Development 122, 3487-3497 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2067

Nerve dependency of regeneration: the role of *Distal-less* and FGF signaling in amphibian limb regeneration

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SUMMARY

***Dlx-3,* a homolog of *Drosophila Dll,* has been isolated from an axolotl blastema cDNA library, and its expression in developing and regenerating limbs characterized. The normal expression pattern, and the changes that occur during experimental treatments, indicate a correlation between *Dlx-3* expression and the establishment of the outgrowth-permitting epidermis. *Dlx-3* is expressed at high levels in a distal-to-proximal gradient in the epidermis of developing limb buds, and is upregulated in the apical ectodermal cap (AEC) during limb regeneration. Expression is maximal at the late bud stage of regeneration, coincident with the transition from the early phase of nerve dependency to the later phase of nerve independence. *Dlx-3* expression in the epidermis is rapidly downregulated by denervation during the nerve-dependent phase and is unaffected by denervation during the nerve-independent phase. We investigated this relationship between nerves and *Dlx-3* expression by implanting FGF-2 beads into regenerates that had been denervated at a nerve-dependent stage. *Dlx-3* expression was maintained by FGF-2 after denervation, and regeneration progressed to completion. In addition, we detected FGF-2 protein in the AEC and in nerves, and observed that the level of expression in both tissues decreases dramatically in response to denervation. We conclude that both limb development and regeneration require a permissive epidermis, characterized by *Dlx-3* and FGF expression, both of which are maintained by FGF through an autocrine loop. The transformation of the limb epidermis into a functional AEC that produces and responds to FGF autocatalytically, is presumed to be induced by FGF. Since nerves appear to be a source of this priming FGF, it is possible that a member of the FGF family of growth factors is the elusive neurotrophic factor of limb regeneration.**

Key words: Limb regeneration; *Dlx;* neurotrophic factor; FGF; apical ectodermal cap

INTRODUCTION

Urodele amphibians are unique among vertebrates in their ability to regenerate lost appendages throughout their lives. The molecular mechanisms controlling this process have not been well characterized, although the basic similarities to limb development suggest that many genes expressed in developing limbs will be reexpressed in regeneration. Homeobox-containing genes are known to be critically involved in limb development (Duboule, 1994; Davis et al., 1995), and recent studies have shown that their involvement in regeneration is likely to be just as crucial (see Gardiner et al., 1995). In a screen for homeobox-containing genes expressed in regenerating limbs (Gardiner et al., 1995), we identified a homolog of *Drosophila distal-less* (*Dll)*, which is the subject of this paper.

In *Drosophila, Dll* expression is required for leg development (Cohen et al., 1989). It's expression is first detected in the precursors of the thoracic imaginal discs that form at the intersection of the stripes of *wingless (wg)* and *decapentaplegic (dpp)* expression (Cohen et al., 1993). Flies mutant for *Dll* are missing progressively more leg segments with increasing severity of the allele (Cohen and Jurgens, 1989). Furthermore, when *Dll* is ectopically expressed in abdominal segments, abdominal legs develop (Cohen et al., 1991; Simcox et al., 1991; Vachon et al., 1992).

Several vertebrate homologs of *Dll* have been identified in the mouse (Price et al., 1991; Porteus et al., 1991; Dollé et al., 1992; Bulfone et al., 1993; Robinson and Mahon, 1994; Simeone et al., 1994), rat (Shirasawa et al., 1994; Zhao et al., 1994), human (Ozcelik et al., 1992; Simeone et al., 1994), frog (Asano et al., 1992; Dirksen et al., 1993; 1994; Morasso et al., 1994; Papalopulu and Kintner, 1993) zebrafish (Ekker et al., 1992; Akimenko et al., 1994) and newt (Beauchemin and Savard, 1992), and these comprise the *Dlx* gene family. Expression of different homologs has been reported in the developing forebrain, branchial arches, facial primordia, limbs, inner ear and numerous parts of the skeleton, and also in adult ovary and skin.

The axolotl *Dlx* gene that we report on in this paper belongs to the *Dlx-3* group. Previously described members of this group are expressed in regenerating and adult skin (Beauchemin and Savard, 1992) and, where this has been analyzed

further, in the epidermis (Morasso et al., 1994; Dirksen et al., 1994). In zebrafish and mice, *Dlx-3* is expressed in the epidermis at the distal edge of the fin and limb bud respectively, and in other structures that rely on epithelial-mesenchymal interactions for their development, such as branchial arches, inner ear, whisker follicles and developing teeth (Akimenko et al., 1994; Robinson and Mahon, 1994; Morasso et al., 1995). Thus *Dlx-3* may have an important role in epidermal differentiation and/or function, including those developmental processes involving interactions between a permissive epidermis and the underlying mesenchyme.

In this paper, we describe the expression of *Dlx-3* in developing and regenerating axolotl limbs. The transcript is upregulated first in the epidermis of developing limb buds, and again in the apical epidermal cap (AEC) during regeneration. Both the permissive epidermis of developing limb buds and the AEC of regenerating limbs function to promote mesenchymal outgrowth and establishment of the proximal-distal (PD) limb pattern (Saunders, 1948; Stocum and Dearlove, 1972). It is possible that *Dlx-3* expression is necessary for this important property of the epidermis.

Despite the many similarities between limb development and regeneration, an intriguing difference is that in developing embryos, limbs can form normally in the absence of nerves, whereas regeneration ceases if the limb is denervated in the early stages regeneration (see Wallace, 1981 for review). Later stages of regeneration, like limb development, are nerve-independent. Since *Dlx-3* expression peaks at the stage when regeneration switches from nerve dependency to nerve independency, we investigated the relationship between its expression and innervation in regenerating limbs. We discovered that *Dlx-3* expression is sensitive to innervation in the nerve-dependent stages, but not at later stages. We also found that FGF-2, which is capable of replacing the function of the permissive epidermis in limb development (Niswander et al., 1993; Fallon et al., 1994; Taylor et al., 1994), can restore *Dlx-3* expression in blastemas denervated at the nerve-dependent stage and, remarkably, can also permit regeneration of these denervated limbs. Finally, we detected the presence of FGF-2 in both the AEC and in nerves, and observed that levels of FGF-2 decrease in response to denervation. These results suggest a link between innervation and the formation of an AEC that is able to sustain distal outgrowth. The FGF-2-mediated rescue of denervated regenerates is the first demonstration of regeneration rescue by any means other than by nerves themselves (Singer, 1978). We suggest that FGF, or a molecule related to FGF, is either the neurotrophic agent of regeneration, or that it functions in the pathway by which nerves exert their effect on regeneration.

MATERIALS AND METHODS

Animal procedures

Axolotls (*Ambystoma mexicanum*) were spawned either at UCI or the Indiana University Axolotl Colony and were maintained at 20-22°C. For RNA isolation, animals were grown to 12-15 cm; animals used for whole-mount in situ hybridization were grown to 4-6 cm. To initiate regeneration, we anesthetized animals in 0.1% MS222 (Sigma) and amputated limbs at proximal (mid-humerus), middle (mid-radius/ulna) or distal (carpals) levels. The amputation surface was trimmed flat.

Isolation and sequencing of axolotl *Dlx-3*

The construction of blastema cDNA libraries, and their screening for homeobox-containing genes using a degenerate oligonucleotide complementary to the DNA sequence encoding the conserved amino acid sequence KIWF(Q/K)NRR, were performed as described in Gardiner et al. (1995). Sequencing identified one clone as a member of the *Distal-less* (*Dll*) class of homeobox-containing genes. This clone encodes a full-length protein and was sequenced in its entirety. Sequence data were analyzed using the GCG Sequence Analysis Software Package and similarity searches were performed using the Blast Programs, NCBI.

RNA isolation and northern hybridization

RNA isolation and northern hybridization were performed as described in Gardiner et al. (1995). The amount of RNA loaded was quantitated spectrophotometrically. To check that equal amounts of RNA were loaded, we visualized the 18S and 28S ribosomal RNA bands by either UV shadowing or by ethidium bromide staining of the gels. Filters were probed with three fragments of the *Dlx-3* clone (Fig. 1A); a 1000 bp *Eco*RI fragment that contains the entire homeobox (fragment R), a 600 bp *Eco*RI/*Xho*I fragment from the 3′ UTR, and a 490 bp *Pst*I fragment containing the coding region 5′ to the homeobox and the first 51 bp of the homeobox (fragment P). All three probes detected the same expression patterns; the data presented are from the R fragment probe. Blots were reprobed with an axolotl EF1-α clone (a gift from Dr D. Dube and Dr L. Lemansky), or an axolotl 33K laminin receptor clone (D. M. Gardiner, unpublished) to normalize the amounts of total RNA loaded for each lane. Autoradiographs were digitized (HP Scanjet IICX) and quantitated using Scan Analysis software (Biosoft).

Whole-mount in situ hybridization

Digoxigenin-labeled RNA probes were prepared according to the manufacturer's protocol (Boehringer). Probes were transcribed from both the P fragment and the R fragment (Fig. 1A).

Our procedure for whole-mount in situ hybridization to axolotl blastemas and limb buds is as reported in Gardiner et al. (1995), with the following variations. Embryos were fixed overnight, while regenerating limbs were fixed for 2-4 hours. Limb buds were treated with 20 μg/ml proteinase K for 15 minutes and regenerating limbs were treated with 30 μg/ml proteinase K for 15 minutes. Specimens were prehybridized at 55°C, and hybridized at 57°C. Post hybridization washes were performed at 59°C. Tissues were cleared and photographed in methyl salicylate.

Retinoid treatment

Systemic and local retinoid treatments were as described in Gardiner et al. (1995). The duration of systemic treatment by swimming animals with regenerating limbs in a solution of retinol palmitate was 1 to 8 days, after which animals were fixed for whole-mount in situ hybridization analysis at various time points. AG1-X2 beads (Biorad) soaked in a solution of 1 mg/ml retinoic acid (RA; all *trans*, Sigma) in DMSO were implanted into the anterior-distal region of blastemas, adjacent to the wound epithelium. Animals were fixed at times between 30 minutes and 2 days after bead implantation, or allowed to regenerate to completion to assess the effect on the skeletal pattern. These limbs were stained with Victoria Blue (Bryant and Iten, 1974).

Denervation of regenerating limbs

To examine the effect of denervation on *Dlx-3* expression, we amputated forelimbs at the mid radius/ulna level and allowed regeneration to progress to the medium bud stage (7 days post-amputation) or late bud stage (9-10 days post-amputation). At those times, limbs were denervated by transecting the 3rd, 4th and 5th spinal nerves at the brachial plexus (Singer, 1974). Contralateral limbs served as sham-operated, innervated controls. Limbs were fixed at various times during the 48 hour period after denervation and *Dlx-3* expression was

analyzed by whole-mount in situ hybridization. The limbs of some animals were redenervated after one week and fixed at later times as described in the Results. These limbs were fixed when the contralateral, control limb had completed regeneration.

FGF treatment of regenerating limbs

Affi-Gel Heparin Beads (BioRad), with a diameter of 200-250 μm, were washed in PBS and incubated for 4 hours in a 2.5 μl drop of 0.2 mg/ml bovine FGF-2 (Sigma) at room temperature. The beads were then washed in PBS and stained with 0.01% Nile Blue just prior to implantation. Beads soaked in PBS alone were implanted into control limbs. A sharpened tungsten needle was use to make a tunnel under the wound epidermis and beads were placed into the distal-most portion of medium bud regenerates adjacent to the apical wound epidermis. Forelimbs were denervated, as described above, immediately following bead implantation. Limbs were fixed at various times during the 48 hour period after denervation and bead implantation, and *Dlx-3* expression was analyzed by whole-mount in situ hybridization. Some limbs were allowed to develop for longer periods up to 15 days after denervation, to determine if FGF would allow denervated limbs to regenerate. These limbs were implanted with a second FGF bead 4 days after denervation, and were redenervated and another bead was implanted after one week.

Immunolocalization of FGF-2

Normal regenerating limbs, and limbs that were denervated at the medium bud stage of regeneration, were fixed in 4% paraformaldehyde in PBS for 4 hours at 4°C. Tissues were dehydrated and embedded in Steedman's polyester wax (Norenburg and Barrett, 1987) at 40°C and cut into 10 μm sections. Wax was removed with 100% EtOH and the tissues rehydrated. Non-specific protein interactions were blocked with TBST-Blotto (Tris-buffered saline with 0.2% Tween 20, 0.1% BSA and 5% non-fat dry milk). Monoclonal antibody to FGF-2 (provided by B. Olwin) was applied at a dilution of 1:500 and incubated overnight at 4°C. Following washing with TBST, alkaline-phosphatase-conjugated anti-mouse secondary antibody was applied. The slides were washed again and antibody localization was visualized using NBT/BCIP.

RESULTS

Cloning and characterization of axolotl *Dlx-3*

From a screen of regenerating axolotl limb blastema cDNA libraries, we isolated 17 different homeobox-containing clones (Gardiner et al., 1995). Comparison of the deduced amino acid sequence of the homeobox of one of these clones (Fig. 1B), with sequence data from other organisms, indicates that it shares 82% identity with *Drosophila Distal-less (Dll)*, and 100% identity with mouse *Dlx-3*, zebrafish *z-dlx3*, *Xenopus X-dll2* and newt *NvHbox-4* (Table 1). It is evident that the axolotl gene is a member of the *distal-less* (*Dll*) class of homeobox-containing genes. Axolotl *Dlx* shares considerable sequence identity with *NvHBox-4* outside of the homeodomain, and to a lesser degree with mouse *Dlx-3* (data not presented), and these appear to be homologous genes.

The axolotl *Dlx-3* clone consists of 1554 bp encoding an open reading frame for the entire *Dlx-3* protein of 280 amino acids (Fig. 1B). The homeodomain is located in the middle of the protein coding region. There is a 3′ UTR of approximately 650 bp.

Analysis of *Dlx* expression by northern hybridization

Using the R fragment (Fig. 1A), we observed a single transcript

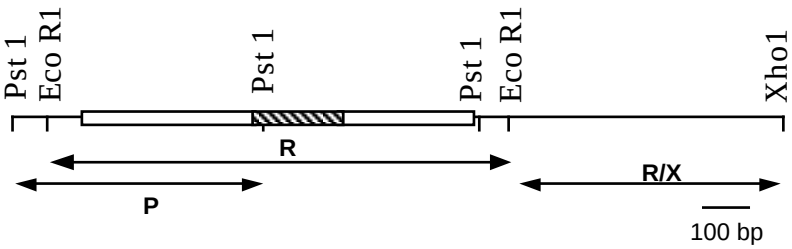
Table 1. Comparison of the deduced amino acid sequence for the homeodomain of axolotl *Dlx-3* to *Drosophila Dll* and vertebrate *Distal-less* (*Dlx*) genes

		10	20	30	40	50	60
axolotl	*Dlx*	IRKPRTIYSS	YQLAALQRRF	QKAQYLALPE	RAELAAQLGL	TQTQVKIWFQ	NRRSKFKKLY
Drosophila	*Dll*	M---------	L--QQ-N---	-RT-------	------S---	----------	-----Y--MM
newt	*NvHBox-4*	----------	----------	----------	----------	----------	----------
zebrafish	*z-dlx3*	----------	----------	----------	----------	----------	----------
Xenopus	*X-dll2*	----------	----------	----------	----------	----------	----------
mouse	*Dlx3*	----------	----------	----------	----------	----------	----------
mouse	*Dlx2*	V---------	F---------	--T-------	------S---	----------	--------MW
human	*Dlx2*	V---------	F---------	--T-------	------S---	----------	--------MW
zebrafish	*z-dlx2*	V--------T	F---------	--T-------	------S---	----------	---------W
Xenopus	*X-dll4*	V---------	F---------	--T-------	------S---	----------	---------W
Xenopus	*X-dll5*	--	F---------	--T-------	------SV--	---	
Xenopus	*X-dll1*	--	F-M-------	--T-------	------S---	---	
mouse	*Dlx4*	----------	L---------	----------	------S---	----------	-
mouse	*Dlx5*	V---------	F---------	--T-------	------S---	----------	-K---I--IM
human	*Dlx5*	V---------	F---------	--T-------	------S---	----------	-K---I--IM
rat	*Dlx1*	V---------	F---------	--T-------	------S---	----------	-K---I--IM
zebrafish	*Dlx4*	V---------	F---------	-NT-------	------S---	----------	-K---L--IM
Xenopus	*X-dll3*	----------	F---------	--T-------	------S---	----------	-K---I--IM
rat	*Dlx3*	----------	F---------	--T-------	------S---	----------	-K---I--IM
mouse	*Dlx1*	----------	L--Q--N---	-QT-------	------S---	----------	-K-------M
human	*Dlx1*	----------	L--Q--N---	-QT-------	------S---	----------	-K-------M
mouse	*Dlx6*	----------	L--Q--NH--	-QT-------	------S---	----------	-K-------L
human	*Dlx6*	----------	L--Q--NH--	-QT-------	------S---	----------	-K-------L
Xenopus	*Xdll*	----------	L--Q--NH--	-QT-------	------S--V	----------	-K---Y---I
newt	*NvHBox-5*	----------	V--Q--NQ--	-QT-------	------H---	----------	-K---Y--IM

dashes (- - -) represent amino acids that are identical to the axolotl *Dlx* sequence at that position.

Axolotl *Dlx-3*

A)



B)

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GGA CCC GCC CCC AAG CGA CCC TTT GCG GGC AGC AGG ACC CCC ACG AGC GGG GGC TTC CAC GGG AAG ATG AGC   72
                                                                                    M   S     2

ACG ATC TTG ACC GAC TTG TCC AGT TCC CTG AGC TGC CAC GCC GCC TCC AAG GAC TCC CCC ACC TTG CCC GAG  144
 T   I   L   T   D   L   S   S   S   L   S   C   H   A   A   S   K   D   S   P   T   L   P   E   26

TCC TCG GCC ACC GAC CTG GGC TAC TAC AGC ACC CAT GGG GGC ACC CAT AGC CCC CAC GAC TAC TTC CAG AGC  216
 S   S   A   T   D   L   G   Y   Y   S   T   H   G   G   T   H   S   P   H   D   Y   F   Q   S   50

CAG CCC TAC CCG CAG CCC ATC AAC CAC CAC TAC CCG TAC CAC CAG TTC AAC CTC AAC GGC CTG GGG GGG CCG  288
 Q   P   Y   P   Q   P   I   N   H   H   Y   P   Y   H   Q   F   N   L   N   G   L   G   G   P   74

GGG ACC TAC TCC CCC AAG TCC GAC TAC CCC TAT GGC GGC AGC TAC CGT CAG TAC GGG CAC TAC CGG GAG TCG  360
 G   T   Y   S   P   K   S   D   Y   P   Y   G   G   S   Y   R   Q   Y   G   H   Y   R   E   S   98

GCG ATG GCG GTG CAG GAG CCA GTT TCA GTG AAG GAG GAG CCC GAG CCG GAA GTG CGG ATG GTG AAC GGC AAA  432
 A   M   A   V   Q   E   P   V   S   V   K   E   E   P   E   P   E   V   R   M   V   N   G   K  122

CCG AAA AAG ATC CGC AAA CCT AGA ACT ATC TAC TCC AGT TAC CAG CTC GCA GCA CTG CAG AGG AGG TTC CAG  504
 P   K   K   I   R   K   P   R   T   I   Y   S   S   Y   Q   L   A   A   L   Q   R   R   F   Q  146

AAG GCG CAG TAC CTG GCA CTG CCA GAG AGG GCG GAG TTG GCA GCC CAG CTT GGA CTC ACC CAA ACT CAG GTG  576
 K   A   Q   Y   L   A   L   P   E   R   A   E   L   A   A   Q   L   G   L   T   Q   T   Q   V  170

AAG ATC TGG TTT CAG AAC CGA CGT TCC AAG TTC AAG AAG CTG TAC AAG AAC GGC GAG GTC CCC GGC ATG GAG  648
 K   I   W   F   Q   N   R   R   S   K   F   K   K   L   Y   K   N   G   E   V   P   G   M   E  194

CAC AGC CCC GAC AAT AGC GAC TCC ATG GCC TGC AAC TCC CCG GCA TCA CCC CCC GTC TGG GAC AGT AAC CCC  720
 H   S   P   D   N   S   D   S   M   A   C   N   S   P   A   S   P   P   V   W   D   S   N   P  218

CCT AGC CGG GTG CCG CAT CCA CAG GCC CAG CCG CTC CCT CAT AAC TCT TCC CCC AGC TAC CTA GAG GAT TAC  792
 P   S   R   V   P   H   P   Q   A   Q   P   L   P   H   N   S   S   P   S   Y   L   E   D   Y  242

AAT CCT GGT ACC ACC ATG AAC AGA ACT TGG CGG GGC ACA CCT GCA GCC GCC CAG CTC GAT GCA CCA TAC CCC  864
 N   P   G   T   T   M   N   R   T   W   R   G   T   P   A   A   A   Q   L   D   A   P   Y   P  266

TCC GGC ACG GGC AGT GTA CTA GCA GGT TGC TGG TGG AAG AAC TAA ATA GCA CAA AGA GAT AAG AGA CTC CGT  936
 S   G   T   G   S   V   L   A   G   C   W   W   K   N                                         280

GCT GGT GTA AGG ACA CAA TGT TCT GAT AGG ACA GAC ACA ACC CTT CCA CTC TCA GGA AAC GAG GCC TCT GCA 1008
GGA GGT ATC CGC AAC TTC AGA AAG GGG TGG CAG CTG GAA TTC CTA CAC TTA TGA GTC GGC CAT GCT CCC ATG 1080
GTG GCC TTC AGA GCC CCG TCT CTA CCG GTC ACA CCA GAG TGG TGT CCA TAT GTT ATC ACT GAA GGT CAT CGC 1152
CGT TTA CTG TAT GCT TTG TAC CAG ATG TTG CGA GTG CCC ATC CTC GCT GGA GCT GGA CCA TTT CTA ATG AGC 1224
AGG ATA CAG GAT TCC TCC TTG CCA CCG AGA TGG GGA GGC AGG GGC TGG GCA TAA GTG GGC GAG GCG CAC CTC 1296
TCC AAC TGG TGA TCG GGA GGA AAC AAG TTG TGG AAA CTT GGT GGT ATA TAA GCT GTA TGT CGT AAA GAA CTG 1368
TGG TGT CAT AGT CTG GTC ACT TAC TAT AAA ATA TCT TCA AGT GGA GAG GGA GTC ACC CCC CCA ACC TCA CCA 1440
CAC GCT CAA ATC TTC CAT GTT TCA CAG GTC ATT CCT TCC CAA CTC AGA AGG TGG GAG CCT TGA GTC ACA CTC 1512
ATG CAA GAG GGC ATT TAA GAT TTA GGG AAA AAA AAA AAA AAA                                       1554
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Fig. 1. Sequence and schematic map of axolotl *Dlx-3* cDNA. (A) Schematic restriction map indicating fragments used for probing northerns (R, P and R/X) and transcribing in situ probes (P). (B) Nucleotide and deduced amino acid sequences of axolotl *Dlx-3* cDNA. The homeodomain is underlined.

of 2.8 kb from developing axolotl embryos as well as from mature and regenerating forelimbs, hind limbs and tails (Fig. 2). The same results were obtained using the P and R/X probes (data not shown). *Dlx-3* expression is detected in embryos as early as the beginning neurulation, or stage 16 of Bordzilovskaya et al. (1989). In mature forelimbs, the transcript is detected at all proximal-distal limb levels (Fig. 2B). Transcripts are abundant in the mature skin (epidermis and dermis), but are not detected on northern blots of total RNA from muscle and associated connective tissue (Fig. 2C). Low levels of expression in these tissues can be detected by RT-PCR (data not shown).

Dlx-3 is expressed at high levels during regeneration of the forelimb, hind limb and tail (Fig. 2D). In the blastema, *Dlx-3* expression is restricted to the wound epidermis; transcripts are not detected by northern analysis in RNA samples from the underlying mesenchyme after the wound epidermis has been manually removed (Fig. 2F). The level of *Dlx-3* expression varies along the proximal-distal limb axis. Expression in the wound epidermis of a distal level blastema is 1.5-fold greater as compared to a proximal level blastema (Fig. 2E). The reason for this difference is apparent from analysis of the whole-mount in situ hybridization results reported below.

Dlx-3 expression is developmentally regulated during regeneration. At the early stages of regeneration, *Dlx-3* expression begins to increase relative to the mature limb (Fig. 2G), such that, by late dedifferentiation, the level of transcripts has doubled. Transcript levels continue to increase and, by late bud stages, they are 4-fold higher as compared to mature limb tissue. At later stages, transcript levels begin to decline (3-fold higher at the palette stage) and, by the late digit stage, are comparable to that of mature tissue.

Dlx-3 expression is inhibited by retinoid treatments that

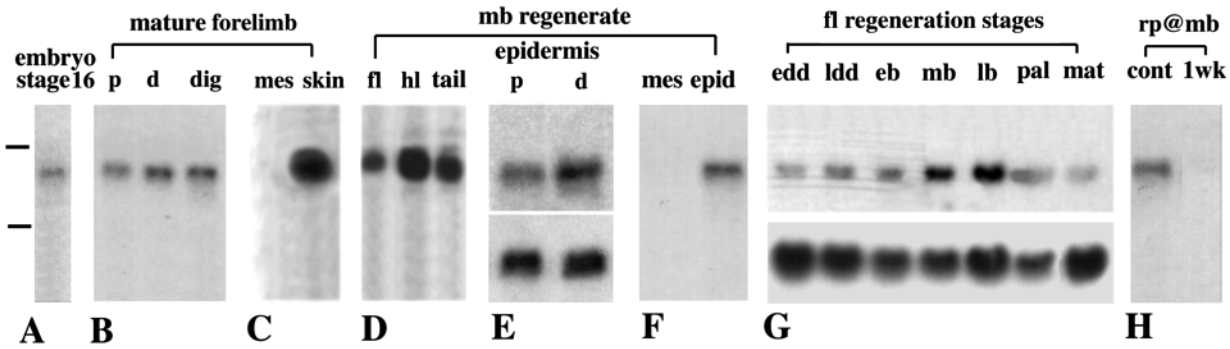

Fig. 2. Northern hybridization analysis of *Dlx-3* expression in developing, regenerating and mature axolotl limbs. Transcript size was determined relative to the mobility of axolotl 28S rRNA (3.9 kb) and 18S rRNA (1.95 kb), as indicated by the hash marks. Equal amounts of total RNA (10 μg/lane) were loaded for each blot as determined spectrophotometrically and verified by quantitation of ethidium bromide staining of the gels. (A) Expression in stage 16 embryos. (B) Expression in mature limb tissues from humerus level (p), mid radius/ulna level (d), or digit level (dig). (C) Expression in skin (epidermis and dermis) removed from a mature limb as compared to expression in the remaining tissues (muscle, cartilage, connective tissue, nerves, blood vessels). (D) Expression in medium bud blastemas from forelimbs (fl), hind limbs (hl) and tails. (E) Expression in the apical epidermis of blastemas from humerus level (p) and mid radius/ulna level (d) amputations. Lower panel is hybridization to the same blot with a probe for axolotl 33 K laminin receptor. (F) Expression in blastemas that had been separated into an epidermal fraction (epid) and a mesenchymal fraction (mes) prior to RNA extraction. (G) Expression at different stages of regeneration from mid radius/ulna level amputations in the forelimbs. The stages of regeneration are early dedifferentiation (edd), late dedifferentiation (ldd), early bud (eb), medium bud (mb), late bud (lb), palette (pal) and mature limb (mat). Lower panel is hybridization to the same blot with a probe for axolotl EF1-α. (H) Expression in response to retinol palmitate treatment (rp) of distal medium bud regenerates after 1 week of treatment (1wk) as compared to control, untreated regenerates (cont).

cause pattern duplications along the proximal-distal limb axis. After one week of treatment with retinol palmitate at the medium bud stage, *Dlx-3* expression is not detected by northern hybridization to 10 μg of total RNA (Fig. 2H). We analyzed retinoid-induced alterations in the pattern of *Dlx-3* expression further by whole-mount in situ hybridization as described below.

In situ hybridization analysis of *Dlx* expression during limb development and regeneration

The spatial and temporal patterns of *Dlx-3* expression were analyzed by whole-mount in situ hybridization using an antisense, digoxigenin-labeled probe transcribed from fragment P (Fig. 1A). A uniform, low level of expression is detected in the epidermis of the prelimb bud larva as early as stage B36 of Bordzilovskaya et al. (1989). This weak, epidermal expression persists throughout development (data not shown). A more intense, and localized expression is associated with the developing forelimb from its earliest appearance (comparable limb bud morphology to stage H36 for *Ambystoma punctatum*, Harrison, 1969; Fig. 3A). This higher level expression is detected in the epidermis covering much of the early limb bud, but is not observed in the most proximal regions or in the adjacent flank epidermis. Expression appears to be graded, with highest levels at the tip of the bud. *Dlx-3* continues to be expressed at high levels in the distal epidermis of later stage limb buds, but not at more proximal levels (Fig. 3B,C). High levels of expression are still detectable during differentiation of the skeletal elements at late digit stages (Fig. 3D). The proximal boundary of *Dlx-3* expression is associated with the middle region of the radius and ulna, and thus expression is associated with the developing digits, hand, wrist and distal zeugopod.

During limb regeneration, a high level of *Dlx-3* expression is not detected during the wound healing and early dedifferentiation stages (Fig. 4A). Expression is first detected at late dedifferentiation and early bud stages when undifferentiated mesenchymal cells begin to accumulate to form a blastema (Fig. 4B). Expression becomes more intense as the blastema increases in size (Fig. 4C), and is most intense at late bud (Fig. 4E,F). When *Dlx-3* expression is maximal, transcripts are detected in the distal-most mesenchymal cells as well as in distal epidermal cells (Fig. 4D). At the early digit stage, *Dlx-3* expression is detected at the tips of the anterior digits (Fig. 4G). Expression is less intense in the undifferentiated, proximal-posterior cells that will form digits 3 and 4. *Dlx* expression decreases further such that expression is not detected in association with fully regenerated limbs.

The temporal pattern of expression of *Dlx-3* is the same during regeneration from both proximal and distal level amputations. In contrast, the size of the *Dlx-3* expression domain relative to the total size of the blastema differs between distal and proximal blastemas. Expression is more distally restricted in blastemas that form at more proximal levels (mid-humerus) as compared to more distal levels (mid-radius/ulna). In distal blastemas, essentially the entire epidermis expresses *Dlx-3*; whereas, in proximal blastemas, the epidermis at the base of the blastema does not express *Dlx-3* (Fig. 4E,F). This difference accounts for the results from northern analysis indicating a higher level of *Dlx-3* expression in distal blastemas (greater proportion of expressing cells) as compared to proximal blastemas. At late stages of regeneration, expression becomes distally restricted to the tips of digits 1 and 2 in proximal blastemas as it does in distal blastemas (data not shown).

Retinoids inhibit *Dlx* expression

The expression of *Dlx-3* is downregulated in response to retinoids. After two days of treating the entire animal with retinol palmitate, expression is considerably reduced compared to blastemas on untreated animals at the same stage (Fig. 5A and data not shown). When blastema cells are treated directly

with RA released from implanted beads, *Dlx-3* expression is downregulated beginning at 3 hours (Fig. 5B), and remains almost undetectable by whole-mount in situ hybridization at 7 days after bead implantation (Table 2).

Dlx-3 expression is upregulated when regeneration recommences. During the first several days after bead implantation, the blastema does not progress beyond the medium bud stage. Regeneration eventually resumes, presumably because all RA has diffused from the implanted bead and been cleared from the limb. *Dlx-3* is again expressed at high levels during this period (Table 2). By 13 days after bead implantation *Dlx-3* is re-expressed in the forming digits (Fig. 5C). Expression is also intense in the more posterior, undifferentiated cells that will give rise to digits 3 and 4. Limbs that are implanted with an RA bead form a regenerate whose pattern is duplicated along the proximal-distal axis (Fig. 5D). Treatment with implanted RA beads can also induce pattern duplication along the anterior-posterior and dorsal-ventral axes of regenerating limbs (Sessions et al., 1989).

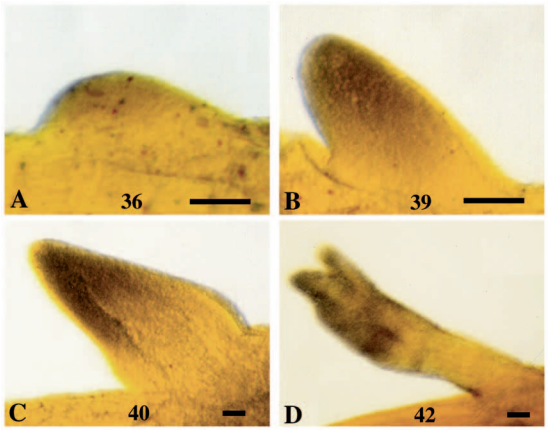

Fig. 3. *Dlx-3* expression in developing axolotl forelimbs visualized by whole-mount in situ hybridization. (A) stage 36; (B) stage 39; (C) stage 40; (D) stage 42 (stages after Harrison, 1969). Scale bar, 50 μm.

Dlx-3 expression and limb regeneration exhibit the same pattern of nerve-dependency

Regeneration is inhibited when a limb is denervated at or prior to the medium bud stage. In contrast, limbs that are denervated at late bud or subsequent stages, go on to form a normally patterned, but smaller regenerate (see Wallace, 1981). In this study, we observed the same results, whereby denervated medium bud blastemas failed to regenerate (Figs 6C, 7), but denervated late bud blastemas form a regenerate of reduced size (Fig. 6F).

Changes in the expression of *Dlx-3* are correlated with the effects of denervation on regeneration. When medium bud blastemas are denervated, *Dlx-3* expression is downregulated within 1 hour and is absent at 6 and 12 hours postdenervation (Fig. 6A; Table 3). *Dlx-3* is eventually reexpressed in most limbs, even though regeneration remains inhibited. The time of reexpression is variable, with half the limbs examined at 24 hours reexpressing *Dlx-3*. Of 12 limbs examined between 2 and 16 days after denervation at early/medium bud, none regenerated but only 2 limbs failed to reexpress *Dlx-3*.

Correlated with the transition from nerve-dependent to nerve-independent regeneration, *Dlx-3* expression in late bud stage regenerates is nerve independent. *Dlx-3* continues to be expressed in denervated late bud blastemas during the initial 24 hour period following denervation (Fig. 6D; Table 3), and regeneration is not inhibited.

FGF-2 prevents the denervation-induced inhibition of *Dlx* expression and rescues regeneration

FGF can mediate the effect of the apical epidermis on limb outgrowth during development (Niswander et al., 1993; Fallon et al., 1994; Taylor et al., 1994; Mahmood et al., 1995). Since *Dlx-3* is expressed in the apical wound epidermis, and its expression is correlated with blastema outgrowth, we tested the effects of FGF on *Dlx-3* expression and regeneration in denervated, nerve-dependent blastemas. In contrast to control denervated limbs, all denervated limbs that received an FGF-2 bead implant continued to express *Dlx-3* during the initial 24 hour period following denervation (Fig. 6G,H; Table 3).

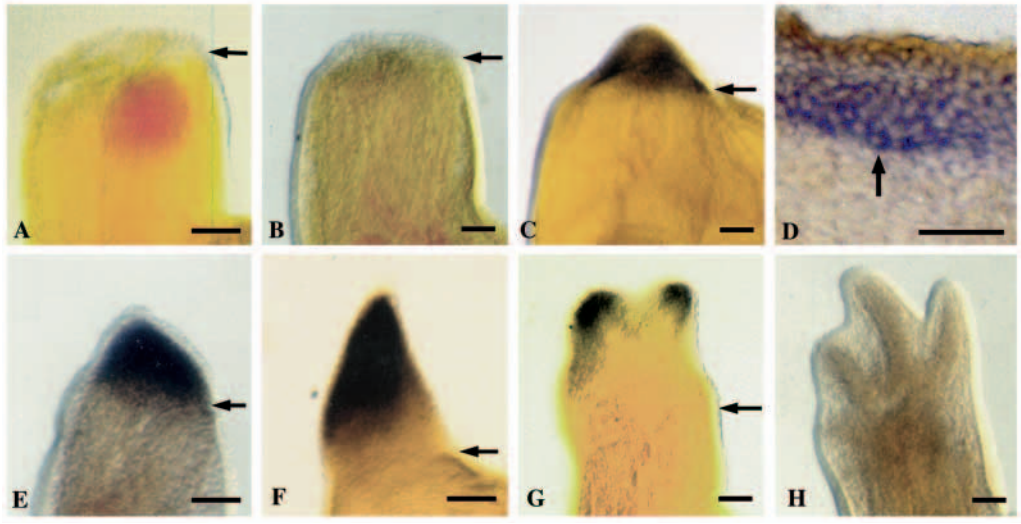

Fig. 4. Expression of *Dlx-3* during axolotl limb regeneration as visualized by whole-mount in situ hybridization. Pictured are forelimbs amputated at the mid radius/ulna level, except for (F) which was amputated at the mid humerus level; amputation plane is indicated by arrows. Limbs are viewed from the dorsal side with posterior to the left and represent progressively later stages of regeneration: (A) wound healing/early dedifferentiation, one day post amputation; (B) early bud stage; (C) medium bud stage; (D) section of a late bud blastema that had been processed for whole-mount in situ hybridization (base of the apical epidermis is indicated by an arrow); (E) late bud stage; (F) late bud stage blastema from a proximal level blastema; (G) early digit stage; (H) late digit stage. Scale bar, 150 μm (except D; scale bar, 50 μm).

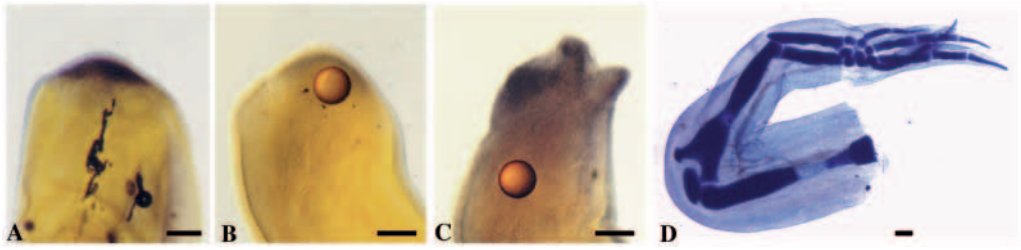

Fig. 5. Expression of *Dlx-3* in response to retinoid treatment as visualized by whole-mount in situ hybridization. All limbs are viewed from the dorsal surface with posterior to the left. For comparison, the control, non-treated medium bud expression pattern is shown (A). RA-containing beads were implanted into a medium bud blastema and expression was assayed 60 hours (B) and 13 days(C) later. Some limbs were allowed to regenerate and were stained with Victoria Blue to visualize the duplicated skeletal pattern (D). Scale bar, 200 μm.

Table 2. Effects of RA on *Dlx-3* expression

Time since RA bead implantation	Limbs with normal exp	Limbs with reduced exp.
1 hr	1	0
3 hr	1	2
6 hr	0	1
1-2 days	0	5
1 wk	0	1
>1 wk	3	0

Table 3. Percent of regenerates expressing *Dlx-3* following denervation with or without without FGF treatment

	Time postdenervation*					
Stage	0 hour	<3 hours	6 hours	12 hours	24 hours	2-16 days
EB/MB	100%(2)	100%(9)†	0%(5)	0%(3)	50%(8)‡	83%(12)
EB/MB with FGF	nd	nd	100%(2)‡	100%(3)	100%(2)	100%(13)§
LB/PAL	nd	nd	100%(2)	100%(2)	100%(5)	100%(7)§
Controls (not denervated)	100%(2)	100%(9)	100%(11)	100%(11)	100%(17)	100%(45)§

*Numbers of limbs examined are shown in parentheses.
†In all nine limbs, expression was reduced compared to normal.
‡One limb had reduced expression compared to normal.
§Many of these limbs had progressed beyond the last stage of strong *Dlx-3* expression.

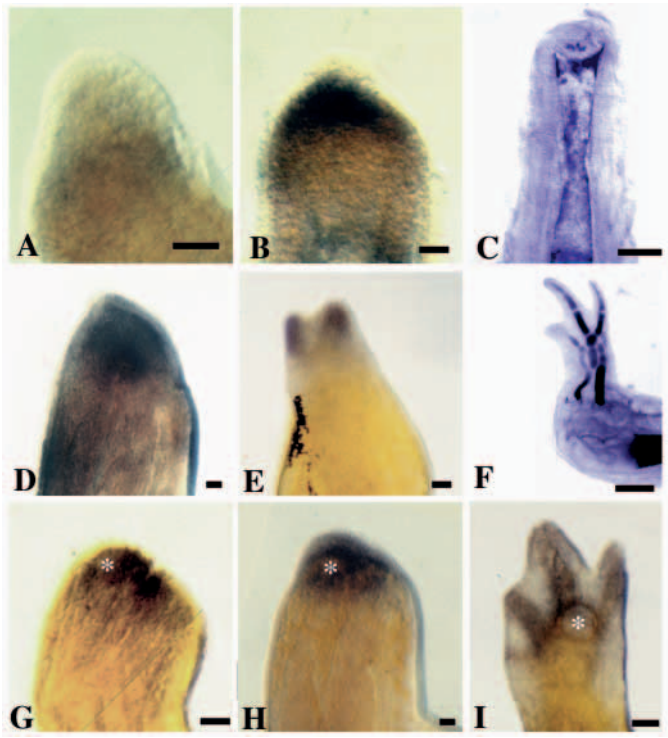

Fig. 6. *Dlx-3* expression in response to denervation and FGF-2 as visualized by whole-mount in situ hybridization. All limbs are viewed from the dorsal surface with posterior to the left. Expression in medium bud blastemas 12 hours (A) and 11 days (B) after denervation. The final truncated pattern resulting from this treatment is illustrated in C which is a limb stained with Victoria Blue to visualize the cartilage pattern. Expression in late bud blastemas 12 hours (D) and 11 days (E) after denervation. The final pattern resulting from this treatment is illustrated in F. Expression in FGF-2-treated, denervated medium bud blastemas 12 hours (G), 24 hours (H) and 16 days (I) after denervation and implantation of an FGF-2 bead. Asterisk marks location of bead. Scale bar, 100 μm (except C,F; scale bar, 500 μm).

In addition, regeneration was not inhibited in denervated limbs treated with FGF-2. Of the four denervated limbs that received a second bead implant and were allowed to regenerate for a longer time period (two weeks), three regenerated to digit stages and one regenerated to a palette stage (Fig. 7). Although there may have been a delay in the progression from medium bud to palette stages of regeneration, the FGF-2 treated limbs reached digit stages of regeneration over the same time period as non-denervated regenerates. The control, denervated regenerates (without FGF-2 beads) did not progress beyond the stage at which they were denervated, except for three cases that arrested at the next stage (late bud stage, Fig. 7).

FGF-2 is present in nerves and the AEC, and is downregulated in response to denervation

Since FGF-2 rescues both *Dlx3* expression and regeneration in denervated limbs, we investigated whether FGF-2 is present in either the apical epidermis or the nerves. Using immunocytochemistry, we detected the presence of FGF-2 in both nerves (Fig. 8A) and the AEC (Fig. 8C). As in developing chick limb buds (Savage et al., 1993), staining in the epidermis is heterogeneous and appears both nuclear and cytoplasmic. Reactivity decreases in response to denervation, and FGF-2 is nearly undetectable in both nerves (Fig. 8B) and the AEC (Fig. 8D) 12 hours after denervation. Studies investigating the regulation of FGF-2 expression during regeneration are in progress.

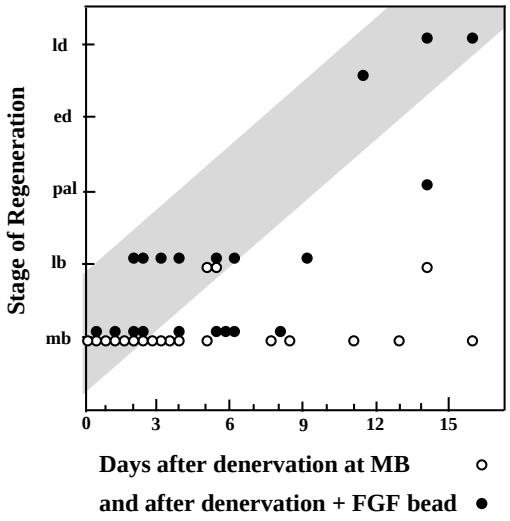

Fig. 7. FGF rescue of regeneration in denervated medium bud blastemas. The normal rate of regeneration (control limbs that were not denervated, *n*=95) is indicated by the shaded area. Open circles represent the stage of regeneration of denervated blastemas at increasing times after denervation. The filled circles represent the stage of regeneration of denervated blastemas that were implanted with an FGF-2 bead at the time of denervation. Each circle represents one animal. The stages are medium bud (mb), late bud (lb), palette (pal), early digits (ed) and late digits (ld).

DISCUSSION

A screen for homeobox genes expressed in regenerating limb blastemas yielded a cDNA homologous to *Drosophila Distal-less* (Gardiner et al., 1995). Several vertebrate homologs of this gene have been reported previously, and they can be grouped into four subfamilies (Akimenko et al., 1994). The clone that we isolated is full-length, and clearly belongs to the group containing zebrafish and mouse *Dlx-3*; *Xenopus Dll-2* and newt *NvHBox-4* (Ekker et al., 1992; Robinson and Mahon, 1994; Morasso et al., 1994; Beauchemin and Savard, 1992).

A single axolotl *Dlx-3* transcript is expressed as early as the neurula stage of development. Expression is elevated in appendages, but is also found in the body ectoderm of the embryo, and persists at a low level in the epidermis of the mature animal. At limb bud stages, axolotl *Dlx-3* is expressed at an elevated level in the ectoderm covering the distal region of the limb bud. *Dlx* genes are expressed in the apical ectodermal ridge (AER) of developing mouse limb buds (Dollé et al., 1992; Bulfone et al., 1993), and of the AER-equivalent of developing zebrafish fins (Akimenko et al., 1994). In axolotls, the AEC performs an equivalent function but is not confined to a narrow stripe of cells bordering the distal rim of the bud. The more extensive area of expression of *Dlx-3* in axolotls correlates with the greater extent of the AEC. A similar broad zone of epidermal expression can be seen in zebrafish pectoral fin buds stained with an antibody to *Drosophila Dll* (Panganiban et al., 1995). *Dlx-3* is expressed in other developing structures where epithelial-mesenchymal interactions are involved, such as in developing axolotl gills (data not shown), in developing mouse ears, whisker follicles and teeth (Robinson and Mahon, 1994), and in zebrafish otic placodes (Ekker et al., 1992).

During regeneration, the AEC covering the blastema shows increasing levels of *Dlx-3* expression, reaching a maximum at late bud. At this stage, expression is also detected in the mesenchyme at the limb tip, as reported for zebrafish fin development (Akimenko et al., 1994). Expression peaks at the stages of maximal growth, and declines to levels seen in mature tissues as growth is reduced and differentiation predominates at palette and later stages. This expression pattern, like that of developing limbs, is consistent with a role of *Dlx-3* in promoting limb outgrowth. Beauchemin and Savard (1992) did not observe upregulation of *NvHBox 4*, the newt homolog of *Dlx-3*, during limb regeneration; however, their observations were limited to northern analysis of whole limb tissues.

Comparison of regenerates formed from distal amputations with those formed from proximal amputations shows that *Dlx-3* expression is associated with distal structures. In regenerates forming at a distal level, where only the hand will be regenerated, *Dlx-3* is expressed in the entire blastema epidermis. Regenerates forming from a proximal level replace proximal as well as distal structures, and here *Dlx-3* expression is localized to the distal third of the blastema. As in developing limbs, expression is strongest distally and declines proximally. The distal region of both proximal and distal blastemas express a distal *Hox* code (Gardiner et al., 1995), and *Dlx-3* expression coincides with the expression of this distal code.

Unlike in developing limbs, where expression remains associated with the hand, wrist and distal part of the lower arm until all digits are formed, in regenerating limbs, expression becomes restricted to the tips of digits one and two, with weaker expression associated with the region in which digits three and four arise. It is unclear whether this difference between developing and regenerating limbs reflects a difference in *Dlx-3* function, but it does indicate that expression at only the extreme distal tip of the limb is sufficient to allow complete formation of the proximal-distal axis.

Retinoid treatment of regenerating limbs induces duplication of the proximal-distal (PD) pattern of the limb (Niazi and Saxena, 1978; Maden, 1982), usually after a pause in regeneration during which mitosis is arrested (Maden, 1983). The response to implanted beads containing RA includes anterior-posterior (AP) and dorsal-ventral (DV), as well as PD duplications (Sessions et al., 1989). Molecular changes that accompany retinoid treatment of regenerating limbs (Simon and Tabin, 1993; Gardiner et al., 1995) indicate that the blastema acquires proximal characteristics. Genes characteristic of distal parts of the pattern are downregulated, and those normally expressed at proximal levels are either not affected, or their expression is upregulated in response to retinoids. The response of *Dlx-3* is consistent with this pattern. *Dlx-3* is normally expressed distally, and is downregulated by retinoids. Expression remains low or undetectable during the period of growth arrest that follows treatment, and upregulation coincides with resumption of outgrowth several days later. Hence, the response of *Dlx-3* to RA is consistent with a role in the function of the epidermis in permitting outgrowth.

A difference between development and regeneration is the influence of nerves. Limb development is independent of nerves, while regeneration exhibits an early nerve-dependent phase, ending at medium bud, and a later nerve-independent stage from late bud onwards. The maximal level of *Dlx-3* expression is correlated with the transition to the nerve-independent phase. We found that *Dlx-3* expression is downregu-

lated within an hour of denervation and is undetectable at 6 and 12 hour after denervation at the nerve-dependent stages. Hence, *Dlx-3* is a target of neurotrophic factors. Expression returns in 50% of limbs by 24 hours, and in most limbs by 2 days, even though regeneration never resumes. This result suggests that a transient, denervation-induced inhibition of *Dlx-3* expression is sufficient to halt regeneration and may be functionally equivalent to loss of the permissive epidermis. Downregulation of *Dlx-3* may be a direct response by the epidermal cells to denervation, rendering them non-permissive such that the underlying mesenchyme cells are unable to participate in outgrowth. Although *Dlx-3* is eventually reexpressed, the epidermis is no longer effective in promoting outgrowth, perhaps because the underlying mesenchyme cells have lost the ability to respond to epidermal signals.

Once a regenerate reaches late bud, it no longer requires the presence of nerves for growth and differentiation. During these nerve-independent stages of regeneration, *Dlx-3* expression is not altered by denervation, a result that is again consistent with a relationship between permissiveness of the epidermis and *Dlx-3* expression. Thus a permissive epidermis and *Dlx-3* expression persist, even in the absence of nerves at these later stages, and limb outgrowth and pattern formation proceed.

The mechanism and consequences of *Dlx-3* downregulation in response to denervation are presumably different from those following retinoid treatment since denervated limbs fail to regenerate and retinoid-treated limbs eventually show extra growth and pattern. Above we propose that the transient inhibition of *Dlx-3* expression induced by denervation results in the loss of the permissive epidermis. It is known that, in the absence of a permissive epidermis, mesenchyme cells at the limb tip lose the ability to participate in further pattern formation, and the limb is truncated (Saunders, 1948). Truncation may be due to the downregulation of genes specifying distal parts of the pattern after AER removal (Hayamizu et al., 1994).

We do not at this time understand why *Dlx-3* downregulation in response to retinoids is not also associated with pattern truncation. One possibility is that the loss of *Dlx-3* expression induced by RA treatment is a secondary effect of a change in the positional identity of mesenchymal cells (Tickle et al., 1989). In this case, since RA converts mesenchymal cells to a positional identity that corresponds to the base of the limb, it may change them to a point in limb formation that precedes the formation of the permissive epidermis and hence the expression of *Dlx-3*. As growth resumes after removal of RA, limb formation will be associated with both the reestablishment of a permissive epidermis and the reexpression of *Dlx-3*.

The permissive epidermis of developing chick and mouse embryos express several different FGFs (Savage et al., 1993; Heikinheimo et al., 1994; Niswander and Martin, 1992). In chicks, the function of the permissive epidermis in promoting limb outgrowth can be replaced by FGF-2 or FGF-4 (Niswander et al., 1993; Fallon et al., 1994; Taylor et al., 1994). More recently it has been shown that FGF-1, FGF-2 and FGF-4 can stimulate the formation of ectopic limbs in the flank of chick embryos (Cohn et al., 1995; Mima et al., 1995). In this study, we discovered that FGF maintains expression of *Dlx-3* after denervation at a nerve-dependent stage, whereas denervated control limbs that did not receive FGF showed a gap in expression of at least 12 hours.

Perhaps the most intriguing result is that denervated, FGF-2-treated limbs are able to regenerate. This represents the first rescue of denervated, nerve-dependent regenerates by any means other than by nerves themselves. FGF-2 can substitute for innervation both in maintaining *Dlx-3* expression and in promoting outgrowth of nerve-dependent blastemas. In addition, we determined that FGF-2 is present in both nerves and the AEC, and that the levels of FGF-2 are decreased in response to denervation. These data suggest that FGF-2, or other members of the FGF family, is the neurotrophic factor required for regeneration, or is a mimic of the endogenous neurotrophic factor operating in the limb. FGF-1 is also present in nerves (Elde et al., 1991), and has been detected in both blastema mesenchyme and epidermis (Boilly et al., 1991). FGF receptors are present in newt blastema mesenchyme and in the basal layer of the epidermis (Poulin et al., 1993; Poulin and Chiu, 1995). Hence, nerves could release FGF into the blastema or, in the case of sensory nerves, into the epidermis, and receptors are appropriately located to respond. FGF has previously been suggested as a candidate neurotrophic agent (Mescher and Gospodarowicz, 1979), based on its ability to stimulate growth of blastemas. When FGF isolated from bovine brain was either slowly injected into denervated regenerates (Mescher and Gospodarowicz, 1979), or added to blastema cultures (Carlone et al., 1981), mitosis was enhanced compared to controls that did not receive FGF.

Our results indicate a functional relationship between nerves and the epidermis in regeneration. Removal of either the permissive epidermis (Stocum and Dearlove, 1972), or of the nerve supply (Singer, 1952) in early regeneration stops growth and pattern formation. There is also a physical connection between the two in normal regenerates, where the apical cap is invaded by fine nerve fibers distributed in a distal-to-proximal gradient (Singer, 1949). Since innervation dramatically affects *Dlx-3* expression, which is also graded in the same direction, it is possible that the nerve gradient and the graded *Dlx-3* expression pattern are related by means of FGF released from nerves.

The evidence that we have presented suggests that both *Dlx-3* expression and the production of FGF in the permissive epidermis are induced by FGF. In developing limbs, and in later stages of regeneration, sufficient FGF would be produced by the permissive epidermis to act as both a paracrine factor, promoting growth in the mesenchyme, and as an autocrine factor maintaining *Dlx-3* expression and stimulating synthesis of FGF by epidermal cells. Since FGF-2 may upregulate its own message (Fallon et al., 1994), it is possible that an FGF feedback loop exists between the mesenchyme and epidermis at the tip of the limb. It is even possible that *Dlx-3* is a transcription factor in this loop that directly controls FGF production, although we have no evidence that addresses this directly.

Such a relationship between FGF, *Dlx-3* and nerves suggests a mechanism accounting for the phenomenon of accessory limb induction in response to nerve deviations in amphibians (see Wallace, 1981). A deviated nerve would provide a source of FGF that would induce *Dlx-3* expression in the overlying epidermis, thus converting it to an outgrowth-permissive epidermis. As discussed above, implantation of an FGF bead, or cells secreting FGF, induces supernumerary limbs in the flank of chick embyros (Cohn et al., 1995; Mima et al., 1995).

Finally, this model provides an hypothesis as to why limb regeneration is dependent on nerves during the early stages, whereas limb development is not. Developing limbs have a per-

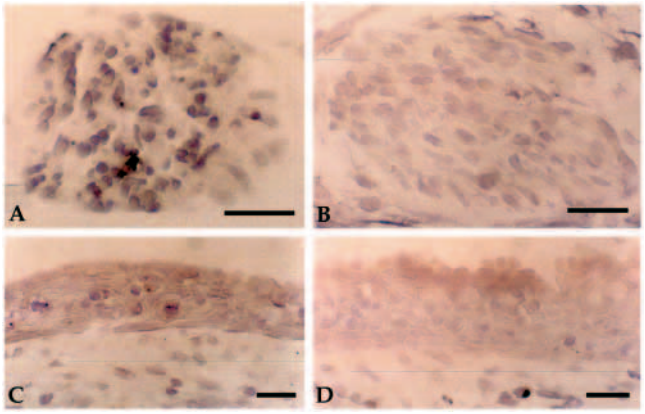

Fig. 8. Immunolocalization of FGF-2 in nerves (A,B) and the AEC (C,D) of normal (A,C) and (B,D) regenerating limbs denervated at medium bud. Tissues in B and D are from limbs denervated 12 hours earlier. Scale bar, 50 μm.

missive epidermis from the outset, which expresses both *Dlx* genes and several forms of FGF. FGF beads implanted into the prelimb bud flank of the chick induce extra limbs (Cohn et al., 1995; Mima et al., 1995). The beads may mimic a natural source of FGF in the flank of the embryo that normally initiates FGF production in the AER, thereby establishing the positive feedback loop of the permissive epidermis, and ensuring a supply of FGF for the growing mesenchyme. By contrast, in the early stages of regeneration, the wound epidermis that forms over the blastema is derived from the mature skin. This epidermis becomes transformed into the AEC, and in so doing acquires new functions and synthesizes a new set of gene products (Tassava et al., 1986; Onda et al., 1991; Klatt et al., 1992). We suggest that FGF and *Dlx-3* upregulation are two changes that are necessary to induce the formation of an outgrowth permitting epidermis, and that the transformation into an AEC needs to be primed by an exogenous source of FGF, supplied by the damaged and regenerating nerves. If the nerve supply is removed during the early stages of regeneration, the level of available FGF will fall, leading to the down-regulation of *Dlx-3* expression and further reduction in FGF. The cells in the blastema can no longer be maintained in a proliferative, pattern formation competent state. However, if the nerve supply is removed later in regeneration (late bud or later), the permissive epidermis maintains *Dlx-3* expression, continues FGF production and therefore maintains blastema proliferation and completes regeneration.

This paper is dedicated to the memory of Marcus Singer, whose lifetime of experiments defined the uniquely important role that nerves play in regeneration. We thank members of the Bryant lab for helpful comments on the manuscript, Dr Brad Olwin for providing the FGF-2 antibody, and Dr Eddy De Robertis for his warm hospitality at UCLA where this project was initiated. Research supported by PHS grants HD25620 and HD 33465 (to S. V. B. and D.M.G.), an allocation of computer resources by the University of California Irvine, and the Indiana University Axolotl Colony, Bloominton, Indiana.

GenBank accession number: The accession number for the sequence reported in this paper is U59480.

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(Accepted 2 August 1996)