Cellular Contribution to Supernumerary Limbs Resulting from the Interaction between Developing and Regenerating Tissues in the Axolotl

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The relationship between limb development and limb regeneration is considered with regard to the mechanisms by which pattern is established during limb outgrowth. In a previous paper (Muneoka, K. and Bryant, S. V. 1982 Nature (London) 298 369-371) the interaction between cells from the developing limb bud and the regenerating limb blastema was found to result in the production of organized supernumerary limb structures. In this paper the relative cellular contribution from developing and regenerating cells to supernumerary limbs resulting from contralateral grafts between limb buds and blastemas has been analyzed using the triploid cell marker in the axolotl. Results show that there is substantial participation from both developing and regenerating limb cells to all supernumerary limbs analyzed. These data lend further support to the hypothesis that developing and regenerating limbs utilize the same patterning mechanisms during limb outgrowth. This conclusion is discussed in terms of patterning models for developing and regenerating limbs and it is proposed that the rules of the polar coordinate model can best explain the behavior of cells during limb development as well as limb regeneration.

INTRODUCTION

Until recently, the field of pattern formation of the vertebrate limb has been divided into two areas of study which have remained relatively isolated from one another. One area of research focuses on understanding how the limb pattern is established during the development of the limb and the other focuses on how the limb pattern is reestablished during the regeneration of the limb. One consequence of this dichotomy has been the formulation of pattern formation models for developing limbs which are very different from those describing patterning of the regenerating limb. Currently, the most prevalent model to account for pattern formation in the regenerating limb is the polar coordinate model (French et al., 1976; Bryant et al., 1981), and in recent years at least one laboratory (see Iten, 1982) has been testing the applicability of this model to limb development. Nevertheless, the most widely accepted view of how the developing limb bud is patterned is based on a combination of two models: the progress zone model (Summerbell et al., 1973) and the polarizing zone model (Tickle et al., 1975), as originally formulated for the developing chick limb, and later applied to amphibian limb buds (Slack, 1977; Meinhardt, 1982, 1983).

These two limb patterning models (polar coordinate model and the progress zone/polarizing zone model) differ not only in the types of mechanisms thought to be at work during limb outgrowth but also in the initial assumptions from which these views have evolved. Briefly, the polar coordinate model assumes that limb cells possess information about their position along the proximal-distal limb axis and around the circumference of the limb. This model proposes that tissues have the general property of intercalary regeneration, i.e., localized growth in response to positional discrepancies. The positional value of a cell is viewed as stable, and new positional values are generated by cell division. Alternatively, the progress zone/polarizing zone model assumes that limb bud cells initially do not possess any information about position along the proximal-distal and anterior-posterior axes of the limb bud and that positional information is assigned to limb bud cells during outgrowth. Two mechanisms are proposed for the assignment of positional information. Limb bud cells are thought to acquire proximal-distal positional information by measuring the amount of time spent in a specialized region (the progress zone) located at the tip of the limb bud. Additionally, limb bud cells are thought to gain anterior-posterior positional information by reading the local concentration of a diffusible morphogen which is produced at the posterior margin (the polarizing zone) of the limb bud.

These two views of limb patterning have evolved from numerous experiments on developing limb buds and regenerating limb blastemas and it has been suggested that pattern formation during limb development and limb regeneration involves completely different cellular mechanisms (Tickle, 1981). However, other interpretations are possible. In a review of limb development in urodeles, Stocum and Fallon (1982) have proposed that the behavior of both limb buds and regeneration
blastemas is consistent with intercalation models, while suggesting that the idea of a polarizing zone could be applicable to the limb field at the time of its specification prior to outgrowth.

In a previous paper (Muneoka and Bryant, 1982), we reported on an experiment designed to test whether cells from a developing limb bud and cells from a regenerating blastema could interact in an organized manner to produce a supernumerary limb. Our findings suggested that similar patterning mechanisms are utilized by both the developing and the regenerating limb cells of the axolotl. In this paper we have used the triploid cell marker in the axolotl to investigate the cellular contributions from developing and regenerating cells to supernumerary limbs resulting from such blastema-limb bud interactions and our results show that the involvement of regenerating and developing limb cells in the formation of these supernumerary limbs is approximately equal. These data lend support to the hypothesis that similar patterning mechanisms are utilized both during limb development and limb regeneration.

MATERIALS AND METHODS

All experiments were performed on the axolotl (Ambystoma mexicanum), spawned at the University of California, Irvine. Animals were maintained at 20 ± 1°C. Newly hatched larvae were kept in 20% Steinberg's solution until they had grown to approximately 5 cm in body length, after which they were maintained in 25% Holtfreter's solution. Prior to grafting, young larvae were kept in pans, and were changed and fed newly hatched brine shrimp or tubifex worms daily. Experimental animals were maintained individually, initially in 100 × 25-mm plastic petri dishes, and were changed and fed tubifex worms daily. When these animals had grown to approximately 5 cm, they were transferred into 1-liter plastic boxes, and were changed and fed tubifex worms three times a week.

Triploid larvae were produced by subjecting fertilized eggs to hydrostatic pressure (6000 psi for 8 min) using the protocol of Gillespie and Armstrong (1979). Treated animals were screened for triploidy following the protocol outlined in Muneoka et al. (1984). Triploid larvae possess cells with three nucleoli per cell while diploid larvae possess cells with two (Fankhauser and Humphrey, 1943). Triploid and diploid larvae were maintained under identical conditions but were kept separately.

Grafting

The grafting procedure is shown in Fig. 1. Hindlimb buds and forelimb blastemas were exchanged contralaterally and positioned such that anterior and posterior tissues were misaligned while dorsal and ventral tissues were aligned (A/P contralateral graft). All grafts were performed between diploid and triploid sibling larval axolotls ranging in size from 30 to 42 mm. Each larva received a single graft. To maximize use of the triploid larvae, both blastemas or both limb buds of each triploid animal were grafted to two separate diploid hosts. One of the two diploid larvae provided the donor graft for the triploid animal. When possible the white (dd)/black (Dd) pigmentation marker was utilized in conjunction with the ploidy marker to distinguish host and donor tissue. The details of the grafting procedures have been described previously (Muneoka and Bryant, 1982). After grafting, larvae were observed daily without anesthesia until the graft was well healed, after which time observations were made two to three times a week.

Tissue Processing

Six to 8 weeks after grafting, well-formed supernumerary limbs were apparent, and limbs were amputated, fixed in Carnoy's fixative, and processed immediately.
Twenty supernumerary limbs (10 experimental limbs each with 2 supernumerary limbs) were analyzed to determine the relative cellular contribution from the stump and the graft. Limbs in which the white/black pigmentation marker was used in conjunction with the triploid/diploid cell marker were drawn with the aid of a camera lucida so as to record the location of the pigment boundary. The dorsal and ventral skin (epidermis and dermis) was removed and the dermis was isolated and stained with bismuth for whole-mount dermal analysis (Muneoka et al., 1984; Muneoka and Bryant, 1984a). The remaining skinned limbs were processed for whole-mount analysis of the skeletal pattern by Victoria blue B staining (Bryant and Iten, 1974).

**Analysis of Data**

Cellular contribution to the dermis was analyzed in bismuth-stained whole-mount dermal preparations of the digits of the supernumerary limbs. The trinucleolate frequency in dermal preparations reflects the number of triploid cells divided by the total number of scorable cells in each digit. All cells, with the exception of red blood cells, of each digit were analyzed and in cases where part of the digit dermis was damaged in processing, cells at the base of the digit were scored. Cells were determined to be ambiguous and unscorable if no nucleoli were evident or if cells were overlapping and there was some question about the number of nucleoli per cell.

The trinucleolate frequency of each digit of a supernumerary limb was compared to its individual control frequency. In cases where triploid blastemas or limb buds were grafted to diploid hosts, the control trinucleolate frequency was determined from cell counts of the contralateral ungrafted triploid limb of the host. We have previously determined that the trinucleolate control frequency in dermal preparations of diploid tissue is zero (Muneoka et al., 1984).

The trinucleolate frequency for each digit, based on cell counts of the dorsal and ventral dermis, was plotted across the anterior–posterior axis of each supernumerary limb (Fig. 2) and the boundaries between triploid- and diploid-derived tissues were determined as previously described (Muneoka and Bryant, 1984a).

**Limb Pattern**

The criteria used to identify the pattern of the supernumerary limbs were those of Pescitelli and Stocum (1980). For the purposes of this analysis, in cases where the graft and the supernumerary limb shared a digit located on the line of symmetry between the limbs (e.g., graft/supernumerary limb digit sequence of 1, 2, 3–4–3, 2, 1), the shared digit was scored as part of the supernumerary limb pattern. Limbs analyzed in this study were selected as those limbs which appeared to have the most complete supernumerary limbs and were maximally expanded in the anterior–posterior plane of the limb. Limbs with a reduced number of supernumerary digits and/or those with supernumerary limbs which became twisted relative to the grafted limb during outgrowth were not analyzed in this study.

**RESULTS**

A total of 20 supernumerary limbs were analyzed from 10 experimental limbs each bearing two supernumerary limbs. Ten supernumerary limbs resulted from blastema to limb bud stump grafts and the remaining 10 super-
Numerary limbs resulted from the reciprocal graft of limb bud to blastema stump. Figure 3 shows examples of limbs resulting from blastema to limb bud stump and limb bud to blastema stump grafts. Supernumerary limbs were found to form at anterior and posterior locations relative to the stump. All 20 supernumerary limbs analyzed in this study were of stump handedness and the grafted limb always maintained its original handedness. Supernumerary limbs generally formed in the autopodial region, appearing fused with the grafted limb and forming an array of digits expanded in the anterior-posterior plane (see Fig. 3).

**Cellular Contribution to Supernumerary Limbs**

Whole-mount preparations of the dorsal and ventral dermis (see Muneoka and Bryant, 1984a) of 20 supernumerary limbs were analyzed for cellular contributions from graft and stump. In this study 12 supernumerary limbs resulted from triploid grafts onto diploid stumps (6 supernumerary limbs from blastema to limb bud stump grafts and 6 from limb bud to blastema stump grafts) and the remaining 8 supernumerary limbs resulted from diploid grafts onto triploid stump (4 supernumerary limbs from blastema to limb bud stump grafts and 4 from limb bud to blastema stump grafts). The cellular contribution to the dermis has been shown to reflect that of internal (cartilage) tissue (Muneoka and Bryant, 1984a), hence, the cellular contribution was analyzed only in the dermis. Since the frequency of trinucleolate cells varies from one triploid animal to another (Muneoka et al., 1984) control cell counts were made from tissues of each individual triploid donor for the experimental limbs analyzed in this study. The control frequency derived from cell counts of dermal preparations from each different triploid animal used in this study varied from 0.50 to 0.68.

Substantial cellular contribution from the graft and the stump was observed in all 20 supernumerary limbs analyzed (Figs. 4 and 5) (Table 1). The relative cellular contribution from stump versus graft was variable from case to case but overall appeared to be approximately equal. The position of the boundary between stump- and graft-derived tissue in the supernumerary limbs generally fell around digits two and three. The position of the dorsal boundary along the anterior-posterior limb axis was either coincident with (5/20) or anterior to (15/20) the position of the ventral boundary, but it was never posterior to the position of the ventral boundary. This indicates that contribution to the supernumerary limb from the cells of the posterior edge of the stump or graft tended to be primarily to posterior and dorsal tissues of the limb, whereas anterior and ventral tissues of the supernumerary limb were derived predominately from cells originating from the anterior edge of the stump or graft. This asymmetrical pattern of cellular contribution to supernumerary limbs was also observed in supernumerary limbs resulting from A/P contralateral limb bud and blastema grafts (Muneoka and Bryant, 1984a).
**FIG. 4.** Summary diagram of the cellular contribution boundaries in the dorsal and ventral dermis of 10 supernumerary limbs resulting from grafts of blastemas onto limb bud stumps. Five supernumerary limbs resulted anterior to the graft (left column) and five posterior to the grafted limb (right column). The experimental limb number which corresponds to the limbs listed in Table 1 is shown on the far left. The boundary position in the dorsal and ventral dermis of each supernumerary limb was derived from data similar to those shown in Fig. 2 and these data are presented for each individual supernumerary limb in Table 1. Complete supernumerary limbs are those with four or five digits and are shown diagrammatically as full ellipses. Incomplete supernumerary limbs possess less than four digits and are shown as partial ellipses. Cross hatching refers to triploid derived tissue.

Sixteen of the 20 supernumerary limbs analyzed in this study resulted from grafts carrying the white/black pigmentation marker as well as the triploid/diploid cell marker. Since pigment cells are less abundant ventrally, analysis was restricted to comparisons between ploidy and pigmentation boundaries in the dorsal dermis. We found that the position of the pigment boundary coincided with the position of the ploidy boundary in a minority of the cases analyzed (6/16). In another three cases the difference between the two boundaries was less than the width of a single digit and in the remaining seven cases this difference was found to be from one to two digit widths or up to approximately one-half the normal anterior-posterior dimension of the limb. This relationship between the relative positions of the pigmentation boundary and ploidy boundaries is discussed in detail elsewhere (Muneoka and Bryant, 1984).

**Limb Pattern**

The completeness of supernumerary limbs and the continuity of the digital pattern of the supernumerary limbs were difficult to interpret in this study. This difficulty stems from the fact that the supernumerary limbs are composed of cells from both the forelimb (possessing four digits) and the hindlimb (possessing five digits) and although cells from fore- and hindlimbs can interact (Rollman-Dinsmore and Bryant, 1982), virtually nothing is known about the detailed homology and distribution of positional values between these two limbs. Thus, the completeness and continuity of the digital pattern of the supernumerary limbs cannot be easily assessed. However, it seems clear that any supernumerary limb with less than four digits is incomplete although some supernumerary limbs with four digits may also in fact be incomplete (see below). In this study 4 of 20 supernumerary limbs possessed less than four digits and were classified as incomplete supernumerary limbs. All 4 of these supernumerary limbs were located anterior to the grafted limb. Three incomplete supernumerary limbs possessed three digits and one possessed two digits.

With regard to the continuity of the digital pattern, most supernumerary limb digital patterns (17/20) were continuous within the supernumerary limb (e.g., digital sequence 1, 2, 3, 4, or 1, 2, 3, 4, 5) regardless of whether the limb was complete or incomplete. The supernumerary limb digital pattern was determined from skeletal analysis in conjunction with the cellular contribution data with regard to whether a digit was of hindlimb (h) or of forelimb (f) origin. For example, the limb in Fig. 3b shows a digital pattern of f1, f2/h2, h3, h4 (anterior supernumerary limb)—h4, h3, h2, h1 (grafted limb)—h1, h2/f2, f3, f4 (posterior supernumerary limb). Note that in this case, as in the rest of the cases in this category both supernumerary limbs form mirror symmetrical patterns with the grafted limb pattern. Note also that with regard to the completeness of the supernumerary limb, the anterior supernumerary limb and the grafted limb were both missing a peripheral digit (h5) and it was impossible to determine whether the posterior supernumerary limb digit pattern was complete or incomplete. The remaining supernumerary limbs (3/20) possessed digital patterns that were discontinuous. All these cases formed the digital pattern f1, f2, f3, h5 or f1, f2, h4, h5. The cellular contribution

![Supernumerary Limbs](image-url)

**FIG. 5.** Summary diagram of the cellular contribution from the stump and graft to 10 supernumerary limbs resulting from grafts of limb buds onto blastema stumps. See Fig. 4 for an explanation of this diagram.
### TABLE 1

| Limb No. (donor/host) | Anterior | Posterior | Control |
|-----------------------|----------|-----------|---------|
|                       | 1 2 3 4 5| 1 2 3 4 5|         |
| 1 D (3N/2N) V         | 0.00 0.43 0.60 — — | 0.63 0.59 0.06 0.01 0.00 | 0.63 |
| 2 D (3N/2N) V         | 0.17 0.58 — — — | 0.57 0.57 0.10 — — 0.01 | 0.58* |
| 3 D (3N/2N) V         | 0.01 0.43 0.68 0.63 — | nd 0.54 0.17 0.00 0.00 | 0.63 |
| 4 D (2N/3N) V         | 0.57 0.19 0.07 — — | 0.01 0.05 0.46 — — 0.62 | 0.61* |
| 5 D (2N/3N) V         | 0.58 0.08 0.06 0.02 — | 0.02 0.02 0.28 — — 0.61 | 0.60* |
| 6 D (3N/2N) V         | 0.01 0.15 0.61 0.65 — | 0.69 0.51 0.07 0.01 — | 0.63 |
| 7 D (2N/3N) V         | 0.56 0.47 0.05 0.03 — | 0.02 0.34 0.51 0.50 — | 0.50* |
| 8 D (2N/3N) V         | 0.68 0.54 0.05 0.01 — | 0.05 0.45 0.63 0.68 — | 0.63* |
| 9 D (3N/2N) V         | 0.00 0.33 0.63 — — | 0.68 0.21 0.04 0.02 — | 0.66 |
| 10 D (3N/2N) V        | 0.00 0.05 0.52 0.52 0.57 | 0.53 0.48 0.02 0.07 — | 0.56 |

**Note.** The data presented in this table correspond to the supernumerary limbs shown in Figs. 4 and 5. The experimental limbs analyzed in this study are listed in the far left column (Limb No.) along with the ploidy of the graft and stump for that individual limb. The far right column lists the control trinucleolate frequency for each individual dermal preparation. In cases where the host was triploid, the control frequency was from cell counts of the contralateral limb and this frequency was used as a control for both dorsal and ventral dermal preparations. The frequency of trinucleolate cells for the dorsal (D) and ventral (V) dermis of each supernumerary digit is listed. Horizontal bars indicate the absence of that supernumerary digit. The bottom of the table lists the total number of cells scored, the mean number of cells scored, and the range of number of cells scored in the experimental and control limbs analyzed in this study. nd, no data collected for that individual digit.

* Control trinucleolate frequency was from the contralateral limb.

### DISCUSSION

Contralateral blastema grafts to appose anterior and posterior limb tissue result in the formation of super-
numerary limbs (Iten and Bryant, 1975; Tank, 1978; Maden, 1980; Stocum, 1982). Similarly, contralateral limb bud grafts to appose anterior and posterior limb tissue in urodèles (as well as in all vertebrate limb buds tested) result in the formation of supernumerary limbs (Maden and Goodwin, 1980; Thoms and Fallon, 1980; Muneoka and Bryant, 1982). Reciprocal grafting exchanges of this type between the hindlimb bud and the forelimb blastema in the axolotl also result in the production of supernumerary limbs suggesting that the cells of the limb bud and the blastema in the axolotl are utilizing similar patterning mechanisms (Muneoka and Bryant, 1982). The results of the analysis of the cellular contribution to supernumerary limbs resulting from limb bud–blastema grafts shown here are virtually identical to those of similar analyses of supernumerary limbs resulting from limb bud–limb bud grafts and blastema–blastema grafts (Muneoka and Bryant, 1984a). This similarity coupled with the demonstration that both blastema- and limb bud-derived cells do participate in the formation of such supernumerary limbs argue strongly for the idea that limb bud and blastema cells are indeed using the same patterning mechanisms during limb outgrowth. Furthermore, these studies show that the graft and stump contribute roughly equal amounts of tissue to the supernumerary limbs, thus suggesting that the cellular interactions involved in pattern regulation and supernumerary limb formation are mutual and cooperative. Such results are not consistent with the idea that polarizing zones might control patterning during embryonic limb outgrowth and limb regeneration. Models of this type require that supernumerary limbs should be formed all, or almost entirely, from responding tissues, i.e., from either the graft or the host, depending on whether the supernumerary limb is formed in an anterior or a posterior location. In fact, a recent polarizing zone model (Meinhardt, 1982, 1983) makes this prediction quite explicitly. However, our results do not rule out the possibility that one or more polarizing zones may play a role in the establishment of pattern very early in the limb field, prior to limb bud outgrowth, as suggested by Stocum and Fallon (1982).

In considering our results in the context of other studies on vertebrate limb development and regeneration, we begin with the assumption that the basic cellular mechanisms governing pattern regulation of all vertebrate limbs are universal (Wolpert, 1969, 1971). Experimental evidence for this assumption stems primarily from the demonstration that a pattern regulatory response can be elicited from tissue grafts between different limbs within an organism (Saunders and Gas seling, 1968; Rollman-Dinsmore and Bryant, 1982), between limbs from evolutionarily diverse animals (Tickle et al., 1976; MacCabe and Parker, 1976; Fallon and Crosby, 1977), and between regenerating and developing limbs (Muneoka and Bryant, 1982). We have argued elsewhere for the validity of Wolpert's universality principle as it applies to the vertebrate limb (Muneoka and Bryant, 1984b).

Next, we focus on what positional information is present in the limb bud and blastema when most patterning experiments are performed. We assume that at the earliest stages of both limb bud and blastema formation, limb cells possess positional information about all three limb axes. Experimental evidence for this assumption comes from experiments on axial determination of the limb field prior to limb bud formation in the embryo of both the chick (Chaubé, 1959; Saunders and Reuss, 1974; Dhouailly and Kieny, 1972) and urodèle (reviewed in Stocum and Fallon, 1982). Evidence that blastema cells possess positional information comes from studies in which very young blastemas are grafted to misalign axes (see Bryant et al., 1981; Stocum, 1982) and from numerous experiments where mature limb tissue rearrangements are followed by limb amputation and result in a position-dependent pattern regulatory response, i.e., supernumerary limb formation (reviewed in Tank and Holder, 1981; Carlson, 1983). The interpretation of these latter studies is that mature limb cells possess a positional memory (positional information) which they recall into use during regeneration.

We propose that intercalary regeneration is a general property of both developing limb bud and regenerating blastema tissues. Intercalary regeneration is defined here as in French et al. (1976): a growth response with the formation of new intermediate positional values in response to a positional disparity. It is clear from many studies that a growth response is elicited after grafting to create a positional discontinuity in both the limb bud (Cooke and Summerbell, 1980, 1981) and the blastema (reviewed in Tank and Holder, 1981) since supernumerary limb structures are ultimately formed. The positional value of a cell is thought to be stable during the initial interactions which lead to recognition of a positional disparity, but may be changed during intercalary cell division such that one or both daughter cells may be able to change positional value (Bryant et al., 1981). Whether one or both daughter cells change positional value may be related to the extent of the positional disparity. For example, using the shorthand of the polar coordinate model, juxtaposing cells with positional values 1 and 6 could result in one of the interacting cells dividing and both of its daughter cells taking on intermediate positional values (Fig. 6). Subsequent intercalary events across smaller positional disparities may only involve one daughter cell changing to a new positional value while the other maintains the original positional value, as is also shown in Fig. 6. Intercalation involving a change in positional value for both daughter
Fig. 6. Model for intercalary regeneration. Using the shorthand of the polar coordinate model, an interaction between cells with positional values 1 and 6 is shown. (a) The interaction between cells of different positional values is thought to result initially in the response of one or both cells. For simplicity, it is supposed that the cell with positional value 6 responds first in this example by dividing to produce two daughter cells both of which take on positional values intermediate between 6 and 1, here shown as positional values 4 and 5. (b) The cell with positional value 1 then interacts with the newly intercalated cell with positional value 4 and responds by dividing to produce two daughter cells both of which take on positional values intermediate between 1 and 4, here shown as positional values 2 and 3. (c) Cells with positional values 12 and 7 now interact with the newly intercalated positional values and are stimulated to undergo intercalary regeneration. Note that at this point both positional values 1 and 6 which represented the initial positional confrontation, are no longer present and positional values 12 and 7 which originally were not exposed at the positional disparity have become involved. (d) All positional disparities have been eliminated through local interactions resulting in the stimulation of intercalary regeneration, but the extent of the cellular involvement has spread away from the original positional discontinuity. Asterisks indicate which cells are responding to any given interaction. Circles around positional values indicate which values have become involved in the patterning response.

cells of a division could account for the propagation of the growth response which is found at a distance from a positional disparity (Cooke and Summerbell, 1980) and thus, for the involvement of cells at a distance from the positional disparity in the formation of supernumerary limbs (Honig, 1981). It can also account for the loss of midline structures along planes of symmetry which is often observed in regenerating limbs (see Bryant et al., 1981).

Finally, we suggest that the capacity to intercalate, because it involves cell division, is related to the degree of differentiation of the cells. Thus, as cells become more differentiated they become less able to undergo intercalary regeneration (Bryant and Iten, 1977). In urodeles the necessary intercalary events for pattern regulation can either occur prior to extensive differentiation of tissues, as in limb buds, or after differentiation given the well-developed capacity for dedifferentiation shown by adult urodele tissues. However, in nonregenerating organisms (e.g., the chick) the ability to undergo intercalary regeneration may be stage (or degree of differentiation) dependent. The failure of older limb buds and mature limbs to engage in regenerative activities may be related to an inability to dedifferentiate. Recently, Iten et al. (1983) have shown that in the chick embryo, grafting experiments using advanced limb buds which are beginning to differentiate do not result in supernumerary limb formation, yet heterochronic grafting of these older limb buds to younger limb bud stumps results in the formation of supernumerary limb structures. These results suggest that the age and degree of differentiation of the graft and the ability to undergo intercalary regeneration and supernumerary limb formation are indeed related. If this is true, then the one-sided cellular contribution to supernumerary limbs following apposition of anterior and posterior tissues in the chick limb bud (Honig, 1983) may be a reflection of the differentiated state of the limb bud cells. Differentiation of the chick limb bud occurs from posterior to anterior and cellular contribution to supernumerary limbs is predominately from the anterior or less differentiated limb bud cells.

Seeing the limb bud as being equivalent to the regeneration blastema leads to the conclusion that the limb field just prior to limb bud outgrowth and the newly amputated limb stump may be similar in the type of positional information present and in the manner by which the limb gains its three-dimensional structure. In both cases a three-dimensional outgrowth is formed from what is essentially a two dimensional sheet of cells with positional information for the anterior–posterior and dorsal–ventral limb axes. In other words, proximal-distal positional information is elaborated starting with anterior–posterior and dorsal–ventral positional values. In the case of the regenerating limb, disparate positional values from around the limb circumference are thought to come together during wound healing, thus stimulating intercalary regeneration and the eventual restoration of the limb pattern (Bryant et al., 1981). In the limb bud it is possible that at the start of outgrowth cells with stable information about the anterior–posterior and dorsal–ventral positions interact beneath the apical ectoderm and intercalate to form the free limb (French et al., 1976; Bryant et al., 1977; Stocum and Fallon, 1982).

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