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# Compatible Limb Patterning Mechanisms in Urodeles and Anurans

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We have experimentally tested the similarity of limb pattern-forming mechanisms in urodeles and anurans. To determine whether the mechanisms of limb outgrowth are equivalent, we compared the results of two kinds of reciprocal limb bud grafts between *Xenopus* and axolotls: contralateral grafts to confront anterior and posterior positions of graft and host, and ipsilateral grafts to align equivalent circumferential positions. Axolotl limb buds grafted to *Xenopus* hosts are immunologically rejected at a relatively early stage. Prior to rejection, however, experimental (but not control) grafts form supernumerary digits. *Xenopus* limb buds grafted to axolotl hosts are not rejected within the time frame of the experiment and therefore can be used to test the ability of frog cells to elicit responses from axolotl tissue that are similar to those that are elicited by axolotl tissue itself. When *Xenopus* buds were grafted to axolotl limb stumps so as to align circumferential positions, the majority of limbs did not form any supernumerary digits. However, in experimental grafts, where anterior and posterior of host and graft were misaligned, supernumerary digits formed at positional discontinuities. These results suggest that *Xenopus*/axolotl cell interactions result in responses that are similar to axolotl/axolotl cell interactions. Furthermore, axolotl and *Xenopus* cells can cooperate to build recognizable skeletal elements, despite large differences in cell size and growth rate between the two species. We infer from these results that urodeles and anurans share the same limb pattern-forming mechanisms, including compatible positional signals that allow appropriate localized cellular interactions between the two species. Our results suggest an approach for understanding homology of the tetrapod limb based on experimental cellular interactions.

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## INTRODUCTION

It has long been recognized that an understanding of developmental principles may enhance our understanding of evolutionary processes (Goodwin, 1982). Historically, studies of phylogeny and homology have been based on analyses of morphological similarities and differences. However, it is not the pattern itself but the genetic information that underlies the generative principles of pattern formation and morphogenesis that is inherited. Ultimately, therefore, a complete understanding of evolutionary processes will depend on an understanding of the generative processes of form and their evolution.

The tetrapod limb represents an opportunity to begin integrating developmental and evolutionary concepts, in part because of the historical background of morphological studies and in part because of the extensive experimental analyses of the mechanisms of limb outgrowth and patterning. The wide diversity seen in limb structure among living and fossil tetrapods is usually interpreted as adaptive variation imposed on a common ground plan inherited from a common ancestor. Nevertheless, differences both in the pattern of skeletal structures and the sequence of their differentiation have been interpreted to indicate that there may be a dichotomy in basic limb pattern and patterning mechanisms within the tetrapods, with anurans and amniotes

on one side and urodeles on the other (Holmgren, 1933; Jarvik, 1980; see reviews by Shubin and Alberch, 1986; Hanken, 1986). This interpretation has been used to support the idea that urodele limb structure and development are unique among tetrapods (Holmgren, 1933; Jarvik, 1980). It is the issue of whether or not a dichotomy in limb patterning mechanisms exists within the tetrapods that we address in this paper.

The well-studied phenomenon that allows for a direct test of the similarity of developmental mechanisms between tetrapods is the ability of limb cells to make supernumerary limbs in response to tissue rearrangements that bring about positional disparities (see Bryant *et al.*, 1987). Hence in urodeles, anurans, chicks, and mammals, when anterior and posterior limb cells are confronted, position-dependent growth and patterning involving communication between cells results in the formation of supernumerary limbs (see Maden, 1981; Wanek *et al.*, 1988). We have used the formation of supernumerary limbs as an assay to determine whether the limb cells of a urodele (*Ambystoma mexicanum*) and an anuran (*Xenopus laevis*) are able to communicate with each other in a position-dependent way. Limb buds of the two species were reciprocally transplanted to create positional disparities. Our results show that position-dependent interactions occur between the cells of the two species and that their cells can cooperate to build recognizable limb skeletal elements. We conclude

that urodeles and anurans share compatible intercellular positional signals that are utilized in a common limb patterning mechanism.

#### MATERIALS AND METHODS

Experiments were performed on Mexican axolotls (*A. mexicanum*) and South African clawed toads (*X. laevis*) spawned at the UCI Developmental Biology Center. Animals were reared at room temperature (20°C) in saline, and were changed to fresh saline and fed three times a week. Axolotls were kept in 25% Holtfreter's solution and were fed tubifex worms. *Xenopus* larvae were kept in 10% Steinberg's solution with 1% humic acid and were fed nettle powder.

Axolotl and *Xenopus* larvae were matched for size and stage of their hind limb buds. *Xenopus* larvae were at stage 52-53 (Nieuwkoop and Faber, 1975) and axolotl larvae were also at a stage just prior to digit formation in the hind limb. Matched pairs of animals were anesthetized in 1:4000 MS222, and transferred to 20% Steinberg's solution with 100 U/ml penicillin and 50 µg/ml streptomycin sulfate. The distal one-third to one-half of the hind limb buds were amputated and then grafted reciprocally to the limb stumps of the other species (Fig. 1). Grafts were made either ipsilaterally to maintain normal orientation (control grafts) or contralaterally to reverse the anterior-posterior orientation of graft and host while maintaining the normal dorsal-ventral orientation (experimental grafts). Grafts were allowed to heal in place for 10-15 min after which time axolotl hosts were placed in individual one-liter plastic boxes containing 25% Holtfreter's solution, and *Xenopus* hosts were returned to similar containers of 10% Steinberg's solution with 1% humic acid. Developing limbs were examined and drawn three times a week using a camera lucida. Limbs representing various developmental stages were collected from 6 days to sev-

eral weeks after grafting when digit formation was judged to be complete or (in *Xenopus* hosts) when the graft tissues began to exhibit signs of immunological rejection by the host. All limbs were preserved in aqueous Bouin's fixative, stained with either victoria blue B or alcian blue, and cleared in methyl salicylate for whole-mount analysis. In scoring the final pattern of digits, only elements that clearly articulated with more proximal elements were counted. Minor bifurcations without segments were not counted. Selected limbs were later embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Mallory's triple stain for histological analysis.

#### RESULTS

##### *Xenopus* Grafts onto Axolotl Hosts

A total of 53 successful grafts of *Xenopus* limb buds onto axolotl host limb bud stumps was performed, of which 27 were contralateral (experimental) and 26 were ipsilateral (control) grafts. The grafts became vascularized within a few days of operation. In 6 cases, we observed that the *Xenopus* graft was displaced by autonomous growth from the axolotl stump, and these cases are not included in the results below.

An analysis of the final pattern of digits of whole-mount preparations of 18 experimental and 20 control limbs revealed that all experimental limbs formed supernumerary axolotl digits (Fig. 2a), whereas the majority of control limbs (60%) formed no axolotl digits (Fig. 2b; Table 1). Taken as a whole, experimental limbs produced nearly four times as many supernumerary axolotl digits as control limbs (Table 1). In addition, all experimental limbs were complete in the proximal-distal axis (Fig. 2a), whereas in the majority of the control limbs (i.e., those that did not form supernumerary digits) the host was truncated proximally (Fig. 2b). The supernumerary digits that were formed by a minority of the control limbs (Table 1) probably arose as a result of small positional mismatches between the host and the ipsilateral graft. Such mismatches presumably also occur when grafting within species, but are most likely resolved by back rotation to align positional values of host and graft (see Harrison, 1921). Back rotation in this grafting combination might be precluded by the enormous difference in cell size between axolotls and *Xenopus* (Figs. 3-6).

In the *Xenopus* graft/axolotl host combination, the *Xenopus* grafts neither grew extensively nor formed well-developed digits, despite the fact that they became well vascularized and innervated (Fig. 3). However, in both whole-mount and histological preparations, where the dramatic difference in cell size between the two species allows for unambiguous identification, it is clear

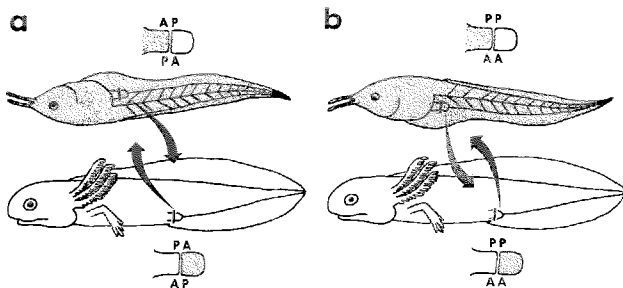


FIG. 1. Diagram of the grafting procedures used in this study. Developing limb buds were reciprocally grafted between young larvae of *Xenopus laevis* (upper, shaded) and *Ambystoma mexicanum* (lower, unshaded). Experimental contralateral grafts (a) result in positional confrontations between anterior and posterior limb tissues of host and donor, while control ipsilateral grafts (b) do not.

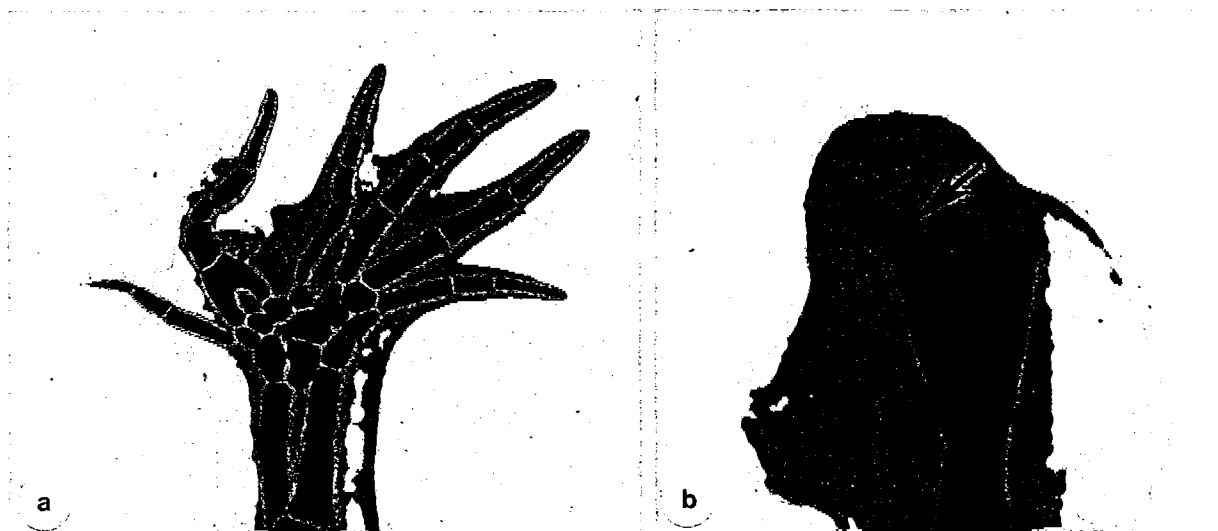


FIG. 2. (a) Whole-mount skeletal preparation of an axolotl host/*Xenopus* donor experimental limb with seven supernumerary axolotl digits, including two that have fused proximally. This limb also has supernumerary tarsals. (victoria blue/methyl salicylate).  $\times 16$ . (b) Whole-mount skeletal preparation of an axolotl host/*Xenopus* donor control limb. The *Xenopus* graft has formed articulated skeletal elements (arrow) and no axolotl skeletal structures have formed distal to the host stylopodium (victoria blue/methyl salicylate).  $\times 30$ .

that the grafts were not rejected but remained healthy and formed small, articulated limb cartilages (Figs. 2b and 4). In many cases the *Xenopus* and axolotl cartilage cells were smoothly integrated into single elements (Fig. 4b).

Pronounced supernumerary outgrowths formed anterior and posterior to the graft-host junction within 1 to 2 weeks after grafting. Histological examination of such early outgrowths showed that the majority (13) were composed primarily of axolotl cells; in four cases a small tongue of *Xenopus* cells also projected into the outgrowth. Two additional outgrowths were composed of *Xenopus* cells alone and three were chimeric and consisted of approximately equal amounts of *Xenopus* and axolotl tissue (Fig. 5). As can be seen from Fig. 6, tissues from both species are actively growing at this stage, and the chimeric border is sharp. Multiple outgrowths from six older limbs were examined histologically, and in these the contribution pattern had changed such that axolotl cells predominated and eventually formed all of the supernumerary structures.

TABLE 1  
RESPONSE OF AXOLOTL HOST LIMB BUDS TO *Xenopus* GRAFTS

	N	Number of axolotl digits								Mean
		0	1	2	3	4	5	6	7	
Experimental	18	0	2	3	1	5	3	2	2	4.00
Control	20	12	2	1	2	3	0	0	0	1.10

#### *Axolotl Grafts onto Xenopus Hosts*

A total of 71 successful limb bud grafts was performed using axolotl donors and *Xenopus* hosts, including 34 contralateral (experimental) grafts and 37 ipsilateral (control) grafts. Grafts became vascularized and innervated within a few days of operation and initially



FIG. 3. Longitudinal section showing innervation and vascularization of a *Xenopus* graft (X) by an axolotl host (A) at 13 days. The axolotl nerve enters at the base of the graft (arrow) and branches extensively; arrowhead designates axolotl blood cells (H & E).  $\times 135$ .

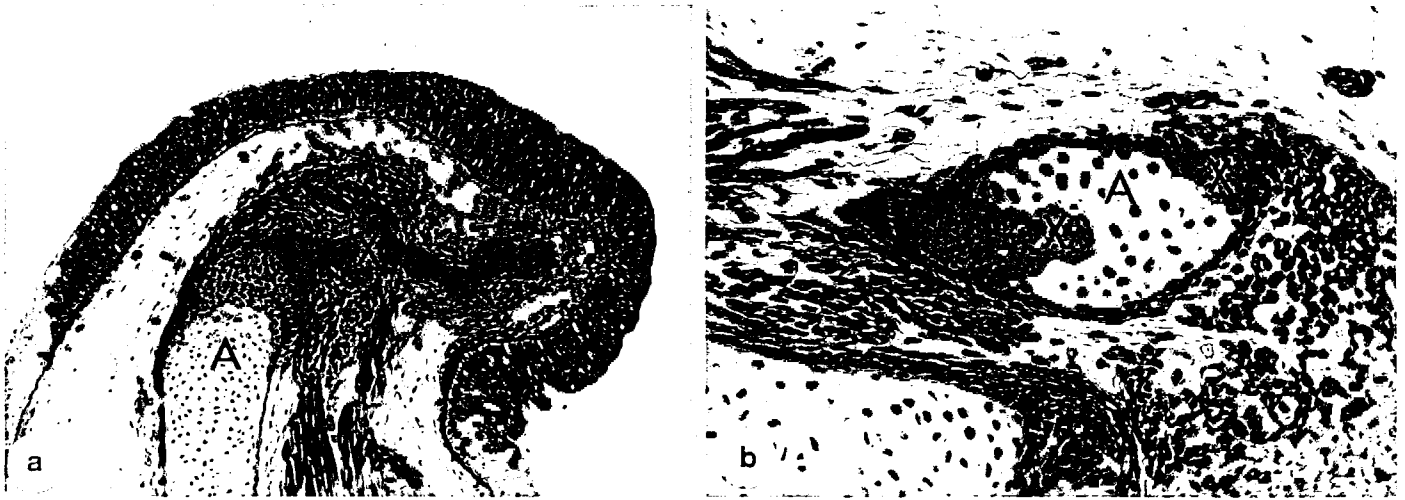


FIG. 4. (a) Longitudinal section through an axolotl host/*Xenopus* donor control limb. The *Xenopus* graft (X) has formed articulated limb cartilages distal to the stylopodium of the axolotl host (A) (Mallory's triple stain).  $\times 50$ . (b) Section through an axolotl host/*Xenopus* donor control limb showing smooth integration of *Xenopus* (X) and axolotl (A) cartilage. *Xenopus* cartilage is identified by smaller cell size (H & E).  $\times 120$ .

appeared to be healthy and rapidly growing. However, all axolotl grafts were eventually rejected by *Xenopus* hosts. With this limitation, we are nevertheless able to

report a clear difference in the behavior of control and experimental axolotl grafts. In both controls and experimentals, axolotl digits appeared in an anterior to posterior sequence, as they do in normal ungrafted limbs. In the grafted limbs, however, rejection occurred prior to formation of all digits, although on average three or four well-developed axolotl digits formed before rejection. In those limbs prepared for whole-mount analysis, the major difference between experimental and control grafts is that in experimental grafts, an additional digit formed anterior (relative to the graft) to the normal axolotl digit 1 in 15 of 17 cases (Fig. 7). This additional

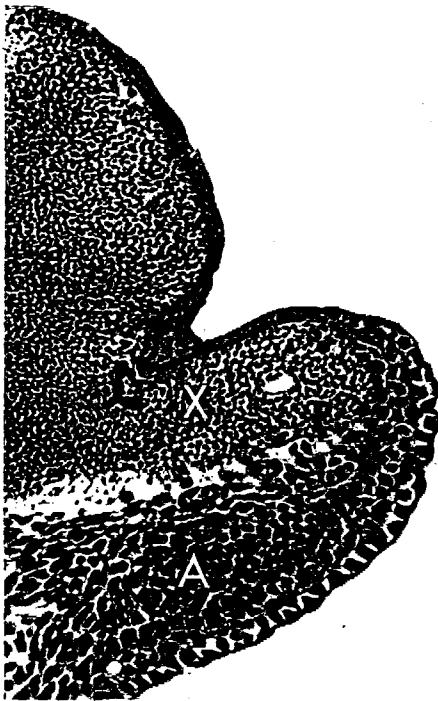


FIG. 5. Longitudinal section through an axolotl host/*Xenopus* donor experimental limb at 13 days. A chimeric lateral outgrowth has formed that is composed of approximately equal amounts of axolotl (A) and *Xenopus* (X) tissues (H & E).  $\times 130$ .

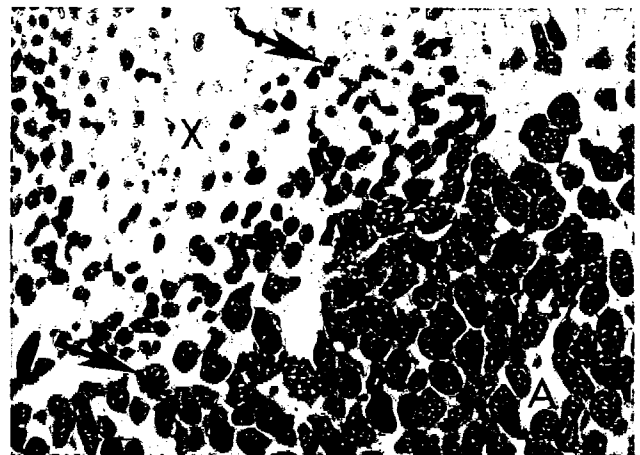


FIG. 6. High power view of junction between *Xenopus* and axolotl cells in chimeric lateral outgrowth showing mitotic figures (arrows) among both the axolotl (A) and *Xenopus* (X) cells (H & E).  $\times 180$ .

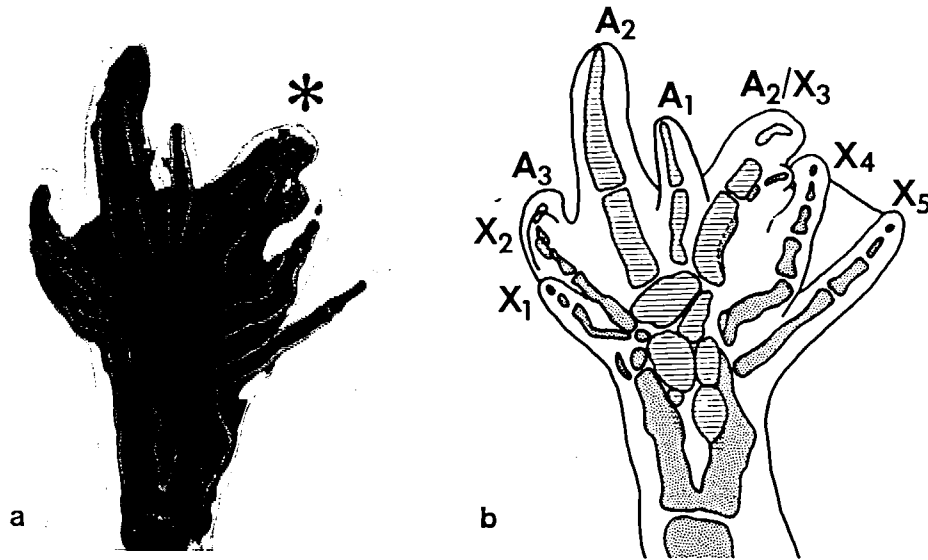


FIG. 7. (a) Whole-mount skeletal preparation of a typical *Xenopus* host/axolotl donor experimental limb with axolotl digits formed by the graft, *Xenopus* digits formed by the host, and a chimeric supernumerary digit (\*) composed of both axolotl and *Xenopus* cells (alcian blue/methyl salicylate).  $\times 35$ . (b) Camera lucida drawing of limb shown in (a) to illustrate contribution of *Xenopus* (stippled) and axolotl cells (hatched) to the chimeric limb pattern. Axolotl digits 1, 2, and 3 are recognized, as are *Xenopus* digits 1, 2, 4, and 5. The supernumerary axolotl digit is actually a composite of an extra axolotl digit 2 and *Xenopus* digit 3.

digit was unambiguously identified in every case as a supernumerary axolotl digit 2 based on three independent criteria: its position relative to the other identified axolotl digits, its time of development (i.e., after the formation of the normal axolotl digits 2 and 1), and its articulation pattern as observed in whole-mount preparations (i.e., the metatarsal articulates on the most anterior tarsal element along with the metatarsals of the normal axolotl digits 2 and 1). In addition, one of these experimental grafts formed a second supernumerary axolotl digit (digit 3) on the anterior edge of the graft. In contrast, none of the 20 control grafts examined as whole mounts formed any supernumerary axolotl digits (Fig. 8).

Axolotl tissues are readily identified in both whole-mount and histological preparations by the much larger size of the axolotl cells (Fig. 9). This cell size difference between axolotl and *Xenopus* enabled us to conclude that half of the supernumerary axolotl digits contained some cartilage elements, including joints, that were chimeric, with the edge closest to the *Xenopus* host consisting of *Xenopus* cells (Fig. 9a). This conclusion was confirmed by subsequent histological examination of four of the whole mount preparations (Fig. 9b). This chimerism was always "appropriate" in that axolotl tarsals formed by the graft (e.g., radiale and tibiale), recognizable by their position and patterns of articula-

tion, were confluent with the equivalent *Xenopus* host element (Figs. 7, 8, and 9b). We have also histologically examined the early changes at the graft-host junction in three control and three experimental limbs fixed after 1 week. Only the experimental combinations showed evidence of lateral outgrowths anterior or posterior to the graft-host junction. Out of a total of four outgrowths, three were clearly chimeric with distinct graft-host boundaries (Fig. 10) and the other was composed only of axolotl cells.

#### DISCUSSION

In this paper, we have experimentally tested the similarity of limb pattern-forming mechanisms in urodeles and anurans. A previous analysis of the cellular contribution to supernumerary limbs in *Xenopus* provided indirect evidence that the patterning mechanism in *Xenopus* is the same as that in axolotl (Muneoka and Murad, 1987). To directly test the compatibility of patterning mechanisms between urodeles and anurans, we compared the results of two kinds of reciprocal limb bud grafts between *Xenopus* and axolotls: contralateral grafts to confront anterior and posterior positions of graft and host, and ipsilateral grafts to align equivalent circumferential positions. Previous studies have consistently shown that positional disparities created by

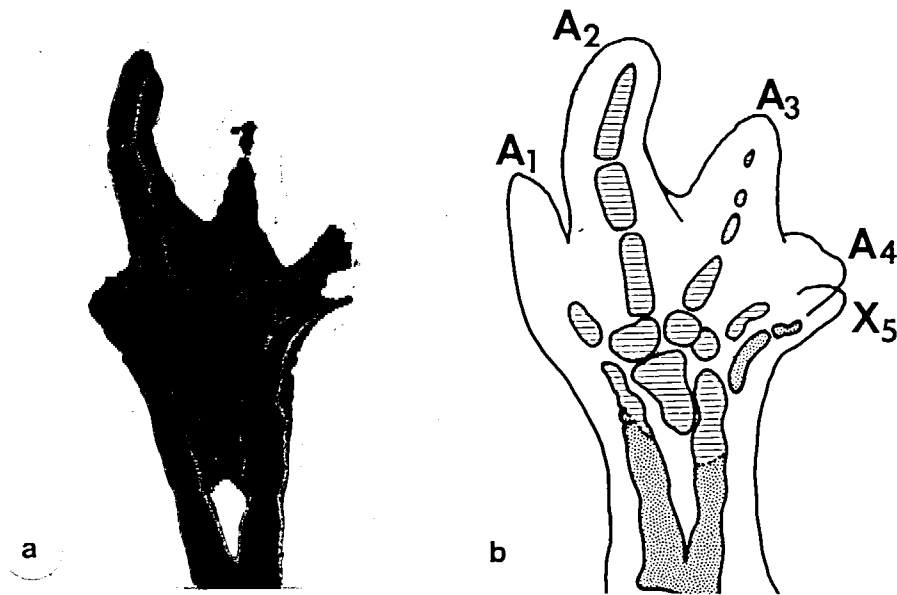


FIG. 8. (a) Whole-mount skeletal preparation of a typical *Xenopus* host/axolotl donor control limb with axolotl digits formed by the graft and one *Xenopus* digit formed by the host. The faintness of some of the distal axolotl cartilages reflects early stages of immunological rejection (alcian blue/methyl salicylate).  $\times 35$ . (b) Camera lucida drawing of limb shown in (a) to illustrate the contribution of *Xenopus* (stippled) and axolotl cells (hatched) to the limb pattern. Axolotl digits 1-4 are identified, as is *Xenopus* digit 5. No supernumerary axolotl digits have formed.

grafts of whole or portions of developing or regenerating limb tissues result in the formation of supernumerary structures in each of a wide variety of organisms including urodeles, anurans, chicks, mammals, and insects (see French *et al.*, 1976; Maden, 1981; Wanek *et al.*, 1988), whereas grafts that do not result in positional

disparities do not form supernumerary structures. Our premise is that if the mechanism controlling growth and pattern specification is the same in urodeles and anurans, then experimental (contralateral) grafts between axolotls and *Xenopus* will develop supernumerary structures and control (ipsilateral) grafts will not.

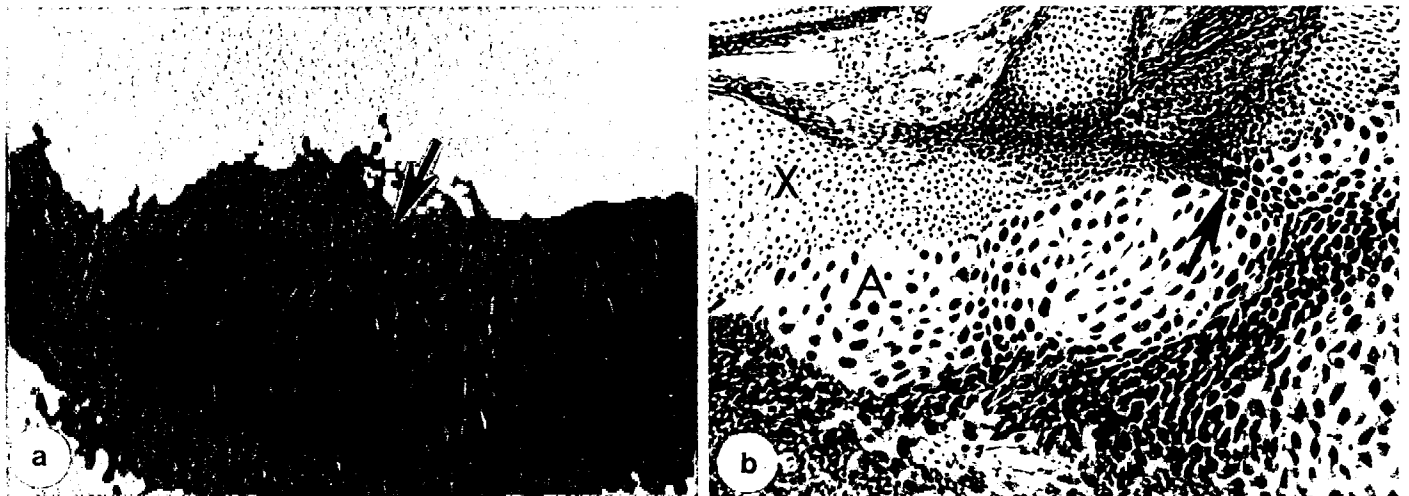


FIG. 9. (a) High power view (Nomarski optics) of a chimeric digit in a whole-mount skeletal preparation of a *Xenopus* host/axolotl donor experimental limb. *Xenopus* cartilage (arrow) is identified by smaller cells (alcian blue/methyl salicylate).  $\times 170$ . (b) Longitudinal section through a *Xenopus* host/axolotl donor experimental limb showing smooth integration of axolotl (A) and *Xenopus* (X) cells to form chimeric cartilages. The base of a chimeric metatarsal (*Xenopus* digit 2) is shown at the upper right (arrow) (H & E).  $\times 150$ .

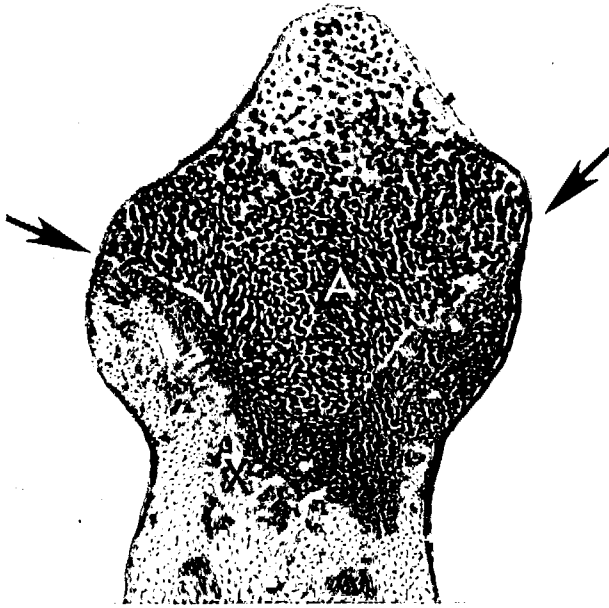


FIG. 10. Longitudinal section through a young (9 day) *Xenopus* host/axolotl donor experimental limb showing early lateral chimeric outgrowths (arrows) and a sharp boundary between graft and host. The axolotl graft is completely covered by *Xenopus* epidermis. A, axolotl cells; X, *Xenopus* cells (H & E).  $\times 85$ .

In contrast, if the mechanisms are different, then the results of experimental and control grafts will be indistinguishable.

Despite the complications arising as a result of xenoplastic grafting (see *Results*), our results lead us to conclude that the mechanism of limb growth and pattern specification in urodeles and anurans is the same. This conclusion is based primarily on the response of axolotl tissue to confrontations with *Xenopus* cells. When axolotls are hosts, contralateral *Xenopus* grafts in all cases lead to the formation of supernumerary axolotl digits. In the majority of ipsilateral grafts (60%), no supernumerary axolotl structures are formed. When axolotl limb buds are grafted to *Xenopus* hosts, contralateral grafts lead in almost all cases (88%) to the formation of supernumerary axolotl digits, whereas none of the ipsilateral axolotl grafts develop supernumerary axolotl digits. Hence axolotl tissue responds to axolotl-*Xenopus* confrontations in a manner that is identical to the way in which it responds to axolotl-axolotl confrontations. When *Xenopus* buds are grafted, they contribute cells to the initiation of outgrowths. However, *Xenopus* buds do not subsequently grow well on axolotl hosts, thereby precluding any analysis of the formation of extra structures in the final limbs. Nevertheless as discussed above, *Xenopus* grafts do stimulate appropriate re-

sponses from axolotl tissues. This result is equivalent to that of Holder *et al.* (1979) who found that although X-irradiated newt limb blastemas do not grow when grafted to unirradiated newt stumps, like the *Xenopus* grafts onto axolotl stumps, they stimulate the stump tissues to form supernumerary digits. When *Xenopus* limbs are hosts, they contribute cells to early outgrowths, but subsequent responses of *Xenopus* tissues are difficult to interpret due to replacement of axolotl digits by *Xenopus* digits during graft rejection. Nevertheless, in this combination, *Xenopus* cells do again evoke an appropriate response from axolotl tissue. Finally, in all graft combinations, chimeric cartilage elements are formed, indicating that *Xenopus* and axolotl cells can cooperate to make recognizable skeletal elements, despite the large cell size differences.

Our results provide direct evidence that urodeles and anurans, representing lineages that have been separate for at least 250 million years (McFarland *et al.*, 1979), share the same limb patterning mechanism including compatible intercellular patterning signals. The differences in structure and sequence of development that have been previously described between urodeles and anurans (Holmgren, 1933; Shubin and Alberch, 1986) cannot, therefore, reflect differences in the basic limb-forming mechanism. Direct evidence that amniotes share a common limb patterning mechanism including compatible intercellular patterning signals was obtained by Fallon and Crosby (1977), who grafted pieces of limb bud tissues from reptiles, mammals, and birds into limb buds of chicks. Our results, in conjunction with those of Fallon and Crosby (1977), suggest that all tetrapods share the same basic limb patterning mechanism inherited from a common ancestor. Furthermore, we can conclude that intercellular positional signals are compatible among amniotes, on the one hand, and between the two groups of limbed amphibians on the other. The common, basic patterning mechanism is apparently ancient, and appears to be based on a fundamental property of cell interaction that is characteristic of epimorphic systems in both vertebrates and invertebrates (Bryant and Simpson, 1984).

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