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Expression of Regeneration-Associated Cytoskeletal Proteins Reveals Differences and Similarities Between Regenerating Organs

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ABSTRACT **The unique events which allow regeneration of an entire organ to occur are formation of a specialized wound epidermis and accumulation of progenitor cells (blastemal cells) at the amputated surface to form a blastema. In order to identify some of the molecular events underlying the early stages of the regenerative process which are either common to different systems or specific to one of them, we have investigated whether molecules which are induced in limb blastemas are also expressed in skin repair and during regeneration of other complex body structures (lower jaws, upper jaws, and tails). In addition, we have addressed the issue of the identity of progenitor cells during jaw development and regeneration by analyzing the expression of limb blastemal markers in the developing head and face. We have focused on cytoskeletal components, and particularly on the epidermal keratin NvKII, the simple epithelial keratins 8 and 18 and 22/18, because they are among the few molecules which have been shown to be associated with regeneration in the limb and may play significant roles in various developmental processes.**

Some important findings emerge from this study: 1) Expression of the epidermal keratin NvKII, unlike that of its mammalian homologue K6, is not simply induced in response to wounding, but is associated with regeneration of specific organs. In fact, NvKII is expressed in regenerating limbs and tails, but not in upper or in lower jaw regenerates, demonstrating the existence of molecular differences in the composition of the wound epidermis in these systems. This, together with the fact that NvKII mRNA is regulated by retinoic acid, which differentially affects patterning of limbs and jaws, argues for distinct inductive abilities of the wound epidermis in different organs. 2) In contrast to the differential expression of the epidermal keratin NvKII, the regeneration-associated cytoskeletal molecules identified in limb blastemal cells are expressed in a similar fashion in jaw and tail blastemas. Therefore, it appears that similar cellular events lead to the establishment of an actively proliferating population of progenitor cells from the stump of different organs. Finally, the mesenchyme of the facial rudiments, unlike that of developing limb buds, expresses simple epithelial keratins. Thus, it appears that mesenchymal progenitor cells of developing and regenerating jaws are alike in regard to their intermediate filament content, and this may be related to nerve-dependent growth control of progenitor cells in different developing and regenerating systems. ***Dev. Dyn. 1997;210:288–304.***

Key words: jaw; limb; keratin; regeneration; skin wounding; development; urodele amphibians

INTRODUCTION

The relationship between regeneration of an entire complex body part (epimorphic regeneration), tissue repair, and response to injury in different organs is of fundamental importance in biology and medicine. Nonetheless, the issue of whether early molecular responses to injury are similar in different regenerating systems, and how these regeneration-associated events relate to developmental processes has not yet been fully elucidated. Because of their ability to regenerate a variety of complex body structures, urodele amphibians represent an excellent model for addressing these issues. The unique events which allow epimorphic regeneration to occur are formation of a specialized wound epidermis and accumulation of progenitor cells, blastemal cells, at the amputated surface to form a blastema. The wound epidermis is identified at a morphological level by its thickness, which is significantly greater than that of normal skin and the lack of a basement membrane, which allows direct interaction with the underlying mesenchyme (Thornton, 1968). Blastema formation is observed neither in organs that cannot regenerate epimorphically nor during tissue repair—for example, after skin injury—either in urodeles or in mammals. It is becoming apparent that patterning of the regenerating appendages is likely to be governed by similar

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mechanisms and the same set of molecules used to build them during development (Akimenko et al., 1995; Gardiner et al., 1995). On the other hand, differences both in the cell phenotype and in the mode of growth of limb buds during development and regeneration have been identified, suggesting that in this respect regeneration does not recapitulate development (Fekete et al., 1987; Fekete and Brockes, 1988; Ferretti et al., 1989).

Both growth and spatial specification of the blastema appear to rely on interactions between the wound epidermis and the underlying mesenchyme (Stocum and Dearlove, 1972), which are believed to resemble classical epithelial–mesenchymal interactions occurring in developing limbs and jaws (Summerbell et al., 1973; Wedden et al., 1988; Tickle, 1991; Richman and Tickle, 1992). In the regenerating limb, proliferation, but not initial accumulation, of the majority of blastemal cells is also under nervous control. The issue of nerve dependency of jaw regeneration is more controversial because of the difficulties in denervating the jaws and in maintaining them denervated (von Szutz, 1914; Guyenot and Vallette, 1925; Vallette, 1929; Finch, 1975). Following the proliferative phase, the blastemal cells differentiate, undergo morphogenesis, and rebuild a faithful copy of the missing part. In adult urodeles this extraordinary regenerative ability is found in limbs (Wallace, 1981; Sicard, 1985), in tails, including the spinal cord and spinal ganglia (Egar and Singer, 1972; Nordlander and Singer, 1978; Géraudie et al., 1988), and in both upper and lower jaws, including associated musculature, teeth, and sensory and secretory epithelia (Vallette, 1929; Goss and Stagg, 1958; Ghosh et al., 1994).

The regenerating limb has been the most studied of these systems, whereas very little is known about the phenotype of jaw blastemal cells (Ghosh et al., 1994). The search for regeneration-associated molecules in the limb has led to the identification of a few blastemal markers, mainly cytoskeletal components (reviewed by Ferretti and Brockes, 1991). Two of the molecules up-regulated at early stages of regeneration are newt vimentin and the 22/18 protein. The latter is an intermediate filament component, yet to be fully characterized, which is expressed in the subpopulation of blastemal cells whose division is nerve dependent (Kintner and Brockes, 1985; Ferretti and Brockes, 1991). Around 4–5 days after amputation, immunoreactivity for keratins 8 and 18, also members of the intermediate filament super-family, is detected in blastemal cells. In addition, significant changes in keratin expression are observed in the wound epidermis, and the mAb LP1K, which recognizes simple epithelial keratins in mammals (Lane et al., 1985; Ferretti et al., 1989), reacts both with the blastema and the wound epidermis (Lane et al., 1985; Ferretti et al., 1989). Therefore, it appears that major changes in the cell cytoskeleton of both wound epidermis and blastemal cells, as compared to the tissues of the stump, are induced following amputation.

We have recently isolated three newt keratins which are up-regulated in regenerating limbs, NvKII, NvK8, and NvK18 (Ferretti et al., 1991, 1993; Corcoran and Ferretti, 1997). NvKII amino acid sequence presents a fairly high percentage of homology with the human epidermal keratin 6 (K6). This keratin is normally expressed in hair follicles and in suprabasal cells of certain internal stratified epithelia, but not in normal epidermis, where it is up-regulated following skin wounding and hyperproliferative states, such as psoriasis and malignant transformation (Tyner and Fuchs, 1986; Ferretti et al., 1991). From RNAase protection analysis it appears that the levels of NvKII mRNA are higher in limb wound epidermis than in the blastemal cells and that NvKII is expressed also in normal distal limbs (Ferretti et al., 1991). However, this type of analysis does not allow precise cellular localization of NvKII in normal and regenerating limbs. In addition, it remains unclear whether NvKII might be simply expressed in response to skin injury, like the mammalian K6, or whether it might represent a response to amputation common to all systems which regenerate epimorphically.

The other two newt keratins we have isolated, NvK8 and NvK18, are clearly homologues of the mammalian keratin pair 8 and 18 (K8 and K18) expressed in simple epithelia (Ferretti et al., 1993; Corcoran and Ferretti, 1997). In lower vertebrates, however, their expression is found in some non-epithelial structures which grow throughout life and have regenerative capability, such as the fish optic nerve and the fin (Druger et al., 1992; Fuchs et al., 1994). In regenerating limbs, NvK8 and NvK18, like 22/18, are expressed in the mesenchymal progenitor cells but not in the wound epidermis, and are not expressed in developing limb buds (Kintner and Brockes, 1985; Ferretti et al., 1993). However, a wave of K8 and K18 expression occurs early in development, before any differentiation is apparent (Jackson et al., 1980; Oshima et al., 1983; Franz and Franke, 1986). In addition, depletion of maternal K8 in *Xenopous* embryos results in abnormal gastrulation, and certain transgenic mice where keratin 8 has been knocked-out die at mid-gestation (Heasman et al., 1992; Baribault et al., 1993). These observations suggest that K8 and K18 may play an important role in cells with a broad developmental potential, and that their up-regulation in blastemal cells, especially if it is indeed common to many different regenerating structures in the same species, may be causally related to blastema growth and the maintenance of the undifferentiated state during the early phases of regeneration.

Since analysis of early regenerates has been so far limited to the limb, in this study we are concerned with identifying early changes occurring in mesenchymal progenitor and epidermal cells during epimorphic regeneration in other organs, with particular emphasis on upper and lower jaws. In addition, we address the issue of the relationship between epimorphic regeneration and tissue repair by assessing whether changes in the phenotype of the wound epidermal cells parallel those occurring during skin repair. We focus on cytoskeletal

TABLE 1. Comparison of Regenerating Jaws, Limbs, and Tails at Different Times After Amputation in *Notophthalmus viridescens

Age of the regenerate	Organ	Morphology[a]	Differentiation markers (muscle, cartilage, Schwann cells)[b]	Innervation (RT97)	Tenascin[c]	NCAM[d]
1 week	Jaw	Small number of blastemal cells	n.d.	Some fine fibers reaching the WE	Blastemal cells	Blastemal cells and some cells in WE
	Limb	=	Not expressed	=	Blastemal cells and some cells in WE	n.d.
	Tail	=	=	=	Blastemal cells	Blastemal cells
2–3 weeks	Jaw	Mound of blastemal cells	Not expressed	Some fine fibers reaching the WE	Blastemal cells	Blastemal cells and WE
	Limb	=	=	=	Blastemal cells and some cells in WE	=
	Tail	=	=	=	=	=
5–6 weeks	Jaw	Blastemal cells present distally/differentiation progressing in proximodistal fashion	Re-expressed proximally	Some fine fibers and regenerating nerves	Blastemal cells, some cells in WE and proliferating cartilage/down-regulated in differentiated muscle and cartilage	Blastemal cells, proliferating cartilage and regenerating nerves
	Limb	=	=	=	=	n.d.
	Tail	=	=	n.d.	=	n.d.

*It should be noted that the times given reflect the average state and behavior of the regenerating structure, since some variability between animals is normally observed; = indicates same behavior as in jaws; n.d., not done.
[a]Goss and study (1958), Iten and Bryant (1976), Wallace (1981), Ghosh et al. (1994).
[b]Kintner and Brockes (1984, 1985), Khrestchatisky et al. (1988: study carried out in the newt *Pleurodeles waltl*), Arsanto et al. (1992: *Pleurodeles waltl*), Ghosh et al. (1994).
[c]Time course in the limb: Onda et al. (1991); time-course in *Pleurodeles* tail: Arsanto et al. (1990); we have observed some tenascin expression also in the tail wound epidermis.
[d]Analyzed by Maier et al. (1986) in limb blastemas.

components, and particularly on the epidermal keratin NvKII, the simple epithelial keratins 8 and 18, and 22/18, because they are among the few molecules which have been shown to be regeneration associated in the limb, and, as discussed above, may play important roles in various developmental processes. The cytoskeletal changes we have identified in the epidermal and mesenchymal components of regenerates from different organs demonstrate significant differences in the epidermal cells during epimorphic regeneration and skin repair and suggest that 1) the wound epidermis from different organs has distinct inductive abilities and 2) that the cytoskeletal composition of blastemal cells may be related to different modes of growth control.

RESULTS

To compare the time-course of the early stages of regeneration in different organs and establish which time points were appropriate to study, we have used the following criteria: 1) morphological analysis both at the gross and microscopic level with particular emphasis on the presence of undifferentiated cells in the regenerate; 2) loss and reappearance of markers of the differentiated state, as they can provide information on the state of the regenerate (Ghosh et al., 1994); 3) expression of molecules which are developmentally regulated during limb and tail regeneration, such as tenascin, which is observed in the majority of blastemal cells and may be associated with de-differentiation and epithelial–mesenchymal interactions (Onda et al., 1991), and NCAM (Maier et al., 1986); 4) occurrence of nerve sprouting in regenerating jaws using the anti-neurofilament antibody RT97 in order to establish whether innervation of jaw regenerates is an early event as in the limb. Molecules believed to play a role in patterning are not useful markers for comparing regeneration stages of structures which will ultimately be very different and will therefore be patterned either by different genes and/or by different spatio-temporal expression of the same genes (Ghosh et al., 1996).

The criteria adopted for the comparison, the literature sources of information, and our own results are summarized in Table 1, and examples of staining of 1- and 2-week jaw blastemas with tenascin and NCAM are given in Figure 1A–D; the plane of the sections is indicated in Figure 1E–G. This comparison shows that although at the gross morphological level jaw blastemas are less pronounced than limb and tail blastemas, their characteristics at the time points used in this study are indeed quite similar, and regeneration proceeds in a proximodistal direction in all systems. Overall the speed at which regeneration of jaws, limbs, and tails proceeds from the time of amputation to the appearance of differentiating structures in the regener-

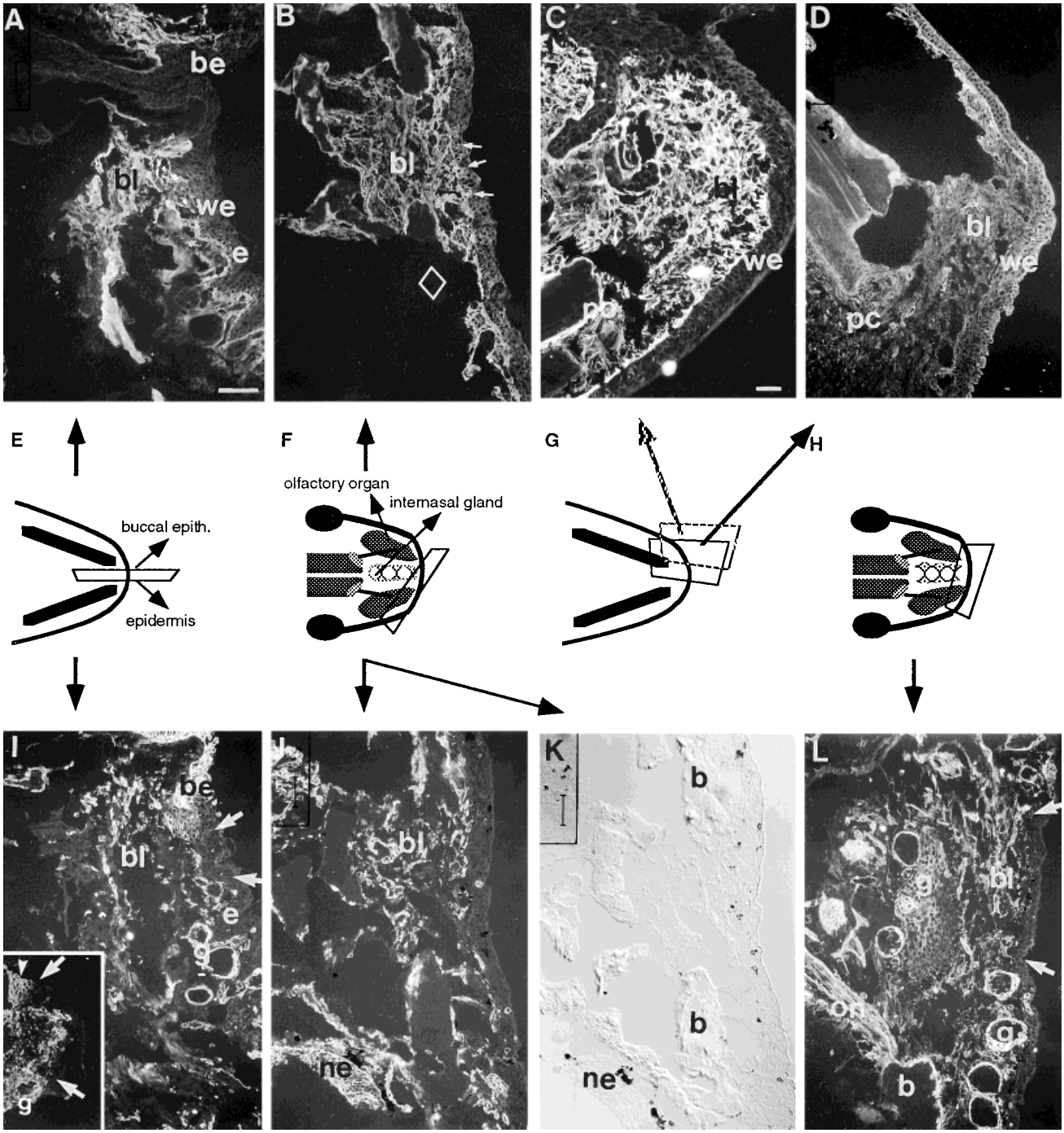

Fig. 1. (Legend on page 292.)

ate does not seem greatly different (Goss and Stagg, 1958; Iten and Bryant, 1976; Wallace, 1981; Ghosh et al., 1994; this study). The fact that completion of regeneration is a longer process in jaws, and particularly in upper jaws, is probably due to the occurrence of more extensive remodeling of these complex structures once the basic scaffolding has formed.

Is NvKII Expression Regeneration Associated or Simply Induced in Response to Injury Like Its Mammalian Homolog K6?

The two newt keratins recognized by LP1K, NvKII and NvK8, are expressed in regenerating limbs (Ferretti et al., 1991). NvK8 expression is restricted to

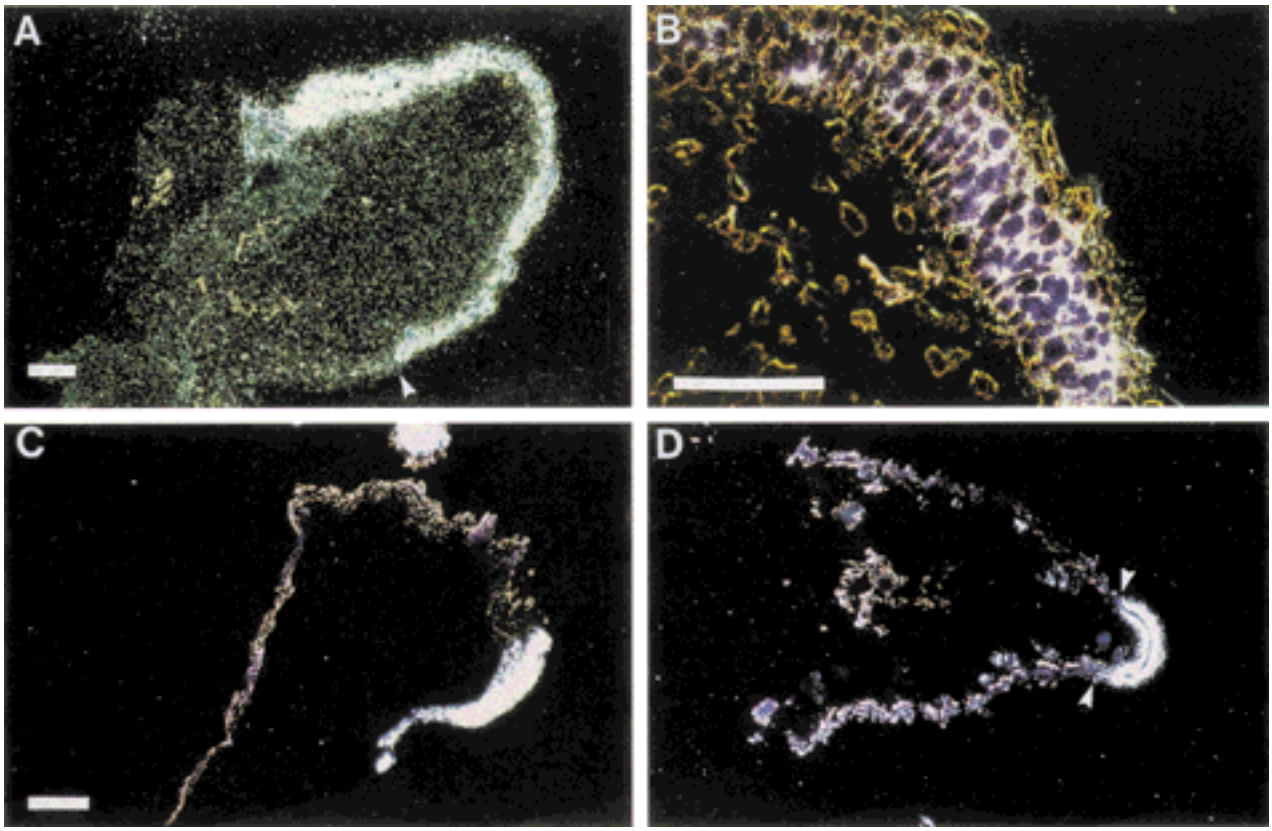

Fig. 2. Analysis of NvKII expression by in situ hybridization in regenerating limb and tails. **A:** Limb blastema. **B:** High magnification of the wound epidermis of a limb blastema. **C:** Digit stage limb regenerate. **D:** Tail blastema. Note that NvKII is localized in the WE of regenerating limbs and tails; the arrowheads in A and D indicate the boundary between NvKII-positive wound epidermis and NvKII-negative normal skin. Scale bars = 100 μm (A,C,D) and 50 μm (B); C and D are at the same magnification.

blastemal cells, but the NvKII transcript is detected by RNAase protection assays both in the WE and in the mesenchyme of limb blastemas, although its levels are significantly higher in the WE (Ferretti et al., 1991). Mechanical dissection of the two tissues, however, might result in some contamination and does not allow cellular localization. We have therefore analyzed the expression of the NvKII transcript by in situ hybridization (Fig. 2). High levels of NvKII mRNA are detected in the WE of tail and limb blastemas (Fig. 2A,B,D) and are

Fig. 1. Tenascin (**A,C**), NCAM (**B,D**), and LP1K (**I–L**) reactivity in 1-week (A,B,I,J), 2-week (C,D), and 6-week (L) jaw regenerates. The planes of sectioning are schematically indicated in E–H, where thicker arrows point to the sections they refer to. The thick black bars in **E** and **G** indicate the lower jaw skeleton. Because of their complexity, the skeletal structures of upper jaws, skull, and palatal bones have not been incorporated in the drawing. For more details on the structures of normal and regenerating jaws, see Ghosh et al. (1994). The large amount of bone present in jaws can cause significant difficulties during cryosectioning; the bone is often displaced, and this can result in some tearing in the section. A: Tenascin reactivity in a 1-week lower jaw regenerate (section perpendicular to the floor of the mouth). Note strong reactivity in the blastema mesenchyme (bl), whereas buccal epithelium (be), normal epidermis (e), and wound epidermis (we) are negative. B: NCAM reactivity in a 1-week upper jaw regenerate (oblique section almost perpendicular to the roof of the mouth). Note positive reactivity in the blastema (bl) and in a few cells of the wound epidermis (arrows); the diamond indicates where bone has pulled off and tearing has occurred. C: Tenascin reactivity in a 2-week lower jaw regenerate (oblique section almost perpendicular to the floor of the mouth). Note strong reactivity in the blastema mesenchyme (bl) and in the stump periosteum (po); the wound epidermis (we) is negative. D: NCAM reactivity in a 2-week lower jaw regenerate. Note positive reactivity in the blastema (bl), in the wound epidermis (we), and precartilage (pc) surrounding the stump of the prearticular bone. **E–H:** Schematic drawing indicating the level of the sections shown in A–D and I–L. I: LP1K reactivity in a 1-week lower jaw regenerate from the same blastema shown in A. Note strong reactivity in the blastema mesenchyme (bl), in the buccal epithelium (be), and glands (g); normal (e) and wound epidermis (edges indicated by arrows) are negative; the insert on the bottom left shows an equivalent area from a different 1-week lower jaw regenerate; the arrowhead points to the buccal epithelium. J: LP1K reactivity in a 1-week upper jaw regenerate from the same blastema shown in (B). Note positive reactivity in the blastema (bl) and in tissues of the stump such as glands (g) and nasal epithelium (ne); the wound epidermis (arrows) is negative. K: Nomarski image of J; some of the stump bone is marked (b). **L:** LP1K reactivity in a 6-week upper jaw regenerate (oblique section almost perpendicular to the roof of the mouth). Note positive reactivity in the blastema (bl), in the regenerating olfactory nerve (on; regenerating nerves are also strongly LP1K-positive in regenerating limbs, Ferretti et al., 1989) and in regenerated glands (g) as in normal jaws; the wound epidermis (arrows) is negative; b, bone. Scale bars are 100 μm; A–D,I–L are at the same magnification.

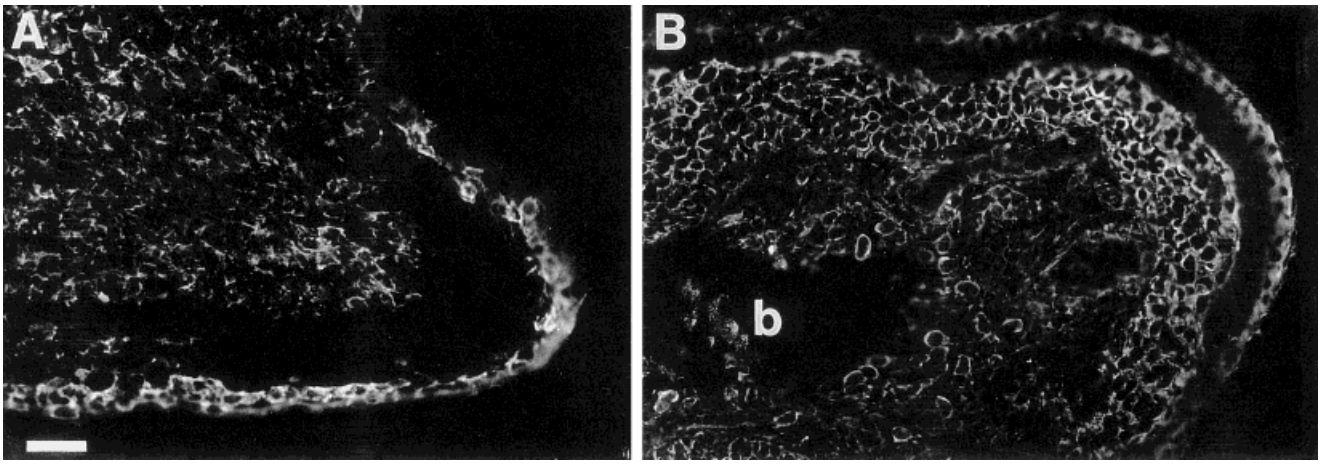

Fig. 3. Reactivity of LP1K in regenerating tail (**A**) and normal fingertip (**B**). A: Strong reactivity is observed both in the epidermis and in mesenchymal cells of the tail blastema. B: strong reactivity is observed both in the epidermis and in mesenchymal cells of the fingertip and in the periosteum. b, location of the bone which has pulled out. Scale bar = 100 μm; A and B are at the same magnification.

maintained at the tip of regenerating digits (Fig. 2C). In contrast, the underlying mesenchyme does not appear to express significant levels of NvKII. Therefore, whereas LP1K reacts both with the WE and blastemal cells in regenerating limbs and tails (Fig. 3A), the NvKII and NvK8 transcripts are selectively expressed in WE and blastemal cells, respectively (Fig. 2; Ferretti et al., 1993). These results indicate that the protein recognized by LP1K which is induced in the WE in response to amputation is indeed NvKII.

Since expression of NvKII is still observed at the digit tip at fairly advanced stages of regeneration, we have examined LP1K reactivity also in unamputated digits (Fig. 3B). Surprisingly, we find that LP1K strongly reacts both with the epidermis at the tip of the digit and with the underlying mesenchyme. These findings indicate that the fingertips are blastema-like, probably as a consequence of continuous injury.

Since expression of the human keratin 6 (K6), which is highly homologous to NvKII (Ferretti et al., 1991), can be induced by wounding, we have addressed the possibility that NvKII expression might simply represent a general response to skin injury, rather than being specifically associated with limb and tail regeneration. Therefore, we have studied NvKII expression by immunocytochemistry and in situ hybridization in regenerating skin 4 days after wounding either the limb or the flank skin (Figs. 4, 5). At this time, although re-epithelialization of the wounded flank has occurred, the process of skin repair has not been completed, and the operated area is still easily identified. This is confirmed also by the weaker and patchier pattern of reactivity in operated than control skin of the epidermal marker LP34 (Fig. 4A,B). Four days after wounding flank skin no LP1K reactivity is induced in the wounded epidermis of the flank (Fig. 4C), but in the limb both the regenerating epidermis and the underlying mesenchyme are brightly stained (Fig. 4D). This suggests that whereas wounding of the limb skin has induced a cellular response comparable to that observed following limb amputation (it should be noted that local limb injury and nerve deviation can induce growth of supernumerary limbs), wounding of the flank skin is progressing through a healing process which does not require NvKII expression. In order to confirm that the LP1K reactivity observed in the wounded limb epidermis is indeed due to up-regulation of NvKII, we have assayed its expression also by in situ hybridization. From Figure 5A,B it is apparent that the NvKII transcript is not detectable in wounded skin from the flank, whereas up-regulation of this RNA has occurred in the wounded skin from the limb (Fig. 5C–E). As shown in Table 2, the differences in NvKII expression in different tissues are not reflected by differences in keratin expression in uninjured epidermis, which become evident only after injury.

Is Expression of Regeneration-Associated Cytoskeletal Components in the Limb Common to Regenerating Jaws?

In order to address this issue, we have studied the expression of molecules which are induced in limb blastemal cells following amputation both in normal and in regenerating jaws by in situ hybridization, immunocytochemistry, and RNAase protection.

Analysis of NvK8 and NvK18 transcripts by in situ hybridization, as compared to the immunohistochemical results, shows a close correlation between RNA and protein expression both in normal and regenerating jaws (Table 3; Figs. 6, 7). Two weeks after amputation (Fig. 6A,B) high levels of NvK8 mRNA are detectable in the blastemal cells which have accumulated at the tip of the cut surface of the upper jaw. Significant up-regulation of NvK8 expression is observed in the nasal

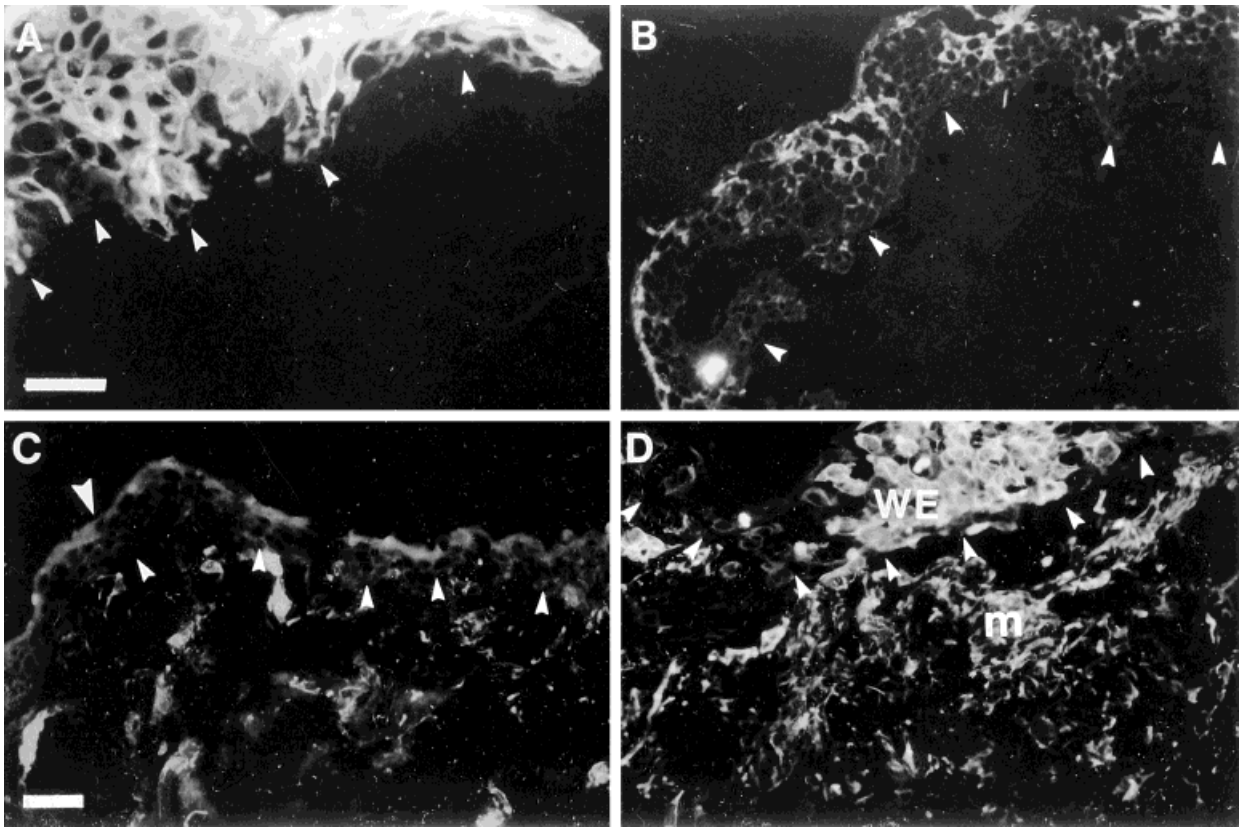

Fig. 4. LP34 (**A,B**) and LP1K (**C,D**) reactivity was evaluated 4 days after wounding either flank (B,C) or limb skin (D); epidermis basal margins are indicated by arrowheads. A: Control flank skin stained with LP34; note that the epidermal marker LP34 strongly reacts with the skin from the flank. B: Wounded flank skin stained with LP34; note that the staining is less intense and more uneven than in the control (the same exposure time was used for photography). C: wounded flank skin stained with LP1K; the margin of the wound is indicated (large arrowhead). Uninjured skin is to the left; no reactivity has been induced in the epidermis. As in normal limbs, some LP1K reactivity is observed in blood vessels and subepidermal glands. D: wounded limb skin stained with LP1K; positive reaction has been induced both in the wound epidermis (WE) and underlying mesenchyme (m). Scale bars = 50 μm (A) and 100 μm (C); A, B, and D are at the same magnification.

cartilage proximal to the plane of amputation, suggesting a significant contribution of this structure to the blastema. In 3-week lower jaw regenerates, areas of cartilage condensation strongly hybridize with the NvK8 probe (Fig. 6C,D). Interestingly, the stump of the Meckel's cartilage is also expressing high levels of NvK8, whereas in normal lower jaws keratin reactivity in Meckel's cartilage is rather weak and mainly restricted to the perichondrium. A significant increase of NvK8 transcript in regenerating jaws has also been observed by RNAase protection (not shown). Finally, clear evidence of the high levels of expression of the NvK8 and NvK18 proteins was demonstrated by immunocytochemistry (Fig. 7). Thus, NvK8 and NvK18 are up-regulated following amputation and are expressed by mesenchymal progenitor cells of both upper and lower jaw regenerates, their expression being apparently controlled at the mRNA level.

NvK8 and NvK18, however, are not the only cytoskeletal proteins up-regulated in jaw blastemal cells. The 22/18 protein, which is undetectable in cryostat sections of normal jaws (not shown), is also up-regulated following jaw amputation (Table 3; Fig. 7C). These results, together with the previous observation that the cytoskeletal antigen 22/31 is also expressed both in jaw and limb blastemas (Kintner and Brockes, 1985; Ghosh et al., 1994), reveal significant similarities in the phenotype of limb and jaw blastemal cells.

In contrast, neither NvKII message nor the NvKII protein could be detected in the wound epidermis of regenerating upper (Fig. 6E,F) and lower jaws by in situ hybridization and immunocytochemistry with LP1K (not shown). As described above this antibody reacts with NvKII in the regenerating limb wound epidermis. In order to further verify that this was not due to insufficient sensitivity of the in situ hybridization technique, we have also assessed the presence of NvKII mRNA by RNAase protection (Fig. 6G). Such analysis confirms that normal and regenerating lower jaws do not express NvKII, although high levels of the transcript are found in limb blastemas, clearly indicating that NvKII expression is restricted to certain regenerating organs.

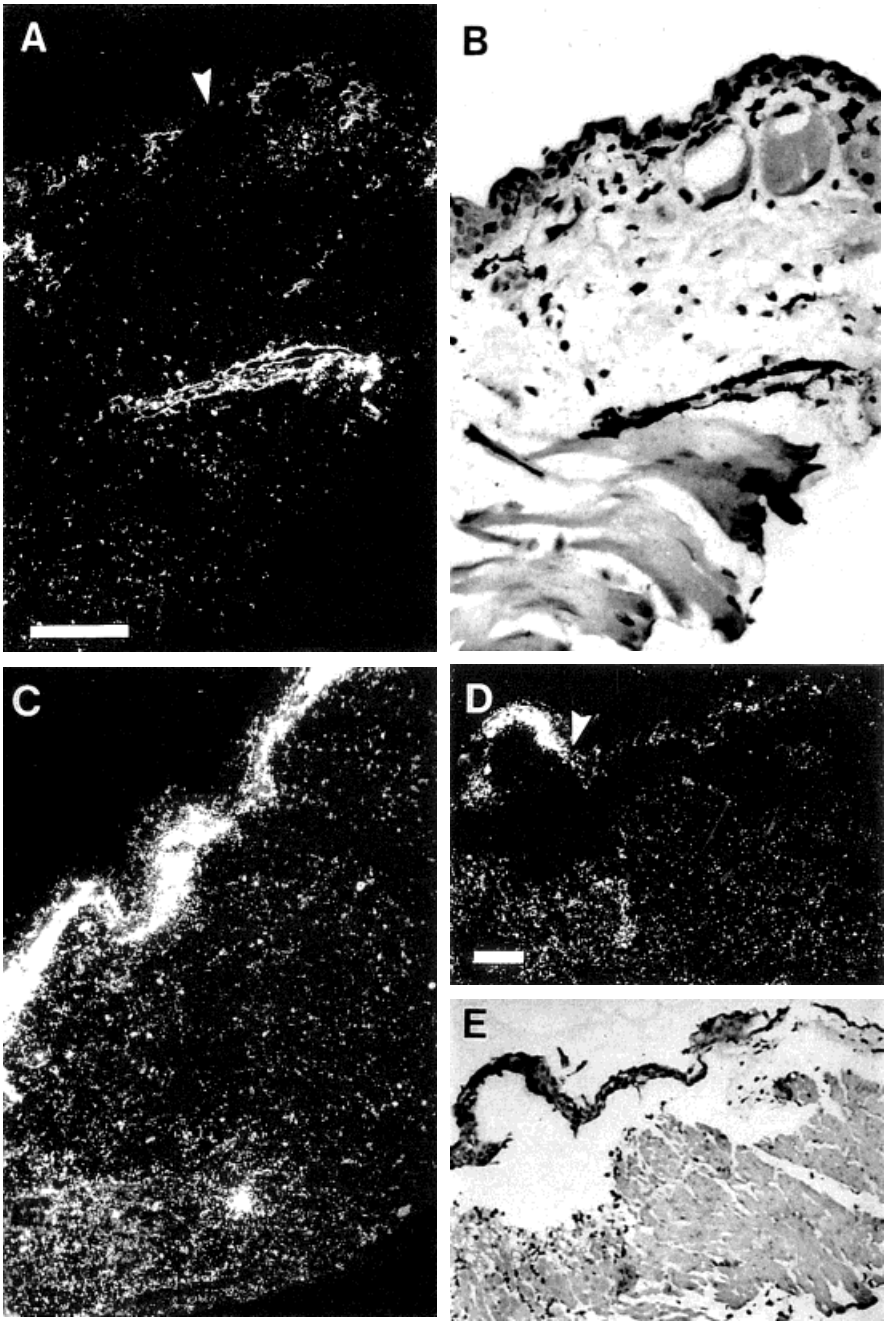

Fig. 5. In situ hybridization with the NvKII probe of wounded skin from the flank (**A,B**) and from the limb (**C–E**); the margin between injured and normal skin in A and D is indicated by an arrowhead. A: NvKII message in wounded flank skin; no reactivity above background is observed either in the epidermis or in the underlying tissues. B: Brightfield image of (A). C: NvKII message in wounded limb skin; NvKII has clearly been induced by wounding. D: another example of NvKII induction following wounding of limb skin. E: Brightfield image of (D). Scale bars = 100 μm; A–C and D and E are at the same magnification.

In order to see if expression of NvK8 in jaw blastemas parallels that in limb blastema, and to confirm that absence of NvKII expression in jaws is not simply restricted to the time points examined, we have used LP1K to examine NvK8 and NvKII expression also in 1- and 6-week jaw blastemas (Fig. 1I–L). By 1 week after lower jaw amputation retraction of the soft tissues from the plane of amputation has occurred (Ghosh et al., 1994) and a small population of blastemal cells, which is LP1K-positive, can be observed (Fig. 1I,J). As we have shown above, these cells are also tenascin and NCAM-positive (Fig. 1A,B). As in normal jaws, LP1K reacts with the buccal epithelium of the stump, but no reactivity is observed either in the wound epidermis

TABLE 2. Summary of mAbs Reactivity in Normal Epidermis From Different Body Regions

	mAb reacting with epidermal keratins			mAbs reacting with simple epithelial keratins		
	LP34 (K1, K5, K6, K18)	KK8.6 (K10, K11)	LP1K (NvKII)[a]	RGE53, CK18.2 (K18)	LP1K, LE41 (K8)	LE64 (K19)
Limb	+	–	–	–	–	–
Jaw	+	–	–	–	–	–
Tail	+	nd	–	–	–	nd
Flank	+	nd	–	–	–	nd

[a]Reactivity in the newt is with epidermal keratin NVKII and simple epithelium keratin NVK8 (see text). In humans and other mammals LP1K reacts with K8 and also with K7.

TABLE 3. Summary of mAbs Reactivity in Normal and Regenerating Jaws and in Regenerating Limbs

mAb	Reactivity	Normal jaw	Regenerating jaw	Regenerating limb
LP1K	NvK8, NvKII HK7,[a] HK8[a]	Glands, nasal epithelium, buccal epithelium, outer enamel epithelium, blood vessels, cartilage (weak), median symphysis sutures	Blastemal cells, regenerating cartilage	Blastemal cells, wound epidermis, regenerating cartilage
RGE53, CK18.2	HK18[a]	Glands, outer enamel epithelium, dental pulp, sutures, cartilage (weak)	Blastemal cells, regenerating cartilage	Blastemal cells, regenerating cartilage
22/18	IF[b]	–	Blastemal cells	Blastemal cells

[a]HK7, human keratin 7; HK8, human keratin 8; HK18, human keratin 18.
[b]IF, intermediate filament component whose identity has not yet been determined (Ferretti and Brockes, 1990).

overlying the blastemal cells or in the stump epidermis of 1-week lower and upper jaw regenerates (Fig. 1I–K). In contrast, as shown in a close section from the same blastema, a few NCAM-positive cells are observed in the wound epidermis at this time point (Fig. 1B), and their number is significantly higher at 2 weeks post-amputation (Fig. 1D). It appears therefore that 1 week after amputation NvK8 expression is already clearly detectable in jaw blastemal cells, as in limbs and tails, whereas in jaw wound epidermis, unlike in limbs and tails, NvKII expression is not induced. A similar picture is observed in 6-week regenerates (Fig. 1L) where LP1K reactivity is still detected in blastemal cells, but not in the wound epidermis of both lower and upper jaws. In these regenerates in which differentiation has occurred, reactivity to NvK8 and NvK18 (not shown) is restricted to tissues which express these keratins in normal jaws (Table 3). In summary, blastemal cells clearly express NvK8 and NvK18 throughout jaw regeneration, and their expression is down-regulated in most tissues at the onset of differentiation. In contrast, NvKII is expressed in limb wound epidermis from 4 to 5 days after amputation but is never detected in normal and regenerating jaws.

Is NvK8 Expressed in Mesenchymal Progenitor Cells During Jaw Development?

Analysis of NvK8 expression in stage 28, 32, and 38 *Notophthalmus viridescens* shows that simple epithelial keratins are highly expressed in the facial primordia and developing jaws (Fig. 8). Expression of NvK18 coincides with that of NvK8 in all the adjacent sections analyzed (not shown). The pattern of keratin expression in the developing newt head is consistent with that reported in *Xenopous* (LaFlamme and Dawid, 1990). At stage 28, NvK8 is detected in most head structures, including the neural tube, but not in the outer ectoderm, which will differentiate into epidermis. High levels of expression are observed in the endoderm surrounding the pharyngeal cavity and in the mesenchyme (Fig. 8A). Also the balancers, which are adhesive organs of ectodermal origin that develop from the mandibular arch, express the NvK8 transcript. At stage 32 (Fig. 8B) the level of expression is still high in mesenchymal cells and lining epithelia but is becoming more restricted in the brain, where only the layers closer to the ventricular cavity now appear to be expressing NvK8. By stage 36–38 most of the head mesenchyme still expresses NvK8, and some NvK8 message is also detectable in the developing Meckel's cartilage and the cartilages of the hyoid apparatus (Fig. 8C,D). Protein expression paralleled closely expression of the transcript (not shown). Thus, it appears that simple epithelial keratins are expressed in mesenchymal progenitor cells of developing head structures, as they are in those of regenerating jaws.

DISCUSSION

We have investigated the pattern of expression of NvKII during epimorphic regeneration and skin repair and have studied the expression of the keratin 8 (NvK8), its natural partner keratin 18 (NvK18), and 22/18 in different regenerating organs. In addition, we have examined NvK8 expression in cells contributing to

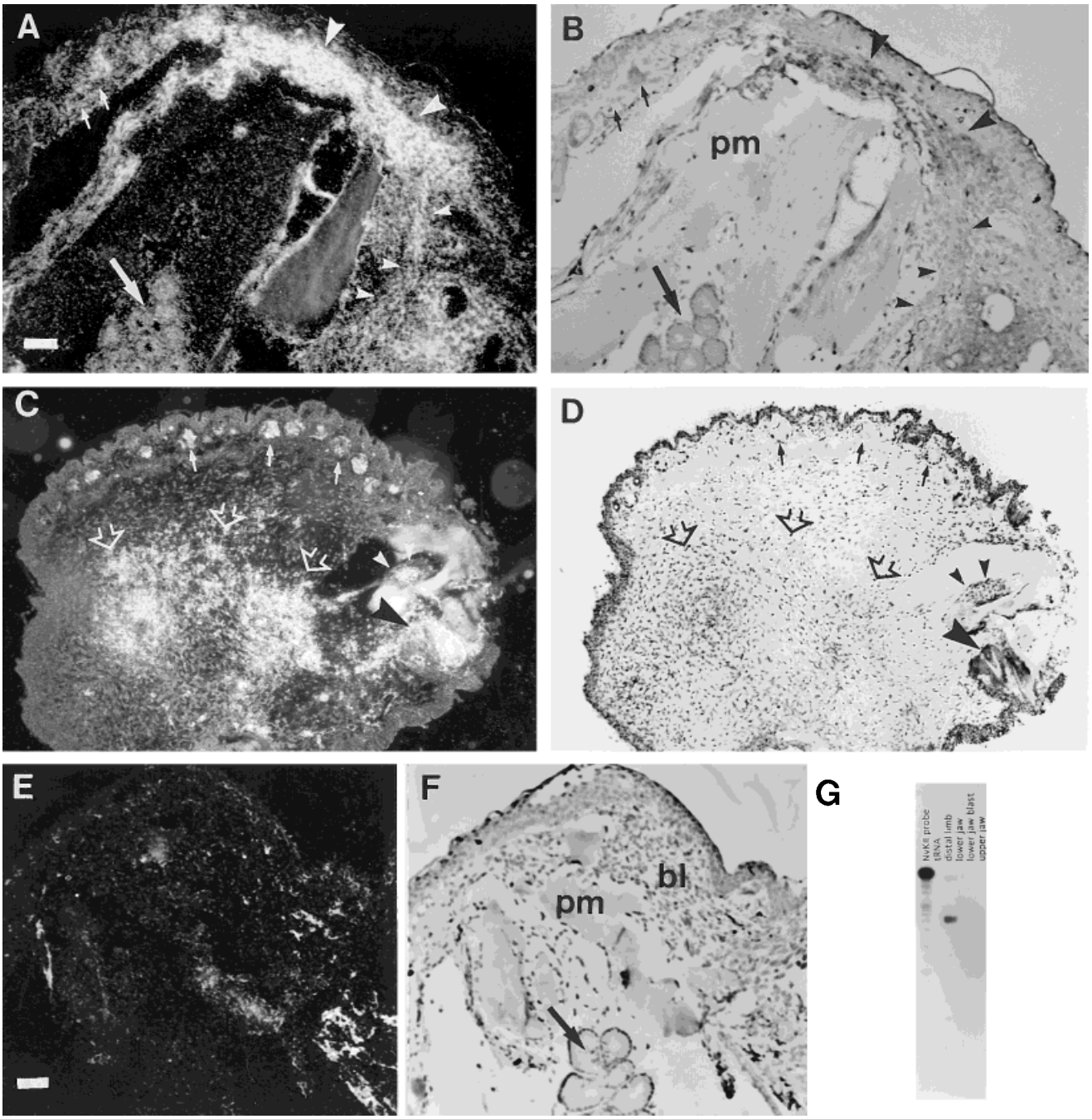

Fig. 6. NvK8 (**A–D**) and NvKII mRNA expression (**E–G**) in normal and regenerating jaws. A: NvK8 in situ hybridization in upper jaw 2-week blastema; strong reactivity is observed in the blastema (large arrowheads) and cartilage of the stump (small arrowheads). Some expression is also detectable in certain mucous glands (small arrows) and in the Bowman's gland (arrow). B: Brightfield image of A. pm, premaxilla. C: NvK8 in situ hybridization in lower jaw 3-week blastema; strong reactivity is observed in the regenerate (open arrows) and in the stump of Meckel's cartilage (small arrowheads). Note also strong reactivity in the tooth (large arrowhead) and glands (arrows). D: Brightfield image of (C). E: NvKII in situ hybridization in upper jaw 2-week blastema; no significant reactivity is detectable either in blastema or stump tissues. F: Brightfield image of (E). bl, blastema; Bowman's gland (arrow). G: RNAase protection with the NvKII probe; note that NvKII is detectable only in distal limb, but neither in normal jaws nor jaw blastema. Scale bars = 100 μm; C and E are at the same magnification.

the formation of facial structures during development and regeneration in order to address the issue of the identity of progenitor cells in regenerating and developing jaws. As summarized in Figure 9, this study has revealed significant differences between wound healing during skin repair and epimorphic regeneration in different organs and interesting similarities in the phenotype of undifferentiated mesenchymal cells.

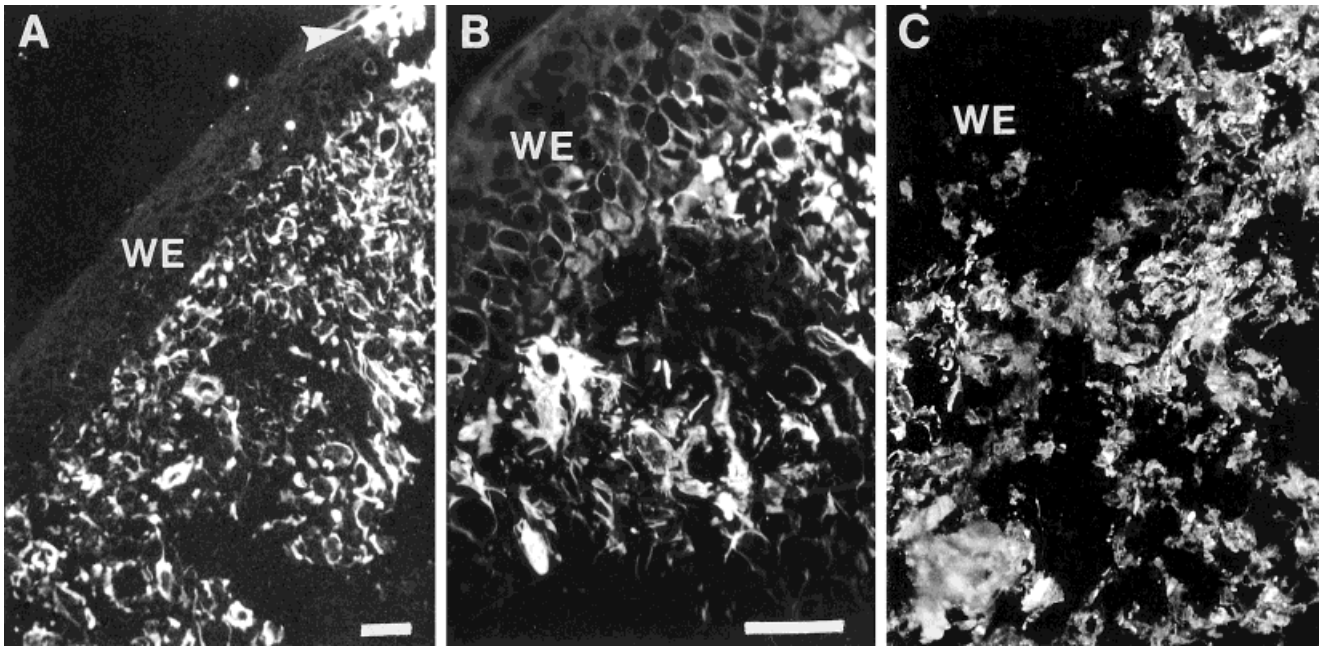

Fig. 7. Immunoreactivity of the anti-keratin antibodies LP1K (**A**), RGE53 (**B**), and of the mAb 22/18 (**C**) in 2-week lower jaw blastemas. WE, wound epidermis. A: LP1K strongly reacts with blastemal cells and the buccal epithelium (arrowhead), but not the WE (note the sharp boundary of reactivity). B: RGE53. C: 22/18 strongly react with blastemal cells. Scale bars = 100 μm; B and C are at the same magnification.

The Epidermal Keratin NvKII Is Induced in Response to Amputation of Specific Structures, and Not Simply in Response to Skin Injury

Analysis by RNAse protection has shown that NvKII is up-regulated in limb blastemas, but its exact cellular localization has not been previously established. Here we show that NvKII is expressed in the specialized wound epidermis of regenerating limbs and tails, but neither in blastemal cells like NvK8, NvK18, 22/18, and vimentin, nor, as indicated also by RNAase protection studies (Ferretti et al., 1991), in normal tissues. Therefore, unlike other wound epidermis markers which are found in a variety of tissues (Tassava et al., 1993), NvKII expression is extremely restricted. Furthermore, we demonstrate that NvKII up-regulation in the wound epidermis is not induced simply in response to skin injury as reported for mammalian keratin 6 (Weiss et al., 1984; Tyner and Fuchs, 1986), which displays a high percentage of amino acid identity with NvKII (Ferretti and Brockes, 1991). To our knowledge this is the first evidence of a molecular difference in flank and limb skin following injury and suggests that NvKII is more than just a wound epidermis marker; its expression may indeed be needed for limb regeneration, since skin grafting experiments have shown that skin from the flank grafted onto a limb prior to amputation inhibits regeneration (Tank, 1984, 1985). In addition, it has also been shown that head skin grafted onto a limb cannot support regeneration (Thornton, 1962).

NvKII up-regulation is not evoked in all systems regenerating epimorphically, but it appears to be restricted to limbs and tails, since it is undetectable both in regenerating upper and lower jaws. The fact that limb and tails, but not jaws, can express this keratin in response to amputation may reflect the relatedness of their respective morphogenetic fields at early developmental stages, as demonstrated by the RA-induced homeotic transformation of tail blastemas to give regenerated limbs (Mohanty-Hejmadi et al., 1992; Maden, 1993).

The differential expression NvKII in the wound epidermis of different regenerating organs is suggestive of different inductive abilities of these epithelia. In addition, since cross-talk between epithelium and mesenchyme is of fundamental importance in controlling gene expression in both face and limb during development (Summerbell et al., 1973; Wedden et al., 1988; Tickle, 1991; Richman and Tickle, 1992), expression of NvKII in limb but not jaw wound epidermis might reflect differences in signaling from the underlying mesenchyme. Grafting experiments will have to be carried out to establish whether limb mesenchyme can induce expression of NvKII in the jaw WE and vice versa. In addition, it will be important to investigate whether ectopic WE can affect gene expression in the blastema mesenchyme of different organs. This will help to establish whether there are indeed "organ-specific" epithelial–mesenchymal interactions which play an important role in regeneration, as suggested by the grafting experiments mentioned above (Thornton, 1962; Tank, 1984, 1985), and the pattern of expression of NvKII (this study).

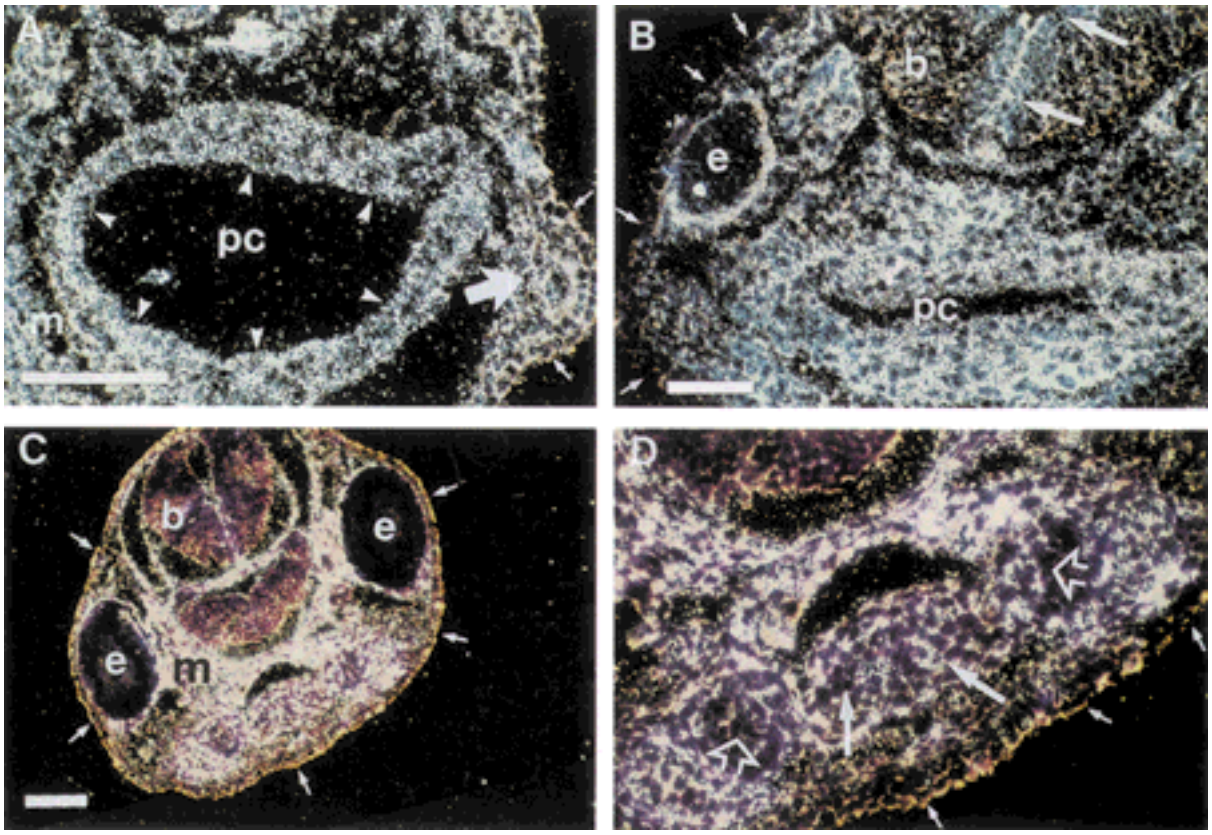

Fig. 8. Distribution of NvK8 transcript in developing facial primordia of *Notophthalmus viridescens* embryos at stage 28 (**A**), 32 (**B**), and 38 (**C,D**). A: NvK8 is expressed at high levels in the pharyngeal endoderm (arrowheads) surrounding the pharyngeal cavity (pc), in the mesenchyme (m), and in the balancer (thick arrow) of stage 28 embryos, but the ectoderm is negative as also observed at later stages (small arrows; see also **B–D**). B: NvK8 is expressed in the mesenchyme and in the pharyngeal endoderm of stage 32 embryos. At this stage expression in the brain (b) is restricted to cells at the midline and cells lining the brain cavity (large arrows). e, eye. C,D: NvK8 is expressed at high levels in head mesenchyme (m) and at lower levels in Meckel's cartilage (open arrows) and cartilages (arrows) of the hyoid apparatus (enlarged in D) of stage 38 embryos. Scale bars = 100 μm; B and D are at the same magnification.

On the basis of the differential induction of NvKII in response to amputation along the proximodistal axis of the limb, and the fact that NvKII mRNA is regulated by retinoic acid, it has been suggested that NvKII-positive wound epidermal cells may play a role in specifying positional information in the limb (Ferretti et al., 1991). The work presented here further supports this possibility, as the expression of NvKII in regenerating limbs, but not in jaws, may be causally related to the different response to RA in these systems, which is also consistent with differences observed in the pattern of expression of RA receptors (Ferretti, 1996; Ghosh et al., 1996). In fact, whereas RA induces proximodistal duplications in regenerating limbs (Niazi and Saxena, 1978; Maden, 1982; Stocum, 1991), a comparable effect is not observed in regenerating jaws, where RA treatment results in truncated upper jaw regenerates which often display a cleft lip and palate-like morphology (Ferretti, 1996; Ghosh et al., 1996).

Surprisingly, reactivity with the mAb LP1K, which recognizes both NvKII and NvK8, is observed in unamputated fingertips. Since other blastemal markers such as 22/18 (not shown) are also detected in the unamputated fingertips, it appears that they are indeed blastema-like. This is probably due to constant damage of the tip of fingers and toes as a consequence of walking on rough substrata both in the wild and in captivity (the bottom of the tanks is covered with gravel), rather than to an embryonic-like phenotype. In fact, the blastemal markers we have studied are not expressed in developing limb buds. Furthermore, it does not seem likely that the newt fingertip is equivalent to the nail bed of higher vertebrates, where high levels of *msx-1* appear to correlate with its significant regenerative capability (Crews et al., 1995; Reginelli et al., 1995), since the entire amphibian limb can regenerate, and indeed expression of *msx-1* in adult newt limbs may not be not restricted to the fingertips (Crews et al., 1995; Reginelli et al., 1995). The analysis of the cellular distribution of NvKII presented here shows that the high levels of NvKII previously observed in normal distal limbs reflect the blastema-like quality of the fingertips, rather than a proximodistal gradient as in regenerating limbs. However, it appears that the difference in NvKII levels

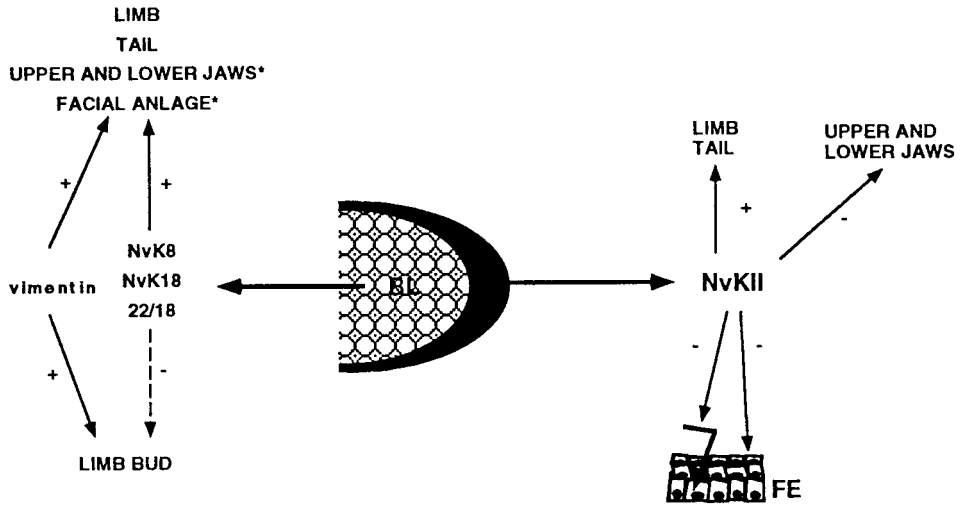

Fig. 9. Cartoon summarizing the pattern of expression of vimentin and regeneration-associated intermediate filaments. BL, blastema; FE, flank epidermis (the large arrow indicates wounding). The dashed arrow pointing to the developing limb bud indicates lack of nerve-dependent growth which is reflected by the lack of NvK8, NvK18, and 22/18 expression. *, Circumstantial evidence of nerve dependency; 22/18 reactivity has not been analyzed at very early developmental stages in the facial anlage of *Notophthalmus viridescens.*

in proximal and distal blastemas previously observed is indeed established as a result of amputating the limb at different axial level (Ferretti et al., 1991).

Induction of Regeneration-Associated Cytoskeletal Proteins in Mesenchymal Progenitor Cells Is Common to Different Regenerating Systems

We have previously shown that jaw blastemal cells do not express markers of the differentiated state (Ghosh et al., 1994), consistent with their morphological appearance, but, like limb blastemal cells, they up-regulate vimentin, whose expression in normal jaws is restricted to dermal fibroblasts and the dental pulp (Kintner and Brockes, 1985; Fekete and Brockes, 1987; Ghosh et al., 1994). Here we have extended our study and addressed the issue of whether cytoskeletal proteins which are associated with limb regeneration are up-regulated also in jaw and tail blastemal cells or are differentially expressed like NvKII.

We show that both simple epithelial keratins, which start to be expressed by blastemal cells at the onset of cell division (4–5 days after amputation: Ferretti et al., 1989; Corcoran and Ferretti, 1997), and 22/18, which is an earlier marker of blastemal cells whose division is dependent on the presence of the nerve (Gordon and Brockes, 1988), are also expressed in progenitor cells of regenerating jaws. They are all down-regulated at the onset of differentiation both in vivo and in vitro. This suggests that their expression is related to the undifferentiated state of the blastemal cell, rather than to a specific regenerating organ, and that the same mechanisms leading to establishment of a population of mitotically active progenitor cells are shared by different adult organs which can regenerate epimorphically.

It is important to point out that expression of keratins in certain tissues of the jaw, such as nasal and Meckel's cartilage, is very low in unamputated jaws. However, high keratin levels are observed not only in blastemal cells, but also in the part of the stump proximal to the amputation surface 2 weeks after amputation. This suggests that cartilage of the stump has been somehow "induced" and significantly contributes to blastema formation over a relatively long time. Whether this process is really analogous to the dedifferentiation that occurs in the muscle of regenerating amphibian limbs (Wallace, 1981; Lo et al., 1993) or reflects activation of a normally quiescent population of "reserve" cells is presently unclear and will require further investigation. What is clear is that in both systems amputation induces a series of events which results in formation of blastemal cells with a common phenotype in respect to intermediate filament expression, presumably reflecting a similar physiological state of the cell.

The embryonic origin of the regenerating tissues in different organs does not seem to be related in any way to the induction of keratin expression following amputation, since the limb forms from lateral plate and somitic mesoderm, whereas most head structures are of neural crest origin, and the regenerating tail has both mesodermal and neuroectodermal components. In addition, the majority of blastema cells in each regenerating organ are simple epithelial keratin positive, supporting the view that there is no relationship between keratin expression and germ layer of origin. Expression of simple epithelial keratins in relation to the physiological state of the cell rather than to the embryonic origin has been reported in other systems (Viebahn et al., 1988; Markl, 1991).

It is well established that both limb and tail regeneration are nerve dependent (Singer, 1952, 1974; Holtzer, 1956), and it has been shown that 22/18 identifies blastemal cells whose division is nerve dependent (Kintner and Brockes, 1985; Ferretti and Brockes, 1991). Therefore, on the basis of 22/18 reactivity in regenerating jaws, it is also likely that jaw regeneration is a nerve-dependent process. In addition there is evidence that also NvK8 and NvK18 keratin expression in developing and regenerating structures is associated with the presence of neural tissue. In fact, the developing limb bud, which among the systems discussed is the only one in which growth is clearly nerve independent, does not express the NvK8 and NvK18 keratins (Ferretti et al., 1989; Corcoran and Ferretti, 1997). While innervation is absent from the keratin-negative developing limb bud, the nervous system is always a major presence in the developing head and face, and they indeed express high levels of keratins 8 and 18 in the mesenchyme surrounding the pharyngeal cavity, in the pharyngeal endoderm, and in developing cartilages, among other tissues. Therefore, in contrast to the limb (Fekete and Brockes, 1987; Ferretti et al., 1989), the phenotype of mesenchymal progenitor cells of developing and regenerating jaws is the same in regard to their simple epithelial keratin content.

The role of the different members of the intermediate filament gene superfamily is still somewhat elusive, but much recent work, and the work reported here, suggests that intermediate filaments are more than "inert" structural proteins and may play a significant role in the control of cell growth and differentiation (Heasman et al., 1992; Baribault et al., 1993; Chu et al., 1993; Chen and Liem, 1994; Lee and Cleveland, 1994; Li et al., 1994; Takahashi et al., 1994; Wang et al., 1996; Corcoran and Ferretti, 1997; Ku et al., 1997) possibly by acting as transcription factors (Traub and Shoeman, 1994). The fact that a marker of mesenchymal differentiation, vimentin, and markers of epithelial differentiation, keratins 8 and 18, are co-expressed both at early developmental stages and in blastemal cells of all the regenerating organs studied may indicate that their up-regulation is critical to the capability of adult newt cells to revert to an undifferentiated state and re-enter the cell cycle. Interestingly, this cell phenotype is also observed in certain tumors (Chu et al., 1993; Garamvoelgyi et al., 1994; Tsarfaty et al., 1994). Finally, in the embryonic chick ectoderm, which appears homogeneous at the morphological level, the existence of sharp anteroposterior and dorsoventral boundaries in keratin expression has been reported (Charlebois et al., 1990a,b). This, together with our observations that NvKII expression in the epidermis is induced only in specific body compartments, supports the view that such differences in intermediate filament content reflect their physiological significance in developmental and regenerative processes. It will be important to re-examine keratin distribution in different skin territories in other species, particularly following injury, since potential regional differences in keratin regulation in the wounded skin may have been overlooked.

Further analysis of intermediate filaments in regenerating systems will help to clarify not only the role of these proteins in cell physiology but also the mechanisms underlying formation and growth of blastema cells and of the wound epidermis, and the relationship between regeneration and repair.

EXPERIMENTAL PROCEDURES

Animals

Red-spotted newts, *Notophthalmus viridescens* (Sullivan & Co, Nashville, TN) were used in all the experiments described. This species is not easily bred in captivity and therefore, as previously described (Ghosh et al., 1994), spawning was induced by injecting HCG (human chorionic gonadotropin, Sigma, Poole, UK) into gravid females collected in the wild on the assumption that some of them had been inseminated before collection. Adult animals were maintained in the laboratory at 19–20°C and fed shredded bovine heart on alternate days. Embryos were grown at 22–24°C in sterile tap water until sacrificed with an overdose of tricaine between stages 28 and 38 and either cryo-mounted for immunocytochemistry or fixed for in situ hybridization (see below). Staging was based on *Pleurodeles waltl* developmental tables (Gallien and Durocher, 1957; Ghosh et al., 1994; Shi and Boucaut, 1995), since appearance of external features in developing *Notophthalmus viridescens* appears to be fairly similar (Ghosh et al., 1994), and no comparable staging of the external features of *Notophthalmus viridescens* has been published.

Surgery

All surgical procedures were performed in adult animals anesthetized by immersion in 0.1% tricaine (3-aminobenzoic acid ethylester methanesulphonate salt, Sigma, Poole, UK). Operated animals were maintained at 25°C after surgery. Amputations of jaws, limbs, and tails were carried out as previously described (Savard et al., 1988; Ferretti et al., 1989; Ghosh et al., 1994) and analyzed at 1, 2, 3, 4, 5, and 6 weeks after amputation (Table 1). Normal and regenerated tissues were either cryoembedded for immunocytochemistry or fixed and processed for in situ hybridization (see below). In some experiments normal and regenerating tissues were collected for RNA extraction. In skin wounding experiments a patch of skin of about 1 mm × 1 mm was removed either from the flank of the animal midway between the forelimb and the hindlimb, since this region is considered morphogenetically "inert" (nerves deflected to this region do not induce supernumerary structures; see Wallace, 1981), or from the thigh. The regenerated skin was collected 4 days later and processed either for immunohistochemistry or in situ hybridization. This time was chosen because expression of the proteins of interest in the regenerating limb was not observed before 4–5 days after amputation.

Immunohistochemistry

Immunohistochemistry was essentially performed as previously described (Ferretti et al., 1989). Reactivity of the monoclonal antibody (mAb) 22/18 (Kintner and Brockes, 1985) and of the polyclonal anti-tenascin antibody (Chiquet Ehrismann et al., 1986) was assayed on 8-µm cryostat sections of unfixed tissue, whereas the sections to be stained with the anti-keratin mAbs LP1K (Lane et al., 1985), RGE53, CK18.2 (Ramaekers et al., 1984; Broers et al., 1986), LP34 (Lane et al., 1985), and KK 8.60 (Huszar et al., 1986), with the anti-neurofilament mAb RT97, and with the polyclonal antibody to amphibian NCAM (Maier et al., 1986) were briefly fixed with cold acid-alcohol (95% ethanol-5% acetic acid). Details of the staining using markers of differentiated tissues are given in Ghosh et al. (1994). The reactivity of the mAbs 22/18, LP1K, RGE53, and CK18.2 in human and newts is summarized in Table 3. Bound antibodies were detected by either a rhodamine-conjugated rabbit anti-mouse–immunoglobulin antibody (Dako, Denmark) or a rhodamine-conjugated swine anti-rabbit–immunoglobulin antibody (Dako, Denmark), and the nuclei were stained with 1.25 µg/ml of Hoechst dye 33258 (Sigma).

RNAase Protection

The guanidine isothiocyanate procedure described by Brown and Brockes (Brown and Brockes, 1991) was used to extract total RNA from different tissues. All the RNA samples were standardized by OD measurements at 260 nm and RNAase protection with the satellite 2 probe pSP6D6 (Epstein and Gall, 1987). For the analysis of NvKII and NvK8 expression riboprobes were prepared from a 231 bp SacI-FokI fragment which encodes part of the C terminus of NvKII (Ferretti et al., 1991) and from a 300-bp PstI-PstI fragment which encodes part of helix 2 of NvK8 (Corcoran and Ferretti, 1997). RNAase protection was performed as previously described (Casimir et al., 1988; Ferretti et al., 1991).

In Situ Hybridization

For in situ hybridization studies jaw blastemas were removed at different times after amputation and fixed overnight in 4% paraformaldehyde in 100 mM phosphate buffer, 120 mM NaCl, pH 7.4 (A-PBS) at 4°C. The jaws were decalcified by treatment with 0.5 M EDTA, pH 7.5, for 3 to 5 days. After rinsing in the same buffer the tissues were dehydrated in graded ethanol, embedded in paraffin wax under vacuum for 3 hr, and 6-µm sections were cut. After dewaxing, sections were fixed in 4% paraformaldehyde for 20 min, treated with 20 mg/ml of proteinase K for 5 min, post-fixed in 4% paraformaldehyde for 5 min, acetylated with acetic anhydride in triethanolamine buffer, and dehydrated. The slides, covered with a coverslip, were hybridized overnight at 55°C with 10^5 cpm/ml of either the NvK8 or the NvKII riboprobe (see above) labeled with ^{35}S-UTP, which had been purified on a Sephadex G-50 drip column, in a hybridization mixture consisting of 50% formamide, 20 mM Tris-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA, 0.5 mg/ml yeast tRNA, 1× Denhardt's solution, 10% dextran sulphate. The slides were washed twice for 30 min in 5× standard saline citrate (SSC), 10 mM DTT at 50°C, the coverslips were removed, and the slides were then washed at high stringency for 30 min at 65°C with 50% formamide, 2× SSC, 10 mM DDT. After three 10-min washings with NTE buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA), the slides were treated with 20 mg of RNAase A for 30 min at 37°C and washed for 15 min with NTE. The high-stringency washing was then repeated, followed by a washing with 2× SSC and one with 0.1× SSC of 15 min each. Following dehydration, the slides were processed for autoradiography and exposed for 5 days at 4°C before being developed and counterstained with toluidine blue. Some of the dewaxed sections were stained either with alcian blue and durazol red or with Harris' hematoxylin and eosin for histological examination. No signal was detected with either NvKII or NvK8 sense probes. Furthermore, the significant differences in the pattern of expression of NvKII and NvK8 in adjacent sections indicated that the hybridization signals observed were highly specific.

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