Determination of axial polarity in the urodele limb regeneration blastema

By DAVID L. STOCUM
From the Department of Genetics and Development, University of Illinois, Urbana

SUMMARY

The state of determination of the anterior-posterior, dorsal-ventral and proximal-distal axes of the undifferentiated limb regeneration blastema was evaluated by heterografting and autografting experiments in which these axes were reversed with respect to the limb stump. Species-specific size differences in skeletal elements were used as markers to trace the origin of regenerate tissues in the heterografting experiments, and differences in the skeletal patterns of hindlimbs and forelimbs were used as markers in the autografting experiments. The resulting primary regenerates fell into two categories, those composed wholly or partly of donor tissues, and those composed entirely of host tissues. Regenerate structures formed from donor tissues always maintained the handedness of origin, while regenerates formed from host tissues always displayed host-side handedness. These results demonstrate that the axes of the blastema are determined from the start of regeneration, and that previous claims of axial lability in reversal experiments are based on an illusion created by resorption of graft tissue, accompanied by regeneration from the host. Reversal of the transverse axes resulted in the formation of supernumerary limbs. Analysis of heterograft cases in which the anterior-posterior axis was reversed showed that 50% of the supernumeraries were constructed partly of donor blastema tissue whose axial polarity was reversed with respect to the adjacent primary regenerate. The vast majority of the primary regenerates in these cases possessed the normal number of digits. It is thus likely that the reprogrammed donor blastema cells used to construct the supernumeraries are derived by division of a thin band of cells at the edge of the graft adjacent to the supernumerary.

INTRODUCTION

Numerous grafting experiments indicate that the structural pattern of a urodele limb regenerate is determined from the earliest stages of blastema formation (see Stocum, 1978; Polezhaev, 1979; Wallace, 1981, for reviews). However, there is some question as to whether the axial polarity of the early regenerate is likewise determined. Milojevic (1924) found, in axial reversal experiments, that the anterior-posterior (AP) and dorsal-ventral (DV) axes of early blastemas of adult Triturus cristatus limbs were not determined, but became determined during a ‘critical period’ from 10–12 days postamputation. Schwidefsky (1934) observed a similar critical period for transverse axial

1 Author's address: Department of Genetics and Development, 515 Morrill Hall, 505 South Goodwin Ave., University of Illinois, Urbana, IL 61801, U.S.A.
A Heterograft

1. AP or DV reverse
   \[\text{Right} \rightarrow \text{Left}\]

2. APDV reverse
   \[\text{Right} \rightarrow \text{Right}\]

3. PD reverse
   \[\text{Right} \rightarrow \text{Left}\]

B Autograft

1. AP or DV reverse
   \[\text{Left} \rightarrow \text{Right}\]

2. APDV reverse
   \[\text{Left} \rightarrow \text{Left}\]

3. PD reverse
   \[\text{[Distal to proximal]} \quad \text{[Distal to distal]}\]

---

Fig. 1. Diagram of heterografting and autografting operations. In the heterografting operations, forelimb or hindlimb blastemas were exchanged between white (and occasionally dark) \textit{A. mexicanum} larvae and \textit{A. maculatum} or \textit{A. texanum} larvae. The stippling indicates the latter two more heavily pigmented species. In the autografting operations, forelimb (FL) and hindlimb (HL) blastemas were exchanged within the same individual of any of the three species. The appropriate rotations were made to produce the various axial reversals. Small arrows indicate polarities of graft and stump after PD reversal. S = stylopodium; B = basipodium.
Blastemal axial polarity

Table 1. Polarity of regenerates after reversal of the blastemal AP axis

<table>
<thead>
<tr>
<th>Graft type and stages</th>
<th>Total cases</th>
<th>*Maintained polarity</th>
<th>†Polarity conformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Heterograft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>11</td>
<td>6 (54.5%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>13</td>
<td>11 (84.6%)</td>
<td>2 (15.4%)</td>
</tr>
<tr>
<td>Medium bud (distal to proximal)</td>
<td>5</td>
<td>4 (80.0%)</td>
<td>1 (20.0%)</td>
</tr>
<tr>
<td>2. Autograft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>14</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>15</td>
<td>11 (73.3%)</td>
<td>4 (26.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>40 (69.0%)</td>
<td>18 (31.0%)</td>
</tr>
</tbody>
</table>

* Graft tissue survived, regenerates were mirror images (disharmonic to) of the host limb.
† Graft tissue did not survive, regenerate had handedness of host limb and tissues.

determination between 16–18 days in regenerating limbs of adult T. taeniatus. More recently, Iten and Bryant (1975) have presented evidence that the AP axis of the limb regenerate of adult Notophthalmus viridescens is gradually determined between the early bud and late bud stages.

However, none of these experiments assessed blastemal axial determination using appropriate species-specific morphological or cell markers to trace the source of the tissues in the regenerate. The possibility therefore remains that the apparent axial lability of reversed early blastemas is an illusion created by graft resorption accompanied by regeneration from the host limb stump. In the present study, blastemal axial determination was tested by heterografting and autografting experiments in which species-specific and limb-type differences in the size and morphology of skeletal elements, and skin pigmentation were used to distinguish graft and host tissues. The results demonstrate that the axial polarity of the regenerate, as well as its fore or hindlimb quality, is determined at the earliest stages of blastema formation.

MATERIALS AND METHODS

1. General

Larvae of Ambystoma maculatum (obtained commercially from Charles Sullivan, Nashville, TN, or kindly provided by Mr. George Guidice, Princeton University), A. texanum (collected from local ponds), and dark or white A. mexicanum (obtained from laboratory spawnings or from the Indiana University axolotl colony) were used for all experiments. The animals were fed freshly hatched brine shrimp every day and their water (1% Holtfreter solution) was changed after each feeding. At the time of operation, the A. maculatum and
*texanum* larvae ranged from 30–50 mm in snout–tail-tip length (2–3 months posthatching), and the *A. mexicanum* larvae ranged from 30–80 mm (2–4 months posthatching).

Forelimbs and hindlimbs were amputated through the midstylopodium and allowed to regenerate to early bud–notch stages (staging according to Stocum, 1979). Prior to transplantation the blastemas were marked with a spot of nile blue sulphate dye on their anterior–dorsal surfaces, thereby providing a reference point for orienting the graft on the host stump.
Blastemal axial polarity

Fig. 2. An early-bud blastema from the left thigh of a white *A. mexicanum* larva was heterografted, with AP reversal, to the right thigh of an *A. maculatum* larva and allowed to develop for 30 days. Digits numbered in A to P order. (a) The contralateral (left) hindlimb of the *maculatum* host, dorsal view. The normal hindlimb has five toes, with a phalangeal formula of 2:2:3:3 (or 4):2 in A to P sequence. (b) Experimental regenerate, dorsal view. The primary regenerate skeleton is a disharmonic (left) hindlimb derived from donor axolotl tissue from the distal end of the stylopodium (arrow) on. The digits of the primary exhibit the foot phalangeal formula. Supernumerary regenerates arose on the anterior and posterior sides of the primary regenerate. The posterior supernumerary \( S_v \) consists of three digits, several basipodial elements, a tibia \( (t) \) and a fibula which is part of a fused tibia–fibula shared by the primary regenerate and the posterior supernumerary in the middle of the zeugopodium. The anterior supernumerary \( S_a \) consists of two digits, several basipodial elements, and a fibula \( (f) \). Both limbs \( \times 60 \).

Fig. 3. A medium-bud blastema from the left upper arm of a dark *A. mexicanum* larva was heterografted, with AP reversal, to the right thigh of an *A. maculatum* larva, and allowed to develop for 30 days. Digits numbered in A to P order. (a) The contralateral (left) hindlimb of the *maculatum* host, dorsal view; \( t \) = tibia. (b) Experimental regenerate, dorsal view. The primary regenerate skeleton is a disharmonic (left) forelimb composed of axolotl tissue from the distal stylopodium on. The normal forelimb has four digits with a phalangeal formula of 2:2:3:2 in A to P sequence. The primary regenerate consists of the first three digits, seven carpals, and a radius \( (r) \) and ulna \( (u) \). Supernumeraries arose at the anterior and posterior sides of the primary regenerate. The anterior supernumerary \( S_a \) is composed of several host-size tarsals and three posterior toes with phalangeal formula 3:4:2. This indicates that the radius and/or anterior soft tissues of the primary regenerate are partially composed of host tissue. The posterior supernumerary \( S_v \) is derived from both host and donor tissue, having a tibia \( (t) \) and the first two digits derived from the host, and an ulna \( (u) \) and digits 3 and 4 derived from the donor. The ulnae of the primary regenerate and \( S_p \) are fused at their bases, and an unidentified mass of cartilage (arrow) lies in the angle between the two. Both limbs \( \times 60 \).

Fig. 4. A medium-bud blastema from the right thigh was autografted to the left upper arm stump in an *A. maculatum* larva, and allowed to develop for 30 days. Digits numbered in A to P sequence. (a) Experimental regenerate, dorsal view. The graft resorbed up to the level of the basipodium, and a host-derived radius \( (r) \) and ulna \( (u) \) with harmonic AP polarity was intercalated. The remaining graft tissue developed as a disharmonic (right) foot with four toes. Supernumerary autopodia were formed on the anterior and posterior sides of the primary regenerate. The posterior supernumerary \( S_v \) consists of three digits with associated basipodialts. The anterior supernumerary \( S_a \) consists of four digits with associated basipodialts. (b) Contralateral (right) forelimb. Both limbs \( \times 60 \).

Fig. 5. Regenerate formed by 30 days after heterografting a medium bud blastema from the distal zeugopodium of a white *A. mexicanum* right hindlimb to the mid-stylopodium of an *A. maculatum* left forelimb, with AP axial reversal. Dorsal view. All of the graft material except one prospective digit (arrow) resorbed. This digit is either toe 3 or 4 (note the three phalanges), but occupies the position of the second finger, thus showing that the graft tissue maintained the polarity of origin. The remainder of the limb was regenerated from host tissue. Host digits are numbered in A to P order. \( \times 60 \).
Table 2. Polarity of regenerates after reversal of the blastemal DV axis

<table>
<thead>
<tr>
<th>Graft type and stages</th>
<th>Total cases</th>
<th>*Maintained polarity</th>
<th>†Polarity conformed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Heterograft</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>4</td>
<td>4 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Medium bud</td>
<td>20</td>
<td>15 (75.0%)</td>
<td>5 (25.0%)</td>
</tr>
<tr>
<td>Medium bud (distal to proximal)</td>
<td>5</td>
<td>0</td>
<td>5 (100%)</td>
</tr>
<tr>
<td><strong>2. Autograft</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>17</td>
<td>3 (17.6%)</td>
<td>14 (82.4%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>10</td>
<td>3 (30.0%)</td>
<td>7 (70.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>25 (44.6%)</td>
<td>31 (55.4%)</td>
</tr>
</tbody>
</table>

* Graft tissue survived, regenerates were mirror images of (disharmonic to) the host limb.
† Graft tissue did not survive, regenerate had handedness of host limb and was composed entirely of host tissues. However, two of these cases, one of early bud (5.9%) and one of medium bud (10.0%) are somewhat uncertain. They had the handedness of the host, but their autopodia had donor morphology (see text).

2. Grafting procedures

Heterografts and autografts were made as illustrated in Fig. 1. For the heterograft operations, right or left fore or hindlimb blastemas were exchanged between the contralateral limbs of *A. mexicanum* and either of the other two species, and rotated so that the AP, DV or proximal–distal (PD) axes were reversed independently of one another. The AP and DV axes were also reversed simultaneously by exchanging blastemas between ipsilateral limbs of the same species combinations, and rotating them 180° around the PD axis. Anterior–posterior, DV, and APDV reversals were done using early and medium-bud-stage blastemas. Proximal–distal reversals were done with the same species combinations, using late-bud to notch-stage blastemas. To effect a PD reversal, the hand plate (approximately the distal half of the regenerate) was removed and the distal end of the remaining half was attached to the host upper arm stump, confronting distal and proximal limb levels. *A. mexicanum* limbs grow more rapidly and to a much larger size than those of either *A. maculatum* or *A. texanum* limbs, and the species-specific size differential of the skeletal elements, plus colour differences between donor and host (when white *A. mexicanum* was used) allowed the origin of the regenerate to be traced (Pescitelli & Stocum, 1980).

The same axial reversals were carried out in the autografting operations, using the same regenerate stages. In these cases, forelimb blastemas were grafted to the contralateral or ipsilateral hindlimb, or hindlimb blastemas were grafted to the contralateral or ipsilateral forelimb. Differences in the number, morphology and pattern of the skeletal elements in the fore and hind
limbs served to trace the origin of the regenerate (Pescitelli & Stocum, 1980). In addition to PD reversals in which different limb levels were confronted, PD reversals were made in which the same limb levels were confronted, by removing the hand plates of upper arm blastemas and attaching the distal end of the remaining piece to the wrist level of the host stump.

In another experiment (not diagrammed), medium-bud-stage blastemas derived from the distal zeugopodium were auto or heterografted to the mid-stylopodium with AP or DV reversal. This distal to proximal shift is known to encourage dedifferentiation of redifferentiating blastemas (Stocum, 1975; Iten & Bryant, 1975), and would thus be expected to provide an even more stringent test of axial determination.

All grafts were allowed to develop for 30–50 days. The limbs were then fixed in Gregg's solution and their skeletal elements stained in toto with methylene blue by the van Wijhe method, as modified by Gregg and Butler (Hamburger, 1960), and the origin and handedness of the resulting primary and supernumerary regenerate skeletons assessed.

Controls for these experiments have been reported previously (Stocum, 1980a; Pescitelli & Stocum, 1980), and consisted of removing blastemas and auto or homografting them without axial reversal. Since those controls invariably developed with the structure, axial polarity, and pigmentation pattern of the donor limb, additional controls were not performed for the present study.

**RESULTS**

(1) **AP Reversal**

(a) **Heterografts.** Table 1 summarizes the results of reversing the blastemal AP axis at early- and medium-bud stages of regeneration. Fifty-five percent of early bud and 85% of medium-bud cases exhibited axiation disharmonic to the host side in all or part of the regenerate skeleton (i.e., maintained the handedness of origin), while the handedness of the remaining regenerates was that of the host side. All of the disharmonic portions of the regenerates were composed of donor-specific tissue. The fact that the extent of the regenerate formed by disharmonic donor tissue ranged from 100% to the formation of only autopodial structures indicates that some grafts were partially resorbed. Those regenerates whose axial polarity conformed to that of the host side (i.e., were harmonic) were always composed entirely of host tissue, indicating that the grafts had completely resorbed, with regeneration taking place from the host stump. Of the five medium-bud blastemas that were shifted proximally in addition to undergoing AP reversal, four suffered partial resorption but developed with the handedness of origin, while the fifth case resorbed completely. Examples of heterografted AP-reversed regenerates are illustrated in Figs. 2, 3 and 5.

(b) **Autografts.** Many of these grafts also exhibited partial resorption, but
the handedness of the structures derived from surviving graft tissue was always disharmonic to the host side. In those cases where regenerate handedness was harmonic to the host side, the regenerate skeletons displayed entirely host limb morphology, indicating complete graft resorption and host regeneration. Fig. 4 illustrates an autografted AP-reversed regenerate.

Summarizing the results of all the transplants, 100% of the surviving early- and medium-bud-stage blastemas retained the handedness of their parent limbs.
(2) **DV Reversal**

(a) **Heterografts.** The results of reversing the DV axis at early- and medium-bud stages of regeneration are summarized in Table 2. Regenerates of donor-specific size, colour and handedness were formed by 100% of early-bud and 75% of medium-bud-stage blastemas. Two examples of DV-reversed regenerates are shown in Figs. 6 and 7. The remaining cases exhibited host-specific size, colour and handedness, indicating total resorption of the grafts in conjunction with host regeneration. All of the medium bud distal blastemas that were shifted proximally concomitant with DV reversal resorbed completely and were replaced by harmonic regenerates derived entirely from the host stump.

(b) **Autografts.** The majority of both early- and medium-bud-stage autografted blastemas resorbed totally in conjunction with host limb regeneration. Only 17.6% of the regenerates formed after grafting early-bud blastemas and

---

Fig. 6. Contralateral limb and experimental regenerate 30 days after heterografting an early bud blastema, with DV axial reversal, from the left upper arm of a white *A. mexicanum* larva to the right upper arm of an *A. maculatum* larva. Dorsal views, digits numbered in A to P order. (a) Contralateral (left) forelimb of *maculatum* host. The digits normally curve ventrally. (b) Experimental regenerate. All of the primary regenerate skeleton is composed of axolotl-size elements, even though *maculatum* pigmentation extends half-way down the zeugopodium, and is a disharmonic (left) limb. It has a harmonic AP axis, but the digits curve dorsally instead of ventrally. Both limbs × 60.

Fig. 7. Experimental regenerate and contralateral limb 30 days after heterografting a medium-bud blastema, with DV axial reversal, from the right upper arm of a white *A. mexicanum* larva to the left upper arm of an *A. maculatum* larva. Dorsal views, digits numbered in A to P order. (a) The primary regenerate skeleton is composed of axolotl tissue from the distal stylopodium on, although *maculatum* pigment is found in the skin covering its anterior edge, and is a disharmonic (right) limb with digits curving dorsally. Digit 3 has only two phalanges instead of the normal three. Three supernumerary (S) digits and associated basipodials arose on the anterior-dorsal side of the primary hand. (b) Contralateral (right) forelimb of *maculatum* host. Both limbs, × 60.

Fig. 8. Regenerate formed by 40 days after autografting an early bud blastema, with DV axial reversal, from the right upper arm to the left thigh in a dark *A. mexicanum* larva. Dorsal view, digits numbered in A to P order. The graft resorbed up to the basipodium. The stylopodium and zeugopodium (tibia, t and fibula, f) regenerated from the host. The remainder of the graft formed a disharmonic (right) hand with digits curving dorsally. × 60.

Fig. 9. Regenerate formed by 40 days after autografting a medium bud blastema, with DV axial reversal, from the right upper arm to the left thigh in a dark *A. mexicanum* larva. Dorsal view, digits numbered in A to P order. The graft resorbed at least up to the basipodium, and a hindlimb stylopodium and zeugopodium (tibia, t and fibula, f) regenerated from the host. The acropodium was composed of four digits in the normal AP sequence, with digit 2 crossing abnormally over digit 3. The DV axis also appeared harmonic, with the digits curving in the normal ventral direction. A small supernumerary digit (arrow) regenerated dorsally at the base of digit 2. × 60.
Table 3. Polarity of regenerates after reversal of the blastemal APDV axes

<table>
<thead>
<tr>
<th>Graft type and stages</th>
<th>Total cases</th>
<th>Maintained polarity</th>
<th>Polarity conformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Heterograft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>5</td>
<td>4 (80.0%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>12</td>
<td>4 (33.3%)</td>
<td>8 (66.7%)</td>
</tr>
<tr>
<td>2. Autograft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>11</td>
<td>2 (18.1%)</td>
<td>9 (81.9%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>9</td>
<td>2 (22.2%)</td>
<td>7 (77.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>12 (32.4%)</td>
<td>25 (67.6%)</td>
</tr>
</tbody>
</table>

* Graft tissue survived, regenerate oriented upside down and backwards.
† Polarity conformed for two different reasons: (1) Graft tissue did not survive and regeneration took place from host stump, or (2) graft tissue survived but derotated.

30% of those formed after grafting medium-bud blastemas were derived wholly or in part from donor tissue. All of these, however, retained the handedness of their origin (Fig. 8). In two other exceptional cases (one early bud, one medium bud) the regenerates possessed zeugopodia of host derivation and autopodia of donor derivation which, however, displayed host-side handedness, the digits exhibiting the normal ventrally directed curvature. These two cases could be interpreted as indicating lability of the blastemal DV axis. However, it is unlikely that they actually represent a change of graft axial polarity, because both cases formed supernumerary structures, a phenomenon associated with the reversal of axially determined blastemas (see below and Discussion). The supernumerary of one regenerate consisted of the duplication, ventrally, of the phalanges of digits 1 and 2, while in the other case, it consisted of an unidentifiable spike of cartilage located dorsally (Fig. 9). Since considerable resorption obviously took place in both grafts, it is possible that the majority of the autopodium in these cases is also derived from the host, but failed to make the normal number of basipodial elements and digits.

To summarize the results of all the transplants, the evidence overwhelmingly indicates that the DV axial polarity of surviving blastemal tissue cannot be altered by adjacent stump tissues.

(3) APDV Reversal

(a) Heterografts. Four of five early-bud blastemas and four of twelve medium-bud blastemas formed regenerates of donor-specific size and colour, while maintaining the backwards and inverted orientation imposed by the operation (Table 3, Fig. 10). All of these regenerates had the same handedness as the host limb, since the grafts were taken from the ipsilateral side of the donor. The remaining cases fell into two categories. Seven grafts (one early bud, six
medium bud) formed regenerates with donor-specific size and colour, and were also oriented normally with respect to the host stump (Fig. 11). These regenerates are assumed to be cases where derotation of the graft took place, a phenomenon which has been well documented for APDV reversed limb-bud anlagen (Harrison, 1921) and regeneration blastemas (Maden & Turner, 1978). Such derotated regenerates are thus equivalent to control, unrotated ones. Two other grafts (both medium bud) resorbed completely, and the regenerate was derived wholly from the host stump.

(b) Autografts. These cases underwent complete resorption or derotation much more frequently than the heterografts (Table 3). Four of the early-bud grafts derotated and five resorbed, while two medium-bud blastemas derotated and five resorbed. The remaining four cases exhibited donor limb morphology and were oriented upside down and backwards.

Summarizing, 100% of those cases which did not totally resorb or derotate maintained their original handedness, and were upside down and backwards.

(4) PD Reversal

(a) Heterografts. Table 4 summarizes the results of reversing the PD axis. All of the heterograft operations confronted distal and proximal limb levels. Three of 17 cases (17.6%) resorbed completely. In five cases (29.4%) the graft maintained the axial polarity of origin, with its originally distal tip (now facing proximal) regenerating digits at an angle from the graft-host junction (Fig. 12). These digits were able to develop because the distal tip of the graft apparently became angled to the host stump, allowing a free wound surface to be maintained. The originally proximal end of the graft (now pointing distally, and covered with fresh wound epidermis) gave rise to another regenerate that was a mirror image of the one formed by the remainder of the graft (Fig. 12). In addition, intercalary regeneration of host-type structures took place between the proximal host level and distal graft levels (Fig. 12).

Maintenance of graft polarity was not detectable in the remaining nine cases (52.9%), and limbs with normal PD axial polarity and segmentation were regenerated. Most of these regenerates were chimaeric, with autopodia composed of donor tissue and the remainder of host tissue. This composition suggests that much of the graft tissue resorbed with the remainder being used for autopodium formation, while the stylopodium and zeugopodium were intercalated from host tissue. In one exceptional case, however, a normal regenerate was derived wholly from donor tissue, suggesting that the whole graft had completely reversed its polarity (Fig. 13). An alternative explanation, however, is that all of the graft except a portion of the stylopodial material may have resorbed. This portion could fuse with the host stylopodium and its original proximal end undergo distal regeneration to produce the observed regenerate. It is to be emphasized that in all cases of normal regenerate formation following PD reversal, I could not determine whether the original polarity of
the graft tissue was reversed or was simply undetectable because there was no good indication of polarity in the structure (other than the distal regenerate parts) formed by the original material.

(b) Autografts. Of the grafts in which distal and proximal limb levels were confronted, 45% resorbed completely, while 10% maintained their polarity and also regenerated from their originally proximal end (Fig. 14). Maintenance of polarity was not detected in the remaining cases. Like their heterograft counterparts, the latter regenerates were chimaeric, having autopodia of donor
limb morphology with the remainder of the regenerate being derived from the host limb.

Eleven additional autografts were made in which the same limb levels were apposed. Three of these grafts resorbed completely, and two exhibited no detectable maintenance of polarity. The latter regenerates were composed of autopodia having donor limb morphology, and stylopodia and zeugopodia derived from the host limb. The remaining six grafts maintained their original polarity and also regenerated from their originally proximal ends (Figs. 15, 16). The high percentage of grafts maintaining polarity in this particular experiment suggests that confrontation of different limb levels is a large factor in decreasing the frequency of cases (probably by encouraging dedifferentiation and resorption) with detectable maintenance of polarity.
Table 4. Polarity of regenerates after reversal of the blastemal PD axis

<table>
<thead>
<tr>
<th>Graft type</th>
<th>Total cases</th>
<th>*Maintained polarity</th>
<th>†Polarity conformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Heterograft (different levels)</td>
<td>17</td>
<td>5 (29.4%)</td>
<td>12 (70.6%)</td>
</tr>
<tr>
<td>2. Autograft (different levels)</td>
<td>31</td>
<td>3 (9.6%)</td>
<td>28 (90.4%)</td>
</tr>
<tr>
<td>3. Autograft (same levels)</td>
<td>11</td>
<td>6 (54.5%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>14 (23.7%)</td>
<td>45 (76.3%)</td>
</tr>
</tbody>
</table>

* Graft tissue survived and a mirror-image supernumerary regenerate developed from its originally proximal end.

† In some cases graft tissue survived and formed autopodial structures of apparent normal polarity (number to the left of colon). In other cases, the graft tissue resorbed completely, and a normal limb regenerated that was derived entirely from host tissues (number to the right of colon).

Fig. 14. Regenerate formed by 40 days after autografting a late-bud-stage blastema, with PD reversal, from the right upper arm to the left thigh in a dark *A. mexicanum* larva. Ventral view, digits numbered in A to P order. Although it cannot be discerned in the photograph, the originally distal end of the graft ulna bends ventrally (toward the reader) and a single digit (arrow) regenerated proximally and ventrally from this end, indicating that the graft ulna and radius maintained their original polarity. The humeral portion of the graft apparently resorbed, and the originally proximal ends of the radius and ulna regenerated a harmonic (left) limb (digits numbered 1–4). × 60.

Fig. 15. A notch-stage blastema from the left forelimb was autografted, with PD reversal, to the right ankle joint of the right hindlimb in an *A. maculatum* larva, confronting the same limb levels. The graft zeugopodium (radius, *r* and ulna, *u*) maintained its original polarity (arrow) but did not regenerate at either end. × 120.

Fig. 16. A notch-stage blastema from the left forelimb was autografted, with PD reversal, to the mid-shank level of the right hindlimb in an *A. maculatum* larva. The distal portion of the blastema was removed by a cut through the mid-radius–ulna level, so the same limb levels were confronted. The radius and ulna (arrow) of the graft maintained their polarity, but did not regenerate from their originally distal end. They did, however, regenerate a harmonic (right) hand with three digits from their originally proximal end. The graft radius and ulna, and the host tibia (*t*) and fibula (*f*) were shifted relative to one another during development of the regenerate. Both tibia and fibula underwent intercalary regeneration, the tip of the tibia finally articulating with the carpals which regenerated from the proximal end of the graft and the tip of the fibula articulating with the mid-radius of the graft. × 120.
Blastemal axial polarity
Table 5. Frequency of supernumerary formation after axial reversal

<table>
<thead>
<tr>
<th>Graft type and stages</th>
<th>Graft survived and maintained polarity</th>
<th>Graft resorbed or derotated*, polarity conformed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AP Reverse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Heterograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>5/6 (83.3%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Medium Bud</td>
<td>7/11 (58.3%)</td>
<td>2/2 (100%)</td>
</tr>
<tr>
<td>Medium Bud (distal to proximal)</td>
<td>1/4 (25.0%)</td>
<td></td>
</tr>
<tr>
<td>2. Autograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>8/8 (100%)</td>
<td>4/6 (66.6%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>11/11 (100%)</td>
<td>2/4 (50%)</td>
</tr>
<tr>
<td><strong>DV Reverse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Heterograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>3/4 (75%)</td>
<td></td>
</tr>
<tr>
<td>Medium bud</td>
<td>7/15 (46.7%)</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>Medium bud (distal to proximal)</td>
<td></td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>2. Autograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>2/3 (66.6%)</td>
<td>3/13 (23.0%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>1/3 (33.3%)</td>
<td>2/6 (33.3%)</td>
</tr>
<tr>
<td><strong>APDV Reversal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Heterograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>3/4 (75%)</td>
<td></td>
</tr>
<tr>
<td>Medium bud</td>
<td>4/4 (100%)</td>
<td>3/8 (37.5%)</td>
</tr>
<tr>
<td>2. Autograft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early bud</td>
<td>2/2 (100%)</td>
<td>3/9 (33.3%)</td>
</tr>
<tr>
<td>Medium bud</td>
<td>2/2 (100%)</td>
<td>4/7 (66.6%)</td>
</tr>
</tbody>
</table>

* Derotated applies to APDV cases only.

(5) Supernumerary Formation

Table 5 summarizes the frequency of supernumerary formation after transverse axial reversal. Supernumeraries arose after each type of transverse axial reversal (Figs. 2, 3, 4, 7, 10), but were somewhat less frequent after DV reversal than after AP or APDV reversal. Supernumeraries were often formed even when a graft derotated or resorbed completely. The degree of completeness of a supernumerary was often correlated with the amount of resorption suffered by the graft. Where little or no resorption occurred, supernumeraries were often comparable in their completeness to the primary regenerate, taking their origin from the original graft-host junction. When only the autopodial material of the graft survived, supernumeraries often consisted only of autopodia or extra digits. The majority of supernumeraries in cases which resorbed totally
Blastema! axial polarity

consisted of extra digits or phalanges, although several cases were observed in which supernumerary autopodium formation occurred after AP reversal and total graft resorption.

The supernumerary autopodia formed by the 13 AP-reversed heterograft cases in which the graft survived were examined to determine their donor:host digit derivation. A total of 23 supernumeraries were formed by these cases, and their derivation fell into two main categories. In the first category were 11 supernumeraries in which the donor:host ratio of digits was 1:2 or 1:1. The second category consisted of 10 supernumeraries in which all the digits were derived from the host. The remaining two supernumeraries were unidentified single digits derived from the donor.

DISCUSSION

Transverse axes

The data presented here demonstrate that the transverse (AP and DV) axes of the limb regeneration blastema are both determined from the start of the regeneration process. One hundred percent of the regenerate structures formed by surviving graft tissue maintained the handedness of their origin after AP, DV and APDV reversal. Even blastemas shifted proximally in conjunction with AP reversal, a procedure resulting in complete dedifferentiation of even redifferentiating blastemas (Stocum, 1975; Iten & Bryant, 1975), maintained the handedness of origin. All regenerates exhibiting host-side handedness were found to be composed entirely of host tissue, indicating total resorption. Early determination of blastemal transverse axes was further indicated by the formation of supernumerary limbs after axial reversal at all stages tested, even when all of the graft tissue resorbed. Supernumeraries arise by intercalary regeneration of positional values between opposed axial poles (French, Bryant & Bryant, 1976) and would not be expected to form if positional information were erased from blastema cells during dedifferentiation.

The data compel the conclusion that enough morphogenetic (positional) information to maintain the transverse tissue pattern and axial polarity of a regenerate is directly inherited by dedifferentiating cells from their parent differentiated cells during blastema formation. The regeneration blastema therefore stands in contrast to the embryonic limb anlage, in which the DV axis, at least, is determined subsequent to determination of the limb as such (Harrison, 1918, 1921; Detwiler, 1933; Swett, 1937; but see Stocum & Fallon, 1982). This conclusion receives support from two other experiments, in which marked blastemas derived from double anterior or posterior limb stumps were exchanged with normal blastemas (Holder & Tank, 1979; Stocum, 1980a). Normal AP axial polarity was not induced in the symmetrical blastemas by normal limb stumps, nor were normal blastemas induced to form symmetrical regenerates by symmetrical limb stumps.
The present results and conclusions are at odds with those of nearly all previous investigators. Only Lodyzenskaja (1928) concluded, from her results of axial reversals, that the transverse axes of axolotl limb regeneration blastemas are determined very early, by 2 days post-amputation. Since dedifferentiation is not yet detectable at that time, it is likely that Lodyzenskaja actually transplanted slices of stump tissue, not blastemas, in her experiments. These tissues could have dedifferentiated completely to give rise to a blastema which maintained the polarity of origin, or the grafts may have been large enough so that differentiated tissues remained proximal to the blastema, thus invalidating the experiment as a test for axial determination. Both Milojevic (1924) and Schwidefsky (1934) claimed that the transverse axes of adult newt (*Triturus cristatus* and *T. taenius*) blastemas become gradually determined during a short period prior to redifferentiation, and Iten & Bryant (1975) have presented evidence that the blastemal AP axis is labile in adult *Notoptthalmus viridescens* at least through the stage of medium bud. None of these studies employed appropriate markers to trace the origin of the regenerates, although Milojevic (1924) did autograft blastemas between forelimbs and hindlimbs to use their morphological differences as a marker. However, he interpreted the host-type regeneration that occurred after grafting early blastemas as a change in limb type and axiation induced by the host stump, rather than graft resorption.

Iten & Bryant (1975) argue that their results cannot be interpreted in terms of graft resorption and host regeneration, for two reasons. First, they did not observe any appreciable reduction in the amount of graft tissues in a histological study of cases fixed at daily intervals after transplantation. Second, they analysed the time required after transplantation for a graft to advance to the next stage of development. Although a delay in graft development was observed, it was less than expected if regeneration had occurred from a freshly amputated stump while the graft resorbed. These arguments can be countered on two grounds. First, evaluating the degree of graft resorption in the absence of cell markers, even in sections prepared at closely spaced intervals, is difficult to do accurately, especially with grafts of early (small) blastemas, because graft cells could be replaced by host cells as fast as they are resorbed. Second, this would be especially true in Iten and Bryant’s experiments because their blastemas were not grafted to freshly amputated limbs, but to limbs which were themselves regenerating. The latter stumps contain tissues still in the process of dedifferentiation. In the event of graft resorption, they could form a new blastema in a much shorter time than a freshly amputated limb, thus accounting for the shorter delay in attaining the next developmental stage.

Iten & Bryant (1975) obtained all harmonic regenerates after transplantation of early-bud blastemas, without supernumerary formation. After grafting medium-bud blastemas, a high percentage of symmetrical or ‘expanded’ hands were recorded. Grafts of later stages produced nearly all disharmonic regenerates.
Blastemal axial polarity

These observations seemed to indicate that the blastema was determined by degrees, the medium-bud stage being a critical period where only partial reversal of AP polarity could be achieved. However, Wallace (1981) has pointed out that the symmetrical hands can be interpreted as cases where supernumerary digits form on either side of the graft digits, and expanded hands can be interpreted as cases where supernumerary digits form on both sides of the graft digits. In both situations, the graft digits maintain the axial polarity of origin.

In view of the present results, it seems certain that previous work indicative of lability in the transverse axes of undifferentiated blastemas can be interpreted in terms of graft resorption in conjunction with host regeneration. Polezhaev (1979) and Wallace (1981) have presented detailed analyses of the data available prior to 1980 and have come to exactly the same conclusion, giving further corroborative examples.

Proximal-distal axis

The data presented here indicate that the PD axis of the blastema is also determined at the earliest stages of regeneration. To reverse this axis, a distal portion of the blastema was removed so that the distal end of the remaining part could be attached to the host stump. When the reversal confronted the same limb levels, 75% of the surviving autograft cases maintained their original polarity. When the reversal confronted different limb levels (enhancing graft dedifferentiation and resorption) only 17.6% of the surviving autografts maintained their polarity, but 35.7% of the heterografts did so. Intercalary regeneration of host-derived intermediate structures took place between the stump and graft levels. In addition, the grafts also regenerated from their originally proximal ends. These results are very similar to those obtained by Wallace (1980) after PD reversal of aneurogenic axolotl limb stumps.

Maintenance of polarity could not be detected in the remainder of the PD-reversed autografts and homografts. In these cases, graft tissue formed all or part of the autopodium of normal-appearing regenerates, and the zeugopodium and stylopodium were composed of host tissues. This result can be explained by assuming that resorption of blastema cells took place from either or both ends of the graft, leaving only a bit of surviving mesenchyme which maintained its polarity. If the surviving material had a positional value more distal than the host stylopodial level, intercalary regeneration would occur between host and graft. If the positional value of the surviving tissue were similar to that of the host stylopodium, little or no intercalary regeneration would take place and the graft would form the distal end of the regenerated stylopodium. In both cases the graft would regenerate from the originally proximal end of its remaining mesenchyme under the influence of the wound epidermis which covers this end. The structures developed from that part of the graft which maintained its polarity would form an insufficient part of the skeletal pattern for their original polarity to be detected. Thus, the normal-appearing limbs
would actually consist of intercalated host-derived structures plus a small
donor-derived portion of reversed polarity, which regenerates another limb
from its originally proximal end. This scheme would also explain the exceptional
heterograft case in which a complete regenerate exhibited normal polarity and
was composed entirely of donor tissue; the whole regenerate would be derived
from a small portion of surviving stylopodium (the rest of the graft having
resorbed) which maintained undetectable polarity.

**Supernumerary formation and blastemal reprogramming**

Although the blastema represents an autonomous morphogenetic field which
more or less faithfully replicates the structure and axial polarity of the original
amputated parts, it is not a mosaic, and its cells will eliminate, by division and
intercalation of normal neighbours, discontinuities made along any axis
(French et al. 1976). Such regulation can be evoked after deleting tissue from
the limb stump or the blastema itself (see Wallace, 1981, for a thorough review),
and by reversal of a blastemal axis with respect to the stump.

One of the most striking responses to tissue deletion occurs when an irradi-
ated limb (which cannot regenerate) is amputated through a region supplied
with a cuff of unirradiated skin. The resulting blastema is derived solely from
the dermal cells of the grafted skin, but some of these cells undergo metaplasia
into cartilage and the regenerate exhibits a normal skeleton (Dunis & Namen-
wirth, 1977). The ability of blastema cells derived solely from dermis to form a
normal skeletal pattern suggests that regenerate form and transverse axial
polarity are determined by the circumferential blastema cells in contact with the
blastemal epidermis. These cells would function as a boundary which specifies
the transverse pattern of redifferentiation of their progeny. Thus, regeneration
of internal transverse structure from circumferential tissue is a matter of
eliminating the discontinuity within the circumference by cell division and
intercalation of transverse positional values (Stocum, 1980b).

The formation of supernumerary transverse axes after reversing the AP or
DV axes of the blastema with respect to the stump is also a case of elimination
of a transverse discontinuity within a circumferential boundary. Supernumer-
ary circumferences are created on both the anterior and posterior, or dorsal
and ventral sides of the blastema–stump junction after axial reversal. Half of a
complete supernumerary circumference can be provided by each half stump
and half blastema that are juxtaposed, or a complete circumference can be
created by intercalation between non-neighbouring points on the apposed half
circumferences according to the shortest intercalation rule of French et al.
(1976). The circumferential cells would then divide and intercalate the trans-
verse pattern, from which a blastema could grow out and form a supernu-
merary regenerate.

The formation and outgrowth of a supernumerary blastema after AP or DV
axial reversal can be assumed to require the presence of a wound epidermis,
Blastemal axial polarity

as does regeneration after simple amputation (Thornton, 1968; Stocum & Dearlove, 1972). Maden (1977) has proposed that the apical epidermis of the blastema acts as the distal boundary of the regenerate (or confers distal boundary properties on subjacent blastema cells (Stocum, 1980b)), while cellular properties specific to the mesodermal cells of each amputation level determine its proximal boundary. The pattern along the PD axis is then regenerated by cell division and intercalation between these boundaries, just as the transverse pattern is regenerated within the circumferential boundary.

It is clear that any kind of regeneration involves the reprogramming of positional values of cells. Since half the supernumeraries formed after AP axial reversal in the present study consisted partially of graft tissue, positional values of blastema cells can be reprogrammed so that the axiation of the supernumerary structures they form is the reverse of the primary regenerate. An important question is how much of the graft tissue has its polarity reversed and what is the nature of the reprogramming? Is an extensive swath of tissue morphallactically repolarized back from the graft edge, with growth subsequently taking place, or, as implied in the mechanism of supernumerary formation outlined above, do cells at just the edge of the graft divide, with polarity reversal taking place in the mass of progeny cells (epimorphosis)? (The same questions can be asked of the host, but host cell programming does not involve reversal of polarity, since the supernumeraries have host handedness.)

Although the present results cannot provide a definitive answer to these questions, they do suggest that it is the progeny of the cells at the edge of the graft that are reprogrammed. A morphallactic polarity reversal in a large mass of graft tissue ought to be accompanied by a loss of anterior or posterior structures that can be identified as belonging to the primary regenerate. However, of the 13 AP-reversed primary regenerates, only 2 that exhibited a contribution to a supernumerary were at the same time missing digits from the primary sequence adjacent to the supernumerary (see Fig. 3b). Digits were also missing from the primary in two other cases, but the adjacent supernumerary was composed entirely of host cells. The latter fact suggests that the graft material for the missing digits was resorbed. Furthermore, in the remaining nine cases, the primary regenerate was not missing any digits (see Fig. 2b). Therefore, it seems likely that the axially reprogrammed graft cells used for supernumerary construction are the progeny of a thin band of cells at the edge of the graft adjacent to the supernumerary.

It is to be emphasized that the interactions between blastema and stump leading to supernumerary formation, as well as the cellular interactions taking place during normal regeneration are not inductive in the classical sense of the word. They are regulative interactions which act to eliminate positional discontinuities between non-neighbouring cells, even if this means the creation of excess patterns.

This research supported by NIH Grant HD 12659-02.
REFERENCES


(Received 18 January 1982, revised 16 March 1982)