Pattern regulation and the origin of extra parts following axial misalignments in the urodele limb bud

By STEPHEN D. THOMS and JOHN F. FALLON

From the Department of Anatomy, The University of Wisconsin

SUMMARY

Pattern regulation following axial misalignments in the stage-38+ to stage-40 urodele limb bud was studied on one newt and two salamander species. Grafts of the distal tip of the limb bud were made to the stump of a host limb bud from which a similar piece had been removed. The grafts were positioned with either their anteroposterior, dorsoventral, or both of these axes reversed with respect to the host axes. Mirror-imaged duplications, positioned posteriorly or both anteriorly and posteriorly, occurred nearly all (96%) of the time when the anteroposterior axis was reversed. Dorsoventral axial misalignment rarely promoted the generation of mirror-imaged duplications (8%) but did affect the organization along the anteroposterior axis by causing a serial repetition of either digit 2 or digit 3. Regulation, therefore, does not always occur along each axis independently of the others. Consistent with the data derived from reversing individual axes, most of the duplications which occurred when both axes were reversed were in the anteroposterior plane. Some were in the dorsoventral plane, and a few had intermediate positions. Of these duplications a few were neither right nor left hands, rather they were of mixed handedness with a change in the dorsoventral polarity from the anterior border to the posterior border.

Whether extra parts which result from axial misalignments arise from the graft, the host, or both the graft and the host was investigated using heteroplastic grafts and grafts exchanged between triploid and diploid axolotls. Duplications were found to have cellular contributions from both the graft and the host. In some cases one source would dominate but usually both made a substantial contribution. The diploid-triploid material suggests that a considerable mixing of host and graft cells may occur in duplications. Additionally, some digits of the graft sequence of digits can be derived from host tissue. The extra digit in those hands displaying a serial repetition was derived from host tissue in some cases and graft tissue in other cases.

INTRODUCTION

Pattern regulation following perturbation of the developing urodele limb has been utilized extensively to study the establishment of pattern within a morphogenetic field. The behavior of the field, following an experimental intervention, is assumed to reflect the processes governing normal morphogenesis. Consistent production of recognizable limb morphology after defined experimental manipulations, supports this assumption. Axial misalignments of the limb field

1 Author’s address: Department of Anatomy, 453 Bardeen Medical Laboratories, The University of Wisconsin, Madison, WI 53706, U.S.A.
district (Weiss, 1939, p. 293) either internally or with respect to the surrounding tissues, have revealed extensive regulative capacities (Harrison, 1921; Swett, 1927). A sequential determination of the polarity of the three cardinal axes has been demonstrated (Detwiler, 1929, 1933; Swett, 1927, 1928, 1930). The anteroposterior axial polarity is determined by the early gastrula stage (Detwiler, 1933). However, the limb field cannot be manipulated prior to this stage, and for this reason the polarity of the anteroposterior axis always differentiates according to its presumptive fate. Dorsoventral axial polarity is determined somewhat later (Swett, 1927, 1930; Hollinshead, 1936) and the proximodistal axial polarity, still later (Swett, 1928).

When a limb rudiment is grafted orthotopically, with the dorsoventral axis reversed with respect to the host animal prior to the determination of its polarity, the prospective polarity of the dorsoventral axis fails to be realized; rather the axis develops in accordance with the polarity of the host (Harrison, 1921). However, if the graft is made to the myotomes the prospective polarity of the axis is realized (Swett, 1939), indicating that axial polarity is instituted in a labile state before it is determined. The factors determining axial polarity, which are probably intrinsic to the field (Swett, 1938a), remain unknown. However, it is well documented that the region surrounding the limb rudiment can direct the institution of polarity (Nicholas, 1958; Swett, 1938b, 1945; Slack, 1977a).

After the determination of axial polarity, the field displays a different behavior. When the limb region is grafted orthotopically, with either the anteroposterior or dorsoventral axis reversed with respect to the host, not only is the resulting axial polarity of the mature limb reversed but also duplications along the reversed axis often appear. The polarity of the duplications mirrors that of the graft's reversed axis (Harrison, 1921; Swett, 1926). Duplications following the grafting of limb rudiments to the flank have been shown to be derived mostly, if not exclusively, from the grafted material (Swett, 1932). Although the polarity of the proximodistal axis is retained when this axis is reversed at late stages of development (Swett, 1928), little is known about the regulative behavior of the limb field following this manipulation.

Much of the above information has been obtained by manipulation of the limb rudiments at a time before the limb protrudes from the body wall as a three-dimensional system. Additionally, most of these experiments realigned the limb region with respect to non-limb regions. This report describes the results of experiments in which the axes of late-stage limb-bud tips were rotated with respect to the base of the limb bud.

Two questions are specifically addressed. First, do reversals of the anteroposterior and dorsoventral axes, both individually and concomitantly, yield the same percentage and types of duplications? Second, do these duplications arise from the graft, the host, or both the graft and the host?

The results show that reversal of the anteroposterior axis of the distal half
Pattern regulation after axial rotations in urodele limb buds

of the urodele limb bud promotes the generation of posteriorly positioned, or both anteriorly and posteriorly positioned, duplications nearly 100% of the time, while reversal of the dorsoventral axis rarely promotes the generation of either dorsal or ventral duplications. However, dorsoventral axial rotation does affect the organization along the anteroposterior axis, but not the polarity. Therefore, the field pattern does not regulate along each axis completely independently of the others. Finally, duplications are shown to have contributions from both host and graft tissue.

MATERIALS AND METHODS

Larvae of *Ambystoma mexicanum*, *Ambystoma maculatum* and *Notophthalmus viridescens* were used in these experiments. All were maintained in 2X Brown–Caston solution (Brown & Caston, 1962) and fed brine shrimp daily.

The operations were carried out on forelimb buds that were of equivalent external morphology to those found on *A. maculatum* at Harrison stages 38+ through 40. Most of the limb buds used were equivalent to stages 39 and 39+. The appearance of axolotl limb buds is delayed in comparison to *A. maculatum*. Therefore the axolotls used were feeding larvae while the *A. maculatum* larvae were a day or two from the initiation of feeding. *N. viridescens* limb buds arise several days before hatching and reach the desired morphology when the animal is still slightly less developed in general than the stage 39+ *A. maculatum*. For convenience, animals are referred to by the Harrison stage number appropriate for the limb-bud morphology present.

Generation of triploid axolotls

Triploid axolotls were produced by a heat-shock method. Eggs were collected every 30 min, held for 45 min at room temperature, then dipped for 8-5 min in 2X Brown–Caston solution maintained at 35-5–36-5 °C. They were then returned to room temperature and allowed to develop at 18 °C. Among the surviving animals, those which were triploid were identified by counting the nucleoli of cells from tail clippings which were observed with a phase-contrast microscope.

Operative technique

Two animals of the desired stage were anesthetized with tricaine methane-sulfonate. All operations were carried out in 100% Holtfreter’s solution. Sometimes one of these larvae was a donor and the other a host. Often, however, each served as a host for a graft donated by the other (exchange grafts).

In all operations the distal one-half to two-thirds of a limb bud was amputated and grafted in the desired orientation, either ipsilaterally or contralaterally, to a limb-bud stump from which a similar piece had been removed. Proper orientation of the graft was facilitated, when either *A. maculatum* or axolotls were used, by inserting a small grain of carmine beneath the epithelium of the
Fig. 1. Illustration of the orientations used when grafting a distal tip of a limb bud to a stump from which a similar piece had been removed. The dot represents a grain of carmine placed under the epithelium on the anterior border. The vertical line through the drawing of the limb bud at the top represents the approximate level of the surgical cut: A, anterior; P, posterior; D, dorsal; V, ventral. Part (a) depicts the control operation in which all axes of graft and stump are normally aligned. Part (b) depicts rotation of the graft anteroposterior and dorsoventral axes by 180° with reference to the stump. Part (c) represents dorsoventral axis rotation by 180°, and part (d) anteroposterior axial rotation.

anterior border. A pigmented blotch consistently found on the distal dorsal surface of the *N. viridescens* limb bud served as a convenient marker in this species. All hosts were checked after 24 h and those in which the graft was either missing or improperly oriented were discarded. Observations of heteroplastic grafts and triploid–diploid exchange grafts indicated that the smaller grafts were destined to form the hand and wrist while the larger grafts also included the distal forearm.

Homoplastic grafts between members of each of the three species as well as heteroplastic grafts exchanged between *A. maculatum* and axolotl larvae were performed. Additionally, grafts were exchanged between triploid and diploid axolotls. Four orientations were used in these experiments and are shown in Fig. 1.

**Preparation of whole mounts and histology**

The animals to be prepared as whole mounts were raised for 28–32 days following the operation, anesthetized and fixed in 10% formalin, stained with Victoria Blue, and cleared.

The axolotls with triploid–diploid exchange grafts were raised for 21 days, anesthetized, fixed in 95% ethanol, 40% formaldehyde, and glacial acetic acid,
in a ratio of 17:2:1, photographed, and embedded in paraffin. Ten μm sections were cut, treated with 0·1 mg DNAse per ml of 0·003 M-MgSO₄ (pH 6·5) for 2 h at room temperature and stained with Azure B (Flax & Himes, 1952).

RESULTS

Homoplastic grafts

Controls

Normal axolotl and *A. maculatum* forelimbs have eight carpal bones and four digits. The digits are numbered 1–4 from anterior to posterior and have a phalangeal formula of 2, 2, 3, 2. *N. viridescens* has only one phalanx in digit 1 and two of the carpals are often fused.

The digits normally curve ventrad and the joints can only be bent in a ventral direction. In these experiments, if the direction of a digit's curvature was in doubt it was manipulated to determine which direction it would bend, in order to establish which side was ventral.

To check the possible effects of the grafting procedure on the resulting pattern, 17 control operations in which the distal aspect of the forelimb bud was grafted to a host with normal orientation were completed on the axolotl. Of these, 16 formed normal limbs indistinguishable from unoperated limbs (Fig. 2). The remaining limb displayed a forked distal phalanx of the first digit. Many of these grafts were exchanges between dark and white animals. In general the hand, wrist and a variable extent of the forearm was of the graft pigmentation. Sometimes the host epithelial pigmentation would extend more distad along the posterior aspect of the limb than the anterior. These results show that the grafting procedure used is not sufficient to bring about abnormal morphogenesis of the developing urodele forelimb.

Anteroposterior and dorsoventral axes reversed

Duplications along the anteroposterior axis were the predominant consequence of rotating both the anteroposterior and dorsoventral axes of the graft by 180° with respect to the host. This operation was done on the axolotl only. Out of 20 cases, 11 contained duplications in the anteroposterior plane (one anterior only, two posterior only, eight both), one had a ventral duplication of digits 1 and 4, and five presented duplications either in both or with an intermediate relationship to the two axes. The three remaining cases had normally oriented hands, possibly the result of derotation (Nicholas, 1924). Of these, one was completely normal, one had a forked digit 2, while the other showed a ventral duplication of digits 1 and 2.

With the exception of an example with 12 digits, the number of digits found on duplicated hands was fairly evenly distributed from six to nine with an average of 7·4. Although most frequently the extra parts were limited to the hand and wrist, one case had an extra distal part of a forearm bone and three
cases had two extra forearm bones, some more complete than others. There was never an indication of more than two extra hands. Frequently digit 4 and occasionally digit 1 of the graft sequence of digits were missing. The sets of digits in the duplications also were frequently incomplete; posterior duplications, when present, were generally more complete than other types.

The curvature of the digits in dorsoventral duplications was always normal for the host, i.e. they always curved ventrad; indicating that their dorsoventral axis mirrored that of the graft. In a similar manner the anteroposterior axis of the anteroposterior duplications mirrored the graft's anteroposterior axis. The extra anterior and posterior hands, however, were not always mirror images of the graft hand. Although in some cases their digits would curve dorsad, like the graft sequence, in other cases they would curve ventrad. Often within a duplication the digits closest to the graft would curve dorsad and the remaining would curve ventrad.

**Figures 2-7**

Fig. 2. Whole mount of an axolotl right forelimb resulting from a homoplastic control operation, ventral view. The anterior to posterior (top to bottom in the photograph) digital sequence is 1, 2, 3, 4 with a normal phalangeal formula of 2, 2, 3, 2 (× 12).

Fig. 3. Whole mount of an axolotl left forelimb resulting from a homoplastic operation with the graft's anteroposterior axis reversed, dorsal view. This limb has a posterior duplication with a displacement dorsad of the middle digit. The anterior to posterior (top to bottom in the photograph) digital sequence is 4, 3, 2, 1, 2, 3, 4. The italicized numbers are the graft sequence of digits (× 12).

Fig. 4. Whole mount of an axolotl right forelimb resulting from a homoplastic operation with the graft's anteroposterior axis reversed, dorsal view. This limb has both an anterior and a posterior duplication. The anterior to posterior (left to right in the photograph) digital sequence is 1, 2, 3, 3, 2, 1, 2, 3, 4. The italicized numbers are the graft sequence of digits (× 12).

Fig. 5. Whole mount of an A. maculatum right forelimb resulting from a homoplastic operation with the graft's anteroposterior axis reversed, dorsal view. This limb has both an anterior and a posterior duplication. The anterior to posterior (top to bottom in the photograph) digital sequence is 1, 2, 2, 1, 2, 3, 4. The italicized numbers are the graft sequence of digits (× 22).

Fig. 6. Whole mount of an axolotl right forelimb resulting from a homoplastic operation with the graft's dorsoventral axis reversed, dorsal view. This limb has an inverted hand displaying no abnormalities (× 12).

Fig. 7. Whole mount of an axolotl right forelimb resulting from a homoplastic operation with the graft’s dorsoventral axis reversed, distal to proximal view. The forearm and upperarm are behind the plane of focus. The graft sequence of digits, extending toward the left of the photograph, curves dorsad with an anterior to posterior (top to bottom in the photograph) order of 1, 2, 3. Digit 1 is forked dorsad. The ventral duplication, extending toward the right of the photograph, curves ventrad with an anterior to posterior digital sequence of 3, 3, 4 (× 12).
Table 1. *Anteroposterior axis rotation: homoplastic grafts*

<table>
<thead>
<tr>
<th>Species</th>
<th>Cases</th>
<th>Reversed hand (Normal 4 digits)</th>
<th>Duplicated (Posterior only)</th>
<th>Duplicated (Anterior and posterior)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. mexicanum</em></td>
<td>15</td>
<td>1 (7%)</td>
<td>3 (20%)</td>
<td>9 (60%)</td>
<td>2 (13%)*</td>
</tr>
<tr>
<td><em>A. maculatum</em></td>
<td>16</td>
<td>0</td>
<td>10 (63%)</td>
<td>4 (25%)</td>
<td>2 (12%)†</td>
</tr>
<tr>
<td><em>N. viridescens</em></td>
<td>7</td>
<td>0</td>
<td>5 (71%)</td>
<td>2 (29%)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>38</td>
<td>1 (3%)</td>
<td>18 (47%)</td>
<td>15 (39%)</td>
<td>4 (11%)</td>
</tr>
</tbody>
</table>

* In these two cases the graft appeared to be displaced ventrad by a posterior duplication.
† One of these had a normal digital sequence with a forked first digit and an extra carpal, the other had an ambiguous digital sequence in the anteroposterior plane so the position of the duplication could not be identified.

**Anteroposterior axis reversed**

Most of the limb buds in which the anteroposterior axis of the graft was rotated 180° with respect to the stump gave rise to duplications (Table 1). Posterior duplications were characteristically found. It is notable that anterior duplications were present only when a posterior duplication was also present, never as a single duplication. The axolotl gave a higher percentage of double duplications than did the other two species (60% v. 25% and 29%). This is reflected in the average number of digits found in duplicated limbs; 7-9 (range: 7-10) digits per duplicated limb in the axolotl, 6-0 (range: 5-7) in *A. maculatum*, and 6-4 (range: 5-9) in *N. viridescens*. The duplicated limbs contained from 9 to 19 carpal bones and a few had extra forearm elements. All of the limbs had a normal dorsoventral polarity and the duplications were mirror images of the graft.

Although in some cases a complete graft sequence of digits was present, often digit 4 or digits 3 and 4 were missing from the graft sequence. Figure 3 shows an axolotl hand with a posterior duplication. The entire set of graft digits is present. Digit 1 is shared with the duplication and has been displaced dorsad. Digit 4 is missing from the graft sequence in the axolotl hand shown in Fig. 4. This hand has both anterior and posterior duplications. The anterior duplication has digits 1, 2 and 3, the graft sequence follows with digits 3, 2 and 1, and finally the posterior duplication made up of a complete set of digits in the normal order. An example with both digits 3 and 4 missing from the graft sequence is shown in the *A. maculatum* hand with both anterior and posterior duplications pictured in Fig. 5. As in the previous example the digits which are missing from the graft sequence are also missing from the anterior duplication.

As these examples show, the entire digital sequence of duplicated hands following an anteroposterior axis reversal always follows the normal digital sequence. In some cases digits are missing. Nevertheless, in all cases, each digit has as its neighbor either a digit which is normally adjacent to it or a duplication
Table 2. Dorsoventral axis rotation: homoplastic grafts

<table>
<thead>
<tr>
<th>Species</th>
<th>Inverted hand (Normal 4 digits)</th>
<th>Inverted hand (5 digits)</th>
<th>Duplicated (Dorsal or Ventral)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. mexicanum</td>
<td>14 (50%)*</td>
<td>6 (21.5%)†</td>
<td>6 (21.5%)‡</td>
<td>2 (7%)§</td>
</tr>
<tr>
<td>A. maculatum</td>
<td>15 (71%)</td>
<td>5 (24%)</td>
<td>0</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>N. viridescens</td>
<td>12 (70%)†</td>
<td>3 (18%)</td>
<td>0</td>
<td>2 (12%)</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>41 (62%)</td>
<td>14 (21%)</td>
<td>6 (9%)</td>
</tr>
</tbody>
</table>

* One of these contained a digit with a forked distal phalanx.
† Two of these contained a digit with a forked distal phalanx.
‡ Three of these were duplications of one digit only.
§ One of these had seven large and irregular carpals and four digits which were straight rather than curved and could not be bent. The second specimen was an inverted five-digited hand with a serial repetition of digit 3, the second of which had a mirror image dorsal duplication giving a total of six digits with three digit 3's.
|| These had serial repetition of digit 3 but in each case digit 4 was missing.

of itself. In all hands resulting from rotation of the anteroposterior axis, if a digit is adjacent to a duplication of itself or has the same type of digit both anterior and posterior to it, the polarity of the digital sequence reverses.

**Dorsoventral axis reversed**

Field behavior following the rotation of the graft's dorsoventral axis by 180° was markedly different from that which followed rotation of the anteroposterior axis or the anteroposterior and dorsoventral axes together (Table 2). The majority of the limbs (62%) developed in accordance with the presumptive fate of the stump and graft; they grew to be normal limbs ending in a dorsoventrally inverted hand (Fig. 6). Often digit 4, and in a few cases, digits 3 and 4 were either straight or curved ventrad rather than displaying a dorsal curvature expected for inverted fingers. Two of the axolotl examples contained a small extra carpal bone.

Only the axolotl yielded duplications. Of the six, one is a dorsal duplication of a single digit. The others are all ventral duplications; two with a single digit, one having two digits, and two with three digits. One of the last is a remarkable case in which two of the three digits in the duplication are digit 3's (Fig. 7), side by side along the anteroposterior axis.

The hands grouped in Table 2 as 'Inverted with Five Digits' also have a serial repetition along the anteroposterior axis. All of the repetitions are of digit 3 (Fig. 8) except two from A. maculatum which repeat digit 2 (Fig. 9). The other digits are all present in the normal order with the extra digit simply inserted. This gives an anterior to posterior digital sequence of 1, 2, 2, 3, 4 or 1, 2, 3, 3, 4. A few of these hands have the normal number of carpals but most have one or two extra (Fig. 8). This is a change in the organization along the anteroposterior
axis, without a change in the polarity of this axis, and is specifically affected by a reversal of the dorsoventral axis.

**Heteroplastic grafts**

When the limb bud of one species is transplanted to another it continues to develop with a timing and to a size which is characteristic of the donor species (Harrison, 1924; Twitty & Schwind, 1931). The noticeable difference in the size of *A. maculatum* (small) and *A. tigrinum* (large) limbs allows the determination of whether the donor or the host contributed to any given part of the limbs following heteroplastic grafts between these two species, particularly when the comparison is made with the unoperated limb of the host (Schwind, 1931; Swett, 1932). Identifying structures which are large or small allows assessment of at least a major contribution by *A. tigrinum* or *A. maculatum*, respectively. Intermediate sizes are thought to be from mixed contribution but this has never been documented. Schwind (1931) has also noticed a slight hypertrophy of *A. maculatum* limb material under the influence of *A. tigrinum* material. Whether this was a direct influence or mechanical is not known. The axolotl is similar to *A. tigrinum* in development and size during the developmental period of the experiments described below and can, therefore, nicely replace *A. tigrinum* in these experiments. Some of the *A. maculatum* hosts used did not grow as vigorously as the axolotl hosts and were still developing the final digits, on both the operated and control sides, when the experiment was terminated.

**Controls**

To determine the fate of the graft on a foreign host without axial misalignments exchanges were made between *A. maculatum* and the axolotl in which the distal one-half to two-thirds of the stage-39 to -40 forelimb bud from one species was grafted with all axes normally aligned onto the other species. The limb buds of the two species are nearly identical in size and shape and each fits well on the stump of the other.

Eleven cases were obtained, six with axolotl hosts and five with *A. maculatum* hosts. Normal hands developed in nine cases, while digit 4 was poorly developed in one case (*A. maculatum* host) and a phalanx was missing from digit one of the other case. Several of the wrists showed some abnormalities but most were normal. Four of the five examples with *A. maculatum* hosts and one of the six with axolotl hosts had hands and wrists solely of donor-type (Fig. 10). Five of the remaining examples had a digit 4 of host-type and the sixth had an intermediate digit 4. When digit 4 was from the host one or two of the posterior carpals were also from the host. In one case with an axolotl host the proximal row of carpals was also host-type.

Some effects on the forearm bones were also present. With *A. maculatum* hosts the forearm bones were often thickened relative to those in the contralateral
Pattern regulation after axial rotations in urodele limb buds

limb, particularly in the distal aspects. A few were slightly elongated and a few axolotl hosts had forearm bones which were shortened.

Anteroposterior axis reversed

All of the 11 cases, seven with axolotl hosts, and four with A. maculatum hosts, in which the anteroposterior axis of the graft was rotated 180° with respect to the host stump, gave duplications. Four (36%) (one with an axolotl host and three with A. maculatum hosts) had only posterior duplications and five (46%) (four with axolotl hosts and one with an A. maculatum host) had both anterior and posterior duplications. The number and arrangements of digits and carpal elements in these were similar to that described for the homoplastic grafts. The two remaining cases could not be easily classified. In one of these not all of the digits could be identified with certainty and in the other there was a confusing array of digits of both axolotl and A. maculatum types.

As assessed by the size of the digits and carpal elements both the graft and the host were found capable of contributing materially to both anterior and posterior duplications. No duplication was found which could be said, with confidence, to be of exclusively host or graft origin. The amount of the contribution from either ranged from little if any, to most if not all, although most duplications had noticeable contributions from both the host and the graft.

An example with both anterior and posterior duplications, each showing contributions from both the host and the graft, is the case of an A. maculatum graft on an axolotl host shown in Fig. 11. The anterior to posterior digital sequence in this example is 1, 2, 2, 1, 2, 3, 4. The middle digit is displaced dorsad. The anterior two digits constitute the anterior duplication, digit 1 of which is host-type and digit 2 is graft-type. The next two digits are the graft sequence with a reversed anteroposterior polarity. Digits 4 and 3 of this sequence are missing. Both digits in the graft sequence are graft-type and the digit 1 is shared with the posterior duplication. Digit 2 of the posterior duplication is graft-type while digits 3 and 4 are host-type.

Figure 12 shows, jointly with the unoperated limb of the A. maculatum host, a case with both anterior and posterior duplications in which the distal aspects of the forearm bones also are involved. The two complete forearm bones found at the anterior and posterior borders of the limb are host-type while the two centrally placed distal parts are graft-type. The anterior to posterior digital sequence in the experimental hand is 1, 2, 3, 3, 2, 1, 1, 2, X; the X represents an unidentifiable digit that was still forming when the experiment was terminated. The first three digits are the anterior duplication, the next three are the graft sequence and the last three make up the posterior duplication. Digits 1 and 2 of the anterior duplication are host-type but the well developed digit 3, although far too small to be considered graft-type, is too large and stout for an A. maculatum digit 3. Therefore, it probably has some contribution from both the host and the graft, but an hypertrophy of pure A. maculatum tissue because
of the nearby axolotl tissue cannot be excluded as an explanation for the size of this digit. Since this digit is about one-half the size expected for an axolotl digit 3, it is unlikely that it is of pure axolotl origin. Interestingly, the digit 3 of the graft sequence is the same size as the anterior duplication's digit 3 and it likely has a considerable contribution from the host. The remaining two digits of the graft sequence are graft-type. Digit 1 of the posterior duplication is graft-type and the last two digits are host-type.

**Dorsoventral axis reversed**

Essentially the same types of hands and wrists were obtained following the rotation of the graft's dorsoventral axis by 180° with respect to the host with heteroplastic exchanges as with homoplastic grafts. Of 19 cases (ten with axolotl hosts and nine with *A. maculatum* hosts), eight (42%) yielded four-digited inverted hands, two of these had an extra carpal bone. The entire hand and wrist and sometimes the distal forearm were graft-type in four of these, all with *A. maculatum* hosts (Fig. 13). In the other four (three with axolotl hosts and one with an *A. maculatum* host) the fourth digit was host-type.

**Figures 8-12**

Fig. 8. Whole mount of an *A. maculatum* right forelimb resulting from a homoplastic operation with the graft's dorsoventral axis reversed, ventral view. This limb has a serial repetition of digit 3 and one extra carpal bone. The anterior to posterior (top to bottom in the photograph) digital sequence is 1, 2, 3, 3, 4 (× 22).

Fig. 9. Whole mount of an *A. maculatum* right forelimb resulting from a homoplastic operation with the graft's dorsoventral axis reversed, ventral view. This limb has a serial repetition of digit 2 but no extra carpal bones. The anterior to posterior (top to bottom in the photograph) digital sequence is 1, 2, 2, 3, 4 (× 22).

Fig. 10. Whole mounts of both the left and right forelimbs from an axolotl host resulting from a heteroplastic control operation on the right side, dorsal view. The experimental limb (on the right in the photograph) displays a normal *A. maculatum* hand, wrist, and distal forearm (× 10).

Fig. 11. Whole mount of a left forelimb from an axolotl host resulting from a heteroplastic operation with the graft's anteroposterior axis reversed, dorsal view. This limb has both an anterior and a posterior duplication; each with contributions from both the graft and host. The anterior to posterior (top to bottom in the photograph) digital sequence is 1-axolotl type-2,2,1,2-*A. maculatum* type-3,4-axolotl type. The second digit 1 lies over the digit 2 anterior to it so that only the distal tip of that digit 1 (arrow) can be seen. The italicized numbers are the graft sequence of digits (× 12).

Fig. 12. Whole mount of both the left and right forelimbs from an *A. maculatum* host resulting from a heteroplastic operation on the right side with the graft's anteroposterior axis reversed, dorsal view. The experimental limb (on the right in the photograph) has both an anterior and a posterior duplication. The anterior to posterior (from left to right in the photograph) digital sequence is 1,2,-*A. maculatum* type-3,3-intermediate (see text)-2,1-axolotl type-2, X *A. maculatum* type. Most of the carpals are *A. maculatum* type but those under the axolotl digits are axolotl type. The complete forearm bones have *A. maculatum* character while the duplicated distal forearm parts have axolotl character. The italicized numbers are the graft sequence of digits (× 16).
Hands with five digits along the anteroposterior axis were found in nine (48%) cases, eight with an extra digit 3 (four with axolotl hosts and four with A. maculatum hosts) and one, on an axolotl host, with an extra digit 2. Five of these limbs had extra carpal elements. The second digit 3 as well as digit 4 was host-type in two cases with axolotl hosts and four cases with A. maculatum hosts (Fig. 14). Both digit 3's were graft-type with only digit 4 showing host character in two cases with axolotl hosts (Fig. 15). In the example with a serial repetition of digit 2, the distal phalanx of the second digit 2 was forked ventrad. Both digit 2's are graft-type but digits 3 and 4 are host-type (Fig. 16).

**Figures 13-18**

Fig. 13. Whole mounts of both the left and right forelimbs from an A. maculatum host resulting from a heteroplastic operation on the right side with the graft's dorsoventral axis reversed, ventral view. The experimental limb (on the left in the photograph) has a normal, inverted axolotl hand, wrist, and distal forearm (× 16).

Fig. 14. Whole mount of a right forelimb from an A. maculatum host resulting from a heteroplastic operation with the graft's dorsoventral axis reversed, ventral view. This limb has a serial repetition of digit 3 and one extra carpal bone. The anterior to posterior (top to bottom in the photograph) digital sequence is 1,2,3-axolotl type-3,4-axolotl type. The posterior carpals have an A. maculatum character while the rest have axolotl character (× 12).

Fig. 15. Whole mounts of both the left and right forelimbs from an axolotl host resulting from a heteroplastic operation on the left side with the graft's dorsoventral axis reversed, dorsal view. The experimental limb (on the left in the photograph) has a serial repetition of digit 3. The anterior to posterior (from right to left in the photograph) digital sequence is 1,2,3,3,-axolotl type-4-axolotl type. The proximal and posterior carpals have axolotl character while the rest have A. maculatum character (× 13).

Fig. 16. Whole mounts of both the left and right forelimbs from an axolotl host resulting from a heteroplastic operation on the left side with the graft's dorsoventral axis reversed, dorsal view. The experimental limb (on the left in the photograph) has a serial repetition of digit 2 with ventral fork on the second digit 2 and two extra carpal bones. The anterior to posterior (from right to left in the photograph) is 1,2,2,-axolotl type-3,4 axolotl type. The proximal and posterior carpals have axolotl character while the rest have A. maculatum character. Digit 1 is missing a phalanx (× 10).

Fig. 17. Whole mounts of both the left and right forelimbs from an axolotl host resulting from a heteroplastic operation on the left side with the graft's dorsoventral axis reversed, dorsal view. The experimental limb (on the left in the photograph) has three digit 3's and one extra carpal bone. The anterior to posterior (from right to left in the photograph) is X-indeterminate-3,3,3,4-axolotl type. The proximal and anterior carpals have axolotl character (× 13).

Fig. 18. Whole mount of both the left and right forelimbs from an A. maculatum host resulting from a heteroplastic operation on the right side with the graft's anteroposterior and dorsoventral axes reversed, ventral view. The experimental limb (on the left in the photograph) has both an anterior and a posterior duplication. The anterior to posterior (from right to left in the photograph) digital sequence is X,2,3,4,3,-axolotl type-2,1,-axolotl type-2,X,-A. maculatum type. The italicized numbers are the graft sequence of digits (× 13).
No dorsal duplications and one (5\%) ventral duplication (on an axolotl host) were found. This limb had an inverted five-digit hand with an extra digit 3 and a ventral duplication of digits 1 and 2. The duplication and digit 4 are host-type; the other digits are all graft-type.

The remaining case, pictured in Fig. 17, is most peculiar in that it has five digits with an anterior to posterior sequence of X, 3, 3, 3, 4. Digit X is abortive and of indeterminate origin. All three digit 3's and digit 4 are graft-type. The most proximal and anterior carpals are probably host-type, and the rest are graft-type.

**Anteroposterior and dorsoventral axes reversed**

Four cases of rotating both the anteroposterior and dorsoventral axes of the graft by 180° were obtained. Normally positioned hands occurred in three cases. One of these, with an *A. maculatum* host, displays a normal four-digit hand and wrist of graft origin. In the second, an axolotl host, also with a normal four-digit hand, only digits 1 and 2 are graft-type, while digits 3 and 4 are host-type. A single digit ventral duplication of graft-type was found with a normal set of digits in the third case (an axolotl host) and again digits 3 and 4 are host-type.

The final example is most instructive for it gave rise to a limb with an anterior and posterior duplication on an *A. maculatum* host (Fig. 18). The anterior to posterior digital sequence is X, 2, 3, 4, 3, 2, 1, 1, 2, X. All of the anterior duplication consists of host-type elements (digits X, 2, 3, 4). Digit 4 of the anterior duplication is shared with the graft sequence of digits. Although slightly large when compared to the contralateral limb, digit 3 as well as digit 4 of the graft sequence of digits are predominantly host-type. Digits 2 and 1 of this sequence are graft-type. The posterior duplication (digits 1, 2, X) has a graft-type first digit while digits 2 and X, an unidentifiable digit, are host-type.

**Triploid–diploid grafts**

The results of the triploid–diploid exchange grafts are consistent with the observations of the heteroplastic grafts. Individual digits, the wrist, forearm, and distal upperarm of two cases with both anterior and posterior duplications in diploid hosts, two cases with posterior duplications on triploid hosts, one case of ventral duplication on diploid host and several cases of inverted 4-digit hands were examined and each area was scored for the presence or absence of identifiable triploid cells. In each case cartilage, muscle, and epidermis were analyzed.

Triploid cells were identified in at least some of the digits of all of the duplications (Fig. 19). In both of the posterior duplications and one of the anterior duplications on diploid hosts no triploid cells could be found in the digit farthest from the graft sequence of digits. Both of the posterior duplications on triploid hosts contained identifiable triploid cells in all of the digits and one of these
Fig. 19. Photograph of a right axolotl limb which resulted from a triploid graft, with its anteroposterior axis reversed, on a diploid host. The two anterior digits (to the left in the photograph) are an anterior duplication. The next three digits are the graft sequence, and the posterior three are a posterior duplication. Triploid cells were found in all digits, except the most posterior one, and in the wrist and anterior distal forearm (T). Region I contained very few identifiable triploid cells and in region D no identifiable triploid cells could be found (×22).

hands had triploid cells in the digit of the graft sequence next to the duplication. The presence of triploid cells in the single ventral duplication with a triploid graft complements the single ventral duplication with a heteroplastic graft showing predominately a host origin. Ventral duplications, therefore, can have contributions from either the host or the graft. The wrists of all limbs with duplications contained triploid cells regardless of whether the host was diploid or triploid. The forearms of these limbs usually showed some triploidy when the host was diploid but the upperarms were always host-type.

When inverted 4-digit hands arose on diploid hosts, triploid cells were found throughout the hand and wrist and often into the distal forearm. In one case triploid cells were found almost as far proximal as the elbow. Triploid cells were never identified in the upperarm. In the complementary cases, when the host was triploid, triploid cells were always found at least as far distal as the mid-forearm and in one case in the wrist to the base of the metacarpals of the two most posterior digits.

Judging from these results it is likely that more mixing of graft and host cells
occurs both in the duplications and at the graft-host junction than is evident from the heteroplastic grafts. Additionally, it is of considerable interest that the ploidy of the epidermal cells did not always match that of the underlying mesodermal derivatives. Therefore, these two elements may move independently during the healing process and subsequent regulation.

DISCUSSION

The data presented in this report demonstrate that reversal of the anteroposterior axis of the distal aspect of the urodele limb bud with respect to the proximal aspect yields mirror-imaged duplication along the anteroposterior axis with a high frequency (96% for both homoplastic and heteroplastic grafts). Although mirror-imaged duplications along the dorsoventral axis occur following reversal of the dorsoventral axis, the frequency with which reversal of this axis yields duplications (8% for both homoplastic and heteroplastic grafts) is strikingly less than the frequency of duplications after reversal of the anteroposterior axis alone or both axes together. Consistent with this, most of the duplications which developed following simultaneous rotation of the anteroposterior and dorsoventral axes are along the anteroposterior axis.

As indicated by the heteroplastic exchanges and triploid–diploid grafts both anterior and posterior duplications arise from both the graft and the stump. Usually a considerable contribution from each was present, but one or the other sometimes gave rise to most, if not all, of a duplication. Therefore, both anterior and posterior mesoderm is capable of making a significant cellular contribution to duplications. This is different from the situation in *Xenopus* (Cameron & Fallon, 1977) and the chick (Fallon & Thorns, 1979) where anterior mesoderm has been shown to be the major cellular source for duplications (see also Saunders & Gasseling, 1968; Wolpert, 1969).

Not only can both the host and graft contribute to the formation of duplications in developing urodele limbs but also in two cases both contributed to the formation of the graft sequence of digits. Host cells which were not originally under the influence of the grafted field pattern, apparently come under its direction. These cells then formed structures which were originally the fate of graft cells. This observation is consistent with Weiss’s (1939) concept of progressive determination, which proposes that although a group of cells may be determined to form an organ, such as a limb, the specific roles which the individual cells will assume in morphogenesis of that organ may yet be undetermined. In this paper determined means that the fate in question cannot be changed. The new fate of the graft cells whose role was performed by host cells and the mechanics of such plastic adjustments awaits further investigation.

The high frequency of duplications after the reversal of the anteroposterior axis is consistent with the results from the limb bud of *Xenopus* (Cameron & Fallon, 1977) and the chick (Saunders, Gasseling & Gfeller, 1958) as well as the
newt and salamander regeneration blastema (Iten & Bryant, 1975; Bryant & Iten, 1976; Tank, 1978). However, the fact of duplications, albeit of low frequency, after the reversal of the dorsoventral axis differs from the results for *Xenopus* and the chick in which no duplications occur following dorsoventral axial rotations. It differs as well from the results for the regeneration blastema in which a high frequency of duplications can be seen after this manipulation. One possible explanation for this intermediate behavior is that the capacity to promote dorsoventral duplications develops with maturation of the limb but only becomes noticeable in systems having the ability to regenerate after histogenesis has been initiated. The low frequency of dorsoventral duplications found with the late-stage limb buds used in our experiments could then signify that this is a transition stage for the establishment of this behavior. In this context it is noteworthy that only the axolotl gave duplications after dorsoventral rotation. Although the limb bud of this species has the same external morphology as the other species, the axolotl has reached a later stage of development by most other criteria (Schreckenberg & Jacobson, 1975). It is possible that limb buds of the same morphology from larvae of *N. viridescens* and *A. maculatum* are less mature than those of the axolotl. This would be consistent with a gradual establishment of the capacity to promote dorsoventral duplications, as proposed above.

This possibility appears to be contradicted by Swett’s (1927, 1930) observations of numerous duplications following dorsoventral axial rotations at stages 32 through 40. In these operations Swett took the dorsoventral axis of the limb disc to be coincident with that of the embryonic body but this may not be accurate. Harrison (1925) describes in detail the evidence that both the anteroposterior and dorsoventral axes differ by at least 45° from the axes of the body prior to and during these stages (see also Tank, Carlson & Connelly, 1976). Swett (1930) disagrees with Harrison about the orientation of the dorsoventral axis but is in complete agreement about the orientation of the anteroposterior axis; i.e. he claims that these axes are not perpendicular. This means that when he did his dorsoventral operations he actually rotated the anteroposterior axis by at least 90°, which may well be sufficient to promote duplications.

Although reversal of the dorsoventral axis usually failed to generate mirror-imaged duplications, it is of special interest that this procedure consistently affected the organization of the anteroposterior axis by promoting a serial repetition in the anterior to posterior digital sequence, without a change in the axial polarity. In some cases both of the repeated digits developed from the graft and in other cases one digit was from the graft and one from the host. Such an arrangement could result from a dorsal or ventral single-digit duplication which moved into line with the other digits. If this were so then one would expect that the extra digit would have a ventral curvature, i.e. a dorsoventral polarity which mirrors that of the graft. However, in some cases both of the repeated digits displayed a dorsal curvature. Additionally, two digit 3’s were found in
a ventral duplication. Because of these observations we think that serial repetitions are not examples of single-digit duplications which subsequently moved, rather they are truly an effect on the organization of the anteroposterior axis. These results indicate, for the first time, a recognition of an interdependence of the dorsoventral and anteroposterior axes during pattern regulation in the developing urodele limb.

Two models have recently been proposed for pattern regulation and pattern formation in the developing urodele limb. One is the polar coordinate model originally developed for the *Drosophila* imaginal disc, the regenerating cockroach leg, and the regenerating urodele limb (French, Bryant & Bryant, 1976; Bryant & Iten, 1976). The other is a diffusible morphogen model based on experiments with the axolotl embryo (Slack, 1977a, b). Both of these models rely on the concept of positional information as put forth by Wolpert (1969, 1971) who asserts that there is a 'mechanism whereby the cell's position within the system... is uniquely specified and this information is used to determine the nature of its differentiation' (Wolpert, 1969).

The polar coordinate model suggests that the arrangement of positional information is best described by a polar coordinate system. The circular sequence of values corresponds to positions about the circumference of the limb while the radial sequence of values corresponds to positions along the proximodistal axis of the limb, with the central position representing the distal limb tip. Duplications result from circular sequences generated by intercalation following axial rotations and the handedness of a duplication depends on the direction of the sequence.

Our experiments apply only to the rules for the circular sequence. If the polar coordinate model can describe regulation and the arrangement of positional information in the urodele limb bud, then there must exist a distribution of circumferential values which would allow the prediction of the results obtained following the operations that were performed. From the occurrence of duplications two aspects of the distribution are obvious. Opposite values must be at or very near the two ends of the anteroposterior axis and opposite values must not be at or very near the two ends of the dorsoventral axis. This distribution differs from the distribution described for the adult *N. viridescens* in which duplications are obtained following rotation of the regeneration blastemas dorsoventral axis with respect to the stump (Bryant & Iten, 1976). Therefore, if the model is applicable to the urodele limb bud then the distribution of the circular positional values must change in *N. viridescens* as the animal develops to maturity.

Severe difficulties are encountered, however, when one tries to find a distribution of circular values which will allow one to predict either the occurrence of an extra digit along the anteroposterior axis following the reversal of the dorsoventral axis or the occurrence of duplications with a mixed handedness following simultaneous rotation of both the anteroposterior and dorsoventral axes. In
Pattern regulation after axial rotations in urodele limb buds

fact, we believe that the constraints of the model do not allow the prediction of either of these results. If this is true, then this model does not adequately describe pattern regulation in the developing urodele limb.

Slack (1979) proposed that the patterns along the dorsoventral and anteroposterior axes are determined independently and he has suggested that the anteroposterior pattern is determined by a monotonic gradient of a morphogen across the limb bud (Slack, 1977b). The high point for the gradient is established by the flank tissue just posterior to the limb region. Such a high point of morphogenetic activity has been described in the limb buds of representative mammalian, avian, reptilian and amphibian species (MacCabe & Parker, 1976; Tickle, Shellswell, Crawley & Wolpert, 1976; Fallon & Crosby, 1977). This high point has been called the zone of polarizing activity or polarizing zone (Balcuns, Gasseling & Saunders, 1970; Cameron & Fallon, 1977; Slack, 1977b). Duplications would then result from an anteroposterior axis rotation because of the establishment of new gradients between the newly juxtaposed anterior and posterior tissues. The data reported here are consistent with this view.

However, as discussed above, the occurrence of a serial repetition along the anteroposterior axis following reversal of the dorsoventral axis contradicts the assertion that each axis is independent of the other. This same observation is also difficult to reconcile with the suggestion of a monotonic gradient of a morphogen. It is not evident how such a gradient can be induced to maintain a signal level within the threshold boundaries for a single digit over a sufficient distance to form a repetition of that digit while exhibiting a normal decline both anterior and posterior to this position.

The results of the experiments reported in this paper are not explained to our satisfaction by existing models. The inadequacies of these models, as well as our own inability to propose a more satisfactory alternative can be attributed to the paucity of information about the cellular events during development in normal and experimentally altered limbs. Specifically, such models assume knowledge about events at the cellular level. To build models which accurately describe pattern formation and pattern regulation one must know: (a) which cells participate; (b) what the variables are to which the cells are responding when, for example, the axes of the field district are misaligned; and (c) what the initial response is and what later responses may be. This report describes initial steps toward identifying which cells participate in the regulation. Little is known about the variables to which the cells are responding or what these responses might be. We suggest that further research be directed toward elucidating the events which occur at the cellular level when operations, such as axial misalignments, are performed at the gross level.

This investigation was supported by NSF Grant No. PCM7903980 and by NIH Training Grant No. 5T32GM07215. We are grateful to Drs S. V. Bryant, A. W. Clark, J. M. Frederick, L. Lemanski, E. Schultz, D. B. Slatterback, D. R. Sonneborn, D. L. Stocum
and P. W. Tank, Ms D. Rowe and Ms E. Boutin for their constructive criticism of this manuscript; and to Ms B. Kay Simandl for technical assistance, Ms L. Taylor for making the drawing, and Ms Donna Leonard for typing the manuscript. We thank Drs D. Stocum, L. Lemanski, T. Poole and R. Tassava for supplying some of the animals used in these studies.

Special thanks are due Dr Stocum for inviting us into his laboratory to learn some of the techniques for these experiments.

REFERENCES


Pattern regulation after axial rotations in urodele limb buds


(Received 9 October 1979, revised 28 April 1980)