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). Its 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; Bullone 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 EcoRI fragment that contains the entire homeobox (fragment R), a 600 bp EcoRI/XhoI fragment from the 3’ UTR, and a 490 bp PstI 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 (Bryan 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 used 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 re-energized limbs to regenerate. These limbs were implanted with a second FGF bead 4 days after denervation, and were re-denervated 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
Axolotl Dlx-3

(A) Schematic restriction map indicating fragments used for probing northemns (R, P and R/X) and transcribing in situ hybridization results reported below.

(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 Bzdilovskaya et al. (1989). In mature forelimbs, the transcript is expressed at high levels during regeneration of 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 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

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.
caused by pattern duplications along the proximal-distal limb axis. After one week of treatment with retinol palmitate at the medium bud stage, \textit{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 \textit{Dlx-3} expression further by whole-mount in situ hybridization as described below.

**In situ hybridization analysis of \textit{Dlx} expression during limb development and regeneration**

The spatial and temporal patterns of \textit{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 \textit{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. \textit{Dlx-3} continues to be expressed at high levels in the distal epidermis of \textit{Dlx-3} expression during regeneration from both proximal and distal level amputations. In contrast, the size of the blastema increases in size (Fig. 4C), and is most intense at late bud (Fig. 4E,F). When \textit{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, \textit{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. \textit{Dlx} expression decreases further such that expression is not detected in association with fully regenerated limbs.

The temporal pattern of expression of \textit{Dlx-3} is the same during regeneration from both proximal and distal level amputations. In contrast, the size of the \textit{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 \textit{Dlx-3}; whereas, in proximal blastemas, the epidermis at the base of the blastema does not express \textit{Dlx-3} (Fig. 4E,F). This difference accounts for the results from northern analysis indicating a higher level of \textit{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 \textit{Dlx} expression**

The expression of \textit{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).

**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).
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.

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

<table>
<thead>
<tr>
<th>Time since RA bead implantation</th>
<th>Limbs with normal exp</th>
<th>Limbs with reduced exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3 hr</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6 hr</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1-2 days</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>1 wk</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>&gt;1 wk</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time postdenervation*</th>
<th>EB/MB</th>
<th>EB/MB with FGF</th>
<th>LB/PAL Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hour</td>
<td>&lt;3 hours</td>
<td>6 hours</td>
<td>12 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>100%(2)</td>
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<td></td>
<td>nd</td>
<td>nd</td>
<td>100%(2)</td>
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<td>100%(2)</td>
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<td>nd</td>
<td>nd</td>
<td>100%(2)</td>
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</table>

*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.
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; Bullone 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 palate 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 blastsma 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 embryos (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-
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 downregulation 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 Edly 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, Bloomington, Indiana.

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