Control of pattern formation in urodele limb ontogeny: a review and a hypothesis

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SUMMARY

From a review of the literature, the hypothesis is advanced that the forelimb region of the urodele embryo acquires its transverse axial polarity and pattern by the action of posterior and dorsal polarizing regions. The anterior-posterior and dorsal-ventral axes are determined simultaneously and their determination is a prerequisite for proximal-distal outgrowth. Outgrowth of the limb bud is accompanied by the generation, between proximal and distal boundaries, of a set of positional values specifying proximal-distal axial polarity and pattern. The proximal boundary is the initial positional value carried by the cells of the limb area. The distal boundary is imposed upon the outermost layer of limb disc cells by the overlying ectoderm.

INTRODUCTION

Developmental biologists in the 20th century have been confronted with two major problems of gene regulation whose domains can be arbitrarily separated at the surface of the cell. The first problem is to understand the sequence of intracellular events that result in a pattern of synthetic activity directed by the genome and associated with a specific differentiation. The second problem is to understand the intercellular interactions which specify regional patterns of gene activity, and thus the regional patterns of differentiation which we recognize as tissues and organs (pattern formation). The problem of how pattern is determined, which historically has proven to be so intractable, is currently a subject of renewed interest, largely because of the detailed theoretical analyses of Wolpert (1969, 1971), French, Bryant & Bryant (1976) and Bryant, French & Bryant (1981).

One of the first and most productive systems for the analysis of pattern formation and regulation was the developing urodele forelimb. The goal of

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investigators working with this system has been to understand the processes underlying (1) the initial segregation of the limb mesoderm from the somatic lateral plate mesoderm, and (2) the development of axial polarity and tissue pattern of the limb mesoderm, which is closely linked to outgrowth of the limb from the body wall. Little is known about the first process, but a large body of experimental data has been accumulated on the second. From these data has emerged the view that the limb rudiment is a harmonious, equipotential system whose three cardinal axes of asymmetry are determined in the following sequence: anterior–posterior (AP), dorsal–ventral (DV), and proximal–distal (PD) (Harrison, 1918, Swett, 1937). The mechanisms of polarization and pattern formation, however, remain unknown. In the present paper, we (1) review the literature on urodele limb development, (2) challenge the classical interpretation of the data relating to axial determination, and (3) propose a hypothesis of axial polarization and pattern development during limb ontogeny which can be integrated with limb regeneration.

Location and map of the forelimb area

The presumptive forelimb rudiment has been located and mapped in several species of *Ambystoma* and *Triturus* by extirpation experiments (Detwiler, 1918; Harrison, 1918; Suzuki, 1928; Rotmann, 1931; Takaya, 1941), vital dye tracking (Detwiler, 1918, 1929, 1933; Swett, 1923), and construction of chimaeric limb rudiments (Schwind, 1928, 1931, 1932). At early gastrula, the presumptive forelimb ectoderm is located bilaterally within the presumptive skin ectoderm region, about 130° anterior to the crescent-shaped blastopore. The presumptive forelimb mesoderm is located bilaterally in the lateral plate mesoderm, a few degrees lateral to the blastopore (Rotman, 1931; Detwiler, 1933). The morphogenetic movements of gastrulation position the prospective limb mesoderm under its corresponding ectoderm. By the medullary fold stage, the limb rudiment is located just posterior to a line dividing the anterior two-thirds and posterior one-third of the embryo (Detwiler, 1918, 1929). At the tailbud stage, the limb rudiment has been classically defined as a 3-5-somite-wide disc whose AP diameter stretches from the anterior border of somite 3 to the middle of somite 6, with its dorsal border overlapping the pronephros (Detwiler, 1918; Harrison, 1918; Takaya, 1941). However, as described below, the actual prospective limb tissue occupies only a part of this disc.

Figure 1 is a map of the Harrison stage-29 forelimb area of *Ambystoma maculatum* (*punctatum*) identifying the regions which will form the free limb and girdle and also the non-limb regions which are capable of regulating to form limb and girdle if prospective limb cells are removed. We stress two points about this map. First, the inner large circle of tissue, 3-5-somites wide, has been classically defined as the 'limb disc'. This is the area that has been transplanted in most experiments investigating the problem of axial determination in limb ontogeny. Second, only slightly more than the anterior half of this disc actually represents
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Fig. 1. Map of the presumptive right forelimb and surrounding areas of the Harrison stage-29 *Ambystoma maculatum* embryo, constructed from the data of Detwiler (1918), Harrison (1921, 1925), Swett (1923), and Schwind (1931). The shoulder girdle develops from the region marked by hatching, while the free limb (FL) is derived from a 2–2.5 somite-wide area (clear circle). The AP and DV axes of the free limb are rotated 30–45° (clockwise for the right limb, counterclockwise for the left) from the corresponding body axes. The stippled area is peribrachial flank tissue (PBF) that does not normally take part in forelimb development. The inner large circle (3-5-somite diameter) encompasses the classical limb disc that has been used in transplantation experiments, even though much of its posterior tissue is not prospective limb. The outer large circle (5-somite diameter) represents the maximum size of the region that can regulate to form limb after actual presumptive limb tissue is removed. Outside this region, flank tissue (F) will form limb only when induced by other tissues such as ear or nasal placodes. S, SS = scapula and suprascapula of the shoulder girdle; C, PC = coracoid and procoracoid of the girdle; a, p, d, v = anterior, posterior, dorsal and ventral poles of the principal transverse axes of the limb; PN = pronephros; S_1–12 = somites; G_1–3 = gills.

prospective free limb and girdle material. The remaining posterior half-ring of limb disc tissue is actually peribrachial flank which does not normally participate in limb development. In our discussion of the literature, the term ‘limb region or disc’ refers to this 3-5-somite-wide area.

The free limb area begins to thicken at stage 29, and becomes visible as a growing bud by stage 36. The limb bud is conical at first and points in a posterior–dorsal direction. At stage 40 the distal half of the bud flattens and undergoes a torsion that orients the radial–ulnar plane approximately 30° to the vertical. As the elbow joint and digits develop, the lower arm twists further to a vertical alignment (Harrison, 1918).

It is commonly held that the limb rudiment is derived solely from the somatopleure of the lateral plate mesoderm in amphibians (Byrnes, 1898; Lewis, 1910; Detwiler, 1918). However, a somitic contribution to the limb does not appear to be ruled out. Recent studies on avian wing development, using marked somite cells, have conclusively demonstrated that wing myogenic cells are derived from the epibrachial somites (Dhouailly & Kieny, 1972; Chevallier, 1975, 1978,
Further, it has been reported that somite cells are required for day-11 mouse limb-bud development in organ culture (Agnish & Kochar, 1977). Circumstantial evidence for a somite contribution to the urodele limb comes from the fact that the neurula-stage limb rudiment of *T. pyrrhogaster* will not develop autonomously *in vitro*, but will develop if cultured with prospective somite tissue (Amano, 1960). Furthermore, supernumerary limb buds can be evoked in this species when ventral splanchnopleure is brought into contact with somite tissue at the neurula stage. However, a clear interpretation of the role of somite-derived cells in limb development is not possible from these experiments because the somite or limb tissues were not marked in any way to determine whether the somites actually contributed cells to the limb rudiments, or provided an environment necessary for limb development. A somite contribution to the limb rudiment is made more likely by the fact that limb buds develop from masses of neurula stage *A. maculatum* somite plates wrapped in ventral ectoderm and cultured *in vitro* (Muchmore, 1957). In view of these results it would be profitable to reinvestigate the question of a somitic contribution to the limb regions in urodeles, using appropriately marked cells.

### Regulative power of the limb region

Data from heterotopic transplantation experiments have suggested that the forelimb of *A. maculatum* is determined as such by late gastrula (Detwiler, 1933). However, the frequency of autonomous differentiation of grafted limb discs at this stage is very low, and Takaya (1941), on the basis of similar experiments with *T. pyrrhogaster*, found that determination of the forelimb mesoderm may not be complete until the medullary fold stage, when the frequency of self-determination rises considerably. The question of when the limb disc is determined as limb is bound up with the question of whether its formation requires a contribution from the epibrachial somite material. If there is such a contribution, self-differentiation may not be possible until the neurula stage because the disc does not contain a critical mass of prospective limb cells until then.

Once determined, however, the limb region exhibits the classic morphogenetic field property of regulation. Any of the four cardinal halves of the stage-29 *A. maculatum* limb disc can regulate to form a whole limb, although regulation is observed more consistently with posterior and dorsal halves (Harrison, 1918). It is noteworthy that the prospective shoulder girdle region is mosaic at this stage (Detwiler, 1918). Hollinshead (1932) found that the dorsal half of the *A. maculatum* limb disc grafted to the flank can regulate to form a normal limb through stage 34, but limbs missing the radius developed with increasing frequency between stages 35 and 37. However, dorsal and posterior halves just of the *free limb* region of the disc developed into radially defective limbs from stage 30 on, while anterior and ventral halves of the *free limb* region...
regulated to produce normal limbs. These results indicate that, starting in the posterior-dorsal quadrant, the prospective free limb cells begin to lose their pleuripotency at stage 30 while the disc cells outside the free limb region retain their potency longer.

A one-somite-wide ring of flank tissue surrounding the limb disc is also competent to form a limb at stage 29 if the disc is completely removed (Harrison, 1918). According to Swett (1941), the replacement cells are derived mainly from the dorsal sector of this tissue. This fact again raises the possibility that the somites contribute some of the replacement cells. The remainder of the flank tissue cannot regulate to form a limb after extirpation of the limb disc plus the peribrachial ring of flank tissue (Harrison, 1918), but can be induced to form limbs if stimulated by transplanted nasal or ear placodes in *Bufo vulgaris* (Filatow, 1927), *Triturus taeniatus*, *Hyla arborea*, *Rana esculenta* (Balinsky, 1925; 1926, 1927a, b, 1933), *Amphibystoma maculatum* (Glick, 1931) and *Triturus pyrrhogaster* (Takaya, 1941; Ichikawa & Amano, 1949), and in *A. maculatum* by implanting mouse kidney or transplantation of tail tissue (Holtfreter, 1955). Balinsky (1933) obtained one case in which an extra limb was induced by implantation of a piece of celloidin to the flank. Limbs were also induced in *A. mexicanum* after grafting haploid forelimb buds to diploid flanks; the induced limbs were diploid and arose after resorption of the transplants (Hertwig, 1925, 1927). Thus, the flank mesoderm outside the immediate peribrachial region can be induced to express latent limb properties.

Limbs induced from the flank were of forelimb character when the inductor tissues were implanted below the 4th to 7th somites, but were of hindlimb character when the inductors were implanted below somites 9–14 (Balinsky, 1933). Either forelimbs or hindlimbs could be formed when the inductors were placed under the 8th somite (Fig. 2). Furthermore, limbs were inducible all along the flank, while pelvic girdles were inducible only under somites 11–13. The frequency of limb formation declined steadily between somites 6 and 13.

**Ectodermal-mesodermal interactions and limb outgrowth**

The developmental specificity of the limb disc resides in its mesodermal component. Stage-29 forelimb ectoderm grafted to the flank does not form limb, whereas stage-29 limb mesoderm grafted under flank ectoderm does so (Harrison, 1918) and experiments involving heteroplastic exchanges of prospective forelimb ectoderm show that the chimaeric limbs develop with the size and morphology of the host species (Rotmann, 1931). During larval stages, however, the limb epidermis apparently does have a small, but noticiable species-specific effect on the limb morphology and size (Rotmann, 1933; Heath, 1953). Aside from this effect, the ectoderm nevertheless has an indispensable role in limb ontogeny and regeneration of all vertebrates. The apical ectoderm of the limb bud of some anurans and all amniote embryos is thickened into a cap or ridge (Saunders, 1948; Milaire, 1965; Tarin & Sturdee, 1971; Raynaud, 1972;
Stebler, 1973; Vasse, 1973; Fallon & Kelly, 1977). A similar ectodermal cap is found on limb regeneration blastemas of larval and adult urodeles (Thornton, 1968), but not on their limb buds (Sturdee & Connock, 1975; Tank, Connelly & Carlson, 1977). Regardless of its degree of thickening, removal of the apical ectoderm or epidermis at progressively earlier stages of limb ontogeny or regeneration results in progressively more truncated distal development (Steiner, 1928; Balinsky, 1935; Saunders, 1948; Goss, 1956; Tschumi, 1957; Amprino, 1965; Stocum & Dearlove, 1972). The commonly accepted role of the ectoderm or epidermis in limb ontogeny or regeneration is one of maintaining subjacent mesenchyme cells in an undifferentiated, dividing condition (Saunders, 1948; Janners & Searls, 1971; Summerbell & Wolpert, 1972; Summerbell, Lewis & Wolpert, 1973; Smith, Lewis, Crawley & Wolpert, 1974; Amprino, 1974; Mescher, 1976). However, in addition to its postulated role in maintaining cells in the cell cycle, the limb bud ectoderm or regenerate epidermis may also function to supply positional information specifying the distal boundary of the limb, as will be elaborated below.

**Determination of axial polarity**

The classical test for the determination of limb axial polarity is to reverse one of the three cardinal axes of the limb disc at different stages of development.
while grafting the disc in either the orthotopic or heterotopic (flank) position. If the limb develops with asymmetry opposite to that of its site of origin, the polarity of the axis in question is not determined at the time of surgery. Using this test, Harrison (1921) and Swett (1927, 1928a) concluded that the axes of the urodele limb disc are determined in sequence. That the limbs developing in these experiments were actually derived from the grafted discs was shown by heteroplastic exchange between *A. maculatum* and *A. tigrinum* (Schwind, 1932). By this test, the AP axis is already determined in *A. maculatum* and *T. pyrrhogaster* when the limb mesoderm becomes determined as limb; i.e. by the late gastrula or medullary fold stages (Detwiler, 1929, 1933; Takaya, 1941). The DV axis becomes determined between stages 33 and 35 in *A. maculatum* (Swett, 1927) and between stages 36 and 38 in *A. tigrinum* (Hollinshead, 1936) and *T. pyrrhogaster* (Takaya, 1941). Although the embryonic stages at which the DV axis is determined in *A. maculatum* and *A. tigrinum* are different, the gross morphological stage of the limb disc is the same in both (Hollinshead, 1936). The PD axis has been investigated only in *A. maculatum*, and becomes determined between stages 35 and 36, about the time the limb region begins outgrowth (Swett, 1927, 1928a). After reversal of the PD axis of a post-stage-37 limb bud, the bud tends to grow medially instead of laterally (Swett, 1927). This direction of growth is blocked by the body wall, and the bud instead turns and elongates in a posterior direction very close to the body wall. There is no regeneration from the original proximal end of the limb, which often remains as a visible swelling anterior to the growing tip. However, larval or adult forelimbs with reversed PD polarity can regenerate mirror image limbs from the formerly proximal end of the reversed limb (Milojevic & Grbic, 1925; Dent, 1954; Butler, 1955; Deck and Riley, 1958; Wallace, 1980).

An important result of the axial determination experiments was the production of duplicated limbs after either orthotopic or heterotopic transplantations (Harrison, 1921; Swett, 1927). The primary limb developed first, maintaining the AP polarity of the graft, while the secondary limbs began development somewhat later, and were mirror images of the primaries. The secondary limbs produced after AP reversal in the orthotopic position arose either on the radial side or ulnar side of the primary, or on both sides. The literature only infrequently contains a quantitative tabulation of the position of secondary limbs, and our statements are based on descriptions given in the texts and figures of Detwiler (1920), Harrison (1921, 1925), Swett (1926, 1927, 1930, 1932), and Blount (1935). After AP reversal in the heterotopic position, the few secondary limbs that developed arose on the radial side of the primary. When the AP axis was not reversed in the heterotopic position, the secondary limbs again arose on the radial side of the primary. These facts argue for some kind of interaction between the limb disc and the adjacent flank tissue, the result of which is different depending on the specific spatial relationships between the disc and the surrounding flank tissue.
Table 1. Frequency of duplication (%) in the orthotopic or heterotopic positions after reversal of the AP or DV axes or both, at different stages of ontogeny. A more complete table may be found in the paper of Bryant & Iten (1976)

<table>
<thead>
<tr>
<th></th>
<th>AP</th>
<th>DV</th>
<th>APDV</th>
<th>No Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthotopic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 29–35a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. mac.</em></td>
<td>85.7</td>
<td>6.3</td>
<td>74.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Post-stage 35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 <em>A. mex.</em></td>
<td>72.9</td>
<td>32.0</td>
<td>37.5</td>
<td>0.0</td>
</tr>
<tr>
<td>1 <em>A. mac.</em></td>
<td>87.5</td>
<td>0.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1 <em>N. virid.</em></td>
<td>100.0</td>
<td>0.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Heterotopic</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Stage 29–35a</td>
<td></td>
<td></td>
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<tr>
<td><em>A. mac.</em></td>
<td>16.7</td>
<td>56.7</td>
<td>7.7</td>
<td>44.7</td>
</tr>
<tr>
<td>Post-stage 35</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2 <em>A. mac.</em></td>
<td>—</td>
<td>90.5</td>
<td>—</td>
<td>41.3</td>
</tr>
</tbody>
</table>

1 Axial reversals carried out on the tips of limb buds at stages 38–46.
2 Axial reversals carried out on whole limb discs or buds at stages 35–42.
3 Average values derived from the data of Detwiler (1920); Harrison (1921, 1925), Swett (1926, 1927, 1930, 1932), and Blount (1935).
4 Average value derived from data of Maden & Goodwin (1980) and Thorns & Fallon (1980).
5 Data from Thorns & Fallon (1980).

Table 1 summarizes the frequency of duplication in *A. maculatum* and three other urodele species after orthotopic or heterotopic transplantation, with or without reversal of the AP, DV or AP and DV axes. The data allow four generalizations: (1) The operation itself is not the cause of duplication. (2) Duplication prior to stage 35 is most frequent in the orthotopic position after reversal of the AP axis, regardless of whether the DV axis is simultaneously reversed. (3) The frequency of duplication in the heterotopic position prior to stage 35 is highest when the AP axis is not reversed, regardless of whether the DV axis is simultaneously reversed. (4) There is a high frequency of duplication in post-stage-35 *A. maculatum* and *A. mexicanum* limb buds after reversal of either their AP or DV axes. Duplication also occurs at a high frequency after either AP or DV orthotopic axial reversal of regeneration blastemas (Milojevic, 1924; Iten & Bryant, 1975; Tank, 1978; Maden & Turner, 1978). When the AP axis is reversed, the secondary limbs have the same relationships to their primaries as are observed after AP reversal of limb discs in the orthotopic position. These results suggest that prior to stage 35, duplication occurs only after changing the spatial relationships of the AP axis of the limb discs with respect to the surrounding tissues. A comparable change of the DV axis alone elicits practically no duplication. After stage 35, however, reversal of the DV
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axis alone also results in the production of supernumerary limbs. The fact that supernumerary limbs can be produced by AP reversal is consistent with the conclusion that the AP axis is determined before the DV axis, if it is assumed that after its determination, the DV axis interacts with surrounding flank tissues differently than it does prior to its determination. In the latter case, the interaction leads to repolarization; in the former case, it leads to supernumerary production (as in the case with the AP axis).

The formation of supernumerary limbs after axial reversal of regeneration blastemas or post-stage-35 limb bud tips can be accounted for by models which postulate that, when confronted, the normally 180° opposite cells of host and graft reciprocally interact to stimulate mitosis and fill in, by intercalary regeneration between the poles, all the positional values specifying pattern in the transverse plane (Carlson, 1975; Bryant & Iten, 1976; French et al. 1976; Bryant & Baca, 1978; Stocum, 1980a). Once these supernumerary values are filled in, distal transformation can create a supernumerary limb. The supernumerary limbs are usually derived from both graft and host cells (Tank, 1978; Holder & Tank, 1979; Stocum, 1980a, b). However, the ability of these models to predict the number and especially the location of supernumeraries has been challenged in studies on regenerating urodele limbs (Maden & Turner, 1978; Wallace & Watson, 1979).

An intercalary regeneration model has also been applied to explain the duplications that occur after grafting embryonic limb discs (Bryant & Iten, 1976). The duplications that occur on the radial side of the primary after AP reversal of the disc in orthotopic position would be due to intercalary regeneration between the anterior edge of the limb disc and the peribrachial flank tissue posterior to it. Duplications forming on the ulnar side would result from intercalary regeneration between the posterior edge of the limb disc and the peribrachial flank tissue anterior to it. The duplications observed on the radial side of the primary after grafting discs to the flank without AP reversal would be the result of intercalary regeneration between the anterior edge of the limb disc with the posterior edge of the flank tissue that is capable of forming forelimb when stimulated by inductors. However, intercalary regeneration models predict no duplications after grafting a limb disc to the flank with reversed AP axiation, a situation where the anterior edge of the forelimb disc would supposedly contact the anterior edge of the hindlimb field. In reality, this operation results in a 16.7% incidence of duplication after reversal of just the AP axis, and a 7.7% incidence of duplication when both AP and DV axes are reversed (Table 1). Finally, intercalary regeneration models of supernumerary limb formation depend upon the assumption that the AP polarity of the embryonic limb rudiment is determined prior to stage 35, a notion we argue against after presenting an alternative model.
Fig. 3. Polarizing model for determination of the AP and DV axes. The classical limb disc, containing limb tissue (FL), shoulder girdle (SG), and posterior peribrachial flank tissue (PBF) is outlined by the large circle. The X's indicate tissue inferred to have a DV polarizing effect, the dots indicate tissue inferred to have an AP polarizing effect. The arrows show the direction of spread of the polarizing signals across the limb mesoderm in the normal embryo. The signals can spread in any direction from the polarizing tissue as shown by experiments in which dorsal polarizing tissue is rotated with respect to the limb tissue (Swett, 1938). PN = pronephros; a, p, d, v = poles of the AP and DV axes; G₁-₃ = gills.

The polarizing model

We propose a polarizing model that can account for both the genesis of axial polarity during limb ontogeny and the known facts about limb duplication after orthotopic or heterotopic transplantation of limb discs. In this model, posterior and dorsal peribrachial flank mesoderm specify AP and DV polarities in the adjacent limb mesoderm by means of signals which spread across the prospective limb mesoderm (Fig. 3). The polarizing tissues need not participate in the development of the limb, nor be reciprocally influenced by the limb tissues, but these possibilities are not ruled out. The AP polarizer is the posterior tissue of the classical limb disc that does not become part of the limb or girdle, and the DV polarizer is the flank tissue in the region of the pronephros. We now develop the argument for this model.

The importance of peribrachial flank tissue to forelimb development has been shown by several studies. Stage-29 limb discs transplanted to the dorsal or ventral midline (Nicholas, 1924a; Takaya, 1941; Finnegan, 1960), to the side of the head between eye and gill (Slack, 1977) or cultured in vitro (Wilde, 1950) fail to develop unless peribrachial flank tissue is included. That this developmental failure is not due to nonspecific effects related to the suitability of the transplantation site is shown by the fact that a limb disc grafted first to the head, then regrafted to the flank with a ring of head tissue around it also fails to develop (Swett, 1945). The postural orientation of the limb corresponds to the orientation of the ring of peribrachial flank tissue, not to the body of the embryo.
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as a whole, and duplication occurs only when the limb disc is disharmonic to the peribrachial ring, not when the ring plus limb tissue is disharmonic to the body (Nicholas, 1924b, 1958; Swett, 1945). Furthermore, duplication effects are not associated with tissues other than flank tissues (Detwiler, 1930; Swett, 1945).

The idea that the developmental importance of flank tissue is due to an axial polarizing activity was first raised in connection with determination of the DV axis of the urodele forelimb. Swett (1938) found that if dorsal peribrachial flank tissue alone was included with a stage-29 A. maculatum limb disc grafted to the flank with its DV axis reversed, repolarization of this axis to harmonize with the DV axis of the body was prevented. When the same experiment was made including ventral, instead of dorsal peribrachial tissue, the DV axis was repolarized. However, dorsal peribrachial tissue transplanted ventral to a flank grafted limb disc having harmonic DV orientation was unable to reverse the DV polarity of the disc. These results led Swett (1938) to view the dorsal peribrachial tissue in these experiments as a physical barrier to other DV polarizing factors in the flank. From his discussion, one would conclude that these factors are ventrally located. The interpretation of Swett’s results is complicated, however, by the fact that the transplantations were made to the flank, the dorsal portion of which he found to have effects similar to those of dorsal peribrachial tissue, thus failing to isolate the dorsal peribrachial tissue as the sole variable in the experiments. Takaya (1941) transplanted limb discs with attached dorsal or ventral peribrachial tissue to the ventral midline (where limb discs alone fail to develop), and found that the discs developed only when the dorsal tissue was included. This result suggests that the dorsal peribrachial tissue is indeed acting as a polarizer, specifying the dorsal–ventral axis, but these ventral midline experiments need to be repeated placing dorsal peribrachial tissue on the ventral side of the disc to see if this operation will reverse the DV polarity of the limb.

A zone of posterior mesodermal tissue which polarizes the AP axis has been postulated for the limb buds of Xenopus (Cameron & Fallon, 1977), reptiles, birds and mammals (Fallon & Crosby, 1977). The major evidence for this zone is that in chick limb buds posterior border tissue grafted to the anterior border stimulates the production of mirror-image supernumerary limb structures, with the most posterior part of the supernumerary arising adjacent to the graft (Saunders & Gasseling, 1968; MacCabe, Gasseling & Saunders, 1973; Fallon & Crosby, 1977; Summerbell & Tickle, 1977). Therefore, this region along the posterior limb bud has been called the zone of polarizing activity or polarizing zone (Balcuns, Gasseling & Saunders, 1970). It is clear that the ectoderm of the posterior border need not be present and that the orientation of the graft itself does not change the result (Fallon & Thoms, 1979). Further, the graft need not take part in the supernumerary outgrowth (Smith, 1979, 1980; Fallon & Thoms, 1979). The wing supernumerary limb elements that do form can be
altered by irradiating quail polarizing zone in increasing doses between 10000
and 90000 rads before grafting to the chick wing anterior border. At low doses,
supernumerary digits 4, 3 and 2 form, at intermediate doses only supernumerary
digits 3 and 2 form, and at high doses, only a supernumerary digit 2 forms.
With the highest doses, no supernumerary limb elements form at all (Smith,
Tickle & Wolpert, 1978). The quail polarizing zone does not contribute cells to
the supernumeraries formed in these experiments. These results suggest the
possibility that at increasing dose levels fewer surviving cells were left to con-
tribute whatever stimulus comes from polarizing zone.

Since the polarizing zone was discovered, there has been controversy about
its role in normal limb development. One major theory is that polarizing zone
is the source of a diffusible morphogen. It is proposed that the morphogen is in
a gradient across the limb bud such that the highest concentration is posterior,
intermediate concentrations are in the middle, and the lowest concentration is in
the anterior bud. This would bring about specification of polarity along the
anteroposterior axis. Thus, grafting a second polarizing zone would set up a
second morphogen gradient resulting in supernumerary outgrowth (reviewed
in Summerbell, 1979; Tickle, 1980a). There are various lines of evidence
supporting this point of view. However, possibly the most convincing data come
from the work of Summerbell (1979) who showed that an impermeable barrier
placed through the limb bud (ectoderm and mesoderm) at right angles to the
body wall caused deletions anterior to the barrier. He interpreted this to mean
that the morphogen from polarizing zone was blocked by the barrier and this
caused the deletions.

The fact that the apical ridge was also blocked, i.e. anterior ridge was separated
from posterior ridge, led to the suggestion that this separation in itself might
cause the observed anterior deletions. It was demonstrated that surgical re-
moval of posterior ridge in the wing bud did result in the failure of anterior
structures to develop even though anterior ridge remained. These results were
similar to those in Summerbell's barrier experiment. Furthermore, there seemed
to be a critical portion of posterior ridge which, once removed, resulted in the
failure of expected anterior structures (Rowe & Fallon, 1981). Equally impor-
tant is the fact that impermeable barriers grafted to the chick leg bud do not
result in deletions anterior to the barrier (Rowe & Fallon, 1982), nor for that
matter does posterior apical ridge removal in the leg result in anterior deletions
(Rowe & Fallon, 1981). One is left with the conclusion that either the barrier
does not block the proposed morphogen as Summerbell (1979) supposed or
that morphogen is not needed for polarization along the AP axis during the
developmental times studied. We stress that, at present, there are no unequivocal
data showing that polarizing zone has any role in polarizing the mesoderm
during the limb bud stages of development. This is consistent with the facts that
the wing bud develops normally following removal of the polarizing zone
(MacCabe, Gasseling & Saunders, 1973) and the zone does not regenerate after

It has been postulated that polarizing zone is not important for limb development at all. Saunders (1977) proposed this because he found polarizing activity in other than limb tissues. Iten and co-workers (Iten & Murphy, 1980; Javois & Iten, 1981; Javois, Iten & Murphy, 1981) claim no special significance for the polarizing zone on the basis of a set of experiments where nonadjacent wedges of limb mesoderm and overlying ectoderm were grafted into slits made into the host limb bud. Supernumerary structures formed even though the graft was of an anterior wedge to an anterior site. She interpreted these and other experiments to mean that the disparity in positional values caused intercalary replacement of the missing positional values and resulted in a supernumerary outgrowth. In this context, Iten sees no special properties associated with the polarizing zone.

There is a question whether Iten's data relate to the issue of whether or not there is a polarizing zone and whether it has a role in limb development. First, polarizing zone mesoderm without ectoderm will cause duplications. Iten always grafts the apical ectoderm with the wedges of mesoderm. Second, if anterior wedges of quail limb bud tissues are grafted to posterior sites in chick limb buds, in the manner described by Iten and co-workers, the supernumerary structures formed are composed entirely or almost entirely of quail cells (L. Honig, personal communication). This result differs from that obtained after grafting polarizing zone anteriorly. Here, the polarizing zone graft need not take part in the supernumerary, nor in fact need it be present after 12–15 h in place (Smith, 1979, 1980; Fallon & Thoms, 1979 and unpublished). Furthermore, if quail limb buds are irradiated in a dose that permits polarizing zone to act (see above) and anterior wedge-shaped pieces cut from them and grafted to posterior locations in unirradiated chick limb buds, no supernumerary structures develop (L. Honig, personal communication). This is different from other systems (Drosophila imaginal discs, regenerating amphibian limbs) in which positional discontinuity between irradiated and unirradiated tissue evokes supernumerary formation from the unirradiated component (Adler & Bryant, 1977; Maden, 1979). Third, as few as 100 polarizing zone cells, disassociated and grafted to the anterior wing border will evoke supernumeraries (Tickle, 1980b). A comparable experiment using as few displaced anterior cells grafted in the same way (Tickle, 1980b) should result in supernumerary outgrowth if Iten is correct. Fourth, polarizing zone will inhibit recombinant limb development (MacCabe, Saunders & Pickett, 1973; Crosby & Fallon, 1975; Frederick & Fallon, 1982). No other limb or flank cells have this property. Finally, Honig (1981) has shown that when polarizing zone tissue and a block of anterior leg tissue are grafted in tandem to the anterior border of the chick wing (with the leg graft posterior to the polarizing zone graft) the host anterior wing tissue next to the leg tissue produces mirror-image supernumerary structures. Anterior
Fig. 4. Frequency of duplication when progressively more posterior flank tissue is placed contiguous to anterior limb rudiment tissue, by (a) autoplastic grafting of the limb disc to progressively more posterior locations on the flank (O—O; A. maculatum) (Detwiler, 1920); (b) homoplastic grafting of the limb disc in the same fashion (●—●; A. maculatum) (Detwiler, 1920); or (c) heterografting progressively more posterior strips of flank tissue (one somite wide) adjacent to the anterior border of the limb rudiment (△—△; A. mexicanum) (Slack, 1976). In the A. maculatum experiments, the limb rudiment was positioned so that its anterior border was at the flank levels indicated by the somite numbers. Note that the duplication frequency is highest from the 5th to the 8th somites, decreasing sharply under the 4th and 9th somites.

leg tissue grafted alone does not result in supernumerary formation. The leg tissue has been grafted with and without apical ridge (L. Honig, personal communication). This result signifies that the posterior border tissue acts on the host wing tissue at a distance, not by intercalation initiated by local interactions between the edges of a positional discontinuity, and constitutes strong evidence for a posterior polarizing region. All of these data suggest it is the graft that grows out in Iten's experiments and this is quite different from the outgrowths caused by polarizing zone, where anterior tissue grows out. The fact remains that there is a demonstrable high point of morphogenetic activity along the mesoderm of the posterior border of the chick and all other amniote limb buds tested to date.
Since it appears that posterior border tissue is not required for AP polarization in the growing chick limb bud, Fallon & Crosby (1977) have suggested that a polarizing region of flank tissue determines the AP axis of the prospective chick limb mesoderm prior to its outgrowth as a bud, and is not thereafter required for normal limb development, although its continued (but residual) activity can be demonstrated by grafting. Fallon & Crosby’s (1977) notion has not been tested on early chick embryos, but is consistent with the fact that the transverse axes of the prospective urodele forelimb are polarized prior to outgrowth of the limb as a bud, and receives support from other studies on urodele limb ontogeny.

First, Swett (1926, 1928b) showed that when the limb disc of a stage-27 to -34 *A. maculatum* embryo was separated into anterior and posterior halves by a thin strip of flank tissue, two primary limbs with the same asymmetry were formed. However, a mirror-image secondary limb usually arose on the radial side of the more posterior primary member, suggesting a selective repolarization of a portion of this member by the grafted flank strip. Second, the posterior edge of the limb disc (the portion which does not participate in the formation of the limb), as well as flank tissues from the 5th to the 8th somites, can stimulate the production of a mirror-image secondary limb on the radial side of a limb disc when in contact with that side (Detwiler, 1920; Slack, 1976). The mesoderm, not the ectoderm, is the effective component. Taken as a whole, the data indicate that the ability of flank tissues to effect duplication is high from somites 5 (non-limb tissue that is part of the classical limb disc) to 8, exhibiting an increase between these two points, and declining sharply under somites 4 and 9 (Fig. 4). Anterior limb disc or anterior peribrachial tissue grafted to the posterior edge of the limb does not result in duplication (Takaya, 1941; Slack, 1976).

The concept of an AP polarizing effect, in which a large region of flank tissue can specify adjacent limb-forming mesoderm as most posterior, can explain the frequency, location, and handedness of duplicated limbs arising after orthotopic and heterotopic transplantations of urodele limb discs (Fig. 5). As seen from the map of the forelimb region (Fig. 1), the transplanted limb discs of most investigations have contained polarizing flank tissue on their posterior edge. The high frequency of duplication on both the radial and ulnar sides of primary limb discs grafted in the AP-reversed orthotopic position would be due to the fact that polarizing tissue is put in contact at both these loci with anterior tissue having limb-forming capability. The high frequency of duplication on the radial side of limb discs grafted to the flank without AP reversal would be the result of placing the anterior edge of the limb disc within a flank region of high polarizing activity. No duplications would arise on the ulnar side of these grafts because the polarizing tissue of their posterior edge does not induce duplications from flank tissue. Duplications would be expected to arise at the observed low frequency on the radial side of AP-reversed limb discs grafted to the flank because the anterior edge of the disc would sometimes lie within the
(A) Orthotopic: axial reversal

(B) Heterotopic: no axial reversal

(C) Heterotopic: axial reversal
rapidly falling gradient of polarizing activity between the 8th and 9th somites, but would lie completely outside the polarizing region in the vast majority of grafts. Similar conclusions based on an AP polarizing action of flank tissue have been drawn by Slack (1976, 1977).

The polarizing model predicts that the secondary limbs produced after grafting limb discs to the flank should all arise from the graft. This prediction has been borne out in experiments by Swett (1932) in which limb discs were heteroplastically exchanged between *A. maculatum* and *A. tigrinum*, although Scharrer (1931), on the basis of similar heterografting experiments using the same species, has claimed that some of these limbs can arise partly from host flank tissue. The model also predicts that secondary limbs produced on the ulnar side of limb discs grafted in the AP-reversed orthotopic position should arise from respondent anterior peribrachial tissue, while those produced on the radial side should arise from respondent anterior limb disc tissue. Although Harrison (1921), Swett (1926), and Detwiler (1930) state, on the basis of external morphology, that secondary limbs always originate from the grafted disc, no cell or tissue markers were employed in their orthotopic transplantations. Scharrer (1931) showed by heteroplastic grafting that secondary limbs produced in the orthotopic position can arise entirely from host or graft tissue, which is in general accord with the prediction. However, he did not make any specific correlation between the tissue origin of the secondary limbs and their radial or ulnar location, and this type of experiment should be repeated with such a correlation in mind. Nevertheless, Slack (1976) has shown that secondary limbs formed after heterografting flank tissue adjacent to the anterior edge of the limb disc in orthotopic position are always derived from the limb disc tissue, as predicted by the polarizing model.

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Fig. 5. Diagrams indicating how the polarizing model accounts for the location and handedness of duplications after orthotopic and heterotopic transplantation of limb discs at stage-29. The upper diagrams of each set depict the grafting operation and the tissues from which the primary (1°) and secondary (2°) limbs are predicted to arise. The arrows show the origin, direction, and extent of penetration of the anterior-posterior polarizing effects of the flank tissue on the posterior side of the disc and the remaining flank tissue surrounding the disc. The dorsal-ventral axis is repolarized to normal at stage-29 whenever it is reversed by the operation (Swett, 1927), and is pictured this way in the diagrams. The lower diagrams of each set show the symmetry relations of the fully developed primary and secondary limbs. a, p, d, v = poles of the anterior-posterior and dorsal-ventral axes; L, R = left and right. (A) Orthotopic transplant of either a right limb disc to the right side, reversing the AP and DV axes, or a left limb disc to the right side, reversing only the AP axis. (B) Heterotopic transplant of right limb disc to the right side, no axial reversal, or a left limb disc to the right side, reversing only the DV axis. (C) Heterotopic transplant of either a right limb disc to the right side, with AP and DV axial reversal, or a left limb disc to the right side, reversing only the AP axis. The frequency of duplication after the latter transplantations is much lower than after any other, because the anterior edge of the grafted disc is in a flank region having little or no polarizing activity.
The polarizing model also offers a possible explanation for a puzzling aspect of the development of limbs induced from flank tissue by nasal placodes and other inducers. These limbs were usually of reversed AP polarity and normal DV polarity (Balinsky, 1933; Ichikawa & Amano, 1949). Furthermore, Takaya (1941) showed that limbs of normal AP and DV polarity were formed after grafting inducers into blocks of flank tissue whose AP axis or AP and DV axes were reversed. Takaya’s flank tissue blocks were 3–5 somites in width and always included tissue posterior to the 8th somite. These facts suggest that the disharmonic AP polarity of limbs induced without flank axial reversal is associated with the AP asymmetry of the steep gradient in polarizing activity posterior to the 8th somite. This asymmetry is reversed in the flank tissue rotation experiments, so that it has the same polarity as the gradient from the 5th to the 3rd somites. Takaya’s reversed grafts did not include dorsal flank polarizing tissue; thus the DV axis of the induced limbs would have been repolarized to normal. This hypothesis suggests that limbs induced between somites 5 and 8, where AP polarizing activity is high, would develop as symmetrical double posterior limbs. Slack (1977) has noted that a number of the flank-induced limbs illustrated in the literature appear to be symmetrical in their AP axis.

The nature of the polarizing signal is unknown. The positional information of the polarizing zones could be transmitted across the prospective limb mesoderm in the form of a gradient of diffusible morphogen, with each concentration specifying a positional value to be interpreted by the cell and transduced into the proper pattern of gene activity (Tickle, Summerbell & Wolpert, 1975). The morphogen could diffuse through the extracellular spaces from its source or from cell to cell via gap junctions (Furshpan & Potter, 1968; Wolpert, 1978; Bennett, Spray & Harris, 1981). Alternatively, the polarizing effect could be in the form of a gradient of cell surface molecular organizations, with the polarizing zones acting as origins of the gradient, which could be developed by local interactions starting at the junction of the zone and the adjacent prospective limb tissue and proceeding in sequence across the prospective limb tissue. Likewise, the interactions between DV and AP polarizing activities are unknown. The same kind of molecules and mechanisms may be involved in establishing both polarities, or the molecules and/or mechanisms may be different in each case.

**Simultaneous determination of AP and DV axes**

The polarizing model raises the possibility that the determination of the AP axis before the DV axis in the prospective forelimb is an illusion. The error would be created by the fact that in the flank grafting experiments used to test this determination, the grafted tissue contains AP polarizing mesoderm and/or is embedded in it. Thus, a stage-29 limb disc with an undetermined AP axis would behave in reversal experiments as if this axis were determined, due to the action of its polarizing tissue, just as the DV axis behaves as if determined when
An interesting aspect of radially duplicating limbs is indicated by one of Swett's (1940) experiments. He found that the number of mitoses observed in the early stages of a radially duplicating limb bud was no greater than the number observed in the contralateral, non-duplicating bud. This observation suggests that the cells of the anterior half of the original limb rudiment are being reassigned AP positional values in a mirror-image sequence by adjacent polarizing tissue. Cells of the posterior half of the original rudiment would also have their positional values reassigned to restore a complete set of AP values of the original asymmetry. Slack (1980) has also stated that half-limb rudiments which regulate to form whole limbs do not show a larger number of mitoses next to the cut edge. The significance of these observations, however, requires further evaluation, for neither Swett nor Slack presented data on the total number of cells, i.e. a mitotic index was not calculated in the experimental and control buds.

Evidence for the plasticity of limb AP polarity throughout tailbud stages comes from studies on the hindlimb region. Stultz (1935) grafted AP-reversed stage-35 *A. maculatum* hindlimb areas homoplastically or heteroplastically (to *A. tigrinum*) to the flank, or in orthotopic position (the development of the hindlimb is 2–3 weeks behind that of the forelimb). In the heterotopic position, the grafted rudiments developed with reversed AP polarity, but in the orthotopic position, they developed with normal AP polarity. These results suggest that at this stage, the AP axial polarity of the hindlimb rudiment can easily be reversed by the tissues immediately surrounding the orthotopic site.

However, the most intriguing evidence for labile AP polarity during tailbud stages, and its determination by polarizing flank tissue, involves a relationship between limb and gill tissues. Detwiler (1922) found that limb discs transplanted in place of gill tissue developed poorly. Wilde (1952a) subsequently carried out a series of ingenious experiments on stage-28 to -38 *A. maculatum* embryos, in which the positions of gills 1–2 and the limb disc were reversed by 180° rotation of the limb–gill complex (leaving gill 3 in place), thereby placing gill tissue anterior and posterior to the limb disc. The operations were done in such a way that the AP and DV axes of both limb and gill were either reversed or remained harmonic with respect to the body. From stages 28–33, when the AP axis is supposedly determined and the DV axis is not, limb development was inhibited. Normal limbs developed from transplants done at stages 35–38. Wilde (1952b) also conducted in vitro studies which demonstrated a similar time course for the acquisition of self-organizational capacity by the limb disc or bud in the presence of gill tissue. The suppressive effect on self-organization was proportional to the amount of gill tissue in the explants, and the limbs exhibited radial symmetry of what little differentiation there was, instead of the expected asymmetry.

Wilde (1952a) noted that the time at which the limb rudiment acquired the
ability to develop in the presence of gill tissue coincided with determination of the DV axis. This fact suggests that outgrowth and morphogenesis of the limb cannot begin until the DV axis has become polarized. We might then conclude that the failure of pre-stage-35 limb discs to develop in these experiments is due to inhibition of DV axial determination by gill tissue, even though the AP axis is already determined. However, Wilde's diagrams show that dorsal peribrachial (polarizing) tissue was included in his grafts, and that this tissue was not in contact with gill tissue. Therefore, the DV polarizing region of pre-stage-35 limb discs should be present in these experiments. We are then led to the conclusion that development of these discs fails because their AP polarity has not yet been established and is prevented by adjacent gill tissue from becoming established. The effect of the gill tissue would be to specifically inhibit the AP polarizing activity of the flank mesoderm in the posterior part of the limb disc. Specific inhibition of posterior disc tissue is indicated by the fact that transplantation of gill tissue to the anterior edge of the hindlimb rudiment does not inhibit hindlimb development (Wilde, 1952a).

The foregoing discussion not only leads to the conclusion that neither the AP nor the DV axes of the prospective limb tissue are determined prior to stage 35, but that both axes must be determined before limb outgrowth and morphogenesis can begin. This conclusion is also supported by the fact that limb discs (which contain AP polarizing tissue) grafted to the ventral midline with attached dorsal peribrachial tissue alone will produce limbs. However, if limb discs are grafted with attached ventral peribrachial tissue alone, they fail to develop (Takaya, 1941). Recently, Slack (1980) has made the very important finding that half or double half limb rudiments will not develop when grafted to the head unless both posterior and dorsal peribrachial flank tissue are present in the graft. In view of these facts, we suggest that the following two concepts are central to the understanding of limb ontogeny: (1) The AP and DV axes of the prospective vertebrate limb mesoderm are determined together, and (2) determination of both these axes is a prerequisite for the outgrowth and polarization of the PD axis.

**Proximal-distal outgrowth**

Since amphibian limb parts are laid down in a proximodistal sequence under the influence of the apical ectoderm (Tschumi, 1957), the dividing cells of the limb mesoderm must change positional value in a distal direction as the disc grows out into a bud. The growing limb bud is a three-dimensional structure with internal pattern, therefore its parts must be specified by at least three positional coordinates (values). Two of these values specify position in the transverse plane, while the third specifies position on the PD axis. Thus, each cell can detect its position relative to each of its neighbours, and the prospective tissue pattern can be represented as a map of positional values. The transverse positional values are specified by the action of the dorsal and posterior polariz-
Fig. 6. Boundary model of proximal-distal limb bud outgrowth. (A) Transverse section through a right limb rudiment (L) whose AP and DV axes have just been determined, but which has not yet begun outgrowth. The mesoderm cells of the rudiment in contact with the overlying ectoderm (E) assume the most distal (Ds) positional value as a result of this contact. The remaining cells have the most proximal (Pr) positional value. These boundaries (stippled cells) represent the proximal-distal axial outline. The discontinuity will be filled in by cell division and continual distal averaging of positional values by the daughter cells (unstippled) (Maden, 1977). (B) Three-dimensional cutaway diagram of a growing undifferentiated right limb bud, showing the boundary shell of mesoderm cells in transverse and longitudinal section. The arrows indicate the distal direction of positional value change. a, p, d, v = poles of anterior-posterior and dorsal-ventral axes.
ing regions, and once this specification has taken place, the limb rudiment can grow out and the PD values can be specified. The morphogenetic field of the limb may thus be viewed as a set of AP, DV and PD boundary positional values which prescribe the structure to be developed (Fig. 6). The AP and DV boundary values will be those represented by the cells at the axial poles of the transverse plane. The ability of the limb area to develop autonomously prior to stage 35 thus requires that the polarizing tissues be included with the prospective limb tissue, whereas self-organization can proceed in their absence after this stage.

The proximal boundary value is that value intrinsic to the limb mesodermal cells prior to the initiation of limb outgrowth, and it specifies the base of the limb. We now make the important provision that cells of the layer of limb mesoderm in contact with the overlying ectoderm must assume the most distal boundary value as a result of that contact (Maden, 1977; Stocum, 1978, 1980a). Further, the dividing cells between the two boundaries or their progeny are free to change their positional values in a distal direction. The PD positional value map is filled in by intercalation between the boundaries, possibly involving a synchronous averaging mechanism (Maden, 1977). All the mesodermal cells are continually monitoring the positional values of their neighbours during limb ontogeny, and are consequently acting as local boundaries. However, the most proximal and most distal boundaries are special in that all changes in positional value are ultimately referenced to them.

We postulate as others do (Maden, 1977) that positional value may be reflected in the molecular organization of the cell surface coat. Thus, in establishing the PD limb positional value map by intercalation, the mesoderm cells interact in an integrated network of strictly local communications. Since the structural details of the limb in the transverse plane are different at different levels of the PD axis, changes in AP and DV positional values during limb outgrowth must be coupled to changes in transverse values. We postulate an intracellular integrating mechanism in which changes in PD value simultaneously effect changes in transverse values so that they correspond to the new PD level. The three cardinal axes of asymmetry thus develop interdependently during limb outgrowth (see also Thoms & Fallon, 1980).

Relationship of limb ontogeny to regeneration

The limb regeneration blastema is formed by the dedifferentiation of stump tissues, and it redifferentiates a replica of the parts lost by amputation. The blastema is a polarized, self-organizing structure that undergoes autonomous redifferentiation and morphogenesis when isolated from its stump (Stocum, 1968, 1978, 1980b; de Both, 1970; Michael & Faber, 1971; Dinsmore, 1974). The morphogenetic field of the regeneration blastema can thus be considered as a set of boundaries of the structure that is to be regenerated, equivalent to that of the limb bud, except that the proximal boundary of the regenerate is deter-
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mined by the positional value of the cells at the level of amputation, and the distal boundary is imposed by the apical wound epidermis (Maden, 1977; Stocum, 1978, 1980a). The supernumerary limbs that form at the graft-host junction after reversing the AP or DV axis of the blastema with respect to the stump can be attributed to the intercalation of new sets of transverse positional values, using the apposed axial poles represented by blastema and stump as boundaries, followed by intercalation of PD values. Likewise, the boundary model explains why tissues internal to the skin (muscle, bone) can be removed from the limb, yet be regenerated distal (but not proximal) to the amputation plane by the blastema derived from the remaining tissue (Bischler & Guyenot, 1925; Weiss, 1926; Goss, 1957; Carlson, 1972). After these operations, either muscle, skin or both are retained in a normal configuration. These tissues contain the positional values representing the entire periphery of the transverse plane of the limb. Therefore, when the cells of these tissues dedifferentiate, opposite positional values will be brought into contact, and intercalation of those values internal to the periphery will ensue.

A major question is whether specification of the map of transverse positional values in the regeneration blastema, or the production of supernumerary regenerates, requires the renewed activity of latent polarizing zones incorporated into the limb during ontogeny. This possibility is unlikely because exclusion of posterior tissue (from which the AP-polarizing zone would be activated) from a limb does not render it incapable of regeneration. Thus, double anterior-half wrist blastemas evoke intercalary regeneration from double anterior-half thighs when grafted to them (Stocum, 1981). The production of both primary and supernumerary regenerates can best be explained as a result of reciprocal stimulation and intercalary regeneration between boundary cells whose positional values were established during ontogeny, and inherited by blastema cells during dedifferentiation, or imposed by contact with apical wound epidermis (distal boundary, Maden, 1977).

CONCLUSIONS

The results of numerous experiments described in the literature of the past 70–80 years suggest the following concepts of urodele limb ontogeny: (1) The AP and DV axes of the limb rudiment are not determined in sequence, but simultaneously between stages 33 and 35, by the action of dorsal and posterior polarizing tissues, and (2) determination of the PD axis and outgrowth of the limb bud cannot begin until the AP and DV axes of the limb rudiment are determined. We emphasize that these assertions are by no means proven; however, their antecedent arguments suggest a variety of experiments to test them.

The morphogenetic field of the limb rudiment is initially unpolarized, or has only a very weak polarity. Dorsal and posterior regions of peribrachial
flank tissue polarize the AP and DV axes of the field, assigning to its cells a set of positional values specifying pattern in the transverse plane. Proximal–distal outgrowth then takes place, generating, between the distal and proximal boundaries of the field, the set of positional values that specifies the PD axis of the limb.

During the regeneration which occurs after amputation of a part of the urodele limb, the distal boundary of the limb must be reconstructed, and is imposed upon the apical layer of blastema cells specifically by the apical wound epidermis. The proximal boundary of the regenerate is the PD value, recorded during ontogeny, of the PD level of origin of those blastema cells not in contact with the apical wound epidermis. As in limb bud outgrowth, a complete set of PD positional values is restored by distal transformation of blastema cells, using the boundaries as reference points.

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