blotting of cytoskeletal fraction from blastema and cultured limb cells with mAb LP1K. The antibody reacts with a 52 and a 57($\times10^3$ M_r) protein in the blastema (Bl), but only with the 52×10^3 M_r protein in cultured cells (C). (B) Schematic representation of the type II keratin (top) translated from the cDNA clones (bottom) purified from a blastema expression library screened with LP1K. The restriction sites used in this study are indicated. The three overlapping clones code for the same polypeptide of the type II cytokeratin family, named NvKII for newt keratin II. None of these clones contains the complete N-terminal sequence, and this is represented in the polypeptide as a dotted line.

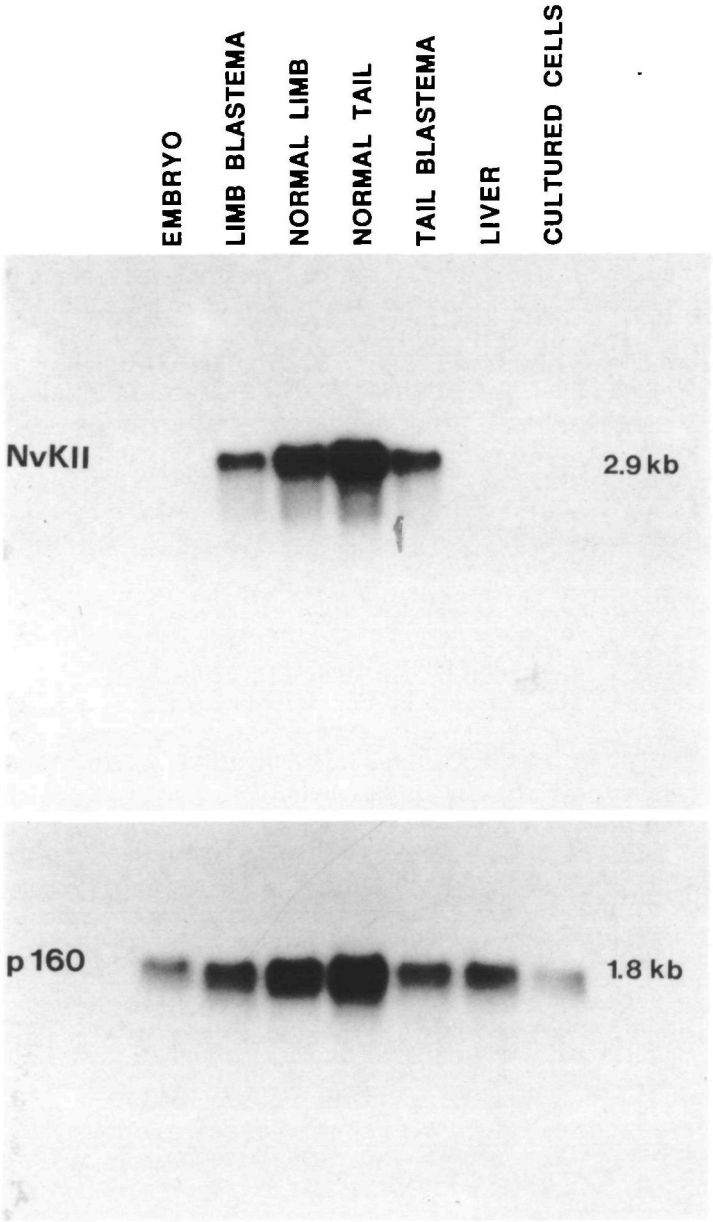

Fig. 2. Northern blot analysis of the NvKII transcript in poly(A)$^+$ RNA from different tissues probed with the *Pst*I–*Pst*I fragment. After probing with *Pst*I–*Pst*I, the blot was stripped and probed with p160 to check for the presence of RNA in all samples. Note that only normal limbs and tails, and their respective blastemas contain a single NvKII mRNA transcript of approximately 2.9 kb. λDNA *Hin*dIII fragments and the transcript of p160 (Savard *et al.* 1988) were used as molecular weight markers.

Comparison with other proteins

When the NvKII polypeptide was compared with type II keratins from different species, the highest percentage of amino acid identity observed was with keratins 5, 6 and 8 (Fig. 4). The identity with each of these keratins was very high in the rod domain, leaving no doubt that NvKII RNA codes for a newt type II keratin (Table 1). The percentage of identity in the entire rod domain was 67–68% for *Xenopus* K5/6 (XK5/6, Foquet *et al.* 1988), *Xenopus* K8 (XK8, Franz and Franke, 1986), human K6B (HK6B, Tyner *et al.* 1985) and human K5 (HK5, Lersch *et al.* 1989); XK8 exhibited a particularly high percentage of identity (100%) in the α-helical tract 1A. At the N-terminal region, the available sequence of NvKII, and, in particular, a stretch of 29 amino acids flanking the rod domain (H1, Table 1) presented somewhat similar percentages of identity with HK5, HK6B and XK8 (66–72%). The amino acid identity between the newt and other type II keratins dropped markedly in the C-terminal region. Analysis of the different subdomains of this region (Table 1) showed significant differences in the percentage of identity between NvKII and various keratins. The H2 subdomain (Steinert and Roop, 1988), a relatively highly conserved stretch of 20 amino acids flanking the rod domain, had higher identity with HK5 and HK6B than with the *Xenopus* keratins. At the C terminus, which usually contains distinctive sequences for different type II keratins, the motive TTTS present in XK5/6 and HK6B was observed. The variable region V2 at the C terminus of NvKII presented very low identity with other type II keratins, but it was rich in inexact glycine and serine repeats which are typical of epidermal keratins in mammals, and they are also found in simple epithelium keratins in goldfish (Giordano *et al.* 1989), *Xenopus* (Franz and Franke, 1986) and chick (Charlebois *et al.* 1990).

Tissue distribution of NvKII mRNA

RNA was prepared from a variety of tissues and organs for analysis by Northern blotting and by RNAase protection. All preparations used in this study were normalized for both procedures by parallel analysis of probes that are ubiquitously expressed (see Materials and methods). The probes for NvKII message hybridized at detectable levels only with normal limbs and tails, and their respective blastemas (Figs 2, 5, Table 2). Among the negative RNA preparations were epithelial tissues such as liver, esophagus, stomach, which are known to express non-epidermal keratins, and tissues such as brain, cultured newt mesenchymal cells and *Pleurodeles* embryos. Preparations from limb skin showed very low but detectable levels of NvKII mRNA, whereas no transcript was detected in skin from the torso and in the normal limb epidermis (Fig. 5,

Table 1. *Comparison of NvKII with other type II keratins expressed as % of amino acid identity*

Keratin	XK5/6	XK8	HK6B	HK5
H1*	55	55	53	58
HELIX 1A	89	100	89	92
LINKER 1	60	45	55	55
HELIX 1B	69	67	73	76
LINKER 2	67	88	67	63
HELIX 2	64	63	63	65
H2*	50	20	65	75

*H1 and H2 refer respectively to the 29 amino acids flanking the rod at the N terminus, and to the 20 amino acids flanking the rod at the C terminus.

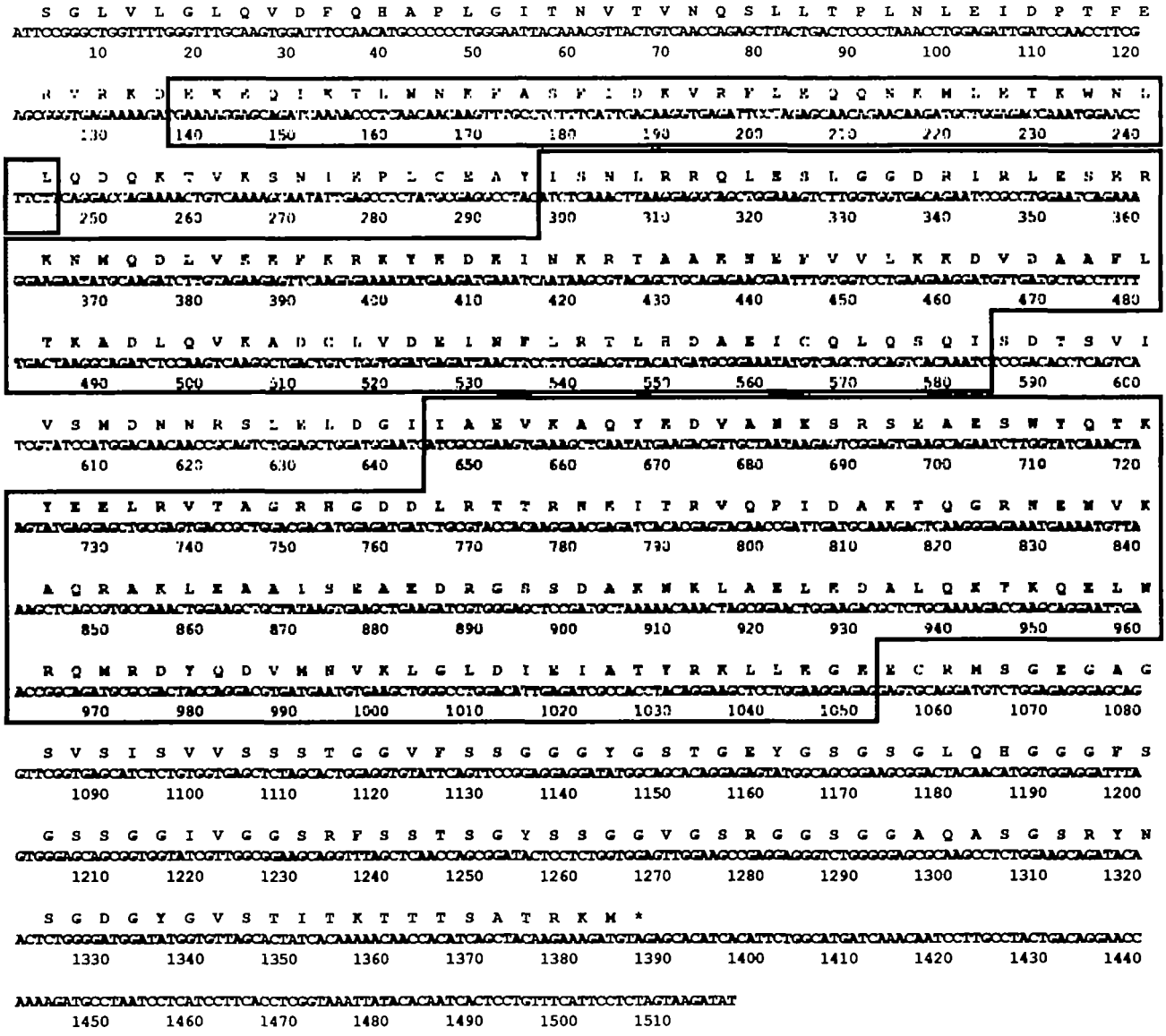

Fig. 3. Sequence and translation of overlapping NvKII cDNA clones. The enclosed blocks define the helical parts of the molecule.

Table 2). The tissue distribution of NvKII transcript in the limb blastema was further analyzed after separating the wound epithelium from the underlying blastemal mesenchyme. RNA preparations from both sources gave a significant signal on RNAase protection analysis, although the blastemal mesenchyme was lower than the wound epithelium. It should be noted that the levels of expression in both blastemal tissues was significantly higher than in limb skin. The RNAase protection analysis of the various RNA samples was repeated using a non-overlapping probe (*Sac*I–*Fok*I, see Fig. 1) with identical results (not shown).

LP1K reactivity in the wound epithelium

Cryostat sections from 3 and 7 day newt limb regenerates were stained with LP1K (Fig. 6). No staining was observed either in the blastemal cells or in the wound epithelium 3 days after amputation (Fig. 6A, B), although, as previously reported, antibody reactivity was observed in the mesenchyme 7 days after amputation and the majority of blastemal cells were positive at 15 days. LP1K reactivity was consistently observed within the wound epithelium in 7-day (Fig. 6C) and 15-day (not shown) regenerates, but with a certain variability among different animals. While the wound epithelium showed little LP1K reactivity in some blastemas, others had many positive cells and, in a few cases, the majority of the epithelial cells within this structure were brightly stained. Immunoreactivity in 15-day wound epithelium was consistent with the presence

Fig. 4. Amino acid comparison with *Xenopus* keratin 5/6 (XK5/6), *Xenopus* keratin 8 (XK8), human keratin 6B (K6b) and human keratin 5 (K5).

Table 2. *Distribution of NvKII mRNA in newt tissues assayed by Northern blotting or RNAase protection*

−	+/−	++
Limb epidermis	Limb skin	Distal limb
Cartilage	Proximal limb	Proximal limb blastema
Muscle		Distal limb blastema
Cultured limb cells		Limb blastema mesenchyme
Tongue		Limb blastema epithelium
Viscera		Normal tail
Esophagus		Tail blastema
Stomach		
Liver		
Spleen		
Heart		
Torso		
Torso skin		
Brain		
Embryos		

of NvKII mRNA as demonstrated by RNAase protection (Fig. 5).

Axial distribution of NvKII transcript and its modulation by retinoic acid

The proximodistal distribution of the NvKII transcript was analyzed by RNAase protection using RNA from paired samples of normal limbs or from blastemas (Brown and Brockes, 1991). In normal limbs, there was a striking difference between proximal samples (mid-humerus to elbow), where a faint signal could only be detected after long exposures, and distal samples (wrist-hand), where the NvKII transcript was readily detectable (Fig. 7). Following amputation, the transcript was expressed at high levels both in proximal and distal blastemas. The proximodistal difference observed in normal limbs was also present in the blastema, although it was less marked (Fig. 7). These results were obtained

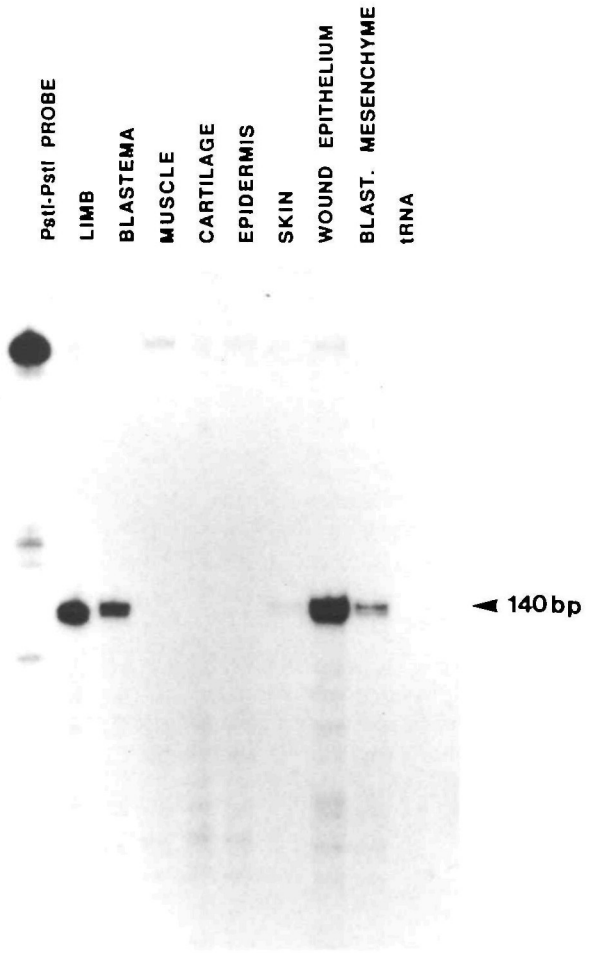

Fig. 5. RNAase protection of total RNA preparations from various limb tissue using the *Pst*I–*Pst*I probe (Fig. 1). NvKII transcript is present in total limbs, but not in muscle, cartilage and epidermis; low levels of the transcript are detectable in the skin. In the 15 day blastema NvKII mRNA is detected both in the wound epithelium and in the mesenchyme.

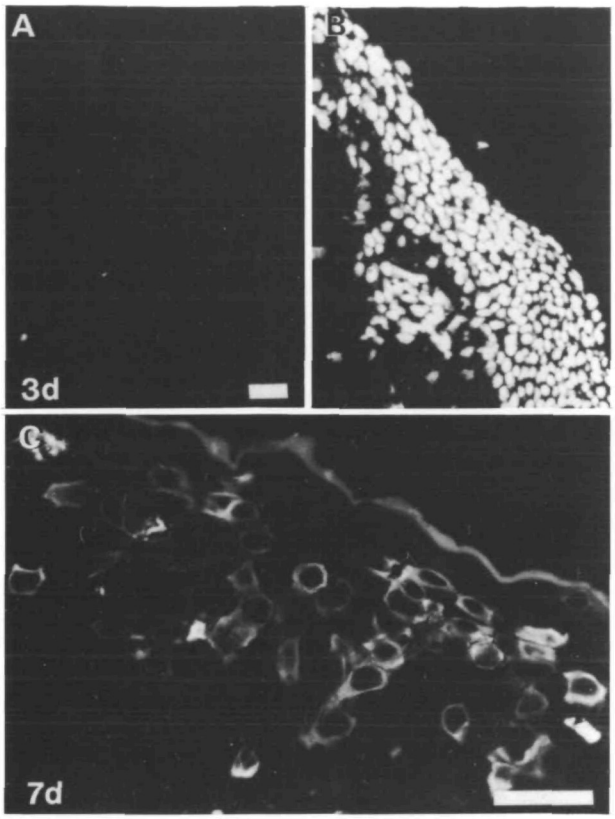

Fig. 6. LP1K immunostaining of the wound epithelium 3 days (A), with corresponding nuclear staining (B) and 7 days (C) after limb amputation. No staining is observed at 3 days, while scattered LP1K-positive cells are present at 7 days. The staining observed at the epidermis edge is due to autofluorescence. Scale bars are 50 μm.

with both the *Pst*I–*Pst*I and the *Sac*I–*Fok*I fragment probes (Fig. 7).

When RNA samples from blastemas were analyzed 7 days after RA or DMSO injection (see Materials and methods), the levels of the NvKII transcript was much lower in blastemas from RA-treated animals than in controls (Fig. 8A). Strong inhibition of NvKII transcription was also observed in unamputated limbs 4 days after RA injection (Fig. 8B). It should be noted that the dose of RA used in these experiments produces proximal respecification of distal blastemas.

Discussion

NvKII is the first newt keratin to be cloned, and its homology with type II keratins in other species further exemplifies the high degree of conservation within this gene family. The NvKII protein is one of two newt keratins recognized by the LP1K mAb. These keratins have apparent relative molecular masses of 52 and 57K and we have previously suggested on the basis of monoclonal antibody reactivities that the 52K keratin is the newt homologue of mammalian keratin 8. This keratin is expressed in the undifferentiated mesenchymal cells of the regenerating limb both *in vivo* and *in vitro*. NvKII message is not detected in newt limb cultures, which express only the 52K keratin (Fig. 1), or in the liver, a tissue that shows LP1K immunoreactivity (Ferretti, unpublished observation). The discrepancy between the presence of antibody reactivity and the lack of NvKII mRNA in these tissues indicates that the NvKII cDNA does not code for the newt keratin 8, but for the 57K keratin.

The percentage of amino acid identity of NvKII with keratin 5 and 6 from various species is very similar, but the fact that the NvKII transcript is not present in normal epidermis suggests a closer relation of NvKII to HK6 than to HK5. Nonetheless, since no other newt keratin cDNA has been analyzed, and since the tissue distribution of NvKII is rather unusual, it is presently unjustified to classify this protein as the homologue of mammalian keratin 6.

Keratin NvKII presents the peculiar feature of being organ specific, since it is detectable only in normal and regenerating limbs and tails, rather than tissue specific

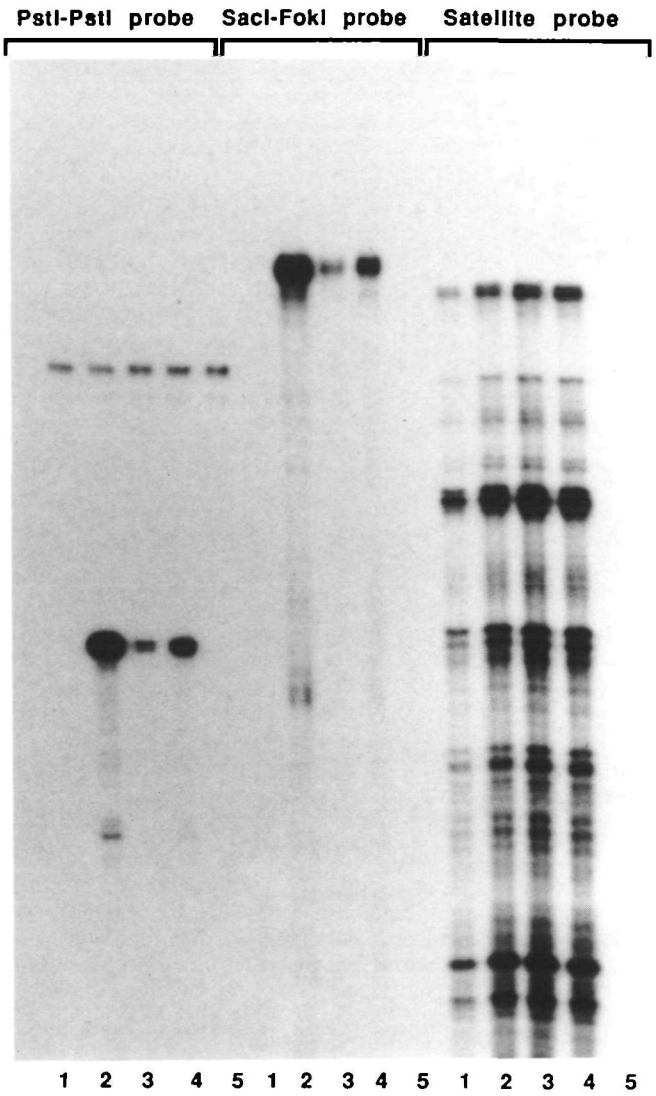

Fig. 7. RNAase protection analysis of proximal and distal fragments from normal limb and proximal and distal limb blastema using the *Pst*I–*Pst*I and *Sac*I–*Fok*I probes (Fig. 1). The same samples were also reacted with the satellite probe pSP6D6 to normalise them. In proximal limbs the NvKII transcript is almost undetectable, while high levels are observed in distal limbs. A proximal–distal difference is also observed in the blastema, but is less dramatic than in normal limbs. Lanes: (1) proximal normal limb; (2) distal normal limb; (3) proximal blastema; (4) distal blastema; (5) tRNA.

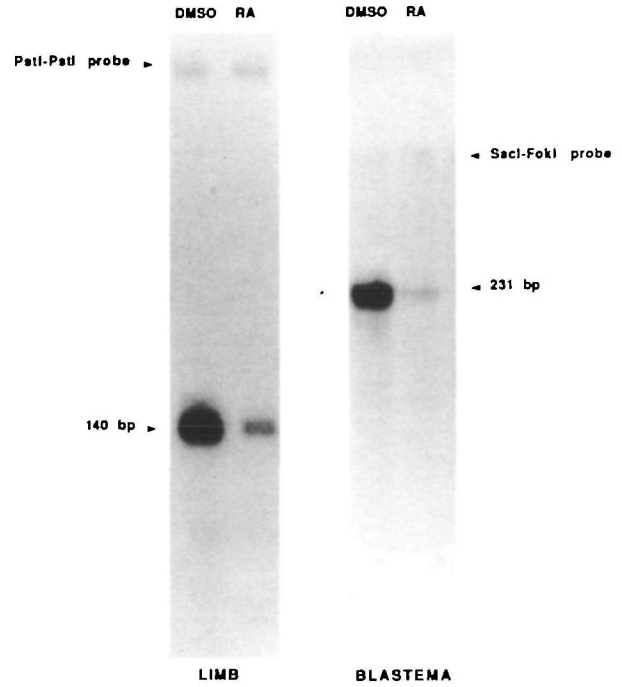

Fig. 8. RNAase protection analysis of total RNA preparation of normal limb and limb blastemas derived from animals treated either with RA or its vehicle, DMSO, using both the *Pst*I–*Pst*I and *Sac*I–*Fok*I probes. Four days after treatment of unamputated newts, NvKII mRNA levels are strongly decreased in the limbs of RA-injected animals. The same result is observed in distal blastemas from animals injected 12 days after amputation, and harvested 7 days later.

as described for intermediate filaments in general. The only other example of a keratin filament that is indicative of regional specialization has been recently described in the chick (Charlebois *et al.* 1990). During neurulation the levels of the type II keratin CHse1 increase dramatically in the trunk ectoderm, while remaining low in the contiguous head ectoderm and in the neural ectoderm. Interestingly, during development this keratin is also expressed in endoderm and in the heart, but its distribution in the adult has not been reported. We do not know if NvKII is more widely expressed at early developmental stages and later becomes restricted to limbs and tails, or whether it is always specific to these organs. The next step will be to analyze NvKII expression at different developmental stages in order to establish whether this keratin is regulated both temporally and spatially during early development as described for some *Xenopus* keratins (Jonas *et al.* 1985; Jamrich *et al.* 1987; Foquet *et al.* 1988; Dawid *et al.* 1988).

Both normal limbs and limb blastemas express a high level of NvKII mRNA as shown by RNAase protection analysis. In the normal limb, NvKII is not expressed in the epidermis, but low levels of mRNA are detectable in the skin, which contains subepidermal glands, capillaries, nerve fibers and smooth muscle embedded in the dermis. The stripped skin might also contain some contamination from the underlying mesodermal tissues. The LP1K mAb does not stain the epidermis, but stains subepidermal glands, blood vessels and perineurium (Ferretti *et al.* 1989), and the presence of NvKII transcript in the skin is consistent with these immunoreactivities. The lack of NvKII message in striated muscle is also consistent with negative antibody staining in this tissue, while the fact that NvKII is not expressed in cartilage from the normal limb, although immunoreactivity is observed in regenerating cartilage, might indicate that this tissue expresses only the 52K keratin. Since LP1K reacts with both the 52 and the 57K

keratins, it is not possible to assess which of the cell types stained by the antibody expresses NvKII. We are currently attempting to analyze the distribution of the transcript by *in situ* hybridization, in order to localize more precisely the cellular distribution of the NvKII mRNA expecially in distal normal limbs (wrist and hand), where dissection of the different tissue components does not give sufficient resolution.

In the limb blastema, NvKII is expressed both in the undifferentiated mesenchymal cells and, at somewhat higher levels, in the wound epithelium. The NvKII transcript is apparently induced after amputation of the limb at the proximal level, where only a very low level of mRNA is detectable prior to amputation. Expression of this keratin appears therefore to be associated with the regenerative process. The presence of NvKII mRNA in the blastemal mesenchyme indicates that a population of blastemal cells expresses this keratin, and that NvKII may be a useful marker for identifying and analyzing such a population in order to gain further insight into the origin and fate of blastemal cells. Since no NvKII mRNA is detected in the normal epidermis, its expression in the wound epithelium appears to be induced following amputation and to be regulated at the level of stable mRNA. Expression of the keratin 6 proteins in human epidermis is induced by wounding but unlike NvKII, HK6A and HK6B mRNAs are also present in normal epidermis, indicating that the expression of these keratins *in vivo* is partly regulated at the translational level (Weiss *et al.* 1984; Tyner and Fuchs, 1986). We have not yet determined if NvKII is induced only in the wound epithelium of the regenerating limb, or also in the epidermis of unamputated limbs after wounding. The time course of NvKII expression, however, suggests that its induction is specific to the wound epithelium, since wound healing occurs within 24 h after amputation, and no antibody staining has been detected in the wound epithelium of a 3 day regenerate. LP1K reactivity within the thickened wound epithelium has been observed at later stages of the regenerative process, when the 57×10^3 M_r protein is detected in the wound epithelium also by Western blotting. These results indicate the presence of NvKII in the wound epithelium, although they do not rule out the possibility that a subpopulation of the LP1K positive cells might express the 52×10^3 M_r keratin.

The pattern of expression of NvKII in the skin identifies a difference in cell composition between the specialized wound epithelium of the regenerating limb and the skin epidermis. To our knowledge, the only other example of a protein with a similar pattern of distribution, is the antigen recognized by WE3 mAb (Tassava *et al.* 1986). Since a difference in molecular composition is likely to reflect the different physiological role of skin epidermis and wound epithelium, NvKII might be a marker for the epithelial cells which play a role in maintaining blastemal cell cycling or their dedifferentiated state.

The NvKII clone seems to be a good molecular marker in the newt limb for the effects of a morphogenetic dose of RA. While neither newt NvHBox 1 nor NvHBox 2 mRNA levels are affected by RA treatment (Savard *et al.* 1988; Brown and Brockes, 1991), NvKII mRNA is significantly reduced in early blastemas harvested 7 days after RA injection, and in normal limbs 4 days after injection. The RA-induced changes in NvKII expression are manifest as changes in the level of stable RNA, but further experiments will be required to demonstrate that they are transcriptional. We do not know if such changes reflect a direct or indirect response to RA, but many reports suggest an indirect regulation of keratin expression in mammalian tissues by RA (Kim *et al.* 1987; Stellmach and Fuchs, 1989), and it is likely that a similar mechanism operates in the regulation of newt keratins. The decrease in NvKII mRNA levels upon RA treatment may reflect a change in the differentiation program in NvKII-positive cells. It is also interesting to speculate that the different proximodistal levels of NvKII transcript observed in normal limbs and blastemas might reflect local differences in the endogenous RA levels along this axis. The negative effect of RA on NvKII is consistent in this respect with the proximalising action of RA, and the observation that NvKII expression is higher in a distal blastema. More work will be necessary to assess whether RA equally affects NvKII expression in the wound epithelium and in the mesenchyme, how its expression is regulated, and which of the recently cloned RA receptors (RARs) mediates this effect (Ragsdale *et al.* 1989; Ragsdale and Brockes, 1990). The fact that the δRAR is preferentially, although not exclusively, expressed in the limb (Ragsdale and Brockes, 1990) makes it an interesting candidate for such a role.

On the basis of the results discussed here, it is tempting to speculate that there might be a relationship between the organ-specificity, the axial distribution of NvKII in the limb and the regenerative potential of newt limbs and tails. NvKII is found both in the wound epithelium and in the blastema mesenchyme, and the fact that RA regulates its expression suggests that NvKII-positive cells are responsive to RA, and might therefore be involved in specifying positional information. Characterization of this cell population should provide additional insights into these important issues.

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