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Vaccinia as a Tool for Functional Analysis in Regenerating Limbs: Ectopic Expression of *Shh*

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Axolotls, with their extensive abilities to regenerate as adults, provide a useful model in which to study the mechanisms of regeneration in a vertebrate, in hopes of understanding why other vertebrates cannot regenerate. Although the expression of many genes has been described in regeneration, techniques for functional analysis have so far been limited. In this paper we demonstrate a new method for efficient overexpression of foreign genes in axolotls. Using vaccinia virus expressing β-galactosidase microinjected into regenerating limbs, we show that vaccinia can infect both dividing and nondividing limb cells. The site of infection remains discrete and there is no secondary spread of infection to nearby cells. β-Gal is expressed at high levels in blastema cells for about a week and in differentiated cells for longer. Blastemas that have been injected with vaccinia at different stages regenerate normally. As a test of the utility of vaccinia for functional analysis in regeneration, we constructed a virus expressing *Shh* and injected it into the anterior of regenerating limbs. Ectopic *Shh* expression caused extra digits, carpals, and tarsals in the hands and feet of regenerating limbs, suggesting that despite differences in the timing of expression and the eventual pattern, the function of *Shh* appears to be similar to that in the developing limbs of other vertebrates. Our results demonstrate that vaccinia virus is an excellent vector for ectopically expressing genes for secreted proteins and is a useful tool to study the function of signaling molecules during the process of regeneration in urodeles.

Key Words: **regeneration; limb; axolotl; vaccinia; Shh; amphibian; urodele.**

INTRODUCTION

Urodele amphibians are unique among vertebrates in their ability to regenerate lost body parts as adults. While many animals can regenerate missing parts as embryos, only urodeles are able to recreate the necessary "embryonic" environment for redevelopment to occur once cells have differentiated. Among the complex structures that can be regenerated in urodeles, it is the limbs about which the most is known. We are interested in using urodeles to discover the signals that trigger and maintain the regeneration response, because these signals will have enormous potential and consequences for improving human health. However, progress in regeneration has been slowed in recent years due to the difficulties of bringing the power of functional analysis to bear on urodeles.

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In this study, we have used vaccinia virus as a vector to ectopically express secreted proteins in regenerating axolotl limbs. Vaccinia virus is a member of the Orthopoxvirus genus of the Poxviridae family (Moss, 1991). It is a DNA virus with a genome of ≈200 kb that encodes the proteins needed for replication and transcription in the cytoplasm. Vaccinia was introduced in 1982 as a vector for transient expression in mammalian cells (Mackett *et al.*, 1985). It can be grown to high titers and can accommodate foreign genes up to 25 kb. Vaccinia virus has been used successfully in *Xenopus laevis* to express CPG-15 and CaMKII genes in the optic tectum of tadpoles (Nedivi *et al.*, 1998; Wu and Cline, 1998; Wu *et al.*, 1995). In our study we show, using vaccinia virus expressing β-galactosidase (Chakrabarti *et al.*, 1985) microinjected into axolotl limbs, that vaccinia has very useful properties for functional analysis in limb regeneration.

To test the utility of vaccinia as a tool for functional analysis in axolotl regeneration, we analyzed the effects of ectopic expression of *Sonic hedgehog* (*Shh*) expression. Of all the signaling molecules described in developing limbs,

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those that control the properties of the signaling region known as the ZPA (zone of polarizing activity), first identified by Saunders and Gasseling (1968), have generated the most interest. First retinoic acid (RA) (Thaller and Eichele, 1987) and later Shh (Riddle *et al.*, 1993) have been cast in the role of the agent that has the property of establishing the pattern of the anterior–posterior axis. ZPA grafts, RA beads, and virally driven *Shh* expression all produce the same digit duplicating effect on the AP pattern when applied to the anterior edge of the chicken limb bud. *Shh* is expressed in the expected location in developing urodele limbs and is reexpressed in limb regeneration in newts (Imokawa and Yoshizato, 1997) and axolotls (Torok *et al.*, 1999), but only for a brief time and in a very small region of the posterior, proximal blastema. This, coupled with reports that another *hh* family member may be broadly expressed in regeneration (Stark *et al.*, 1998), raised doubts about whether Shh was playing a role in patterning the regenerating limb.

We show that ectopic expression of *Shh* in the anterior of regenerating limbs leads to the duplication of digits and carpals/tarsals, showing both a functional role for Shh in limb regeneration and the utility of vaccinia as a tool for transgenesis in urodeles.

MATERIALS AND METHODS

Animal Procedures

Axolotls (*Ambystoma mexicanum*) were spawned either at the Indiana University Axolotl Colony or at UCI. Larvae were maintained at 20–22°C in 40% Holtfreter's solution. Animals (5–7 cm) were anesthetized in 0.1% MS222 solution for amputations through the upper arm to induce regeneration and for virus injections. At the beginning of our study, amputations were also made in the lower arm/leg levels and identical duplications were observed; since upper level amputations were easier to inject we continued with these amputations, and the vast majority of the results reported here are from upper arm/leg amputations. Animals were euthanized at the end of the experiment. For Victoria blue staining, samples were fixed overnight in Bouin's fixative and then processed as in Bryant and Iten (1974). For X-gal staining (Miller, 1972), samples were fixed for 30 min in phosphate-buffered saline containing 1% formaldehyde, 0.2% glutaraldehyde, 0.5 mM EDTA, 0.02% NP-40, and 2 mM $MgCl_2$ for 30 min.

Preparation of Recombinant Shh Vaccinia Virus

Vaccinia virus and the pSC65 shuttle vector containing *lacZ* driven by a strong early/late viral promoter (provided by Hollis Cline, with permission from Bernard Moss, NIH) were grown and purified as described previously (Mackett, 1985). Full-length chicken *Shh* cDNA was a gift from Juan Carlos Izpisúa-Belmonte. *Shh* cDNA was excised from Bluescript II SK plasmid (Stratagene, La Jolla, CA) and cloned into the *Sal*I and *Sma*I sites of the shuttle vector, pSC65. The resulting plasmid, pSC*Shh*, was used to drive homologous recombination after being transfected into RK13 cells (ATCC CCL-37) infected with the wild-type vaccinia virus. Recombinant viruses were then selected by plaque assay and purified as described previously (Mackett *et al.*, 1985).

Viral Injection

Albino or white axolotls were used for our experiments to facilitate the detection of the X-gal. Viral injections were made either subcutaneously into the intact limb or into the regeneration blastema. Single (300–500 nl) or multiple (1–2 μl total volume) injections of either control (β-gal) or experimental (*Shh*) virus (10^9 pfu/ml) were made into a single site in the blastema or subcutaneously in the intact limb. Regenerating limbs at the early dedifferentiation, early bud, medium bud, late bud, and palette stages were injected in the anterior of the blastema with virus expressing *Shh*. Mature limbs were also injected with *Shh*-expressing virus (on the anterior) 2 days prior to amputation. As controls, blastemas and limbs were injected with β-gal-expressing virus. To determine the earliest time of marker gene expression after infection, virus was added to the culture medium of an axolotl limb connective tissue cell line (ACT1; Gardiner, unpublished).

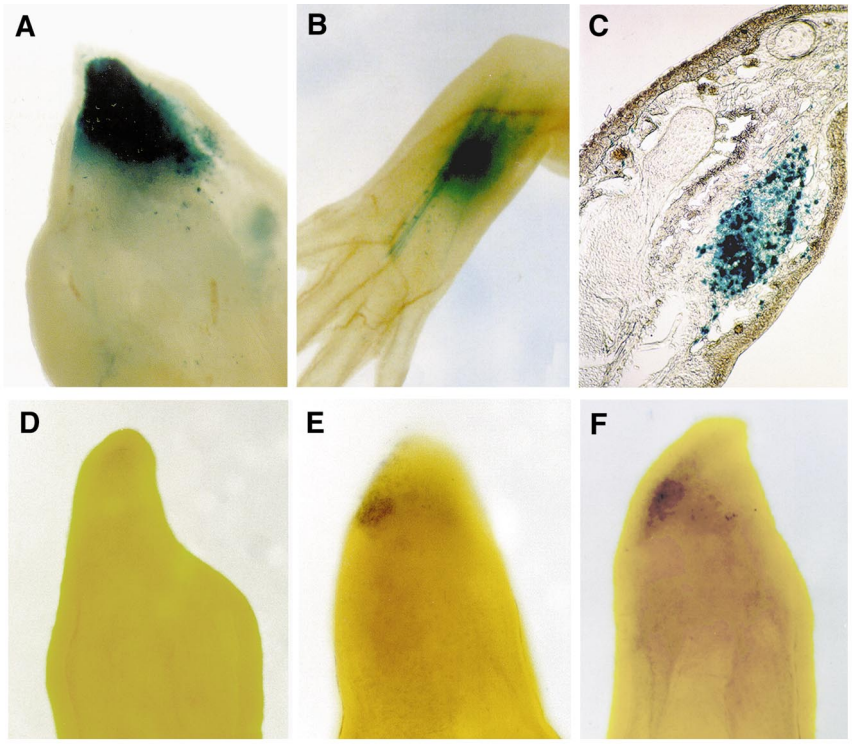
Whole-Mount in Situ Hybridization

The procedure for whole-mount *in situ* hybridization was as previously described (Gardiner *et al.*, 1995) with a few modifications. The injected limbs were fixed in MEMFA for 48 h and then transferred in 100% methanol. The limbs were treated with 30 μg/ml proteinase K, first for 30 min on ice and then for 30 min at 37°C. Prehybridization and hybridization were at 65°C.

RESULTS

β-Galactosidase Vaccinia Virus

Vaccinia virus with the *lacZ* gene inserted into its genome was able to infect axolotls cells *in vivo*. When injected at several sites in a regenerating medium bud blastema, virtually all cells were positive for β-galactosidase activity with X-gal as substrate for the enzyme (Fig. 1A). A time course experiment showed that β-galactosidase activity was detectable for 7 days in regenerates, with very strong blue staining between 1 and 5 days, after which staining intensity declined rapidly (Table 1). By teasing apart injected blastemas, it was possible to detect expression as early as 18 h. In cultures of axolotl cells, expression was also readily seen at 18 h after addition of medium containing vaccinia. No expression was seen in cultured cells at 3 h, but at 6 and 12 h scattered expressing cells were observed. By dropping virus solution onto an open wound, we found that vaccinia was able to infect cells in the wound bed and margins (data not shown). Differentiated cells (muscle cells and fibroblasts) became infected with vaccinia following subcutaneous injection into mature limbs (Figs. 1B and 1C). These infections persisted for longer than in blastemas, and cells were still clearly expressing β-gal 12 days after injection (Table 1; Fig. 1B). In all of these sites, β-gal activity was localized to the site of injection and showed minimal spreading, even in the vicinity of the injection site (Fig. 1C). Even when virtually the entire blastema was infected, as in Fig. 1A, virus did not spread into the overlying epidermis. As controls for the nonspecific effects of vaccinia virus infection, fore- and



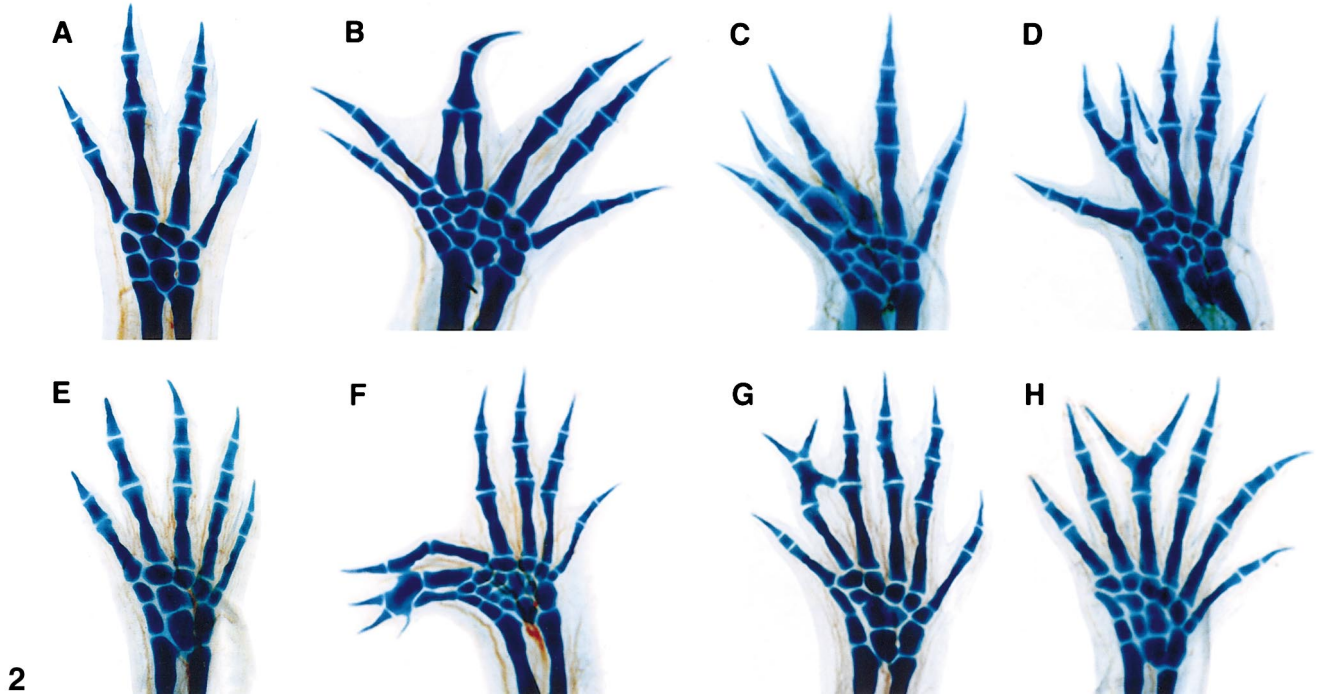


FIG. 1. X-gal staining and *Shh* expression in regenerating forelimb blastemas and mature limbs injected with β-gal vaccinia or *Shh* vaccinia. (A) X-gal staining in a medium bud blastema 4 days after β-gal vaccinia injection. (B) X-gal staining in a mature limb 12 days after β-gal vaccinia injection. (C) Section through a region of X-gal staining in mature limb tissues 4 days after β-gal vaccinia injection. (D) Control, uninjected, forelimb blastema probed with exon 3 of chicken *Shh* cDNA. (E) Medium bud blastema 24 h after *Shh* vaccinia virus injection and (F) late bud blastema 72 h after *Shh* vaccinia virus injection. For A and D–F the orientation is anterior to the left and posterior to the right.

FIG. 2. Skeletal preparation of forelimbs and hindlimbs injected with *Shh* vaccinia virus. (A and E) Control fore and hindlimbs. (B–D) *Shh* vaccinia virus-injected forelimbs displaying extra digits and carpals. (F–H) *Shh* vaccinia virus-injected hindlimbs displaying extra digits and tarsals. All the limbs (A–H) are oriented with the anterior to the left and posterior to the right.

TABLE 1
β-Gal Activity after Ectopic Expression of *lacZ*

Regenerating limbs (MB and LB)								
Day:	1	2	3	4	5	6	7	8
Activity	++	+++	+++	+++	+++	+	+/−	−

Mature limbs (subcutaneous injections)			
Day:	4	8	12
Activity	+++	+++	++

hindlimb blastemas were injected with β-gal vaccinia at early bud, medium bud, late bud, and palette stages. As can be seen from Table 2, all of these limbs regenerated normally. In all other respects, virus-injected animals showed no signs of disease or illnesses, and they sustained normal behavior, feeding, and growth.

Sonic Hedgehog Vaccinia Virus

Given the highly conserved nature of signaling molecules across species, and the unavailability of full-length axolotl *Shh,* we used the chicken *Shh* cDNA to make a recombinant vaccinia. As shown by Torok *et al.* (1999), the known portion of the axolotl cDNA sequence is 93% identical at the amino acid level with the chicken sequence. *Shh* virus was injected into the anterior of regenerating fore- (Figs. 1E and 1F) or hindlimb blastemas. Blastemas received either multiple injections into the same position (1–2 μl) or single-site injections (300–500 nl). Using exon 3 from the chicken sequence to generate a probe for whole-mount *in situ* hybridization, we examined the expression of chicken *Shh* in limbs injected 1 (Fig. 1E), 2, or 3 days (Fig. 1F) earlier with chicken *Shh*-expressing vaccinia virus. Injected limbs showed the presence of *Shh* RNA on the anterior side of the blastema, at the site of injection (Figs. 1E and 1F), demonstrating that the ectopic *Shh* was being actively transcribed. Uninjected blastemas at a stage known to express endogenous *Shh* did not react with the chicken probe (Fig. 1D), and neither did the injected blastemas show expression on the posterior side (Figs. 1E and 1F). These results indicate that the chicken probe is specific for exogenous *Shh* and does not cross-react with endogenous axolotl *Shh*. The final results, as determined by digit patterns, were the same, regardless of whether single injections or multiple injections into the same site were used.

We found that at three stages of regeneration (medium bud, late bud, and palette), *Shh* virus caused digit and carpal/tarsal duplications in the majority of cases (Table 2; Fig 2). The frequency of duplications increased from 54% at medium bud to a maximum of 66% at palette stages. No duplications were observed after anterior injection of *Shh* virus into early bud blastemas, preamputation limbs, or mature limbs. As mentioned earlier, regenerates injected with β-gal virus never formed duplications (Table 2), and the control, uninjected, contralateral limbs of *Shh*-injected animals were also all completely normal.

Analysis of the skeletal patterns of the duplicated limbs showed that they contained one to three extra digits and up to six extra carpals/tarsals (see Fig. 2). The normal number of digits and carpals in the forearm is four and eight, respectively, and of digits and tarsals in the hindlimb, five

TABLE 2
Phenotype after Ectopic Expression of *Shh* or *lacZ*

Gene	Stage	No. of limbs	No. with extra digits[a] (% extra)	No. with extra carp/tars[b] (% extra)	Lb. with extra digits or carp/tars	% extra digits or carp/tars
Shh	Preamp	7	0	0	0	0
	EB	10	0	0	0	0
	MB	13	7 (54)	4 (31)	7	54
	LB	10	6 (60)	4 (40)	6	60
	Pal	9	4 (44)	5 (56)	6	66
	Mature	4	0	0	0	0
lacZ	EB	4	0	0	0	0
	MB	10	0	0	0	0
	LB[c]	8	0	0	0	0
	Pal[d]	10	0	0	0	0
	Mature	4	0	0	0	0

[a] Number of extra digits ranged from 1 to 3.
[b] Number of extra carpals/tarsals ranged from 2 to 6.
[c] 1 limb was missing a digit.
[d] 2 limbs were missing 1 digit.

and nine, respectively. Regardless of whether the blastema arose at a proximal limb level, where it would regenerate forearm/leg and hand/foot, or at a distal level where it would only regenerate a hand/foot, no structures more proximal in the pattern than carpals/tarsals were ever duplicated. The digital patterns of all duplicated limbs were similar. Rather than the mirror-image duplicated patterns seen in developing chick limbs (Riddle *et al.*, 1993), these limbs formed expanded hands. In expanded hands, as defined by Iten and Bryant (1975), the most anterior and posterior digits and carpals/tarsals appear normally organized, but there are a variable number of extra digits (and carpals/tarsals) in between. These extra digits are difficult to identify with certainty, but the most posterior of the internal set in any of these limbs, based on criteria described in Iten and Bryant (1975), is either digit 3 or digit 2 (see Fig. 2).

DISCUSSION

In this paper we describe an efficient system to functionally overexpress proteins in axolotls. Although several other methods for functional analysis in regenerating limbs have been attempted in recent years, none have been able to generate large numbers of transfected cells in a localized region. Biolistic delivery, leading to a scattering of transfected cells, has been innovatively employed to test the function of different isoforms of the retinoic acid receptor in regeneration (Pecorino *et al.*, 1994), but it is not broadly useful for the functional analysis of other genes, including signaling molecules. Recent successes in electroporation in chicken embryos have been reported (Muramatsu *et al.*, 1997a,b), but we have found it difficult to target specific regions of the blastema or to obtain homogeneous expression within the affected area, at the same time as preserving the integrity of the blastema. Among possible viral vectors, adenovirus is known to infect a broad range of different species and is a useful vector in chicks and mice (Tsukui *et al.*, 1996; Yamagata *et al.*, 1994). However, in our tests, adenovirus did not infect axolotl cells (unpublished results). A pantropic retrovirus pseudotyped with the vesicular stomatitis virus G glycoprotein has been reported to infect newt cells in tissue culture (Burns *et al.*, 1994). A recent report has made use of this retrovirus to generate newt cell lines expressing chimeric retinoid receptors (Cash *et al.*, 1998), but there have been no reports of successful use of this vector for *in vivo* functional tests.

Vaccinia virus has been successfully used to overexpress foreign genes in the developing *Xenopus* nervous system (Nedivi *et al.*, 1998; Wu and Cline, 1998; Wu *et al.*, 1995). We adapted the techniques used in *Xenopus* (Wu *et al.*, 1995) to test the efficacy of the virus in axolotl limbs and found that axolotl blastema cells were readily and uniformly infected by vaccinia at the site of microinjection. Overexpression of β-gal in infected cells in blastemas was strong for about a week. Nonproliferating cells of the stump were also readily infected by vaccinia, and here expression of β-gal persisted for longer than 12 days. Infection did not spread from the site of injection, and even when injection was directly beneath the epidermis, it did not spread into it. This is presumably because this strain (WR) of virus binds tightly to the cell membranes of the secreting cells (Blasco and Moss, 1992). Injection of β-gal-expressing virus into the blastema at different stages of regeneration did not affect the differentiation or pattern of the regenerates. Two limitations of vaccinia are that transcription and translation of host genes are impaired after infection (Ausubel, 1987), rendering it unsuitable for overexpression of transcription factors, and that ectopic expression is transient rather than permanent.

Having determined that vaccinia had the potential to conduct tests of function, we engineered a virus to overexpress the *Shh* protein. In developing chicks, *Shh* expression colocalizes with the zone of polarizing activity on the posterior edge of the limb and causes digit duplication when overexpressed on the anterior side (Riddle *et al.*, 1993). In the axolotl and newt, *Shh* is similarly expressed on the posterior side of developing and regenerating limb blastemas (Imokawa and Yoshizato, 1997; Torok *et al.*, 1999). If *Shh* function in regenerating limbs is similar to that in developing amniote limbs, we would expect digit duplications when *Shh* vaccinia virus is injected on the anterior side of the blastema.

Ectopic *Shh* expression caused digit and carpal/tarsal duplication in the majority of the injected limbs. No structures proximal to the carpals/tarsals were ever observed, suggesting that the role of *Shh* in limb formation may be limited to the hand/foot, consistent with the findings from mice null for *Shh* (Chiang *et al.*, 1996). However, when *Shh*-expressing fibroblasts were grafted into chick limbs, duplications of the lower arm and even the upper arm were sometimes observed (Riddle *et al.*, 1993). One difference between the chick and the axolotl experiments is that in the chick, a pellet of cells was grafted. It is possible that these additional cells may have mechanically split the anlage of the developing radius and/or humerus.

The function of ectopically expressed *Shh* during regeneration was restricted temporally as well as spatially. Duplications were induced only at the stages when endogenous *Shh* was also expressed. Torok *et al.* (1999) demonstrated that *Shh* is expressed only in a limited time window from medium bud to the palette stage of regeneration. Our results suggest that not only *Shh*, but also other components of the signaling pathway are not functional prior to medium bud, since limbs respond only to ectopic *Shh* at medium bud and later. In developing chick limbs, in contrast, the *Shh* signaling pathway is functional from the start of limb outgrowth (Riddle *et al.*, 1993). A number of other comparisons between developing and regenerating limbs also suggest that a medium bud regenerate is equivalent to an early limb bud. We have proposed that the earlier phases of regeneration, prior to medium bud, are the unique

preparation stages necessary to develop a blastema capable of limb formation from the mature cells of the limb stump (Gardiner *et al.*, 1999) and that there is not an equivalent stage during limb development.

The hand/foot structures regenerated in these experiments are similar in appearance to those obtained as a result of implanting RA beads into the anterior of a blastema (Sessions *et al.*, 1989; unpublished data). This is not surprising since RA has been shown to lead to ectopic *Shh* expression in chicks (Riddle *et al.*, 1993), as well as in axolotls (Torok *et al.*, 1999). However, the details of the digital patterns in response to ectopic *Shh* expression, as well as ectopic RA exposure, differ from those in chick limbs. The mirror-image duplications seen in chicks following anterior placement of ZPA grafts, RA beads, and ectopic *Shh* expression are not seen in the axolotl experiments. Instead, the limbs consist of expanded hands (Iten and Bryant, 1975), in which the anterior and posterior edges exhibit a normal pattern, and extra digits are present in the center of the limb.

The different responses of chick and axolotl limbs appear to be a consequence of differences in the apical epidermis that is required for limb outgrowth. In chick limbs, the outgrowth-permissive apical ectodermal ridge (AER) is spatially restricted to a narrow region extending along the proximal–distal margin of the limb bud. When the ectopic signal is placed under the anterior edge of the AER, only cells posterior to the signal can respond because only cells on the posterior side of the signal are beneath the AER. This leads to limbs with posterior digits at both anterior and posterior margins. In axolotls, in contrast, the apical ectodermal cap (the AER equivalent) is more extensive in area and can form anywhere on the limb (Bryant and Muneoka, 1986). Hence, when an ectopic posterior signal is placed at the anterior side of a regenerating blastema, cells anterior to the signal as well as posterior to it respond, leading to expanded hands. Expanded hands have been described in chick limbs after ZPA grafts are placed in a more posterior position, under the central region of the AER (Tickle *et al.*, 1975). In this case, cells both anterior and posterior to the signal lie beneath the AER, and both develop digits, leading to expanded hands.

In our experiments, the induced digits do not have the typical characteristics of the most posterior digit (digit 4 in the forelimb and digit 5 in the hind limb). Although they cannot be identified with certainty, the most posterior of the centrally positioned digits is either digit 3 or digit 2. In normal digit development, digit 1 forms first, and digit 4 (or 5) last. In terms of the length of time of exposure to the *Shh* signal, the cells in digit 1 have the least and digit 4 (or 5) the most. It is possible that we fail to see induction of a digit 4 or 5 because the signal, which is transiently expressed, is only present long enough to stimulate the formation of digits 1, 2, and possibly 3. Similar conclusions about the relative exposure times needed for different digits to develop in response to Shh have been described from experiments in chick limbs (Yang *et al.*, 1997).

A recent paper has described the expression pattern of another hedgehog family member, *Banded hh* (*Bhh*), as being uniformly expressed in the mesoderm of newt limb regenerates (Stark *et al.*, 1998). Since it is believed that many members of the *hh* family signal via the same receptor, it was hypothesized that the uniform distribution of *Bhh* precluded a role for *Shh* in regeneration. Our results indicate that there are stages of regeneration at which a functional *Shh* pathway is present in regenerating limbs.

In summary, the present study demonstrates for the first time that vaccinia virus can be used successfully as a vector to overexpress secreted proteins in axolotls. We also provide experimental evidence that Shh affects the AP axis of regenerating limbs in a manner similar to its effects in developing limbs, despite differences in the timing of normal expression and in the final pattern. This suggests conservation of Shh function between limb regeneration in urodeles and limb development in other vertebrates.

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