Retinoic acid coordinately proximalizes regenerate pattern and blastema differential affinity in axolotl limbs

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Summary

An assay that detects position-related differences in affinity of axolotl regeneration blastema cells in vivo was used to test whether retinoic acid, which proximalizes regenerate pattern, simultaneously proximalizes blastema cell affinity. The assay involved autografting or homografting late bud forelimb blastemas derived from the wrist, elbow or midupper arm levels to the dorsal surface of the blastema-stump junction of an ipsilateral, medium-bud-stage hindlimb regenerating from the midthigh level. The grafted blastemas consistently displaced to their corresponding levels on the proximodistal axis of the host regenerate, indicating the existence of level-specific differences in blastema cell affinity. Retinoic acid proximalized the pattern of donor forelimb regenerates to the level of the girdle and abolished their displacement behaviour on untreated host hindlimbs. Conversely, untreated forelimb donor blastemas displaced distally to their corresponding levels on host ankle regenerates, that had been proximalized to the level of the girdle by retinoic acid. These results indicate that positional memory in regenerating limbs is directly related to blastema cell affinity, and that very similar or identical sets of level-specific affinity properties are shared by forelimb and hindlimb cells.

Key words: differential affinity, positional memory, retinoids, axolotl, limb, regeneration, pattern formation.

Introduction

Mesenchymal cells of the amphibian limb regeneration blastema inherit from their parent limb cells a memory of their level of origin. This positional memory (Carlson, 1975) or value (Wolpert, 1971) specifies the proximal boundary of the regenerate by preventing blastema cells forming structures proximal to their level of origin (Stocum, 1984). Since the limb regenerates exactly what is amputated from any level, its proximodistal (PD) axis can be viewed as a series of such potential regenerate boundaries. Similar sets of level-specific memories appear to exist along the anteroposterior (AP) and dorsoventral (DV) axes of the limb (Carlson, 1975). The boundary function of positional memory in regeneration is seen in experiments that juxtapose cells from different axial levels (i.e. cells with different positional memories). The result is the dedifferentiation and/or mitosis of cells on either side of the interface, followed by the restoration, via intercalary regeneration, of the pattern normally occupying the space between the two levels (Iten & Bryant, 1975; Pescitelli & Stocum, 1980; Muneoka & Bryant, 1984).

Despite the importance of positional memory in reestablishing a normal pattern of cell neighbours during regeneration, little is known of its cellular or molecular basis. One approach to this problem is to identify cellular properties that exhibit position-related differences along a limb axis and which therefore might be directly related to positional memory. Demonstration of a direct relationship requires showing that experimental modification of regenerate pattern is accompanied by modification of the cellular property.

Cell affinity is one property that might be related to positional memory, as shown by an assay of blastema cell adhesiveness in vitro (Nardi & Stocum, 1983). In this assay, binary combinations of wrist, elbow or upper arm blastema mesenchyme were cultured in vitro, with one member of each pair being marked by
This result is consistent with Steinberg's (1963, 1978) differential adhesion hypothesis, and suggests the existence of position-related differences in cell adhesiveness along the PD axis of the limb.

Vitamin A and its derivatives (retinoids) are agents that modify regenerate pattern. The effect of retinoids is to proximalize regenerate pattern in the PD axis, resulting in serial repetition in the regenerate of structures proximal to the amputation plane (Niazi & Saxena, 1978; Maden, 1982; Thoms & Stocum, 1984). In the AP axis, retinoids posteriorize the pattern, resulting in mirror-image duplications (in regenerating anuran limbs and embryonic chick limbs; Niazi & Saxena, 1978; Maden, 1983; Tickle et al. 1982), or the completion of the missing posterior pattern following amputation of an anterior half limb (in urodeles; Kim & Stocum, 1986a). All these effects have been interpreted as a reprogramming of the positional memory of blastema cells to a new level. Measurements of endogenous retinoic acid in embryonic chick limb buds have yielded results suggesting that this retinoid might be a morphogen that specifies anteroposterior axial pattern in the limb bud (Thaller & Eichele, 1987).

Retinoids modify the phenotypic differentiation of a variety of cultured normal and tumour cells. These modifications are accompanied by quantitative changes in cell–cell and cell–substrate adhesiveness that are correlated with qualitative and/or quantitative changes in cell surface glycoconjugate composition (Lotan, 1980; Shapiro & Mott, 1981; Roberts & Sporn, 1984, for reviews). Taken in conjunction with the effects of retinoids on regenerate axial pattern, these findings suggest that positional memory and blastema cell differential affinity might be directly related.

A direct test of this hypothesis requires an assay that can reveal changes in both blastema cell affinity and in regenerate pattern in response to retinoid. The major disadvantage of an assay for blastema cell adhesiveness in vitro (Nardi & Stocum, 1983) is that the blastema mesenchyme does not undergo morphogenesis in vitro, so correlations between changes in adhesiveness and pattern in response to retinoid cannot be made. Therefore, we developed an assay which detects level-specific differences in blastema cell affinity in vivo, and in which blastema morphogenesis takes place. Here we describe this assay and use it to show that retinoic acid coordinately proximalizes blastema cell affinity and regenerate pattern.

### Materials and methods

#### Animals and maintenance

Larval axolotls (Ambystoma mexicanum) were provided by the Indiana University Axolotl Colony or were obtained from spawnings of our laboratory stock. They were reared individually in 50% Holtfreter solution in waxed paper cups, at room temperature (21°C), and fed freshly hatched brine shrimp or frozen brine shrimp every other day. At the time of amputation, the animals weighed 2–5 g and were 6–9 cm in length.

#### Surgical operations

1. **General procedures**

   Animals were anaesthetized in Benzocaine (Sigma) dissolved in 100% Holtfreter solution (0.007% w/v). Operations were performed with sharp watchmaker’s forceps and indectomy scissors. Amputations were done in air after placing anaesthetized animals on Benzocaine-soaked filter paper. Grafting was done in deep Petri dishes lined with filter paper, with the animal submerged in anaesthetic solution. After grafting, the animals were maintained in the anaesthetic solution for 5–6 h at room temperature to facilitate healing, then placed in 100% Holtfreter solution. They were left undisturbed in the dark for 2 days and special precautions in feeding and changing water were observed for an additional 5 days postgrafting to avoid dislodging grafts. All grafts healed in 24 h and blood circulation in the grafts was observed by 1–2 days post-operation.

2. **Differential affinity assay in vivo**

   Fig. 1 diagrams the assay used to detect level-specific differences in blastema cell affinity in vivo. Forelimbs were amputated bilaterally through the wrist, elbow or distal midupper arm levels, and hindlimbs of the same animal were amputated bilaterally through the midthigh. The forelimbs were allowed to regenerate to the late bud stage, at which time the hindlimb regenerates were at medium bud (staging according to Stocum (1979)). The forelimb blastemas were autografted to the blastema–stump junction of the ipsilateral hindlimb after removing a piece of blastema epidermis and stump skin from the dorsal surface of the junction. The AP axis of the grafted blastema was aligned with that of the host limb. Late bud blastemas rather than earlier stages were chosen as donors because they were easier to manipulate and they exhibit a high frequency of autonomous development when grafted to the dorsal fin (Stocum, 1988). The prediction was that level-specific differences in blastema cell affinity would be detected as displacement of the grafts to their corresponding levels on the PD axis of the host regenerate.

3. **Test for coordinate proximalization of regenerate pattern and blastema cell affinity**

   For each experiment, a fresh stock solution of all-trans retinoic acid (RA, Sigma) was made by dissolving 50 mg of RA in 1 ml of dimethyl sulphoxide (DMSO) under subdued light (to minimize photo-isomerization). Two homografting experiments were done, in which either donor or host
Cellular basis of positional memory

Donor forelimbs regenerating from wrist, elbow or midupper arm

Host hindlimbs regenerating from midthigh

Displacement of grafts to their corresponding host levels

Fig. 1. Scheme and result of the differential affinity assay in vivo. Wrist (W), elbow (E), or midupper arm (UA) blastemas were autografted to the blastema-stump junction of hindlimbs regenerating from the midthigh. The dashed oval indicates the area where a patch of blastema epidermis and stump skin was removed from the dorsal surface of the host limb, and the line bisecting this oval indicates the blastema-stump junction. The grafted blastemas displaced to their corresponding host levels. A, ankle; K, knee; T, midthigh of the host hindlimb.

Groups of animals were treated with RA prior to their use in the differential affinity assay. The animals were injected intraperitoneally, via microlitre syringe, with 100 ng of RA per g body weight, at 4 days postamputation (during the accumulation blastema stage). This dose and time of injection have been shown previously to maximize proximalization of pattern in regenerating forelimbs and hindlimbs (Thoms & Stocum, 1984; Niazi et al. 1985; Kim & Stocum, 1986b).

(a) RA-treated forelimb blastemas homografted to untreated hindlimbs (Fig. 2). The forelimbs of the donor animals were amputated bilaterally through the wrist, elbow or midupper arm levels and their hindlimbs were amputated bilaterally at the ankle, knee or midthigh levels. Host hindlimbs were amputated bilaterally through the midthigh. 4 days later, the donor animals were injected with RA to proximalize positional memory. When the donor forelimb regenerates reached the late bud stage, they were homografted to the blastema-stump junction of ipsilateral, medium-bud-stage host hindlimbs, as described in (2), and their displacement behaviour was correlated with their final morphogenesis. As a control for proximalization of pattern, the donor hindlimbs were allowed to regenerate in situ.

Regeneration is inhibited during RA-induced proximalization by approximately 10 days in animals of the size used here, and the late bud stage of an RA-treated donor forelimb was not attained until 20–22 days postamputation instead of the normal 10–12 days postamputation. Host hindlimbs were, therefore, amputated 10 days later than the donor forelimbs, so they would reach the medium bud stage at approximately the same time that the donor forelimbs reached the late bud stage.

(b) Untreated forelimb blastemas homografted to RA-treated hindlimbs (Fig. 3). In this experiment, host hindlimbs were amputated bilaterally at the ankle level. The animals were injected with RA 4 days postamputation to proximalize regenerate positional memory and allowed to regenerate to the medium bud stage. Donor forelimbs were amputated bilaterally through the wrist, elbow or midupper arm levels and allowed to regenerate to late bud. The forelimb blastemas were then homografted to the ipsilateral blastema-stump junction of the RA-treated host hindlimb and their displacement behaviour correlated with their final morphogenesis.

Due to the inhibitory effect of RA, host hindlimbs did not reach the medium bud stage until 20–22 days postamputation instead of the usual 10–12 days postamputation.

Fig. 2. Scheme and result of homografting RA-treated wrist elbow or midupper arm blastemas to the blastema-stump junction of a host hindlimb regenerating from the midthigh. Symbols as in Fig. 1. Displacement behaviour was abolished in the grafted wrist and elbow blastemas.
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Untreated donor forelimbs regenerating from wrist, elbow, or midupper arm RA-treated host hindlimbs regenerating from ankle

Displacement of grafts to their corresponding host levels

Fig. 3. Scheme and result of homografting untreated wrist, elbow or midupper arm blastemas to the blastema–stump junction of a RA-treated host hindlimb regenerating from the ankle level (A). ST, SK, SA refer to the stump thigh, knee and ankle, respectively. DT, DK, DA refer to the thigh, knee and ankle, respectively, of the serially duplicated regenerate. All other symbols as in Fig. 1.

Donor forelimbs were therefore amputated at the wrist, elbow or midupper arm 10 days later than the host hindlimbs, so they would attain the late bud stage when the host regenerates were at medium bud.

(c) Controls. Two sets of control experiments were done. First, animals regenerating from the wrist, elbow or midupper arm of the forelimbs, and from the ankle, knee or midthigh of the hindlimbs, were injected with 2 μl of DMSO per g body weight at 4 days postamputation and allowed to regenerate in situ. This control determined whether DMSO alone had any effect on normal regeneration. Second, animals regenerating from the wrist, elbow or midupper arm levels were injected with 2 μl of DMSO per g body weight at 4 days postamputation. When the limbs of these animals had regenerated to the late bud stage, the blastemas were homografted to the blastema–stump junction of ipsilateral, medium-bud-stage hindlimb hosts regenerating from the midthigh, and their displacement behaviour correlated with regenerate morphology. This control determined whether DMSO by itself would affect blastema cell affinity.

Staining of limb skeletons

The grafted-host combinations were allowed to regenerate for 5–7 weeks, harvested and fixed in Gregg's fixative. The limb skeletons were stained toto for cartilage according to the method of Fox (1982), substituting methylene blue for Victoria Blue B. Donor forelimb regenerates were distinguished from host hindlimb regenerates by their different skeletal patterns (Pescitelli & Stocum, 1980).

Results

Assay for blastema cell differential affinity

All of the surviving forelimb blastema grafts developed as recognizable forelimb regenerates. 83% of the wrist and 74% of the elbow blastemas displaced distally from the host midthigh level to their corresponding levels on the PD axis of the host regenerate, thus eliminating the positional discontinuity between graft and host (Table 1). Hence the fully developed wrist regenerates articulated with the host ankle, and the elbow regenerates articulated with the host knee (Figs 1, 4A, B). 75% of the midupper arm blastemas did not displace and their regenerates articulated with the host midthigh, which is their corresponding level (Figs 1, 4C). Supernumerary autopodial structures were induced between the ventral surface of the graft and dorsal surface of the host regenerate in two cases of wrist blastema grafts.

In a few cases, the donor regenerates displaced to a level that was either distal to (elbow or midupper arm blastema grafts) or proximal to (wrist or elbow blastema grafts) their corresponding host level (Table 1). Displacement of elbow and midupper arm grafts distal to their corresponding host level most likely reflects resorption of graft cells destined to form proximal parts, allowing displacement to the level established by the resorption. The reasons why some grafted wrist and elbow blastemas failed to displace or only partially displaced are unknown. This failure may be related to stage variations in which either the graft or the host, or both, were developmentally too advanced to allow displacement. In several of these cases, however, intercalary regeneration occurred, restoring the pattern between graft and host levels.

Visual inspection of the limbs 48 h after grafting often revealed signs that were correlated with displacement. Wrist and elbow blastemas appeared to be further distal on the host regenerate than upper arm blastemas, which remained at the blastema–stump junction. In addition, the initial 90° angle between the ventral surface of the graft and dorsal surface of the host was frequently reduced in wrist and elbow grafts, but remained constant in upper arm grafts. These observations indicated that displacement began by 48 h postgrafting, and possibly earlier.
Cellular basis of positional memory

Table 1. Displacement behaviour of forelimb wrist, elbow and upper arm blastemas autografted to hindlimbs

<table>
<thead>
<tr>
<th>Donor</th>
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<th>F</th>
<th>K</th>
<th>T</th>
</tr>
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<tr>
<td>Wrist</td>
<td>32</td>
<td>1</td>
<td>(3-3)</td>
<td>2 (6-7)*</td>
<td>2 (6-7)†</td>
</tr>
<tr>
<td>Elbow</td>
<td>23</td>
<td>1</td>
<td>(4-3)</td>
<td>1 (4-3)§</td>
<td>17 (74)</td>
</tr>
<tr>
<td>Upper arm</td>
<td>12</td>
<td>9</td>
<td>(75)</td>
<td>0</td>
<td>3 (25)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are percentages of the total number of surviving grafts.
0 = no displacement.
F, K, T = displacement to the level of the distal femur, the knee and the tarsus, respectively.
* Both cases intercalated zeugopodial and distal stylopodial structures.
† Both cases intercalated zeugopodial structures.
‡ Two cases formed supernumerary hands.
§ Intercalated a distal stylopodial element.

Table 2. Displacement behaviour of RA-treated elbow, wrist and upper arm blastemas homografted to untreated hindlimbs

<table>
<thead>
<tr>
<th>Donor</th>
<th>Total</th>
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<th>F</th>
<th>K</th>
<th>T</th>
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<tr>
<td>Wrist</td>
<td>15</td>
<td>12</td>
<td>(80)*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Elbow</td>
<td>11</td>
<td>11</td>
<td>(100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper arm</td>
<td>16</td>
<td>16</td>
<td>(100)*†</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate percentage of total surviving grafts.
0 = no displacement.
F, K, T = displacement to the level of the distal femur, the knee or the tarsus, respectively.
* Two grafts developed as unidentifiable hypomorphic structures.
† Four grafts developed as unidentifiable hypomorphic structures.

Effect of RA on blastema morphogenesis and differential affinity

(1) RA-treated forelimb blastemas homografted to untreated hindlimbs

100% of RA-treated elbow and midupper arm blastemas, and 80% of RA-treated wrist blastemas failed to displace from the graft site (Table 2). 17% of the nondonor wrist blastemas and 25% of the nondonor upper arm blastemas either redifferentiated no cartilage or hypomorphic cartilage elements. The remainder of the nondisplaced wrist blastemas developed forelimb regenerates that were proximalized to the level of the shoulder girdle; i.e. serially repeated a girdle, stylopodium and zeugopodium (Figs 2, 5A,B). The control hindlimb regenerates of the donor animals were also proximalized to the level of the pelvic girdle, indicating that the grafted regenerates developed just as they would have in situ. Hence failure of graft displacement was strongly correlated with proximalization of regenerate pattern.

20% of the grafted RA-treated wrist blastemas and their in situ hindlimb controls serially repeated only a zeugopodium. These grafts displaced to the knee level of the host regenerate, thus behaving like native elbow blastemas and showing that the degree of displacement matched the level of proximalization (Fig. 5C).

(2) Untreated forelimb blastemas homografted to RA-treated hindlimbs

Host ankle regenerates were proximalized by RA to the level of the proximal femur or pelvic girdle, resulting in the serial repetition of girdle, stylopodium and zeugopodium in the regenerate. 100% of the wrist blastemas were displaced to the ankle level of the host regenerate and 59% of the elbow blastemas were displaced to the knee level of the host regenerate (Table 3; Figs 3, 6A,B). 69% of the upper arm blastemas displaced only slightly, to the proximal or midfemur level of the host regenerate (Figs 3, 6C). 41% of the elbow blastemas and 31% of the upper arm blastemas were displaced beyond their corresponding level on the host regenerate. In these cases, it is likely that resorption of cells established a more distal basal level in the graft, allowing it to displace to a correspondingly more distal level on the host regenerate.

(3) Controls

All of the blastemas of DMSO-treated animals regenerated normally in situ. DMSO-treated blastemas tested in the differential affinity assay behaved like untreated blastemas and displaced distally to their corresponding level on the host regenerate in the majority of cases (Table 3; Figs 3, 6A,B; compare with Fig. 4A–C). The frequency of incomplete displacement, however, was over twice that for untreated donor blastemas in the wrist and elbow groups (compare Tables 1 and 3). Intercalary regeneration eliminated the pattern discontinuity in all cases of...
incomplete displacement. A single upper arm graft displaced to the distal femur and formed a lower arm and hand, again suggesting the establishment of a more distal graft basal level by resorption.

Discussion

The in vivo assay reveals position-related differential affinity of blastema cells

The first objective of our experiments was to develop an assay that detects level-specific differences in blastema cell affinity in vivo, and simultaneously allows the morphogenesis and redifferentiation of regenerate pattern to take place. This objective was accomplished by grafting donor forelimb blastemas from three different PD levels to the blastema-stump junction of a host hindlimb regenerating from a standard midthigh level. The grafted blastemas developed into normal forelimb regenerates, and each made host neighbour choices that specifically matched its own level of origin, thus eliminating
positioned discontinuity between host and graft. Thus midupper arm blastemas remained at the graft site, whereas elbow and wrist blastemas were displaced to the knee and ankle of the host regenerate, respectively.

This assay is a modification of an experiment designed by Shuraleff & Thornton (1967) to test another hypothesis, namely that grafting a given distal structure onto an axolotl hindlimb amputated through the midthigh would suppress the development of that structure during regeneration of the hindlimb. They grafted ankle-level tissue onto the dorsal half of the amputation surface of the midthigh and sealed the ventral half of the amputation surface.

Fig. 4. Displacement and development of regenerates after autografting blastemas derived from different levels of the forelimb to the blastema-stump junction of host hindlimbs regenerating from the midthigh. All magnifications are x8. The arrows indicate the original position of the graft on the host.

(A) Right wrist blastema autografted to right hindlimb, dorsal view of hindlimb. The blastema displaced to the tarsal (t) level of the host regenerate and formed several carpals (c) and three digits, indicated by the asterisks. The anterior-to-posterior sequence of host toes is indicated by the numbers 1–5. The tibia, fibula, femur and pelvic girdle of the host limb skeleton are indicated by tb, fb, f and g, respectively.

(B) Left elbow blastema autografted to left hindlimb, anterior view of hindlimb. The blastema displaced to the knee level of the host regenerate (arrowhead) and formed a radius and ulna (u), several carpals (c) and four digits, plus a supernumerary digit (x). The host femur and tibia/fibula are indicated by f and f, respectively.

(C) Left midupper arm blastema autografted to left hindlimb, anterodorsal view. The most proximal element formed by the graft was the distal end of the humerus (h), and it displaced slightly to the corresponding level on the host regenerate. The graft radius and ulna are indicated by ru. The host femur, tibia and fibula are indicated by f, t and fb, respectively.

Fig. 5. Result of homografting RA-treated blastemas derived from different levels of the forelimb to the blastema-stump junction of untreated hindlimbs regenerating from the midthigh. All magnifications are x8 except the insert in (C), which is x6. The arrows indicate the original position of the graft on the host.

(A) Right RA-treated wrist blastema homografted to right hindlimb, anterior view of hindlimb. The proximal boundary of the blastema was reset to the level of the shoulder girdle (g), so that the regenerate now formed a complete forelimb. The graft did not displace. The arrowhead indicates the host tarsals, the level to which an untreated wrist blastema would normally displace.

(B) Left RA-treated elbow blastema homografted to left hindlimb, anterior view of hindlimb. The graft was proximalized to the girdle level, formed a complete limb and failed to displace. The arrowhead indicates the knee level, to which an untreated elbow blastema would normally displace.

(C) Right RA-treated wrist blastema homografted to right hindlimb, anterior view of hindlimb. The graft was proximalized to the elbow level and formed radius and ulna (u), carpals (c) and four digits. Accordingly, it displaced to the knee level (arrowhead) of the host regenerate. The insert below shows the in situ ankle regenerate of the donor animal. This regenerate was proximalized to the level of the knee. In this limb, the bar indicates the regenerate–stump junction and the stump tibia and fibula are indicated by t and f, respectively.
The results are consistent with Steinberg's (1963, 1978) differential adhesion hypothesis, which predicts that cell populations differing in adhesivity will exchange neighbours until the intensity of like-cell cohesions is maximized. Clearly, very similar or identical sets of PD level-specific affinity properties are shared by forelimb and hindlimb cells. Because donor blastemas from different levels 'sort out' along the PD axis of a host regenerate according to their affinity for cells of corresponding host levels, we have termed the differential displacement behaviour affinophoresis.

Position-related differential cell affinity has also been demonstrated in a number of studies on insect development. Nardi & Kafatos (1976a, b) found that squares of *Manduca* pupal wing epithelium grafted to different axial positions in the wing rounded up and invaginated, indicating increased cohesion among graft cells and their decreased adhesion to surrounding cells. The degree of rounding up and invagination was proportional to the distance between the original and final positions of the grafts, suggesting the existence of gradients of adhesiveness along the wing.

When dorsal and ventral imaginal discs of *Drosophila* were dissociated, reaggregated in binary combinations and cultured within an adult female host, cells derived from different discs at the same DV level (leg–leg or wing–haltere) failed to sort out, whereas disc cells from different DV levels (wing–leg) sorted out (Fehon & Schubiger, 1985). Identical sorting behaviours were obtained in binary dorsal plus ventral reaggregates of notum, wing and leg discs cultured in vitro (Fausto-Sterling & Hsieh, 1987). Finally, Gauger et al. (1985) showed that dissociated disc cells cultured with whole embryos bound preferentially to their body segments of origin. These data suggest the existence of dorsal–ventral and segment-specific cell affinities in *Drosophila*.

Our experiments do not address the question of the mechanism whereby blastema displacement occurs.

Fig. 6. Result of homografting blastemas derived from different forelimb levels to the blastema–stump junction of RA-treated hindlimbs regenerating from the ankle level. All magnifications are ×8. In each case, the host regenerate was proximalized to the level of the proximal femur or pelvic girdle and formed a complete limb angled 90° to the stump. The femur, tibia and fibula of the host regenerates are indicated by f, t and fb, respectively.

(A) Left wrist blastema homografted to left RA-treated hindlimb, dorsal view of host regenerate. The blastema displaced to the tarsus level (arrowhead) of the host regenerate and formed carpals and digits. The graft digits are marked with asterisks.

(B) Left elbow blastema homografted to left RA-treated hindlimb, dorsal view of host regenerate. The blastema displaced to the knee level (arrowhead) of the host regenerate and formed radius (r), ulna (u), carpals and digits.

(C) Right midupper arm blastema homografted to right RA-treated hindlimb, dorsal view of host regenerate. The blastema failed to displace from the graft site and formed a distal humerus (h), radius (r), ulna (u), carpals and digits.
Cellular basis of positional memory

Fig. 7. Result of homografting DMSO-treated blastemas derived from different forelimb levels to the blastema-stump junction of host hindlimbs regenerating from the midthigh. All magnifications are ×8. The arrows indicate the original position of the graft on the host.

(A) Right DMSO-treated wrist blastema homografted to right hindlimb, posterior view of host regenerate. The graft (G) displaced to the level of the host tarsus (arrowhead) and formed carpals and digits.

(B) Left DMSO-treated elbow blastema homografted to left hindlimb, dorsal view of host. The grafted forelimb regenerate (G) formed radius, ulna, carpals and digits, and shifted with respect to the host so that its ventral surface is visible. The ulna of the graft and fibula of the host regenerate have fused proximally (arrowhead).

(C) Right DMSO-treated midupper arm blastema homografted to right hindlimb, dorsal view of host regenerate. The blastema displaced slightly toward the distal end of the femur, and formed a distal humerus (h), radius (r), ulna (u), carpals and digits.

on the host regenerate. The grafted blastema might be passively pushed along the host blastema by distal sliding movements of the host epidermis until the corresponding host level is reached, where the strength of cohesion between graft and host mesenchyme cells and/or extracellular matrix is strong enough to anchor the graft. Distal epidermal sliding has been reported to occur in *Xenopus* and chick limb buds (Tschumi, 1956; Amprino, 1965). Alternatively, active movements of mesenchymal cells at the graft–host interface might provide the motive force. For example, Ettensohn & McClay (1986) have shown that sea urchin primary mesenchyme cells injected into the blastocoel at the animal pole migrate to their normal site of ingestion, join host primary mesenchyme cells and develop normally into spicules.

**RA coordinately proximalizes regenerate pattern and blastema cell affinity**

The second objective of our experiments was to test, using the affinophoresis assay, whether retinoic acid coordinately proximalizes blastema cell affinity and regenerate pattern, and hence to determine whether positional memory is directly related to blastema cell affinity. The results showed clearly that RA-treated wrist and elbow blastemas behaved in the assay like native midupper arm blastemas; they failed to displace from the midthigh level of the untreated host regenerate. Likewise, untreated wrist, elbow and upper arm blastemas displaced on RA-treated host ankle regenerates that were proximalized to the girdle in a manner identical to their displacement on native midthigh regenerates. These data demonstrate that RA coordinately proximalizes level-specific blastemal cell affinity and positional memory, and allow us to conclude that the two are correlative.

There is considerable evidence that cell surface and matrix glycoconjugates (glycoproteins, glycolipids, proteoglycans) mediate cell recognition and affinity during development (Edelman, 1985; Thiery et al. 1985; Gallin et al. 1986; McClay & Ettensohn, 1987). Retinoids alter cell–cell or cell–substrate adhesive-ness of fibroblasts, chondrocytes, epithelial cells and various types of carcinoma cells concomitantly with changes in their differentiation or morphology. These effects are correlated with qualitative and/or quantitative changes in cell surface glycoconjugates, including hormone and growth factor receptors (for reviews, see Lotan, 1980; Shapiro & Mott, 1981; Roberts & Sporn, 1984). In addition, transcriptional and translational studies have shown that retinoids alter patterns of keratin gene transcription in cultured human keratinocytes and conjunctival cells (Fuchs & Green, 1981; Eckert & Green, 1984). Similarly, it has been shown that the enhanced synthesis of laminin
### Table 4. Displacement behaviour of control DMSO-treated wrist, elbow and upper arm blastemas homografted to untreated hindlimbs

<table>
<thead>
<tr>
<th>Donor</th>
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<td>2 (13)*</td>
<td>4 (27)†</td>
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<tr>
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</tr>
<tr>
<td>Upper arm</td>
<td>10</td>
<td>9 (90)</td>
<td>0</td>
<td>1 (10)</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to percentages of total surviving grafts.

0 = no displacement.

F, K, T = displacement to the level of the distal femur, the knee and the tarsus, respectively.

* Intercalated a zeugopodium and distal tip of stylopodium.

† Intercalated a zeugopodium.

‡ Intercalated a zeugopodium and the distal half of a stylopodium.

subunits in RA-treated F9 embryonal carcinoma cells is under transcriptional regulation (Carlin et al. 1983; Wang et al. 1985).

Taken in conjunction with our data, the evidence from studies on retinoid-induced changes in cell morphology, adhesion and transcription suggest that positional memory in amphibian limb cells might be the result of level-specific differences in gene expression that affect the molecular structure of the cell surface, and that RA modifies positional memory by affecting this expression. Many retinoid-sensitive cells contain two ligand-specific cytoplasmic proteins (cellular retinol binding protein = CRBP, and cellular retinoic acid binding protein = CRABP) that bind retinol and RA (Liau et al. 1981; Takase et al. 1986; Crow et al. 1987; Chytil & Ong, 1984, for review). These binding proteins shuttle retinol and RA to the nucleus and transfer them to a nuclear retinoid receptor that is distinct from the cytoplasmic binding proteins themselves (Daly & Redfern, 1987), which are recycled to the cytoplasm (Chytil & Ong, 1984). A cDNA encoding a retinoid nuclear receptor in human breast cancer cell lines has been cloned; the receptor is a RA-inducible trans-acting enhancer factor that is structurally related to steroid and thyroid hormone receptors (Petkovich et al. 1987).

A cytoplasmic CRABP has recently been detected in limb regenerates of axolotls (Keeble & Maden, 1986; McCormick et al. 1988). Hence a plausible hypothesis regarding the mechanism by which exogenous retinoids modify positional memory of blastema cells is that after being delivered to the nucleus by CRBP or CRABP, they bind to and activate a nuclear receptor, allowing it to bind to the enhancer region of genes that are involved in specifying positional memory. Currently, however, there is no direct molecular evidence for this hypothesis.

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### References

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