Title
Retinoic acid, local cell-cell interactions, and pattern formation in vertebrate limbs

Journal
Developmental Biology, 152(1)

ISSN
0012-1606

Authors
Bryant, SV
Gardiner, DM

Publication Date
1992-07-01

DOI
10.1016/0012-1606(92)90152-7

DEVELOPMENTAL BIOLOGY 152, 1-25 (1992)

REVIEWS

Retinoic Acid, Local Cell–Cell Interactions, and Pattern Formation in Vertebrate Limbs[1]

S. V. BRYANT AND D. M. GARDINER

Developmental Biology Center, University of California, Irvine, Irvine, California 92717

Accepted April 1, 1992

Retinoic acid (RA), a derivative of vitamin A, has remarkable effects on developing and regenerating limbs. These effects include teratogenesis, arising from RA's ability to inhibit growth and pattern formation. They also include pattern duplication, arising as a result of the stimulation of additional growth and pattern formation. In this review we present evidence that the diverse effects of RA are consistent with a singular, underlying explanation. We propose that in all cases exogenously applied RA causes the positional information of pattern formation-competent cells to be reset to a value that is posterior-ventral-proximal with respect to the limb. The diversity of outcomes can be seen as a product of the mode of application of exogenous RA (global versus local) coupled with the unifying concept that growth and pattern formation in both limb development and limb regeneration are controlled by local cell–cell interactions, as formulated in the polar coordinate model. We explore the possibility that the major role of endogenous RA in limb development is in the establishment of the limb field rather than as a diffusible morphogen that specifies graded positional information across the limb as previously proposed. Finally, we interpret the results of the recent finding that RA can turn tail regenerates into limbs, as evidence that intercalary interactions may also be involved in the formation of the primary body axis.

INTRODUCTION

Developing and regenerating limbs respond in remarkable and diverse ways to retinoic acid (RA). In different organisms and under different conditions, these responses lead to various types of pattern duplications as well as to teratogenesis. Over the last year or two, there has been a flurry of reviews that have focused either on limb development or limb regeneration (see for example: Brockes, 1990; Thaller and Eichele, 1990b; Duboule, 1991; Maini and Solursh, 1991; Stocum, 1991a; Tabin, 1991; Tickle, 1991). All have drawn attention to some of the known and interesting facts about the effects of RA on limbs. Our article differs from the previous reviews by providing a unified view of the mode of action of RA and in presenting evidence that the apparently varied RA responses can be comprehensively understood in terms of the local cell-cell interactions that drive limb outgrowth and pattern formation. Further, we propose that the principle function of endogenous RA in limb development is in the establishment of the limb field. We begin with the two premises concerning limb development and regeneration that underlie our interpretation.

A. PREMISES

1. Developing and Regenerating Limbs of All Vertebrates Use the Same, Basic Mechanisms for Limb Outgrowth and Pattern Formation

There are several lines of evidence that support this premise and none that argue against it. This issue is also addressed in Muneoka and Sassoon (1992). First is the fact that the basic features of limb outgrowth, such as proximal to distal elaboration of the pattern and the requirement for a permissive epidermis, are the same for the developing limbs of all the different classes of vertebrates and for regenerating amphibian limbs (see Bryant and Muneoka, 1986).

Next is the fact that all vertebrate limbs share a common repertoire of regulative responses to experimental interventions (Bryant and Muneoka, 1986) (Fig. 1). These include the development of supernumerary limbs in response to positional disparities, the ability to intercalate missing parts of the pattern along the proximal-distal axis, and the ability to regenerate amputated distal parts of the pattern. In urodele amphibians these responses can be evoked from limb cells at any time

[1] This article is dedicated to the memory of Howard A. Schneiderman, a friend and colleague for close to 25 years, whose support for the work and ideas at the heart of this article never wavered.

0012-1606/92 $5.00

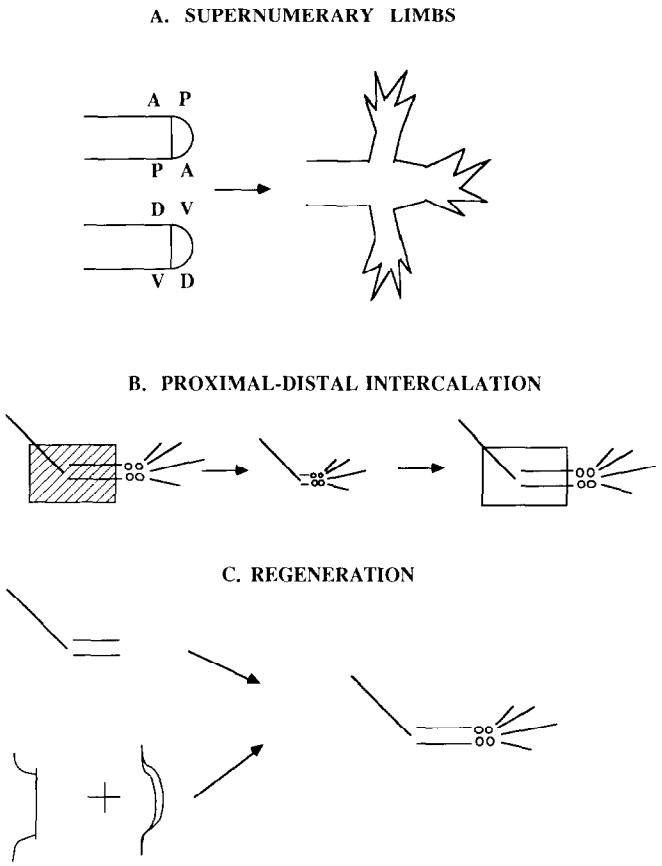

FIG. 1. Spectrum of regulative abilities of vertebrate limbs. (A) Formation of supernumerary limbs. Urodeles form supernumerary limbs in response to anterior–posterior as well as dorsal–ventral confrontations. The ability to form D and V supernumerary limbs is restricted in higher vertebrates due to the restricted location of the apical ectodermal ridge along the anterior–posterior distal edge of the limb bud (see text). A, anterior; P, posterior; D, dorsal; V, ventral. (B) Intercalation along the proximal-distal axis. Regenerating urodeles show this behavior. The midsection of the limb (boxed region on the left limb) is removed by transplantation of a blastema from a distal to a proximal limb level. Intercalary regeneration replaces the removed segment of the limb. Developing limbs of higher vertebrates show this behavior at early stages, when cells are still within the progress zone at the limb tip (see text). Stick diagrams show a single element in the stylopod, two elements in the zeugopod, and four digits with their associated carpals (or tarsals) in the autopod. (C) Regeneration. Mature urodele limbs (above) regenerate completely following amputation. Developing limb buds of higher vertebrates (below) can regenerate if provided with a permissive distal ectoderm (see text). Stick diagrams are as in B.

during the life of the animal. In all other tetrapods, the regulative responses are more restricted, both spatially and temporally.

In the developing chick limb, for example, only cells in the distal mesenchyme (progress zone) under the apical ectodermal ridge (AER) exhibit these behaviors (Summerbell *et al.*, 1973; Summerbell, 1977; Kieny and Pautou, 1977). Once cells leave the progress zone during limb outgrowth, they no longer participate in the creation of new pattern. The regulative response of distal limb bud cells is further restricted by the limited spatial extent of the permissive AER. For example, supernumerary limbs develop in response to anterior–posterior confrontations because the site of the positional disparity is beneath the AER, which is present as a narrow distal band extending anterior to posterior (Tickle *et al.*, 1975). In contrast, if dorsal and ventral cells are confronted by bud tip grafting, the positional disparity does not lay beneath the AER, and supernumerary limbs do not develop. Despite the absence of a full supernumerary response, supernumerary muscles are generated in response to dorsal–ventral confrontations (Javois and Iten, 1982). Similarly, the lack of regenerative ability in chick limb buds can be viewed as a consequence of a lack of a permissive epidermis. Numerous experiments have shown that if the AER is removed in chicks, the epidermis heals but an AER is not reformed. As a consequence, further pattern formation ceases (Saunders, 1948). We have recently confirmed earlier reports that if amputated chick limb buds are resupplied with an intact AER, they are capable of complete regeneration (Rubin and Saunders, 1972; Muneoka *et al.*, 1992). Hence, cells that have recently exited the progress zone can be caused to reenter it. In mice, a distinct AER does not form until after outgrowth is well underway (see Wanek *et al.*, 1989b), and it is possible that this lesser dependence upon a maximally developed AER could account for the ability of early stage rodent limb buds to "regenerate" in culture (Deuchar, 1976; Chan *et al.*, 1991) and of older limb buds to regenerate their peripheral digits (Wanek *et al.*, 1989a).

The limitations discussed above can account for all the deficits in regulative behavior displayed by higher vertebrate limbs when compared to the spectrum shown at its most complete by the limbs of urodele amphibians. In urodeles, outgrowth permitting epidermis covers the entire outgrowth, and it is readily reformed after removal (Stocum and Dearlove, 1972). Further, limb cells that leave the progress zone at the limb tip can reinitiate pattern formation in response to injury.

A third line of evidence that outgrowth of developing and regenerating limbs involves the same mechanisms comes from a direct test that asks whether developing and regenerating limb cells can interact appropriately with one another to form a limb (Fig. 2). This test has been carried out in urodeles, the only group to both develop and regenerate their limbs. Grafts to confront anterior cells of a developing limb with posterior cells of a regenerating limb and vice versa lead to the formation of supernumerary limbs of normal structure (Muneoka and Bryant, 1982). Further, these limbs are derived in equal parts from cells that originated on the developing

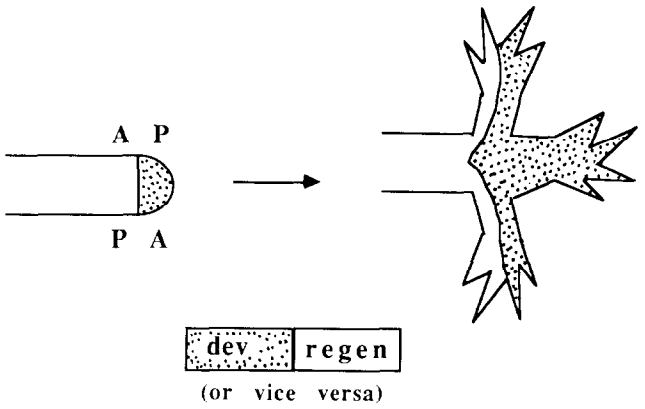

FIG. 2. Direct test of the similarity of mechanism in limb development and regeneration. When grafts are made between developing limb buds and regenerating blastemas of urodeles, developing and regenerating cells on either side of the confrontation interact to form supernumerary limbs. The contribution pattern is indistinguishable from the result of grafting within developing or regenerating limbs.

limb side of the confrontation and cells that originated on the regenerating limb side (Muneoka and Bryant, 1984b) (Fig. 2). In other words, the interaction of cells from regenerating and developing limbs at an anterior-posterior positional disparity is no different than the interaction that occurs at a similar disparity within developing or regenerating limbs tested separately (Muneoka and Bryant, 1984a).

A final line of evidence concerning the universality of limb outgrowth and pattern formation mechanisms is that genes considered likely to be involved in pattern formation are expressed in both developing and regenerating limbs. Although we are only now at the threshold of uncovering the genes involved in limb pattern formation, sufficient evidence is already available to support this conclusion. Candidate pattern formation genes that have been reported to date include the various nuclear and cytoplasmic receptors for RA (see Mendelsohn *et al.*, 1992), as well as various homeobox genes (see Izpisúa-Belmonte and Duboule, 1992; Muneoka and Sassoon, 1992). Although data for only two homeobox genes from regeneration blastemas have been published (*Hox 3.3:* Savard *et al.*, 1988; *Hox 4.6:* Brown and Brockes, 1991), it is clear that several more are expressed. In our own efforts to isolate and characterize homeobox genes expressed during regeneration, we have thus far identified the axolotl homologues of more than a dozen additional homeobox genes (Gardiner and Blumberg, unpublished data).

The differences that do exist between limb development and limb regeneration all seem to involve aspects of cell biology that are not directly related to the control of growth and pattern formation. Most of these differences are associated with events involved in dedifferentiation of mature limb tissues leading to the creation of a group of undifferentiated cells from which outgrowth and pattern formation can proceed (see Wallace, 1981). Differences have been noted in a variety of cytoskeletal components of cells in limb buds versus regenerates (Ferretti and Brockes, 1991). These differences presumably reflect the fact that cells in regenerating limbs have to leave their organized and differentiated tissues and migrate toward the center of the amputation surface (Gardiner *et al.*, 1986). There are also differences between limb development and limb regeneration in the characteristics of the respective epidermal cells. The apical cap of regenerating limbs, which has outgrowth-permitting properties similar to the AER, arises by dedifferentiation from stump epidermis (Tassava *et al.*, 1986). Another often cited difference between the two types of outgrowth is that blastema cells, unlike limb bud cells, are dependent on a mitosis-permitting trophic factor supplied by nerves during the early stages of the regeneration process (Singer, 1978). This trophic factor, whose nature is at present unknown, does not control the amount of growth or the nature of the pattern, but rather functions solely as a permissive growth requirement (see Muneoka *et al.*, 1989). As Kiffmeyer *et al.* (1991) have pointed out, the blastema is only nerve-dependent during the early phase of regeneration, a time during which it is also avascular. It is possible that the nerves provide a required factor that is also present in the blood. The required factor would therefore be available to developing limb buds and to older regenerates by means of the vascular supply; the nerves would supply the required factor to regenerates during the avascular phase (Kiffmeyer *et al.*, 1991).

The experiments that are most frequently cited to argue that limb development and limb regeneration are different processes are those of Scadding and Maden (1986a,b). In these experiments, animals with both developing and regenerating limbs were exposed to RA in the aquarium water, yet developing and regenerating limbs gave different responses. As we show in detail below (Section B4), this difference in response can readily be accounted for as a result of the dedifferentiation response in regenerating limbs, which continues to add pattern formation-competent cells to the base of the blastema. In contrast, when developing limb buds (no amputation) are treated with RA (Section B3), all pattern formation-competent cells (cells in the progress zone) are affected by RA and no new, unaffected cells are available to participate in pattern formation. Thus, the difference in response shown by developing and regenerating limbs is a consequence of whether or not dedifferentiation is stimulated, rather than being a consequence of differences in the basic properties of pattern formation and growth regulation in the two situations.

In summary, in all essential features concerning growth and pattern formation, limb development, and limb regeneration are alike.

2. Local Positional Differences Drive Growth and Pattern Formation; in the Absence of Positional Differences, Growth and Pattern Formation Cease

The clearest demonstrations that positional differences are essential for growth and pattern formation come from grafting experiments to generate positional disparities (French *et al.*, 1976; Bryant *et al.*, 1981). For example, when posterior cells are placed next to anterior cells, growth and pattern formation are stimulated and a new limb forms between the confronted cells. In higher vertebrates, the pattern of cellular contribution has become very one-sided, with anterior cells contributing the majority of the cells for the new outgrowth (Honig, 1983; Javois and Iten, 1986). Hence, when a small group of posterior chick wing bud cells is grafted into an anterior (responding) location, a full supernumerary set of digits is obtained (Tickle *et al.*, 1975). In contrast, when small grafts of anterior (responding) cells are made into a posterior site, the supernumerary response is more limited, undoubtedly due to the small pool of cells available to respond (Iten and Murphy, 1980; Honig, 1983). Nevertheless, when the whole tip is rotated, thereby bringing anterior and posterior regions of the bud into contact on both edges of the limb bud, two full supernumerary limbs are produced as expected (Saunders *et al.*, 1958; Javois and Iten, 1986).

In amphibians, which we assume represent the more generalized condition, it has been clearly demonstrated that the cells that form supernumerary limbs are contributed equally from anterior and posterior parts of the confrontation (Muneoka and Bryant, 1984a,b; Muneoka and Murad, 1987). Even when small clumps of cells are transplanted from posterior to anterior and vice versa, the frequency and completeness of supernumerary outgrowths are the same (Rollman-Dinsmore and Bryant, 1982; Groell and Gardiner, unpublished data). These results require that the pattern formation mechanism involves both anterior cells and posterior cells in both signaling and responding. In the simplest case, cells will respond by growth and intercalation when they are confronted with neighbors from a distant part of the pattern. If some condition has occurred to prevent one of the partners in the interaction from contributing, as appears to be the case in chick limbs and probably also in mice (see Wanek *et al.*, 1989a), the pattern is generated by the remaining partner. Equivalent one-sided contribution can be demonstrated experimentally in amphibians, where the pattern can be generated by either anterior or posterior cells if the other partner has been prevented from participating by X-irradiation (Holder *et al.*, 1979).

An additional feature of the growth response to positional confrontations that has been demonstrated in amphibians is that the response has an inherent directionality or polarity, with anterior cells contributing more to the ventral and posterior cells contributing more to the dorsal parts of the new pattern (Muneoka and Bryant, 1984a; Muneoka *et al.*, 1986b). The basis for this polarity in the patterning process is presently unknown, but it could be linked to an obligatory polarity in the spatial and temporal pattern of relevant gene expression, similar to that described for the *Hox-4* complex of homeobox genes in developing limbs (Dollé *et al.*, 1989a; Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991). Regardless of its basis, the existence of directional intercalation provides a possible mechanism whereby the limb field could be generated at the interface between two oppositely specified cell populations: posterior-ventral and anterior-dorsal. This idea is explored further in Section D2 (see also Fig. 12).

In addition to evidence indicating that whenever positional disparities are created, growth and pattern formation occur, there is reciprocal evidence that when positional disparities are reduced or eliminated, growth and pattern formation are concomitantly curtailed. Evidence of this type came from surgically created limb stumps in newts that were symmetrical, double half limbs (Bryant, 1976). These limb stumps were lacking half of the normal circumference of positional values, and the other half was present twice, arranged in mirror symmetry. After allowing sufficient time for these limbs to be fully healed (reintegrated, revascularized, and reinnervated), they were amputated to study their regenerative ability. As predicted, due to the lack of positional disparities, regeneration was either very reduced (and symmetrical) or it did not occur at all. Subsequent experiments have confirmed and extended these results (reviewed in Bryant *et al.*, 1982).

Perhaps the most graphic demonstration of the relationship between positional disparities, growth, and pattern formation comes from experiments by Emile Lheureux on X-irradiated salamander limbs (Lheureux, 1975) (Fig. 3). In these experiments, limbs were X-irradiated to inhibit regeneration and then supplied with various types of skin grafts to determine the minimal number of different circumferential qualities of positional information (i.e., anterior, posterior, dorsal, ventral) that are required for outgrowth. As illustrated in Fig. 3, a full cuff of skin containing information from the entire limb circumference is able to support the regeneration of a normally patterned limb. Parenthetically, this experiment shows that dermal fibroblasts alone are capable of generating the entire limb pattern.

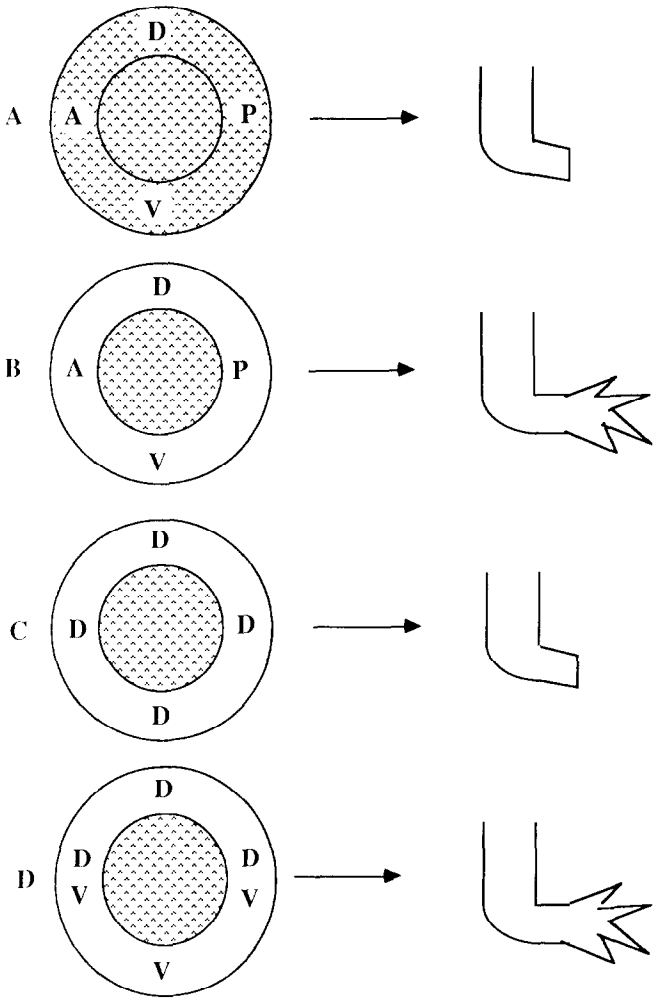

FIG. 3. Relationship between positional disparities, growth, and patterning. In this experiment limb stumps are shown in cross section on the left, and the outcome after regeneration on the right. The outer ring represents skin; the inner circle represents the core of the limb. Hatched tissues have been X-irradiated to prevent participation in regeneration. In A, when both the skin and the core of the limb are X-irradiated, limbs do not regenerate. In B, limbs with an X-irradiated core can regenerate when provided with a normal circumference of unirradiated skin. In C, if the unirradiated skin graft is oriented such that only one quality of circumferential information is present at the amputation plane, limbs with an X-irradiated core do not regenerate. In D, when a minimum of two qualities of circumferential information are present at the amputation plane, limbs with an X-irradiated core can regenerate. Abbreviations used are as in the legend for Fig. 1. After Lheureux (1975).

The telling experiment is the one in which a cuff of skin derived from only one circumferential position is wrapped around the irradiated limb (Lheureux, 1975). Despite the presence of unirradiated fibroblasts, regeneration does not occur. Only when at least two different qualities of positional information from the limb circumference are present (e.g., dorsal and ventral or anterior and posterior) does regeneration take place.

In summary, positional disparities are essential for growth and pattern formation; without them, growth and pattern formation do not occur. In normal limb outgrowth, in both development and regeneration, the circumferential positional disparities essential for pattern formation (French *et al.*, 1976; Bryant *et al.*, 1981) exist within the progress zone. As cells divide in response to these positional disparities, they adopt a more distal positional identity (Bryant *et al.*, 1981), thereby accounting for the properties that characterize the progress zone (Summerbell *et al.*, 1973).

The above two premises form the basis of our interpretation of the responses of limb cells to RA.

B. INTERPRETATION OF RESPONSES OF LIMBS TO EXOGENOUS RA

In this section we will focus on the effects of exogenously applied RA on limbs and defer until later a discussion concerning the role that endogenous retinoids might play in normal development.

1. Exogenously Applied RA Changes the Positional Values of Limb Cells to a Value That Is Posterior-Ventral-Proximal with Respect to the Limb

This conclusion is based on experiments in both developing and regenerating limbs (anterior–posterior axis: Tickle *et al.*, 1982; Kim and Stocum, 1986b; dorsal-ventral axis: Ludolph *et al.*, 1990; proximal–distal axis: Niazi and Saxena, 1978; Maden, 1982). At the present time, very little is known concerning how this change is effected, and even less is known regarding the molecular nature of the positional values that are changed by RA. As is apparent from other reviews in this issue (e.g., Mendelsohn *et al.*, 1992), molecules likely to be involved in mediating the effect of RA in limbs include cellular retinoic acid binding proteins (CRABPs), as well as DNA-binding retinoic acid receptors (RARs; RXRs). Homeobox genes presently are the best candidates for limb pattern formation genes (Duboule, 1991), and thus would be likely targets of RA, a subject also reviewed in this issue (see Izpisúa-Belmonte and Duboule, 1992; Muneoka and Sassoon, 1992). Representatives of all these classes of molecules have been shown to be present in developing and regenerating limbs, and hence could be involved in the change in positional value effected by RA.

In several instances an apparent dose response to RA has been observed, with higher doses for longer periods leading to more extreme results than lower doses for shorter periods of time (Tickle *et al.*, 1985). At present, it is not possible to distinguish between two possibilities for the dose effect. The first possibility is that cells are converted from one extreme *toward* the other (e.g., from

anterior toward posterior) along a continuum, the higher the dose or the longer the exposure, the further the change along this continuum. For example, in the chick limb it has been suggested that a RA concentration of 0.9 n*M* specifies digit 2, a concentration of 2.5 n*M* RA specifies digits 3 and 2, and 25 n*M* RA specifies digits 4, 3, and 2 (Tickle *et al.*, 1985). It has also been hypothesized that digit 2 must be specified before digit 3 and similarly, specification of digits 2 and 3 must precede that of digit 4. According to this view, it would obviously take a longer period of exposure, and a higher dose of RA, to specify a digit 4 than a digit 2. Evidence for a sequential activation of expression of homeobox genes in EC cell lines in response to different RA concentrations (Boncinelli *et al.*, 1991) has been cited as indirect evidence for the likelihood of a graded response to RA in limbs (Stocum, 1991b; Tabin, 1991). However, the RA concentrations (10^{-5} *M*) and duration of exposure (several days) necessary to obtain sequential expression of *Hox* genes *in vitro* (Boncinelli *et al.*, 1991) are not consistent with the kinetics of the RA effects on limb pattern formation (less than 5×10^{-8} *M* for less than 20 hr; Eichele *et al.*, 1985). In addition, a recent report indicates that the 5′ members of the *Hox-4* complex that are sequentially expressed during limb development (and are considered to be involved in anterior–posterior specification), are not activated but *inhibited* by RA in the *in vitro* model system (Simeone *et al.*, 1991).

The other interpretation is that cells are switched from anterior to posterior at some threshold level of RA, and that higher doses and longer exposures generate more switched cells (Wanek *et al.*, 1991; also see Tickle *et al.*, 1985). Evidence that RA converts anterior cells to posterior edge cells but not to cells with intermediate positional values is presented and discussed below (Section B2). Cheryl Tickle has presented direct evidence that a graded patterning response can be stimulated by varying the numbers of posterior (ZPA) cells (Tickle, 1981). Hence, grafts of large numbers of ZPA cells lead to the stimulation of full duplications (digits 4, 3, and 2), whereas grafts of small numbers of ZPA cells lead to the formation of only a digit 2. An intermediate number of ZPA cells yields an intermediate result (digits 3 and 2). This graded, partial response to variable numbers of stimulating cells could occur as follows: During positional interactions (intercalation) between cells with different anterior–posterior positional values, the progeny of the interacting cells take up a positional value that is intermediate between those of the cells around them, i.e., a value that is intermediate between the confronted anterior and posterior (ZPA) cells. When many ZPA cells are present, those that are at a distance from the site of interaction will maintain their most posterior positional value and hence will serve to stabilize the posterior boundary of the outgrowth. When few ZPA cells are present, as they divide and take up positional values that are the average of their surroundings, the most extreme posterior part of the pattern will be lost. Hence, it will appear as though the cells had been only partially posteriorized. As discussed below (Section B4), this view can also account for the equivalent, dose–response phenomenon in RA-treated regenerating amphibian limbs.

Recent investigations of the molecular basis of graded, inducible responses at the level of gene transcription provide evidence consistent with this second interpretation (Ko *et al.*, 1990). These studies have been carried out in a model system for the dose-dependent transcription of a reporter gene with a glucocorticoid response element in its enhancer/promoter region. The glucocorticoid nuclear receptor, along with those for steroid and thyroid hormones and retinoids, belongs to the steroid/thyroid hormone receptor superfamily of ligand-dependent transcription factors (see Evans, 1988; Green and Chambon, 1988). In these studies it was found that individual cells do not respond in a graded way to increasing doses of dexamethasone; rather the dose dependence is a cell population phenomenon, in which increasing numbers of cells initiate transcription in response to increased concentrations of inducer.

Experiments designed to distinguish between these or other possibilities would provide an important, missing piece to the puzzle of how RA changes a cell's positional value.

2. Locally Applied RA Causes Local Changes in Positional Values and Is Equivalent to Grafting

Although the pattern-duplicating effects of RA were first described for the proximal–distal axis of regenerating amphibian limbs (Niazi and Saxena, 1978; Maden, 1982), it has been the effect of RA on the anterior–posterior pattern of chick wing buds that has received the most attention. The experimental paradigm is to load RA onto ion-exchange resin beads and then to implant a single bead under the anterior edge of the AER (Tickle *et al.*, 1985). The bead acts as a slow release source of RA. When the limb is examined several days later, a supernumerary set of digits is found with its posterior edge adjacent to the site of the bead. Hence, the RA–bead appears to mimic a graft of posterior cells into an anterior location. We have confirmed the generality of this phenomenon by eliciting similar responses in axolotl limbs (Sessions *et al.*, 1989, and manuscript in preparation). Hence, when RA–beads are implanted into the anterior of axolotl limbs, either developing buds or regenerates, they cause pattern duplications similar to those described for the chick.

There are at least two explanations for pattern dupli-

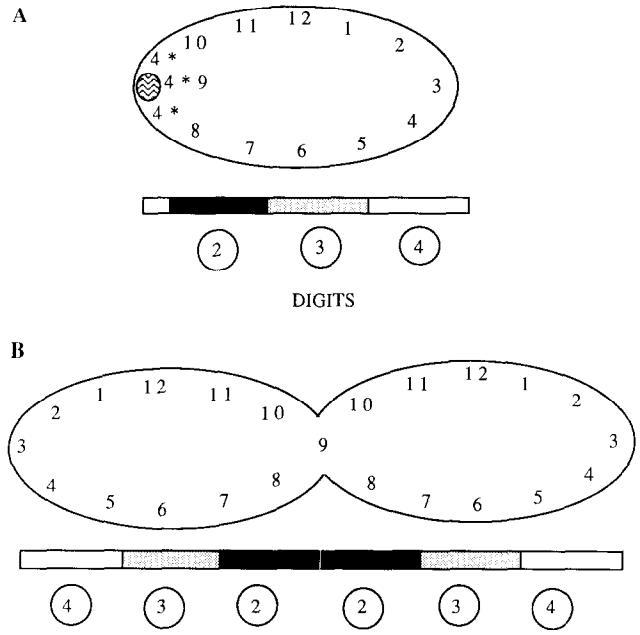

FIG. 4. RA-induced pattern duplication in chick limb buds. (A) An RA-bead in the anterior progress zone of a chick wing bud, 15–24 hr after grafting. The bud is shown end on; numbers represent positional values in the periphery of the limb mesenchyme. Cells adjacent to the bead have been converted to posterior-ventral-proximal positional value by exposure to RA (see text). Asterisks indicate where intercalation will be stimulated by the proximity of posterior cells with anterior cells. The bar below indicates the digits that are represented by the positional information shown. (B) After intercalation, a fully duplicated limb pattern is formed.

cation in the anterior–posterior axis in response to RA. The first is that exogenous RA, which becomes distributed as a gradient from a high point at the bead, directly respecifies the positional identity of cells along the anterior–posterior limb axis in a graded, concentration-dependent fashion. Hence, in chick limbs, RA would directly respecify the cells in the 200–300 μm of tissue adjacent to the bead as digits 4, 3, and 2 (Eichele *et al.*, 1985).

A second explanation is that RA first converts cells adjacent to the bead into posterior edge (ZPA) cells (Wanek *et al.*, 1991; see also Tickle *et al.*, 1985). Since these converted posterior cells are surrounded by anterior cells, the positional disparity thereby created results in the stimulation of growth and the intercalation of new pattern in the same way as proposed for posterior to anterior tissue grafts (Fig. 4). As a test of whether cells next to an RA-bead become functional ZPA (posterior edge) cells, we removed RA-beads from chick limb buds at various times after implantation and assayed the wedge of tissue next to the bead for ZPA properties by grafting into the anterior of a host wing bud (Wanek *et al.*, 1991). We found that the cells next to the bead become functional ZPA cells beginning at about 15 hr after bead implantation. Further, by using the chick–quail marker, we found that all of the cells in the 200- to 250-μm wedge adjacent to the bead become ZPA cells; i.e., the grafted wedge of RA-treated anterior cells makes the same small contribution to the posterior of digit 4 as does a ZPA graft. In other words, the region of the limb bud in which the rudiments of the respecified digits 4, 3, and 2 should be found according to the RA-as-morphogen view, consists only of cells that behave like ZPA cells.

Based on the results from this and other (Summerbell and Harvey, 1983; Noji *et al.*, 1991; Tickle, 1991) experiments, we interpret the sequence of events that lead to RA-bead-induced pattern duplication in chick limbs as follows. Cells next to the bead are exposed to RA which initiates changes that lead eventually to changes in positional value. We propose that the cells acquire a posterior-ventral-proximal positional identity. In chicks because pattern regulation only occurs along the anterior–posterior axis (see Section A1), the experiments only reveal the posteriorization of the cells. In order to see unambiguous proximal–distal effects, limbs that had already formed distal parts of the pattern would need to be treated. As we discuss later, the time required for the response to RA to be completed by the pattern formation-competent cells precludes the possibility of this test. Nevertheless some evidence of a proximal–distal effect has been noted by Oliver *et al.* (1990). In addition, as discussed above (Section A1), dorsal–ventral effects are expected to be masked if pattern regulation is only possible beneath the AER. After the start of RA exposure, no stable change in positional value is achieved before 15 hr of exposure. Removal of the bead at this time leads to some partially duplicated limbs (Eichele *et al.*, 1985). At this time point, grafts of cells that were adjacent to the bead into an anterior site in a host wing bud show ZPA activity, although the frequency of full duplications increases the longer the cells are exposed to RA prior to testing. By 24 hr after RA exposure, cells next to the bead have developed near-normal ZPA activity. We have proposed that, left *in situ*, such cells will interact with adjacent anterior cells to generate the parts of the pattern that normally lie between the newly generated extreme posterior edge and digit 2 (Wanek *et al.*, 1991) (Fig. 4).

The time of conversion of cells adjacent to the bead into posterior edge cells preceeds the onset of ectopic expression of the *Hox-4* genes that are normally expressed in a restricted posterior domain. Posterior edge cells are present after 15–24 hr of exposure to RA. Cells next to the bead initially express *Hox-4.6* at about 20–30 hr, and later express *Hox-4.8* at about 24–48 hr (Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991). The timing of the *Hox* gene expression relative to the timing of con-

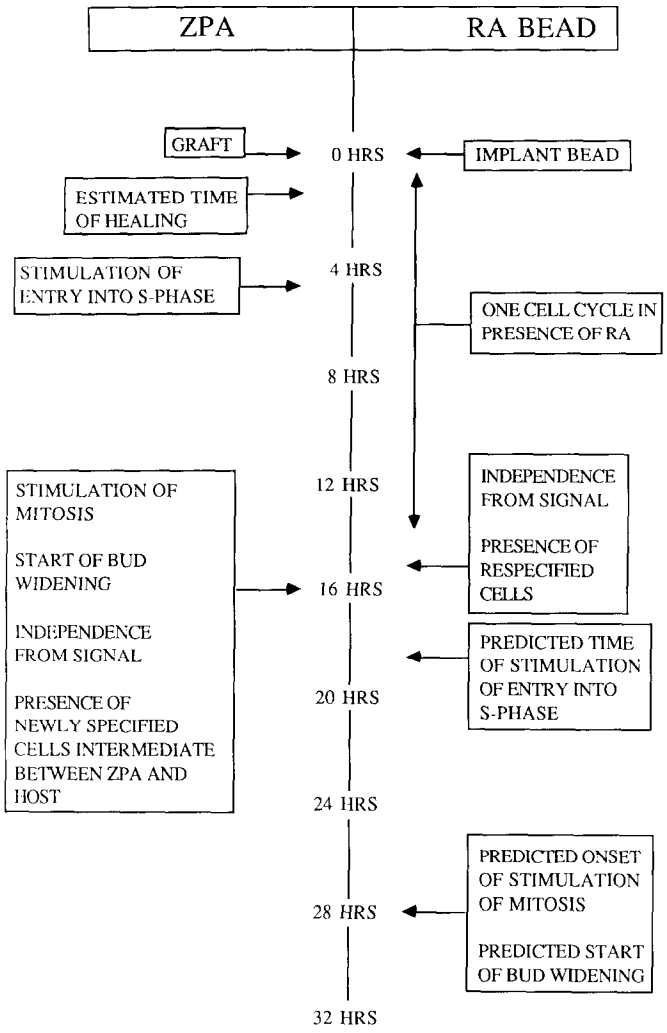

FIG. 5. Proposed sequence of events leading to pattern duplication after a ZPA graft and an RA-bead. Additional detail is provided in the text.

version to posterior suggests that the initiation of *Hox-4* gene expression is an indirect result of RA exposure, with RA initially converting cells to posterior identity. As new cells are intercalated, they begin to express the posterior *Hox-4* genes.

One aspect of the comparison between ZPA- and RA-bead-induced duplications that has generated confusion is that, in each case, the duplication-inducing stimulus must be present for about 15 hr in order for it's effect on limb pattern to become irreversible (Tickle and Brickell, 1991). This similarity in timing has contributed to the conclusion that the ZPA is in fact a source of endogenous RA, and that RA acts as a natural morphogen to specify graded positional values across the normal anterior–posterior axis of the chick limb. It is also possible to view the similarity in time to independence from the stimulus in a different way (Fig. 5). In both RA-bead- and ZPA-induced duplications, we define the time to independence as the time after which positionally altered cells have been generated next to the bead or graft. The presence of these cells allows for the generation of the remaining intermediate positional values after the removal of the stimulus.

Let us first consider what happens after a ZPA graft (Fig. 5). Following an interval for graft healing (most likely less than an hour), local interactions between graft and host cells stimulate cells to enter S-phase of the cell cycle. An increase in S-phase cells next to a ZPA graft has been documented as early as 4–5 hr after grafting (Cooke and Summerbell, 1980), which would correspond to 3–4 hr after healing-in of the graft. Twelve hours later, i.e., 16 hr after grafting, the increase in S-phase cells results in an increase in mitosis (Cooke and Summerbell, 1980) and the start of the widening of the limb bud (Smith and Wolpert, 1981). We view this as the time when the intercalated pattern begins to be generated. The limb becomes independent of the graft after 15–17 hr (Smith, 1980), presumably because newly intercalated cells with positional values intermediate between the graft and the host have been generated in the host tissue. The remaining parts of the duplicated pattern can be generated by intercalation after the limb bud has become independent from the graft stimulus.

In the case of RA-beads, the time to independence also suggests an involvement of the cell cycle. We propose that cells only become reprogrammed by RA as a result of going through some critical part of the cell cycle in the presence of RA. Once enough of the cells are reprogrammed, beginning at about 15 hr, they are independent of the stimulus (Eichele *et al.*, 1985; Wanek *et al.*, 1991), and can therefore go on to interact with adjacent host cells to generate the new pattern through intercalary growth. As the population of cells in the vicinity of the bead is exposed to RA for longer periods, increasing numbers of cells pass through the critical phase of the cell cycle in the presence of RA, and thus the population of reprogrammed cells can continue to expand with time. As discussed above (Section B1) we predict that the increase in the number of reprogrammed cells will eventually result in a more complete supernumerary response. This view predicts that the growth necessary for the expanded pattern in RA-induced duplications will take place *after* and as a consequence of the initial respecification interval (Fig. 5). In other words the similarities in time to independence reflect different processes, both of which involve the cell cycle and both of which result in the generation of some threshold number of respecified cells that are intermediate between anterior and posterior extremes. Hence, the increase in S-phase observed after ZPA grafts at 4–5 hr is expected to occur at about 18–20 hr after bead im-

plantation (a 15-hr respecification interval plus 3–4 hr to recruit cells into S-phase). By the same reasoning, an increased mitotic rate and the onset of bud widening is expected to occur another 10–12 hr after that, or at about 28–30 hr after initial bead implantation (Fig. 5).

A puzzling difference between ZPA graft- and RA-bead-induced duplications becomes interpretable in the context of this view. Although limbs up to stage 24 can respond to ZPA grafts by generating extra digits (Iten *et al.*, 1983; Summerbell, 1974), their response to RA-beads is much reduced at stages 21–22, and absent at stage 23 (Eichele *et al.*, 1985; Tickle and Crawley, 1988). We suggest that this difference is related to the prediction that RA-bead-induced duplications require a longer time, on the order of 12–24 hr, for completion of the intercalary growth necessary for the generation of the new pattern. After stage 20/21, there is insufficient time remaining in the limb pattern formation period for cells next to a bead to first become reprogrammed by RA and then to complete intercalation before cells lose their responsiveness to positional signals. We thus far have been unsuccessful in attempts to generate pattern duplications in developing mouse limbs by implanting RA-beads, despite being able to obtain a limited regulative response from grafts to confront anterior and posterior cells (Wanek *et al.*, 1989a). It has not been possible to extend these studies to earlier time points due to the technical limitations of *exo utero* surgery (Muneoka *et al.*, 1986c). It is likely that the amount of time available in the mouse studies for pattern duplication by locally applied RA (in contrast to the time needed for duplication as a result of direct anterior-posterior confrontations created by grafting) is insufficient for the reasons outlined above.

In summary, we propose that locally applied RA in developing chick limbs (as well as in the limbs of other vertebrates) causes pattern duplication via a two-step process. In the first step, cells adjacent to the RA-bead are reprogrammed to posterior-ventral-proximal with respect to the limb. The time required for this step suggests an involvement of the cell cycle. Once reprogrammed cells have been generated, they interact with adjacent anterior cells stimulating growth and the intercalation of new pattern by the same means as a graft of posterior cells.

3. Globally Applied RA Leads to Uniformity of Positional Values and Hence to Hypomorphic Outgrowth in Limb Development

Before RA was found to cause interesting pattern duplications in amphibians and chicks, retinoids were known to be teratogens in mammals (see Lammer *et al.*, 1985). Indeed, at the present time, a retinoid used in acne medication is considered one of the most potent of human teratogens. When retinoids are supplied to the fetus via the placenta, a number of different organ systems, among them limbs, develop abnormally (Kochhar, 1977; Kochhar *et al.*, 1984). The types of limb defects encountered are varied, and are sensitive to dose, to the particular form of retinoid administered and to the time of exposure relative to stage of limb development (Howard and Willhite, 1986; Kochhar, 1977). However, in almost all instances, the affected limbs are hypomorphic, ranging from complete truncations to missing or reduced skeletal elements at different proximal-distal levels. The effects of systemic administration in mammals can be replicated in chicks by adding retinoids to the amniotic cavity (Larsen and Janners, 1987) or by implanting very high dose RA-beads into limb buds (Tickle and Crawley, 1988). Similar results can also be generated in developing (but not regenerating, see Section B4 below) axolotl and *Xenopus* limbs by exposure to retinoids in the aquarium water (Scadding and Maden, 1986a,b).

Various explanations for the teratogenic effect of retinoids have been proposed, among them cell death in the mesenchyme and destruction of the AER. However, recent studies have shown that teratogenic doses of RA do not increase the amount of cell death in mouse limb buds above normal (Abbott *et al.*, 1990). In chicks treated to give truncated limbs, the AER is affected and becomes flat. However, this appears to be a secondary consequence of RA-induced changes in the mesenchyme, since transplants of AERs that have been flattened by RA exposure, to untreated mesenchymes, leads to the restoration of the AER and the outgrowth of a normal limb (Tickle *et al.*, 1989).

The teratogenic effects of RA can be interpreted as a consequence of the ability of RA to change limb cell positional information to posterior-ventral-proximal. As described in the preceding section, *local* reprogramming generates positional disparities that in turn lead to extra pattern. The converse is true for global application of RA. If a sufficient dose of RA is administered, then all or most cells in the progress zone will be reprogrammed. Rather than generating positional diversity, globally applied RA will reduce positional diversity. Since positional differences are required to sustain growth and pattern formation (see Section A2), reduction of positional diversity will lead to reduced or truncated structures (Fig. 6). Studies in mouse limb buds have documented an inhibition of growth in response to RA exposure *in vivo* (Abbott *et al.*, 1990), and we have observed a growth reduction in cultures of mouse limb bud cells treated with RA (Gardiner *et al.*, 1992). The view we have presented here predicts that teratogenic effects in limbs would be expected at similar stages to

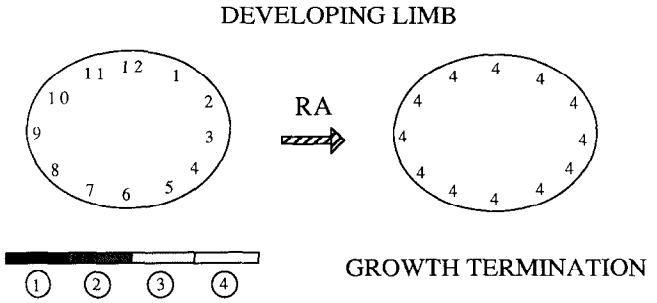

FIG. 6. Teratogenic effect of systemically applied RA to developing vertebrate limbs. RA affects all cells in the pattern-forming region of the limb bud similarly by converting them to cells with posterior-ventral-proximal positional information. Without positional disparities, limb outgrowth ceases, leading to limb defects. Details of the diagrams are the same as described in the legend for Fig. 4.

those at which pattern duplication can be elicited, as well as at later stages, since no enhanced growth response to RA-induced changes in positional information is required in order to observe the effect. Studies in chick limbs show that, whereas RA-induced duplications do not occur beyond stage 21/22, RA-induced truncations and reductions occur from prelimb bud stages (Larsen and Janners, 1987) at least through stage 24, the latest stage tested (Tickle and Crawley, 1988). Another prediction of this view is that it should be possible to rescue limbs whose cells have been reprogrammed to positional uniformity by removing any further influence of RA and providing anterior cells as a source of positional diversity.

An outstanding exception to the discussion above concerning the effects of global application of RA can be found in regenerating amphibian limbs. This exception is discussed below, where we present arguments that this is the exception that proves the rule.

In summary, we propose that global application of RA in developing vertebrate limb buds causes all cells to be reprogrammed toward uniform positional values. Lack of positional diversity in the progress zone leads to the failure of growth and pattern formation, and to the formation of reduced or truncated limbs.

4. Globally Applied RA in Regenerating Amphibian Limbs Leads to Uniformity of Positional Values in the Blastema; Outgrowth Is Rescued by Dedifferentiating Cells of the Stump

All the arguments presented above for the teratogenic effects of systemically applied retinoids in developing limbs might also be expected to apply to the regenerating limbs of amphibians. Indeed, a significant growth inhibition in the blastema during the period of exposure to retinoids has been well documented (Maden, 1983b; Pietsch, 1987). However, when urodele limbs are amputated through the lower arm and the animals are swum in a retinoid solution, or when they are injected with a retinoid in the first few days after amputation, the striking result is the development of limbs with serially duplicated elements along the proximal-distal axis (Maden, 1982; Thoms and Stocum, 1984). The result is similar to what would be expected if a proximal blastema were developing on a distal stump.

In contrast to the conclusions of others (Scadding and Maden, 1986a,b; Stocum, 1991a), we view the difference in the eventual outcome of exposure to RA in developing and regenerating limbs as attributable to the special circumstances of regeneration, not to any difference in the fundamental mechanisms of limb outgrowth and pattern formation in the two systems. The special circumstances we are referring to are associated with the process of dedifferentiation of mature limb tissues, leading to the creation of a group of undifferentiated cells from which outgrowth and pattern formation can proceed. Of particular significance is that dedifferentiation continues to progress back from the amputation plane during the early phases of blastema outgrowth, causing the addition of new pattern formation-competent cells to the base of the growing blastema (Tank, 1977).

The observation that the period of maximal sensitivity to retinoids coincides with the period during which dedifferentiation is occurring (Maden, 1983b; Maden *et al.*, 1985; Thoms and Stocum, 1984) indicates to us that retinoids are only affecting the undifferentiated cells of regenerating limbs. This parallels the finding that it is cells in the progress zone of developing limbs that are sensitive to RA (see Tickle and Brickell, 1991). In regenerating limbs, administration of RA either too soon, before cells have dedifferentiated, or too late, after cells have redifferentiated, results in little or no pattern duplication (Maden, 1983b; Thoms and Stocum, 1984; Maden *et al.*, 1985; Niazi *et al.*, 1985). We propose that the effect of retinoid treatment in regenerating limbs is the same as that in developing limbs; namely, blastema cells are converted to a posterior-ventral-proximal positional value (Fig. 7). We propose that in the presence of retinoids, growth and pattern formation are arrested due to the uniformity of positional values present in the blastema. During the exposure interval, any newly dedifferentiating blastema cells that enter the blastema from the stump will be reprogrammed along with the original blastema cells. However, after retinoids are withdrawn, newly dedifferentiating cells entering the base of the blastema will be able to maintain their original positional values. These newcomers are therefore able to provide the necessary circumferential positional diversity (anterior and dorsal) to stimulate outgrowth

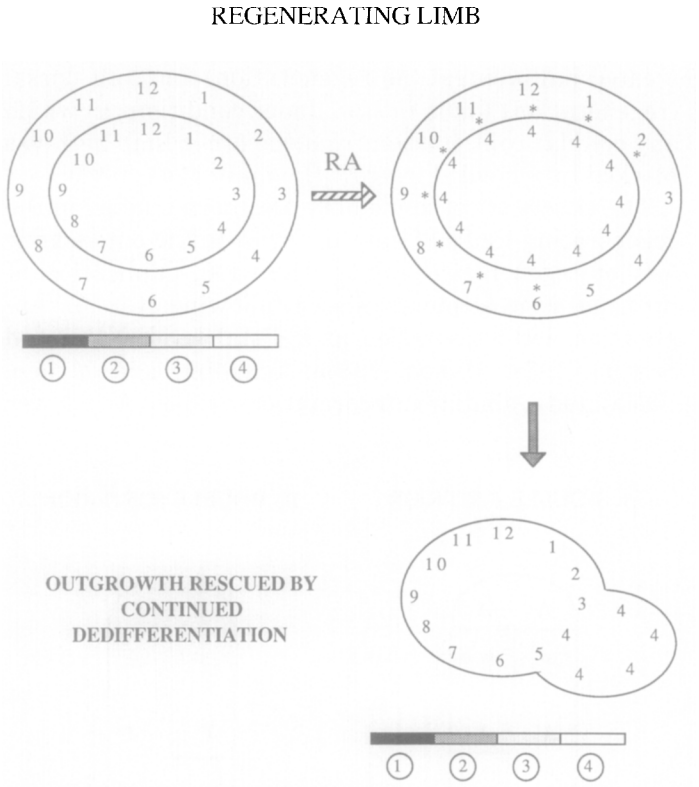

FIG. 7. Rescue of outgrowth by dedifferentiating cells in regenerating limbs exposed systemically to RA. The details of these diagrams are similar to those described in the legends for Figs. 4 and 6, except that in this figure cells in the blastema are represented by the inner circle and stump cells that dedifferentiate and enter the base of the blastema are represented by the outer ring. The bar below represents digits as depicted in Figs. 4 and 6. RA is shown converting blastema cells uniformly to posterior-ventral-proximal positional values. After withdrawal of RA, newly dedifferentiating cells enter the base of the blastema and create the positional disparities (*) necessary for outgrowth (see text).

from the RA-treated posterior-ventral-proximalized cells of the blastema (Fig. 7). The positional confrontations necessary to promote outgrowth of an RA-treated blastema will be expected to occur on the anterior edge of the blastema, and Kim and Stocum (1986a) have reported that RA-treated blastemas appear to originate from the anterior part of the limb stump. The view that we present makes it unnecessary to propose the existence of a unique population of cells in the peripheral anterior-dorsal part of the blastema, whose properties render them refractory to the effects of RA (Stocum, 1991a,b).

At later stages of regeneration, there is still a distal region of undifferentiated cells; dedifferentiation in the stump has ceased; and the proximal regions of the blastema have left the progress zone and are beginning (or will shortly begin) to redifferentiate. Administration of RA at this stage leads to the formation of limbs that are truncated distally but normal proximally (Niazi *et al.*, 1985). We interpret this result as follows: the proximal part of the blastema is unaffected by RA because it has left the progress zone at the time of exposure; the distal cells are reprogrammed as expected, but in the absence of newly dedifferentiated adjacent cells, they are not able to proceed with outgrowth and thus the distal part becomes hypomorphic or truncated.

According to the hypothesis presented above, the base of an RA-treated regeneration blastema is expected to consist of a mixture of proximal and distal cells. This leads to the prediction that intercalation will generate a small, reversed-polarity segment between the distal limb stump and the proximal boundary of the reprogrammed blastema cells (Fig. 8). The occurrence of such reversed-polarity segments has been noted by Kim and Stocum (1986a) and is evident in illustrations in other papers (e.g., Maden, 1983b). We have found that the vast majority (83%, n = 29) of RA-duplicated regenerates have reversed-polarity segments (Fig. 9).

Demonstration that RA posteriorizes and ventralizes regenerating limb cells comes from studies of double half limbs in urodeles. We illustrate these results by reference to double anterior and double posterior limbs (Fig. 10), but similar conclusions can be drawn from the results of double dorsal and double ventral limbs. It has

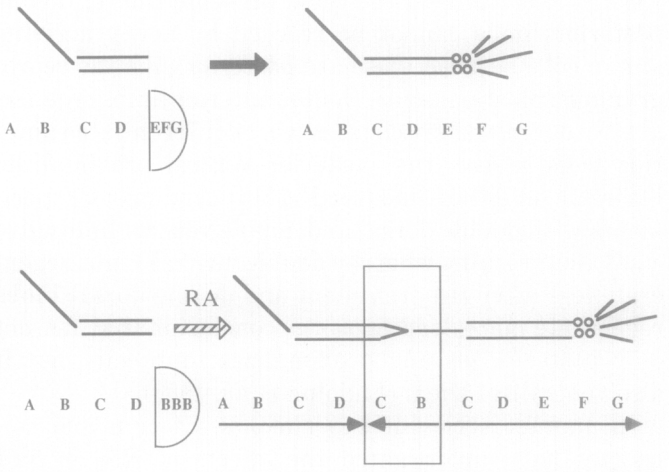

FIG. 8. Proximal-distal duplication in regenerating limbs. The details of these diagrams are similar to those described in the legend for Fig. 1. At the top, a limb amputated through the lower arm forms a blastema that replaces the missing parts of the proximal-distal sequence of information (shown by A-G). Below, when a regenerating limb is treated systemically with RA, the blastema is converted to posterior-ventral-proximal positional values (posterior and ventral not shown here, see text). Interactions between the proximalized blastema and the distally specified dedifferentiated cells from the stump lead to the formation of a reverse-polarity segment (region inside box) between the stump and the most proximal structure in the regenerate (see text). Arrows indicate proximal-distal polarity of the segments (see text).

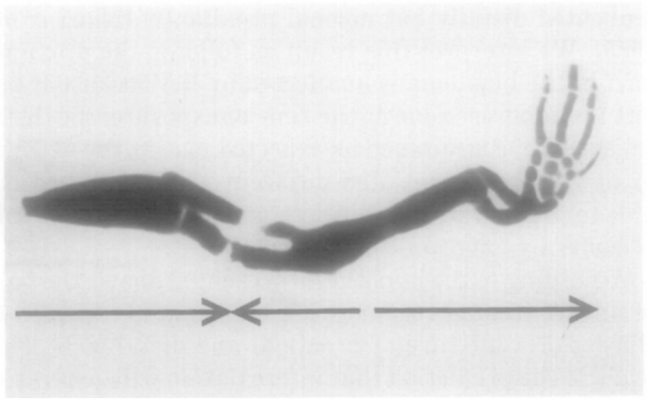

FIG. 9. Whole-mount skeletal preparation of RA-treated regenerate. The pattern of elements formed in this specimen is very similar to that illustrated diagramatically in Fig. 8. Arrows indicate the proximal-distal polarity of the segments (see text).

been argued that when double anterior or double posterior limbs are amputated through the lower arm in the absence of RA, the reduced positional diversity at the amputation plane leads to regeneration of tapering symmetrical limbs with zero to three digits (Stocum, 1978; Krasner and Bryant, 1980; Kim and Stocum, 1986b). After RA treatment, double posterior limbs no longer regenerate at all, and double anterior limbs regenerate much more than before, forming double, mirror-imaged outgrowths (Stocum and Thoms, 1984; Kim and Stocum, 1986b). According to the view presented here, double posterior limbs cannot be rescued by newly arriving stump cells because these are posterior, like the reprogrammed blastema cells. Double anterior limbs regenerate two outgrowths because newly dedifferentiated anterior cells rescue the posterior-ventral-proximalized blastema on either side (see Fig. 10). Analogous experiments with double dorsal and double ventral limbs give equivalent results, whereby double ventral limbs regenerate less after RA treatment and double dorsal limbs regenerate more, leading to the conclusion that RA not only posteriorizes and proximalizes limb cells, but it also ventralizes them (Ludolph *et al.*, 1990).

In another series of experiments, Stocum and colleagues have investigated the effects of RA on half limbs (Kim and Stocum, 1986b; Ludolph *et al.*, 1990). These studies have provided results that can also be accounted for using the principles described here. In the case of half limb stumps grafted to the orbit to isolate them from all influences from the other half of the limb stump, anterior but not posterior halves can regenerate after retinoid treatment. Hence, outgrowth from the posterior-ventral-proximal blastema cells on an anterior half limb stump can be rescued as predicted by newly dedifferentiating anterior stump cells. Half posterior limbs cannot be rescued by newly arriving cells because these do not add any positional diversity to the blastema. Similar results have been obtained from RA-treated half ventral (no regeneration) and half dorsal (regeneration) limbs *in situ*, under conditions in which any cellular contribution from the other limb half was blocked by a head skin graft (Ludolph *et al.*, 1990).

The effects of retinoids have also been studied in the regenerating limbs of anurans prior to the ontogenetic loss of regenerative ability. Here too, duplication of proximal limb elements is seen following systemic application and amputation at a distal level (Niazi and Saxena, 1978; Maden, 1983a; Scadding and Maden, 1986b) and a similar interpretation applies. At a lower

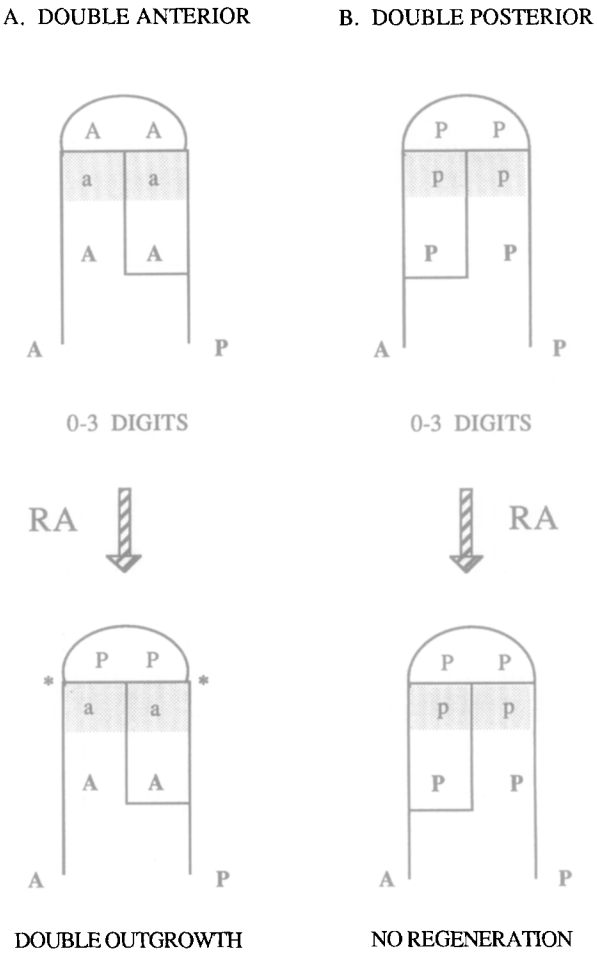

FIG. 10. RA effects on regeneration of double half limb stumps. At the top, surgically created double anterior (A) and posterior (B) limb stumps each regenerate 0–3 digits after amputation through the lower arm. Below, after RA treatment, double posterior limbs fail to regenerate and double anterior limbs regenerate double outgrowths. Dedifferentiating cells (hatched, lower-case letters) from the stump provide positional diversity to the posteriorized blastema in double anterior limbs, but fail to add any positional diversity in double posterior limbs (see text). Asterisks indicate sites of intercalation. Bold letters represent stump cells. Abbreviations used are as described in the legend for Fig. 1.

frequency, retinoids also induce duplication in the transverse axes in frogs, leading to the formation of mirror-imaged hands (Niazi and Saxena, 1978; Maden, 1983a; Scadding and Maden, 1986b). Similar results are also obtained in urodeles (Lheureux *et al.*, 1986) but only when limb buds rather than mature limbs are amputated. From the available descriptions, it appears that such duplicated limbs are arranged in mirror symmetry, joined by their anterior or anterior–ventral edges (Maden, 1983a; Lheureux *et al.*, 1986).

At present, it is not clear why the formation of mirror-imaged limbs is a response of amputated limb buds, but not of mature limbs, to RA treatment. However, in a recent study to map the organization of positional information in the interior of the axolotl limb, we were surprised to find that cells with anterior and ventral positional values predominate in the center of the limb (Gardiner and Bryant, 1989). Assuming that in limb buds, cells from central as well as peripheral regions of an amputated bud contribute equally to the regenerate, we suggest that anterior–ventral cells will be released into the center of an RA-treated blastema that consists of posterior–ventral–proximal cells. This would lead to the regeneration of mirror-imaged limbs. In contrast, it is known that the cellular contribution to the blastemas of mature limbs is dominated by peripheral cells (Muneoka *et al.*, 1986a) and that the organization of positional information in the periphery dictates the pattern of the regenerate regardless of the organization of the central tissues (Tank, 1979; Slack, 1980). Hence, cells in the center of mature limbs and of limb buds may differ in the degree to which they are able to influence pattern formation, leading to the absence of mirror-imaged regenerates from RA-treated mature limbs.

As in the case of duplication in the anterior–posterior axis in chicks following local application of RA, the effect on the proximal–distal axis in regeneration is dose dependent, with higher doses and longer durations leading to more proximal duplications than lower doses for shorter times (Maden, 1983b; Kim and Stocum, 1986a). As with the chick results, it is not possible at present to decide whether with a lower dose fewer cells are converted to extreme proximal values or whether more cells are converted part of the way toward extreme proximal. As recognized in the "rule of distal transformation," distal parts of the pattern are generated from more proximal parts, but not the reverse (Pescitelli and Stocum, 1980). During intercalation between cells with different proximal–distal positional values, we assume that progeny of the proximal cells take up a positional value that is intermediate between those of the neighboring cells. When many proximal cells are present, those that are at a distance from the site of interaction will maintain their most proximal positional value, and hence will stabilize the most proximal boundary of the regenerate. When few proximal cells are present, as they divide and take up intermediate positional values, the most extreme proximal part of the pattern will be lost. Hence, it will appear as though the cells had been partially proximalized. As discussed above (Section B1), recent evidence regarding the molecular mode of action of glucocorticoid hormones (Ko *et al.*, 1990) is consistent with the interpretation that the dose dependence is a consequence of increasing numbers of cells being converted at higher doses. As in the case of the effect on chick limbs, it is important to distinguish between these possibilities if we are to understand the way in which retinoids affect the expression of positional information.

In summary, we have shown that the wide range of results that have been obtained in amphibian limbs using retinoids can be accounted for by virtue of the fact that in order to regenerate, limb tissues have to dedifferentiate. Further, dedifferentiation continues after the initial establishment of the blastema, providing a new source of cells migrating into the base of the blastema. There is no need to invoke different mechanisms for developing and regenerating limb outgrowth and pattern formation in order to accommodate the results. The recent results concerning the apparent homeotic effect of RA on amphibian tail regeneration (Mohanty-Hejmadi *et al.*, 1992) are also interpretable by similar arguments, and are discussed in detail in Section D3 (see also Figs. 13 and 14).

C. RA AS MORPHOGEN

The view we present above is clearly at odds with the RA-as-morphogen idea that has dominated thinking in the limb field for much of the last decade. In this section we look at the origins of this idea and where it stands in the face of current knowledge about vertebrate limbs.

1. The Basic Idea

The idea that pattern formation across the anterior–posterior axis of developing limb buds is controlled by a diffusible morphogen (Tickle *et al.*, 1975) grew out of Lewis Wolpert's conceptualization of how positional information might, in theory, be specified (Wolpert, 1969, 1971). The one-sided nature of the interaction between anterior and posterior cells in the chick wing bud following grafting (most of the new growth is from the anterior partner: Honig, 1983; Javois and Iten, 1986) was consistent with the view that posterior cells signal and anterior cells respond. Reflecting the view that posterior cells can affect limb polarity, the name ZPA ("zone of polarizing activity") was coined for the posterior, distal region of the bud with the ability to stimulate the

development of supernumerary outgrowths after transplantation to an anterior site (Balcuns *et al.*, 1970).

As we have argued elsewhere (Bryant and Muneoka, 1986), while this view may work in principle for the anterior–posterior axis of the chick limb, it does not accommodate the data for other vertebrate limbs for several reasons, the most obvious of which is that in other developing and regenerating limbs, anterior as well as posterior cells both signal and respond at a graft interface (Muneoka and Bryant, 1984a,b; Muneoka and Murad, 1987). Nevertheless, the idea of a simple gradient of a diffusible molecule that specifies positional information has gained widespread acceptance.

When it was reported that RA-beads in chick limbs apparently mimic ZPA grafts (Tickle *et al.*, 1982), RA began to be viewed as the putative endogenous morphogen (Eichele *et al.*, 1985). A pivotal paper in the development of the RA-as-morphogen story was that of Thaller and Eichele (1987; see Slack, 1987) who reported on the levels of endogenous RA in chick limb buds. In that paper, they reported that while the level of retinol, the precursor to RA, is uniform and high in limb buds, RA itself is asymmetrically distributed with a 2.5-fold enrichment in the posterior one-fourth as compared to the anterior three-fourths. They subsequently reported evidence that posterior limb cells are capable of synthesizing RA from retinol (Thaller and Eichele, 1988). The discovery of several nuclear receptors for RA belonging to the steroid hormone receptor family (see Mendelsohn *et al.*, 1992), as well as homologous receptors with unknown ligands (Mangelsdorf *et al.*, 1990), provided a possible mechanism by which levels of RA could be transduced into the sort of differential gene expression that might account for pattern formation. However, considerations of physical chemistry led to the realization that these receptors would be saturated, even in anterior cells, given the levels of RA present and the shallow nature of the calculated gradient (see Smith *et al.*, 1989). These concerns were overcome by the report that cellular retinoic acid binding protein (CRABP) was also distributed as a gradient across the anterior–posterior axis, but with more in the anterior than the posterior (Maden *et al.*, 1988). It was thus concluded that a high level of CRABP in the anterior would bind up RA and thereby steepen the gradient of available RA (Maden *et al.*, 1988).

In summary, the field of chick limb development was predisposed to conclude that RA functions as an endogenous morphogen, and the major evidence in support of the idea came from direct measurements of RA levels.

2. The Picture Is Not So Simple

The idea of RA-as-morphogen, and in fact the idea of any diffusible morphogen specifying positional information, has never been able to accommodate the data for amphibian limbs (discussed in Bryant and Muneoka, 1986). Rather, pattern formation in amphibian limb regeneration is thought of as occurring via local cell–cell interactions and intercalation (French *et al.*, 1976; Bryant *et al.*, 1981). RA itself has the additional problem that it affects positional information in all three limb axes; thus, it is difficult to conceptualize how a gradient of RA could specify graded positional values in three dimensions simultaneously. Since pattern regulation in the chick limb is basically one dimensional (for the reasons discussed in Section A1 above), a one-dimensional gradient of RA has not presented problems, provided that the discussion of mechanism is restricted to the chick limb.

In the last year or so, several findings have contributed to a reevaluation of the view that RA acts as an endogenous morphogen in chick limbs. The most direct evidence that is inconsistent with this view comes from experiments in which it was shown that the wedge of tissue next to an RA-bead contains only the most posterior edge of the pattern after 24 hr of RA exposure (Wanek *et al.*, 1991). The extent of positional information present in that wedge of tissue corresponds to that contained in the ZPA, rather than to the anlage for digits 4, 3, and 2 as predicted from the RA-as-morphogen view (Eichele and Thaller, 1987). Other reports (although lacking cell lineage markers) have also provided evidence that RA converts cells next to the bead into ZPA cells (Summerbell and Harvey, 1983; Noji *et al.*, 1991; Tickle, 1991). In this way, RA is not a mimic of the ZPA since the tissue next to a ZPA graft does not acquire ZPA activity (Smith, 1979).

It is not feasible to propose that once the ZPA is made, it then becomes a source of RA that reprograms cells. Since anterior cells failed to become respecified to form graded positional information in response to the exogenous RA, there is no reason to propose that they would do so in response to endogenous RA after a new ZPA has been generated. In addition, in order for the induced ZPA to become a RA source, anterior limb cells would be responding to RA autocatalytically. Such a response would preclude the development of a RA gradient that could specify graded positional information (Wanek *et al.*, 1991).

Other evidence that is inconsistent with the idea of RA as an endogenous, diffusible morphogen comes from studies of the expression pattern of retinoic acid receptor-β (RARβ). The promoter of this gene contains an RA-responsive element within it, and therefore its expression would be expected to be elevated in places where there are elevated levels of RA. The gene has in fact been shown to be responsive to elevated levels of exogenous RA in chick limbs; however, there is no evi-

dence of a graded distribution of RARβ across the anterior–posterior axis as predicted by the RA-as-morphogen idea. Furthermore, ZPA tissue grafted to the anterior margin does not lead to any increase in expression of RARβ in the adjacent cells (Noji *et al.*, 1991). Two recent papers have examined the expression patterns of constructs in transgenic mice consisting of the RA-response element of the RARβ promoter fused to *lacZ* (Mendelsohn *et al.*, 1991; Rossant *et al.*, 1991). Expression of *lacZ* was essentially absent from the limb buds, although prominent in the adjacent trunk regions (Fig. 11). These results raise critical questions about the presence and relevance of endogenous RA for limb pattern formation.

Since the original description of a shallow gradient of RA in limb buds, an additional, endogenous retinoid (3,4-didehydroretinoic acid; ddRA) that is six times more abundant than RA and just as potent in inducing extra structures, has been identified in limb buds (Thaller and Eichele, 1990a). Since no data have been reported about the distribution of this retinoid, the current picture of the gradient of active retinoids is unclear.

The status of the reported gradient of CRABP has also become more complicated. Subsequent to the original report in the chick (Maden *et al.*, 1988, 1989), it was reported that CRABP protein is present in mouse limb buds, but that it is not differentially distributed along the anterior–posterior limb axis (Dencker *et al.*, 1990). In addition, studies in the mouse show that CRABP transcripts have a similar proximal–distal distribution to that of chick limb buds but they are not present in an anterior–posterior gradient (Dollé *et al.*, 1989b). Another study on the mouse limb bud however does report an anterior–posterior gradient of CRABP transcripts (Perez-Castro *et al.*, 1989). Most recently this issue has been further complicated by questions about cross-reactivity of the heterologous antibody used in the original study of the chick limb bud (Maden *et al.*, 1990). Because the current view of RA as an endogenous morphogen in the chick limb is dependent on the existence of an anterior–posterior gradient of CRABP, this issue needs to be unequivocably resolved. Since there is an undisputedly nonuniform distribution of CRABP along the proximal–distal axis, either whole-mounts or longitudinal sections showing proximal and distal as well as anterior and posterior on the same section would dismiss the nagging possibility that in even a slightly oblique cross section, a proximal–distal gradient could give the misleading appearance of an anterior to posterior gradient.

Finally, the original report that posterior limb cells can convert retinol into RA was incomplete in that it did not report on whether anterior cells are the same or different in this regard (Thaller and Eichele, 1988). The RA-as-morphogen view requires that ZPA cells synthesize high levels of RA from retinol, and that RA diffuses across the field of non-ZPA cells which act as dispersed sinks (Eichele and Thaller, 1987). Accordingly, the non-ZPA cells do not synthesize high levels of RA, but be-

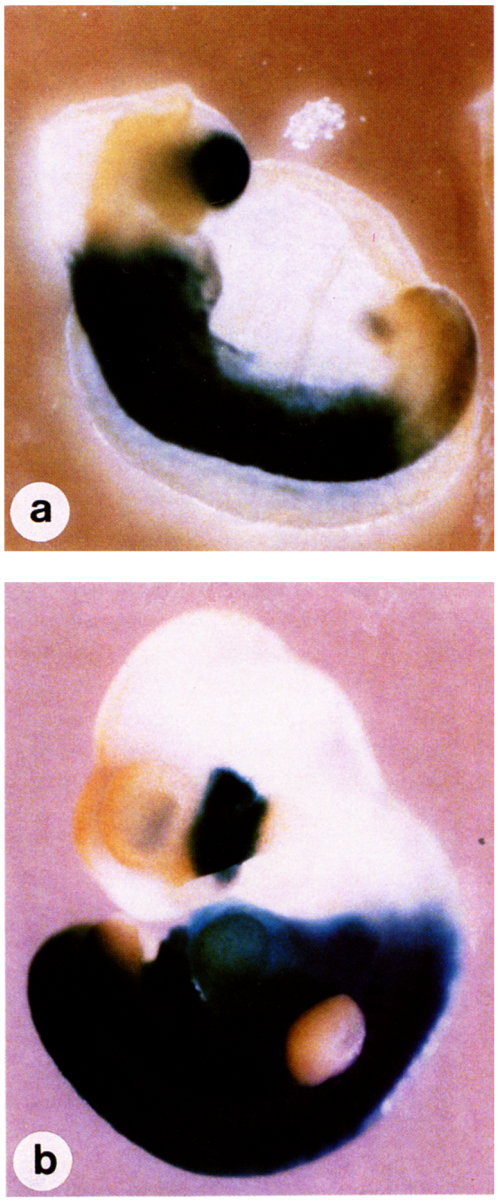

FIG. 11. Expression of *RARE-hsplacZ* transgene in mouse embryos. Reproduced, by permission from the publisher, from Rossant *et al.* (1991). (a) Mouse embryo with ~9–10 somites showing transgene expression in the trunk and its apparent absence in the head and tail regions. An interpretation of this staining pattern is that RA is present in the trunk but not in the head or the tail. (b) Later embryo with stage 3 limb bud (see Wanek *et al.*, 1989b, for limb stages). Note the maintenance of a sharp boundary of transgene expression between the head and the trunk and the absence of transgene expression in the limb buds.

come specified as to anterior–posterior positional value by exposure to different concentrations of RA generated posteriorly. However, recent studies indicate that anterior and posterior tissue extracts synthesize RA at the same rate (see Tabin, 1991). Hence, a gradient of RA would be a *reflection* of intrinsic differences between anterior and posterior cells, rather than being the cause of such differences as originally proposed in the RA-as-morphogen view.

In summary, in addition to the fact that the RA-as-morphogen idea cannot accommodate the data from amphibians, where RA affects all three axes similarly, the idea that it acts as a diffusible morphogen that specifies anterior-to-posterior positional information in developing chick limb buds has not withstood the test of time.

3. *Where Does That Leave the Diffusible Morphogen?*

As we have argued above (see also Saunders, 1977; Saunders and Gasseling, 1983), the chick limb field was primed to find a diffusible morphogen by the concepts developed by Wolpert in the late 1960s (Wolpert, 1969). However, since that time it has become apparent that we cannot look only at developing chick limbs if we want to deduce the likely mechanisms of limb pattern formation. Other vertebrates show a much more complete spectrum of regulative abilities (see Section A1) and hence provide more information about the nature of the mechanisms involved. The cumulative evidence from all vertebrate limbs cannot be accommodated by the idea that positional information in the anterior–posterior axis is specified with reference to a diffusible morphogen (Bryant and Muneoka, 1986).

Rather than seeing the emergence of graded positional qualities in limbs or other developing systems as the result of underlying gradients of diffusible molecules, we suggest that graded properties arise in development as a consequence of interactions at the interfaces between cells with different qualities. In other words, we propose that developmental processes in limbs and possibly elsewhere are driven by discontinuities. Discontinuities are resolved by the generation of a graded series of intermediate properties that provide a smooth, seamless transition between the original discontinuities. According to this view, development consists of a successive series of events in which discontinuities are first generated then resolved. For limbs, the polar coordinate model (French *et al.*, 1976; Bryant *et al.*, 1981), in which the pattern of the limb is seen as being generated in the progress zone as a result of intercalation between discontinuities, continues to provide a good fit for the available data (see also Iten, 1982; Saunders and Gasseling, 1983; Javois, 1984; Wanek *et al.*, 1991). It is possible, as we discuss below (Section D3), that the same principles, namely the emergence of graded pattern as result of discontinuities, will prove to have more widespread applicability to development as a whole.

D. RA AND THE ESTABLISHMENT OF THE LIMB FIELD

1. *RA and the Primary Body Axis*

It has recently become clear that RA, in addition to its effects on limb development, has profound effects on pattern formation in the primary embryonic axis (Durston *et al.*, 1989; Mitrani and Shimoni, 1989; Sive *et al.*, 1990; Ruiz i Altaba and Jessell, 1991). Hence, *Xenopus* embryos treated with RA are missing the most rostral-dorsal parts of the pattern and, as reported by Cho *et al.* (1992), in severe examples (known as "squdgy" embryos) the tail is also shortened. In addition, it has been shown that genes that are normally expressed in rostral structures are repressed by RA treatment, whereas those that are normally expressed in the trunk are expressed more strongly in the response to RA (Cho and De Robertis, 1990; Sive *et al.*, 1990; Cho *et al.*, 1991). Finally, implantation of RA-beads into gastrula stage chick embryos leads to the development of duplicate axes (Chen and Solursh, 1990).

It is conceivable that RA not only affects primary pattern formation when it is exogenously applied, but also that it plays a role in normal axis formation (see Sive *et al.*, 1990; Durston and Otte, 1991). A likely source of RA would be Hensen's node in chicks (and by homology, the blastopore of amphibian gastrulae) as suggested by the results of experiments involving grafts of Hensen's node into limb buds (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990). When grafted to the anterior of the chick wing bud, Hensen's node causes duplicated digits, consistent either with it having a posterior specification similar to that of the posterior limb field or with it being a source of RA. The node shows this activity from the definitive primitive streak stage to the time that it has completed its regression (Hornbruch and Wolpert, 1986).

The transgenic mouse studies discussed earlier, which fail to provide support for a role for RA in limb development (Mendelsohn *et al.*, 1991; Rossant *et al.*, 1991), at the same time provide intriguing data concerning a possible role for RA in the primary axis. In both studies, the *lacZ* constructs report the presence of RA in the trunk, but not in the head or the tail of early postgastrulation embryos. The rostral and caudal boundaries of expression are impressively sharp (Fig. 11). These results lend support to a role for RA in axis development and suggest that the timing of RA synthesis in gastrulation divides the body into three domains: head, trunk, and tail. Consistent with this idea is the finding discussed above that RA treatment of *Xenopus* embryos tends to reduce the

extent of both head and tail development. Other studies in *Xenopus* in which the amount of organizer tissue has been reduced (Stewart and Gerhart, 1990) or in which gastrulation is halted prematurely (Gerhart *et al.*, 1989) lead to rostral truncations. These results have been interpreted as showing that in order for rostral structures to form, they need to escape from a caudalizing influence in the region of the marginal zone that invaginates last (Gerhart *et al.*, 1989). It is conceivable that this caudalizing influence is RA.

In summary, a variety of different types of evidence is accumulating that suggests that RA is involved in the specification of pattern in the primary body axis. Of interest is the finding that while evidence suggests that the trunk region contains endogenous RA, both heads and tails appear to lack it. Further, heads and tails, but not trunks, are reduced in the presence of added RA.

2. RA and the Establishment of the Limb Base

An interesting body of data that provides an important piece of the RA puzzle comes from the studies of Hornbruch and Wolpert (1991) in which they mapped the distribution of posterior activity on the flank of the prelimb bud chick embryo at different stages. In these studies, posterior activity was assessed by the ability of the graft to stimulate the formation of supernumerary pattern after grafting to the anterior of a host limb bud. The earliest stage at which flank tissue shows posterior activity overlaps with the last stage at which Hensen's node shows activity (stage 9). At stages 10 and 11, posterior activity is found in a positionally contiguous stripe that spans the region from 2–4 somites in front of the position of the future wing, back to the node, which is 2–4 somites posterior to the future position of the wing at this stage. At later stages, the rostral extent of the posterior activity appears to move progressively more caudal. At stage 12 the rostral limit is at somite 15; at stages 13 and 14 it is at somite 17; at stage 15 it is at somite 18; and at stage 16, the beginning of visible outgrowth (Hamburger and Hamilton, 1951), the rostral limit of activity is at the level of somite 19, where it remains. Hence, during these stages the rostral limit of posterior activity shifts from opposite somite 15 to opposite somite 19. This shift matches in both magnitude and direction the backward shift of the prospective wing region mapped by Chaube (1959). The caudal extent of posterior activity has not been as completely mapped, but at stage 16, when the wing is starting to be visible, there is a 3-somite-wide region behind the wing bud that shows no posterior activity. The position of this region corresponds to the middle of the hind limb field. Hence, at this stage, posterior activity extends from the posterior of the wing bud, through the intervening flank region and into the anterior third of the leg bud region. These puzzling findings can be reconciled by the following hypothesis.

We suggest that RA is involved in the specification of positional information in the flank mesenchyme during gastrulation, with Hensen's node the most likely source of endogenous RA. As prospective flank cells invaginate through the primitive streak they are exposed to high levels of RA emanating from the nearby Hensen's node. We hypothesize that flank cells are set by this exposure to RA to a positional value that can be described as posterior-ventral-proximal with respect to the (future) limb, just as occurs experimentally when older limb bud cells are exposed to RA. We have argued that positional diversity is essential for limb outgrowth; hence, we propose that limb outgrowth is initiated by the arrival of migrating cells into the region of the future limb buds. We propose that these cells arrive from more anterior-dorsal regions and bear corresponding anterior-dorsal positional values. The interaction of the new arrivals with the posterior-ventral-proximal cells of the flank is predicted to lead to intercalation of the limb base and subsequent limb outgrowth as described previously (French *et al.*, 1976; Bryant *et al.*, 1981, 1987). The directional intercalation discussed earlier (Section A2) would make it possible for interactions at the interface between two oppositely specified groups of cells to generate a complete limb base that was oriented appropriately with respect to the main axes of the body (Fig. 12). The idea that fields can arise at the interfaces between differently determined regions has also been explored by Meinhardt (1991) to account for the origin of the limb field. The idea that cells with anterior-dorsal positional value arrive on the flank could account for the change in distribution of cells with posterior activity, as migration and intercalation displace them to their final locations. Hence, in the case of the forelimb, interaction between the posterior-ventral and anterior-dorsal cells will result in the caudal displacement of the posterior-ventral cells. In the case of the hindlimb, the arrival and growth of anterior-dorsal cells will lead to the apparent gap in the otherwise continuous stretch of cells with posterior properties.

Consistent with these ideas is the fact that although posterior limb properties can be shown to be present on the flank of the chick embryo from stage 9 onward (Hornbruch and Wolpert, 1991), the prospective limb regions are incapable of forming a limb after isolation from their normal surroundings before stage 15 for the wing, and later for the leg (Kieny, 1969, 1971; Pinot, 1969, 1970). Between stages 10 and 15 wings can develop from the relevant flank region only if the normally adjacent somites are included with the graft. Somites from other regions are ineffective substitutes in these experi-

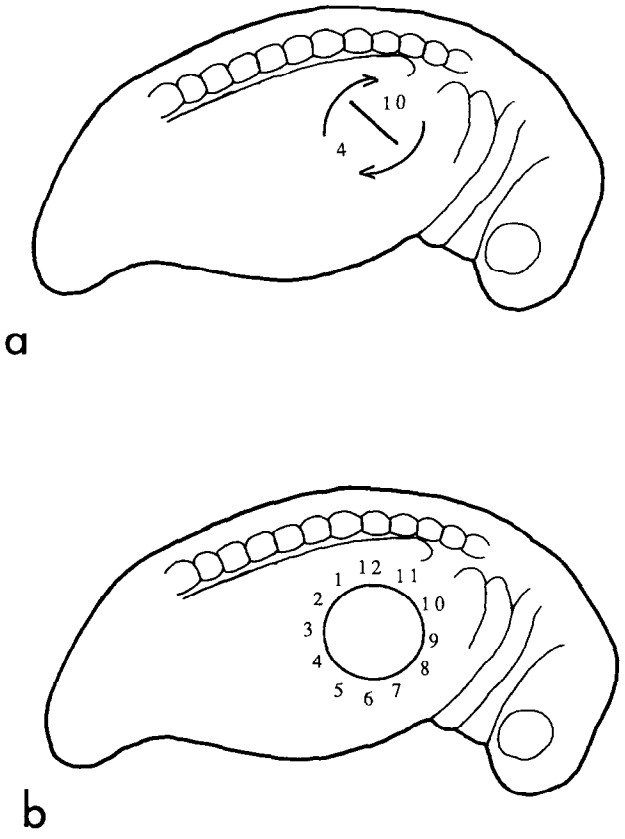

FIG. 12. Limb bud initiation. (a) Interaction at a boundary between prelimb bud flank cells with a posterior-ventral-proximal positional identity (4) and recently immigrating cells with a more anterior-dorsal-proximal position value (10). Arrows indicate directional intercalation inferred from several experiments. See text for details. (b) After intercalation, a limb base with a full circumference has been generated. Distal outgrowth can proceed from this base as described previously (Bryant *et al.*, 1981).

ments. Other experiments have shown that foil barriers separating the prospective wing region in the lateral plate from the somites between stages 10 and 15 inhibit wing development *in situ* (Stephens *et al.*, 1991). Hence, despite the presence of posterior properties, something further is needed before limb outgrowth can occur. Additional experiments (Stephens *et al.*, 1991) have pinpointed the location of the required material to the intermediate mesenchyme lying between the edge of the somites and the lateral plate mesoderm between stages 13 and 15. Further, it appears that the missing ingredient has arrived in the limb field by stage 15, when grafts of prospective limb fields to the coelom can differentiate into limbs (Kieny, 1969, 1971; Pinot, 1969, 1970). This stage immediately preceeds the first detectable signs of limb outgrowth at stage 16. We propose that the ingredient that is missing between stages 9 and 15 is cells that migrate into the limb field from more anterior-dorsal regions of the embryo. The newly arriving cells interact with the posterior-ventral-proximal cells already present, stimulating intercalation and the onset of limb outgrowth.

Three migratory cell populations are candidates for involvement in the establishment of the limb base: neural crest, somitic, and nephric. Although there are not many studies of this region using modern cell tracing techniques, Chevallier (1977) demonstrated a level-specific cellular contribution from the somitic mesoderm to the shoulder girdle in chicks using the chick-quail cell marker. The level specificity of this contribution is in contrast to the lack of specificity in the contribution from the somites to the limb musculature (Chevallier *et al.*, 1977). Since all three of the candidate cell types are located close to one another and are to some degree intermingled, this finding does not specifically rule out either neural crest or nephric cells carried along with the somites as the relevant cells.

In molecular terms, it is possible that the homeobox gene *XlHbox 1* identifies the proposed anterior-dorsal cell population that migrates to the forelimb base to initiate outgrowth. Oliver *et al.* (1988) describe the expression of the *XlHbox 1* long protein in a rostral-caudally restricted zone in the prelimb bud region of the flank of *Xenopus*. Scattered cells expressing *XlHbox 1* protein are present in the myotomes of the prelimb bud region, and these have been tentatively identified as neural crest cells (Oliver *et al.*, 1988), lending credence to the suggestion that *XlHbox 1* cells could migrate ventrally toward the flank. As the limb forms, cells that are positive for *XlHbox 1* protein are found in the anterior half of the bud. A similar pattern of protein expression has been documented in the limbs of mice, chick, and zebrafish (Oliver *et al.*, 1988; 1990; Molven *et al.*, 1990). Protein expression is graded within limb buds, with the highest expression in proximal-anterior cells and lowest in posterior and distal cells. This graded distribution could reflect a dilution of the *XlHbox 1* protein as *XlHbox 1*-positive cells are stimulated to divide as a result of their proposed interactions with the posterior-ventral-proximal cells of the flank. Expansion of the expression domain of *XlHbox 1* protein in chick limbs that will later duplicate their pattern after exposure to RA-beads or ZPA grafts (Oliver *et al.*, 1990) could result secondarily from the interaction between the cells that express *XlHbox 1* protein and the ZPA cells that are either grafted or converted into ZPA by the RA-bead (Wanek *et al.*, 1991). Duplication of the shoulder girdle is associated with expansion of the expression domain of *XlHbox 1* (Oliver *et al.*, 1990), as expected if this gene identifies cells that contribute to the girdle during normal development.

In summary, we suggest that the primary role of RA in limb development is most likely to be in the establish-

ment of flank mesenchyme with posterior-ventral-proximal information with respect to the limb. It is possible that Hensen's node is the source of endogenous RA and that invaginating lateral plate mesoderm cells are exposed to this source, thereby acquiring a posterior-ventral-proximal positional value. A subsequent influx of cells into the limb region from a more anterior-dorsal location is proposed. There is evidence for the forelimb that this population originates in the somites adjacent to the limb region and contributes to the formation of the girdle. It is possible that this population of cells is the same as that which expresses *XlHbox 1.* This anterior-dorsal cell population provides the necessary level-specific positional diversity to stimulate intercalation and the formation of the entire limb pattern by mechanisms outlined in detail elsewhere (French *et al.*, 1976; Bryant *et al.*, 1981, 1987).

3. RA and the Transformation of Tails into Limbs

The most recent listing in the catalogue of the developmental consequences of RA is the most amazing to date. Amputated tails of marbled balloon frog tadpoles (*Uperodon systoma*) exposed to RA during the first few days of regeneration regenerate legs from the tail blastema (Mohanty-Hejmady *et al.*, 1992) (Fig. 13).

Based on the ideas we have outlined in this article, we offer an interpretation, shown in schematic form in Fig. 14, where we have identified different positions along the rostral-caudal axis of the body with numbers I through VII. We have proposed that the normal role of RA is in the establishment of a trunk region (i.e., level IV) that is distinct from the RA-negative head and tail (see Fig. 11). In development RA exposure converts the gastrulating flank cells of the trunk to a positional value that is equivalent to level IV, also described as posterior-ventral-proximal (with respect to the limb). Limb cells exposed to exogenous RA later in development also acquire this same positional value, with the consequences that we have discussed in earlier sections of this article.

In the balloon frog experiments, we propose that RA affects tail blastema cells the same way that it affects limb blastema cells, developing limb cells, and gastrulating flank cells: the cells acquire a positional value that is posterior-ventral-proximal *with respect to the limb;* in other words, they acquire the flank positional value (level IV), even though they are located at the tail tip. Uniformity of positional value within the blastema is predicted to bring regeneration to a halt, and indeed previous reports of exposure of tail regenerates to RA have shown inhibition of growth (Pietsch, 1987) and truncation (Niazi and Saxena, 1979). In the balloon frog experiments, the animals were removed from RA after a few days. We suggest that after removal from RA, dedifferentiation of tail cells continues, bringing cells with level VI positional value into contact with the blastema cells with level IV value. In previous studies we have demonstrated that intercalation occurs along the rostral-caudal axis of newt tails (Iten and Bryant, 1976). In the case of the balloon frog, rostral-caudal intercalation between blastema and stump will lead to a reversed-polarity intercalated region that spans the region from which hind limbs normally develop, shown here as lying between body positions IV and V. Further, the tail regenerate will be able to resume outgrowth using the diverse positional information from the dedifferentiated stump cells that are added to the blastema after RA treatment is ended, in much the same way as proposed for limb regenerates. In this case, regeneration in a caudal direction from level IV will generate another region that spans the position from which hind limbs originate (between IV and V). The prediction that more than a single pair of hindlegs develops in these experiments is borne out by the results (Mohanty-Hejmadi *et al.*, 1992). We interpret the multiple legs illustrated in Fig. 13 as basically two sets of hindlegs (as in the example in Fig. 13a) which are then able to duplicate

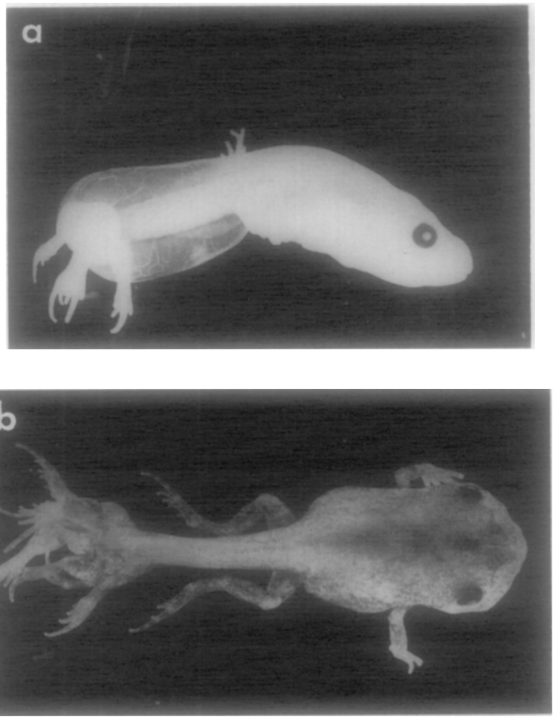

FIG. 13. Legs regenerating from tail after RA exposure. Reproduced, by permission of the publisher, from Mohanty-Hejmadi *et al.* (1992). Balloon frog tadpoles were treated with RA during the first few days after tail amputation. Sets of hindlegs develop from the tail blastema. (a) A tadpole with two ectopic sets of hindlimbs. The most caudal pair appears to be fused along the midline. (b) A tadpole in which the extra sets of legs have themselves duplicated. See text for details.

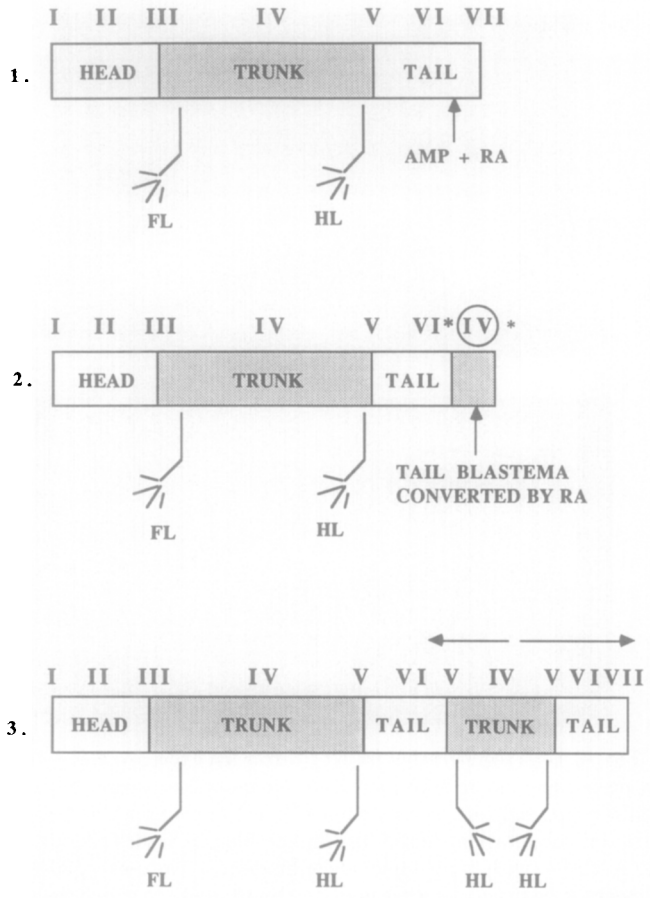

FIG. 14. Interpretation of transformation of tails into legs by RA in marbled balloon frogs (see Fig. 13). (1) Diagram of the body of the tadpole showing the head, trunk, and tail regions. The trunk is shaded to indicate the presence of RA as described in the legend for Fig. 11. Positions along the rostral-caudal axis are designated I–VII. The positions at which legs will develop are indicated (forelimbs, between III and IV; hindlimbs, between IV and V). The arrow indicates that the tail was amputated and the animal was exposed to RA for several days. (2) Diagram as in 1 above, except that the tail blastema has been converted to trunk positional value (IV) by RA. Asterisks indicate where growth will occur. (3) After removal from RA, newly dedifferentiating tail cells will enter the base of the blastema. Intercalation between positional values VI (tail tip) and IV (tail blastema converted to flank positional value) will lead to the formation of a reversed-polarity segment that spans the positional values associated with hindlimb formation. A second hindlimb region will be generated by regeneration from the tail blastema at level IV. Hence, it will appear as if RA has caused a homeotic transformation of the tail blastema into limb.

during regeneration (as in the example in Fig. 13b), much as leg regenerates in anurans have been reported to do (Niazi and Saxena, 1978; Maden, 1983a; Scadding and Maden, 1986b) (see Section B4).

The description above provides a logical explanation for an otherwise mysterious result, and it is fully consistent with what has been shown previously for both limbs and tails. It also raises the provocative question of whether intercalation along the rostral-caudal axis of the body plays a role in the development of the primary axis. The idea that graded positional qualities could arise from initial discontinuities is not inconsistent with some features of primary axis formation. Axis development is preceeded by the generation of a major discontinuity at gastrulation. Several lines of data suggest that the body is initially divided into three regions, head, trunk, and tail (discussed in Section D1 above). Slack (1991) has recently shown for the origin of the mesoderm in amphibians that when inducing and responding cells are separated by a filter, the induction is incomplete, suggesting that local cell-cell interactions are normally involved. Blumberg *et al.* (1991) showed that in addition to the homeobox gene *goosecoid,* thought to be involved in the unique properties of the organizer itself (Cho *et al.*, 1992), the only other classes of homeobox-containing genes present in the organizer region of *Xenopus* embryos are homologs of *labial* and *caudal,* which are involved in specification of the ends of the *Drosophila* body axis. This led Blumberg *et al.* (1991) to speculate that the ends of the body axis might be established first, followed by intercalation to generate the middle regions of the axis.

In summary, the apparent homeotic change of tail to limb in regenerating frogs can be understood in terms of the proposed unitary effect of RA in changing pattern formation-competent cells to a posterior–ventral–proximal (i.e., flank) positional value, followed by intercalation along the rostral-caudal axis of the body to generate two additional pairs of hind limb sites on the tail. We raise the issue of a role for intercalation in primary axis formation for further consideration.

E. CONCLUSIONS

In this Review we have described a mechanism by which the diverse effects of RA on limbs can be understood. According to this view, RA has a single effect on pattern formation-competent cells: it converts them to a posterior–ventral–proximal positional value (with respect to the limb) that is synonymous with the positional value of the prelimb bud flank. Short-range interactions between cells with discontinuous positional information, followed by growth and the intercalation of intervening qualities of positional information can account for the diverse outcomes of RA treatment. The view we have presented can account for the teratogenic effects of RA, as well as the different effects of RA on the anterior-posterior, dorsal-ventral, and proximal-distal axes of the limb, and for the apparent homeosis from tail to limb. In normal development, RA appears to be involved in the division of the primary body axis into head, trunk, and tail regions and in the establishment of

a population of flank cells with posterior-ventral-proximal specification. Both limb and tail cells retain the ability to respond to added RA throughout the period that they are actively engaged in pattern formation. Focusing on how RA changes the positional identities of cells to posterior-ventral-proximal might be expected to lead to insights into the molecular basis of positional information.

The explanatory power of the polar coordinate model and the role of cell-cell interactions in the development of insects have successfully served as guides for the exploration of the molecular basis of pattern formation (Martinez Arias, 1989; Wilkins and Gubb, 1991). In vertebrates, on the other hand, there has been a tendency to move directly from the tissue level to molecular explanations with the result that analysis at the cellular level has been more or less bypassed. We suggest that the behavior of cells, particularly local cell-cell interactions, and the principles of the polar coordinate model provide a workable cellular level of explanation for the phenomena of limb pattern formation. The challenge is to use the predicted cellular properties to assist in the discovery of the molecules involved. Hence, we need to discover the receptors and ligands that allow cells to detect similarities and differences between themselves and their neighbors, the mechanisms by which this information is transduced into the inhibition or stimulation of growth, the mechanisms by which cells in the cell cycle are able to acquire a molecular identity that is intermediate between those of the surrounding cells, the molecular basis of directional and shortest route intercalation, and the mechanism of distal transformation. All of these and more are direct questions about the molecular basis of pattern formation that arise from an understanding of the underlying cellular properties of the system and that can be used to guide an exploration into the molecular basis of pattern formation.

We thank Laurie Iten, Terry Hayamizu, Ken Muneoka, and Nancy Wanek for their various roles in the development of the views presented here. We also thank Ken Cho for bringing "squdgy" embryos to our attention, Bruce Blumberg and Kaz Umesono for helpful discussions, and the reviewers of this article for their constructive critiques. Research supported by PHS Grant 25620.

REFERENCES

Abbott, B. D., Hill, L. G., and Birnbaum, L. S. (1990). Processes involved in retinoic acid production of small embryonic palatal shelves and limb defects. *Teratology* **41,** 299-310.

Balcuns, A., Gasseling, M. T., and Saunders, J. W., Jr. (1970). Spatiotemporal distribution of a zone that controls antero-posterior polarity in the limb bud of the chick and other bird embryos. *Am. Zool.* **10,** 323A.

Blumberg, B., Wright, C. V. E., De Robertis, E. M., and Cho, K. W. Y. (1991). Organizer-specific homeobox genes in *Xenopus laevis* embryos. *Science* **253,** 194-196.

Boncinelli, E., Simeone, A., Acampora, D., and Mavilio, F. (1991). Hox gene activation by retinoic acid. *TIG* **7,** 329-334.

Brockes, J. P. (1990). Retinoic acid and limb regeneration. *J. Cell Sci. (Suppl.)* **13,** 191-198.

Brown, R., and Brockes, J. P. (1991). Identification and expression of a regeneration-specific homeobox gene in the newt limb blastema. *Development* **111,** 489-496.

Bryant, S. V. (1976). Regenerative failure of double half limbs in *Notophthalmus viridescens*. *Nature* **263,** 676-679.

Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration and symmetry. *Science* **212,** 993-1002.

Bryant, S. V., Gardiner, D. M., and Muneoka, K. (1987). Limb development and regeneration. *Am. Zool.* **27,** 675-696.

Bryant, S. V., Holder, N., and Tank, P. (1982). Cell-cell interactions and distal outgrowth in amphibian limbs. *Am. Zool.* **22,** 143-151.

Bryant, S. V., and Muneoka, K. (1986). Views of limb development and regeneration. *TIG* **2,** 153-159.

Chan, W. Y., Lee, K. K. H., and Tam, P. P. L. (1991). Regenerative capacity of forelimb buds after amputation in mouse embryos at the early-organogenesis stage. *J. Exp. Zool.* **260,** 74-83.

Chaube, S. (1959). On axiation and symmetry in transplanted wing of the chick. *J. Exp. Zool.* **140,** 29-77.

Chen, Y. P., and Solursh, M. (1990). Retinoic acid can induce secondary axis formation in the chick embryo. *J. Cell Biol.* **111,** 236A.

Chevallier, A. (1977). Origine des ceintures scapulaires et pelviennes chez l'embryon d'oiseau. *J. Embryol. Exp. Morphol.* **42,** 275-292.

Chevallier, A., Kieny, M., Mauger, A., and Sengel, P. (1977). Developmental fate of the somitic mesoderm in the chick embryo. *In* "Vertebrate Limb and Somite Morphogenesis" (D. A. Ede, J. R. Hinchliffeand, and M. Balls, Eds.), pp. 421-432, Cambridge Univ. Press, London.

Cho, K. W. Y., Blumberg, B., and De Robertis, E. M. (1991). Cooperation between mesoderm-inducing growth factors and retinoic acid in *Xenopus* axis formation. *Sem. Dev. Biol.* **2,** 393-403.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H., and De Robertis, E. M. (1992). Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *goosecoid* in gastrulation. *Cell* **67,** 1111-1120.

Cho, K. W. Y., and De Robertis, E. M. (1990). Differential activation of *Xenopus* homeobox genes by mesoderm-inducing growth factors and retinoic acid. *Genes Dev.* **4,** 1910-1916.

Cooke, J., and Summerbell, D. (1980). Cell cycle and experimental pattern duplication in the chick wing during embryonic development. *Nature* **287,** 697-701.

Dencker, L., Annerwall, E., Busch, C., and Eriksson, U. (1990). Localization of specific retinoid-binding sites and expression of cellular retinoic-acid-binding protein (CRABP) in the early mouse embryo. *Development* **110,** 343-352.

Deuchar, E. M. (1976). Regeneration of amputated limb-buds in early rat embryos. *J. Embryol. Exp. Morphol.* **35,** 345-354.

Dollé, P., Izpisúa-Belmonte, J.-C., Falkenstein, H., Renucci, A., and Duboule, D. (1989a). Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature* **342,** 767-772.

Dollé, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C. M., Gudas, L. J., and Chambon, P. (1989b). Differential expression of genes encoding α, β and γ retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature* **342,** 702-705.

Duboule, D. (1991). Patterning in the vertebrate limb. *Curr. Op. Gen. Dev.* **1,** 211-216.

Durston, A. J., and Otte, A. P. (1991). A heirarchy of signals mediates neural induction in *Xenopus laevis*. *In* "Cell-Cell Interactions in Early Development" (J. Gerhart, Ed.), Wiley-Liss, New York.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J.,

de Vries, N. J., Heideveld, M., and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340,** 140–144.

Eichele, G., and Thaller, C. (1987). Characterization of concentration gradients of a morphogenetically active retinoid in the chick limb bud. *J. Cell Biol.* **105,** 1917–1923.

Eichele, G., Tickle, C., and Alberts, B. M. (1985). Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: Temporal and spatial aspects. *J. Cell Biol.* **101,** 1913–1920.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889–895.

Ferretti, P., and Brockes, J. P. (1991). Cell origin and identity in limb regeneration and development. *Glia* **4,** 214–224.

French, V., Bryant, P. J., and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193,** 969–981.

Gardiner, D. M., and Bryant, S. V. (1989). Organization of positional information in the axolotl limb. *J. Exp. Zool.* **251,** 47–55.

Gardiner, D. M., Gaudier, C., and Bryant, S. V. (1992). Mouse limb bud cells respond to retinoic acid *in vitro* with reduced growth. *J. Exp. Zool.*, in press.

Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1986). The migration of dermal cells during blastema formation in axolotls. *Dev. Biol.* **118,** 488–493.

Gerhart, J., Danilchick, M., Doniach, T., Roberts, S., Rowning, B., and Stewart, R. (1989). Cortical rotation of the *Xenopus* egg: Consequences for the anteroposterior pattern of embryonic dorsal development. *Development (Suppl.)*, 37–51.

Green, S., and Chambon, P. (1988). Nuclear receptors enhance our understanding of transcription regulation. *TIG* **4,** 309–314.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Holder, N., Bryant, S. V., and Tank, P. W. (1979). Interactions between irradiated and unirradiated tissues during supernumerary limb formation in the newt. *J. Exp. Zool.* **208,** 303–309.

Honig, L. S. (1983). Does anterior (non-polarizing region) tissue signal in the developing chick limb? *Dev. Biol.* **97,** 424–432.

Hornbruch, A., and Wolpert, L. (1986). Positional signalling by Hensen's node when grafted to the chick limb bud. *J. Embryol. Exp. Morphol.* **94,** 257–265.

Hornbruch, A., and Wolpert, L. (1991). The spatial and temporal distribution of polarizing activity in the flank of the pre-limb-bud stages in the chick embryo. *Development* **111,** 725–731.

Howard, W. B., and Willhite, C. C. (1986). Toxicity of retinoids in humans and animals. *J. Toxicol. Toxin Rev.* **5,** 55–94.

Iten, L. E. (1982). Pattern specification and pattern regulation in the embryonic chick limb bud. *Am. Zool.* **22,** 117–129.

Iten, L. E., and Bryant, S. V. (1976). Regeneration from different levels along the tail of the newt, *Notophthalmus viridescens*. *J. Exp. Zool.* **196,** 293–306.

Iten, L. E., and Murphy, D. J. (1980). Pattern regulation in the embryonic chick limb: supernumerary limb formation with anterior (non-ZPA) limb bud tissue. *Dev. Biol.* **75,** 373–385.

Iten, L. E., Murphy, D. J., and Muneoka, K. (1983). Do chick limb bud cells have positional information? *In* "Limb Development and Regeneration. Part A" (J. F. Fallon and A. I. Caplan, Eds.), pp. 77–88, A. R. Liss, New York.

Izpisúa-Belmonte, J.-C., and Duboule, D. (1992). Homeobox genes and pattern formation in the vertebrate limb. *Dev. Biol.* **152,**

Izpisúa-Belmonte, J.-C., Tickle, C., Dollé, P., Wolpert, L., and Duboule, D. (1991). Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. *Nature* **350,** 585–589.

Javois, L. C. (1984). Pattern specification in the developing chick limb. *In* "Pattern Formation: A Primer in Developmental Biology" (G. M. Malacinski and S. V. Bryant, Eds.), pp. 557–579. Macmillan, New York.

Javois, L. C., and Iten, L. E. (1982). Supernumerary limb structures after juxtaposing dorsal and ventral chick wing bud cells. *Dev. Biol.* **90,** 127–143.

Javois, L. C., and Iten, L. E. (1986). The handedness and origin of supernumerary limb structures following 180° rotation of the chick wing bud on its stump. *J. Embryol. Exp. Morphol.* **91,** 135–152.

Kieny, M. (1969). Sur les relations entre le mésoderme somitique et le mésoderme somatopleural avant et au cours de l'induction primaire des membres de l'embryon de Poulet. *C. R. Acad. Sci. Paris.* **268,** 3183–3186.

Kieny, M. (1971). Les phases d'activité morphogène du mésoderme somatopleural pendant le développment précoce du membre chez l'embryon de Poulet. *Ann. d'Embryol. Morphogen.* **4,** 281–298.

Kieny, M., and Pautou, M.-P. (1977). Proximo-distal pattern regulation in deficient avian limb buds. *Wilhelm Roux's Arch. Dev. Biol.* **183,** 177–191.

Kiffmeyer, W. R., Tomusk, E. V., and Mescher, A. L. (1991). Axoplasmic transport and release of transferrin in nerves of regenerating amphibian limbs. *Dev. Biol.* **147,** 392–402.

Kim, W.-S., and Stocum, D. L. (1986a). Effects of retinoids on regenerating limbs: Comparison of retinoic acid and arotinoid at different amputation levels. *Wilhelm Roux's Arch. Dev. Biol.* **195,** 455–463.

Kim, W.-S., and Stocum, D. L. (1986b). Retinoic acid modifies positional memory in the anteroposterior axis of regenerating axolotl limbs. *Dev. Biol.* **114,** 170–179.

Ko, M. S. H., Nakauchi, H., and Takahashi, N. (1990). The dose dependence of glucocorticoid-inducible gene expression results from changes in the number of transcriptionally active templates. *EMBO J.* **9,** 2835–2842.

Kochhar, D. M. (1977). Cellular basis of congenital limb deformity induced in mice by vitamin A. *Birth Defects* **13,** 111–154.

Kochhar, D., Penner, J. D., and Tellone, C. (1984). Comparative teratogenic activity of two retinoids: Effects on palate and limb development. *Teratogen. Carcinogen. Mutagen.* **4,** 377–387.

Krasner, G., and Bryant, S. V. (1980). Distal transformation from symmetrical forearms in the axolotl, *Ambystoma mexicanum*. *Dev. Biol.* **74,** 315–325.

Lammer, E., Chen, D., Hoar, R., Agnish, N., Benke, P., Braun, J., Curry, C., Fernhoff, P., Grix, A. J., Lott, I., Richard, J., and Sun, S. (1985). Retinoic acid embryopathy. *N. Engl. J. Med.* **313,** 837–841.

Larsen, H. L., and Janners, M. Y. (1987). Teratogenic effects of retinoic acid and dimethylsulfoxide on embryonic chick wing and somite. *Teratology* **36,** 313–320.

Lheureux, E. (1975). Régénération des membres irradiés de *Pleurodeles waltlii* Michah. (Urodèle). Influence des qualités et orientation des greffons non irradiés. *Wilhelm Roux's Arch. Dev. Biol.* **176,** 303–327.

Lheureux, E., Thoms, S. D., and Carey, F. (1986). The effects of two retinoids on limb regeneration in *Pleurodeles waltl* and *Triturus vulgaris*. *J. Embryol. Exp. Morphol.* **92,** 165–182.

Ludolph, D. C., Cameron, J. A., and Stocum, D. L. (1990). The effect of retinoic acid on positional memory in the dorsoventral axis of regenerating axolotl limbs. *Dev. Biol.* **140,** 41–52.

Maden, M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature* **295,** 672–675.

Maden, M. (1983a). The effect of vitamin A on limb regeneration in *Rana temporaria*. *Dev. Biol.* **98,** 409–416.

Maden, M. (1983b). The effect of vitamin A on the regenerating axolotl limb. *J. Embryol. Exp. Morphol.* **77,** 273–295.

Maden, M., Keeble, S., and Cox, R. A. (1985). The characteristics of local application of retinoic acid to the regenerating axolotl limb. *Wilhelm Roux's Arch. Dev. Biol.* **194,** 228–235.

Maden, M., Ong, D. E., and Chytil, F. (1990). Retinoid-binding protein distribution in the developing mammalian nervous system. *Development* **109,** 75–80.

Maden, M., Ong, D. E., Summerbell, D., and Chytil, F. (1988). Spatial distribution of cellular protein binding to retinoic acid in the chick limb bud. *Nature* **335,** 733–735.

Maden, M., Ong, D. E., Summerbell, D., and Chytil, F. (1989). The role of retinoid-binding proteins in the generation of pattern in the developing limb, the regenerating limb and the nervous system. *Development (Suppl.),* 109–119.

Maini, P. K., and Solursh, M. (1991). Cellular mechanisms of pattern formation in the developing limb. *Int. Rev. Cytol.* **129,** 91–133.

Mangelsdorf, D., Ong, E., Dyck, J., and Evans, R. (1990). Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345,** 224–229.

Martinez Arias, A. (1989). A cellular basis for pattern formation in the insect epidermis. *TIG* **5,** 262–267.

Meinhardt, H. (1991). Determination borders as organizing regions in the generation of secondary embryonic fields: The initiation of legs and wings. *Sem. Dev. Biol.* **2,** 73–75.

Mendelsohn, C., Ruberte, E., and Chambon, P. (1992). Retinoids in vertebrate limb development. *Dev. Biol.* **152,**

Mendelsohn, C., Ruberte, E., LeMeur, M., Morriss-Kay, G., and Chambon, P. (1991). Developmental analysis of the retinoic acid-inducible RAR-β2 promoter in transgenic animals. *Development* **113,** 723–734.

Mitrani, E., and Shimoni, Y. (1989). Retinoic acid inhibits growth in agarose of early chick embryonic cells and may be involved in regulation of axis formation. *Development* **107,** 275–280.

Mohanty-Hejmadi, P., Dutta, S. K., and Mahapatra, P. (1992). Limbs generated at the site of tail amputation in marbled balloon frog after vitamin A treatment. *Nature* **355,** 352–353.

Molven, A., Wright, C. V. E., Bremiller, R., De Robertis, E. M., and Kimmel, C. (1990). Expression of a homeobox gene in normal and mutant zebrafish embryos: Evolution of the tetrapod body plan. *Development* **109,** 279–288.

Muneoka, K., and Bryant, S. V. (1982). Evidence that patterning mechanisms in developing and regenerating limbs are the same. *Nature* **298,** 369–371.

Muneoka, K., and Bryant, S. V. (1984a). Cellular contribution to supernumerary limbs in the axolotl, *Ambystoma mexicanum. Dev. Biol.* **105,** 166–178.

Muneoka, K., and Bryant, S. V. (1984b). Cellular contribution to supernumerary limbs resulting from the interaction between developing and regenerating tissues in the axolotl. *Dev. Biol.* **105,** 179–187.

Muneoka, K., Bryant, S. V., and Gardiner, D. M. (1989). Growth control in limb regeneration. *In* "Developmental Biology of the Axolotl" (J. B. Armstrong and G. M. Malacinsky, Eds.), Oxford Univ. Press, New York.

Muneoka, K., Fox, W., and Bryant, S. V. (1986a). Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls. *Dev. Biol.* **116,** 256–260.

Muneoka, K., Holler-Dinsmore, G., and Bryant, S. V. (1986b). Pattern discontinuity, polarity and directional intercalation in axolotl limbs. *J. Embryol. Exp. Morphol.* **93,** 51–72.

Muneoka, K., and Murad, E. H. B. (1987). Intercalation and the cellular origin of supernumerary limbs in *Xenopus. Development* **99,** 521–526.

Muneoka, K., and Sassoon, D. (1992). Limb development and regeneration. *Dev. Biol.* **152,**

Muneoka, K., Wanek, N., and Bryant, S. V. (1986c). Mouse embryos develop normally *exo utero. J. Exp. Zool.* **239,** 289–293.

Muneoka, K., Wanek, N., Taylor, G., Hayamizu, T. F., Trevino, C., Shi, C., Gardiner, D. M., Anderson, R., and Bryant, S. V. (1992). Regeneration of the chick limb bud is stimulated by the apical ectodermal ridge. *Science,* submitted.

Niazi, I. A., Pescitelli, M. J., and Stocum, D. L. (1985). Stage-dependent effects of retinoic acid on regenerating urodele limbs. *Wilhelm Roux's Arch. Dev. Biol.* **194,** 355–363.

Niazi, I. A., and Saxena, S. (1978). Abnormal hind limb regeneration in tadpoles of the toad, *Bufo andersoni,* exposed to excess vitamin A. *Folia Biol. (Krakow).* **26,** 3–11.

Niazi, I. A., and Saxena, S. (1979). Relationship between inhibiting influence of vitamin A and developmental stage of tail in toad tadpoles (*Bufo andersonii*). *Indian J. Exp. Biol.* **17,** 866–868.

Nohno, T., Noji, S., Koyama, E., Ohyama, K., Myokai, F., Kuroiwa, A., Saito, T., and Taniguchi, S. (1991). Involvement of the Chox-4 chicken homeobox genes in determination of anteroposterior axial polarity during limb development. *Cell* **64,** 1197–1205.

Noji, S., Nohno, T., Koyama, E., Muto, K., Ohyama, K., Aoki, Y., Tamura, K., Ohsugi, K., Ide, H., Taniguchi, S., and Saito, T. (1991). Retinoic acid induces polarizing activity but is unlikely to be a morphogen in the chick limb bud. *Nature* **350,** 83–86.

Oliver, G., De Robertis, E. M., Wolpert, L., and Tickle, C. (1990). Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J.* **9,** 3093–3099.

Oliver, G., Wright, C. V. E., Hardwicke, J., and De Robertis, E. M. (1988). A gradient of homeodomain protein in developing forelimbs of *Xenopus* and mouse embryos. *Cell* **55,** 1017–1024.

Perez-Castro, A. V., Toth-Rogler, L. E., Wei, L.-N., and Nguyen-Huu, M. C. (1989). Spatial and temporal pattern of expression of the cellular retinoic acid-binding protein and the cellular retinol-binding protein during mouse embryogenesis. *Proc. Natl. Acad. Sci. USA* **86,** 8813–8817.

Pescitelli, M. J., Jr., and Stocum, D. L. (1980). The origin of skeletal structures during intercalary regeneration of larval *Ambystoma* limbs. *Dev. Biol.* **79,** 255–275.

Pietsch, P. (1987). The effects of retinoic acid on mitosis during tail and limb regeneration in the axolotl larva, *Ambystoma mexicanum. Wilhelm Roux's Arch. Dev. Biol.* **196,** 169–175.

Pinot, M. (1969). Mise en évidence d'un rôle du mésenchyme axial sur la morphogenèse précoce des membres de l'embryon de Poulet. *C. R. Acad. Sci. Paris* **269,** 201–204.

Pinot, M. (1970). Le rôle du mésoderme somitique dans la morphogenèse précoce des membres de l'embryon de Poulet. *J. Embryol. Exp. Morphol.* **23,** 109–151.

Rollman-Dinsmore, C., and Bryant, S. V. (1982). Pattern regulation between hind- and forelimbs after blastema exchanges and skin grafts in *Notophthalmus viridescens. J. Exp. Zool.* **223,** 51–56.

Rossant, J., Zirngibl, R., Cado, D., Shago, M., and Giguère, V. (1991). Expression of a retinoic acid response element-*hsplacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5,** 1333–1344.

Rubin, L., and Saunders, J. W., Jr. (1972). Ectodermal-mesodermal interactions in the growth of limb buds in the chick embryo: Constancy and temporal limits of the ectodermal induction. *Dev. Biol.* **28,** 94–112.

Ruiz i Altaba, A., and Jessell, T. (1991). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. *Genes Dev.* **5,** 175–187.

Saunders, J. W., Jr. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of ectoderm. *J. Exp. Zool.* **108,** 363–403.

Saunders, J. W., Jr. (1977). The experimental analysis of chick limb bud development. *In* "Vertebrate Limb and Somite Morphogenesis" (D. A. Ede, J. R. Hinchliffe, and M. Balls, Eds.), pp. 1–24. Cambridge Univ. Press, London.

Saunders, J. W., Jr., and Gasseling, M. T. (1983). New insights into the

problem of pattern regulation in the limb bud of the chick embryo. *In* "Limb Development and Regeneration. Part A" (J. F. Fallon and A. I. Caplan, Eds.), pp. 67–76, A. R. Liss, New York.

Saunders, J. W., Jr., Gasseling, M. T., and Gfeller, M. D., Sr. (1958). Interactions of ectoderm and mesoderm in the origin of axial relationships in the wing of the fowl. *J. Exp. Zool.* **137,** 39–74.

Savard, P., Gates, P. B., and Brockes, J. P. (1988). Position dependent expression of a homeobox gene transcript in relation to amphibian limb regeneration. *EMBO J.* **7,** 4275–4282.

Scadding, S. R., and Maden, M. (1986a). Comparison of the effects of vitamin A on limb development and regeneration in the axolotl, *Ambystoma mexicanum*. *J. Embryol. Exp. Morphol.* **91,** 19–34.

Scadding, S. R., and Maden, M. (1986b). Comparison of the effects of vitamin A on limb development and regeneration in *Xenopus laevis* tadpoles. *J. Embryol. Exp. Morphol.* **91,** 35–53.

Sessions, S. K., Wanek, N., and Bryant, S. V. (1989). Effects of localized application of retinoic acid on pattern in developing and regenerating salamander limbs. *Am. Zool.* **29,** 73A.

Simeone, A., Acampora, D., Nigro, V., Faiella, A., D'Esposito, M., Stornaiuolo, A., Mavilio, F., and Boncinelli, E. (1991). Differential regulation by retinoic acid of the homeobox genes of the four HOX loci in human embryonal carcinoma cells. *Mech. Dev.* **33,** 215–228.

Singer, M. (1978). On the nature of the neurotrophic phenomenon in urodele limb regeneration. *Am. Zool.* **18,** 829–841.

Sive, H. L., Draper, B. W., Harland, R. M., and Weintraub, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. *Genes Dev.* **4,** 932–942.

Slack, J. M. W. (1980). Morphogenetic properties of the skin in axolotl limb regeneration. *J. Embryol. Exp. Morphol.* **58,** 265–288.

Slack, J. M. W. (1987). We have a morphogen! *Nature* **327,** 553–554.

Slack, J. M. W. (1991). The nature of the mesoderm-inducing signal in *Xenopus:* a transfilter induction study. *Development* **113,** 661–669.

Smith, J. C. (1979). Evidence for a positional memory in the development of the chick wing bud. *J. Embryol. Exp. Morphol.* **52,** 105–113.

Smith, J. C. (1980). The time required for positional signalling in the chick wing bud. *J. Embryol. Exp. Morphol.* **60,** 321–328.

Smith, J. C., and Wolpert, L. (1981). Pattern formation along the anteroposterior axis of the chick wing: The increase in width following a polarizing region graft and the effect of X-irradiation. *J. Embryol. Exp. Morphol.* **63,** 127–144.

Smith, S. M., Pang, K., Sundin, O., Wedden, S. E., Thaller, C., and Eichele, G. (1989). Molecular approaches to vertebrate limb morphogenesis. *Development (Suppl.),* 121–131.

Stephens, T. D., Spall, R., Baker, W. C., Hiatt, S. R., Pugmire, D. E., Shaker, M. R., Willis, H. J., and Winger, K. P. (1991). Axial and paraxial influences on limb morphogenesis. *J. Morphol.* **208,** 367–379.

Stewart, R. M., and Gerhart, J. C. (1990). The anterior extent of dorsal development of the *Xenopus* embryonic axis depends on the quantity of organizer in the late blastula. *Development* **109,** 363–372.

Stocker, K. M., and Carlson, B. M. (1990). Hensen's node, but not other biological signallers, can induce supernumerary digits in the developing chick limb bud. *Wilhelm Roux's Arch. Dev. Biol.* **198,** 371–381.

Stocum, D. L. (1978). Regeneration of symmetrical hindlimbs in larval salamanders. *Science* **200,** 790–793.

Stocum, D. L. (1991a). Limb regeneration: A call to arms (and legs). *Cell* **67,** 5–8.

Stocum, D. L. (1991b). Retinoic acid and limb regeneration. *Sem. Dev. Biol.* **2,** 199–210.

Stocum, D. L., and Dearlove, G. E. (1972). Epidermal-mesodermal interaction during morphogenesis of the limb regeneration blastema in larval salamanders. *J. Exp. Zool.* **181,** 49–62.

Stocum, D. L., and Thoms, S. D. (1984). Retinoic-acid-induced pattern completion in regenerating double anterior limbs of urodeles. *J. Exp. Zool.* **232,** 207–215.

Summerbell, D. (1974). Interaction between the proximo-distal and antero-posterior co-ordinates of positional value during the specification of positional information in the early development of the chick limb-bud. *J. Embryol. Exp. Morphol.* **32,** 227–237.

Summerbell, D. (1977). Regulation of deficiencies along the proximal distal axis of the chick wing-bud: A quantitative analysis. *J. Embryol. Exp. Morphol.* **41,** 137–159.

Summerbell, D., and Harvey, F. (1983). Vitamin A and the control of pattern in developing limbs. *In* "Limb Development and Regeneration. Part A" (J. F. Fallon and A. I. Caplan, Eds.), pp. 109–118. A. R. Liss, New York.

Summerbell, D., Lewis, J. H., and Wolpert, L. (1973). Positional information in chick limb morphogenesis. *Nature* **244,** 492–496.

Tabin, C. J. (1991). Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. *Cell* **66,** 199–217.

Tank, P. W. (1977). The timing of morphogenetic events in the regenerating forelimb of the axolotl, *Ambystoma mexicanum*. *Dev. Biol.* **57,** 15–32.

Tank, P. W. (1979). Positional information in the forelimb of the axolotl: Experiments with double-half tissues. *Dev. Biol.* **73,** 11–24.

Tassava, R. A., Johnson-Wint, B., and Gross, J. (1986). Regenerate epithelium and skin glands of the adult newt react to the same monoclonal antibody. *J. Exp. Zool.* **239,** 229–240.

Thaller, C., and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327,** 625–628.

Thaller, C., and Eichele, G. (1988). Characterization of retinoid metabolism in the developing chick limb bud. *Development* **103,** 473–483.

Thaller, C., and Eichele, G. (1990a). Isolation of 3,4-didehydroretinoic acid, a novel morphogenetic signal in the chick wing bud. *Nature* **345,** 815–819.

Thaller, C., and Eichele, G. (1990b). Toward a molecular understanding of vertebrate limb morphogenesis: Retinoids and the specification of the anteroposterior limb pattern in the chick embryo. *In* "The Cellular and Molecular Biology of Pattern Formation" (D. L. Stocum and T. L. Karr, Eds.), pp. 111–125. Oxford Univ. Press, New York.

Thoms, S. D., and Stocum, D. L. (1984). Retinoic acid-induced pattern duplication in regenerating urodele limbs. *Dev. Biol.* **103,** 319–328.

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289,** 295–298.

Tickle, C. (1991). Retinoic acid and chick limb development. *Development (Suppl.)* 113–121.

Tickle, C., Alberts, B., Wolpert, L., and Lee, J. (1982). Local application of retinoic acid to the limb bond [sic] mimics the action of the polarizing region. *Nature* **296,** 564–566.

Tickle, C., and Brickell, P. M. (1991). Retinoic acid and limb development. *Sem. Dev. Biol.* **2,** 189–197.

Tickle, C., and Crawley, A. (1988). The effects of local application of retinoids to different positions along the proximo-distal axis of embryonic chick wings. *Wilhelm Roux's Arch. Dev. Biol.* **197,** 27–36.

Tickle, C., Crawley, A., and Farrar, J. (1989). Retinoic acid application to chick wing buds leads to a dose-dependent reorganization of the apical ectodermal ridge that is mediated by the mesenchyme. *Development* **106,** 691–705.

Tickle, C., Lee, J., and Eichele, G. (1985). A quantitative analysis of the effect of all-*trans*-retinoic acid on the pattern of chick wing development. *Dev. Biol.* **109,** 82–95.

Tickle, C., Summerbell, D., and Wolpert, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254,** 199–202.

Wallace, H. (1981). "Vertebrate Limb Regeneration." Wiley Chichester/New York.

Wanek, N., Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1991). Conversion by retinoic acid of anterior cells into ZPA cells in the chick wing bud. *Nature* **350,** 81–83.

Wanek, N., Muneoka, K., and Bryant, S. V. (1989a). Evidence for regulation following amputation and tissue grafting in the developing mouse limb. *J. Exp. Zool.* **249,** 55–61.

Wanek, N., Muneoka, K., Holler-Dinsmore, G., Burton, R., and Bryant, S. V. (1989b). A staging system for mouse limb development. *J. Exp. Zool.* **249,** 41–49.

Wilkins, A. S., and Gubb, D. (1991). Pattern formation in the embryo and imaginal discs of *Drosophila:* What are the links? *Dev. Biol.* **145,** 1–12.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. Theor. Biol.* **25,** 1–47.

Wolpert, L. (1971). Positional information and pattern formation. *Curr. Top. Dev. Biol.* **6,** 183–224.

Yokouchi, Y., Sasaki, H., and Kuroiwa, A. (1991). Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* **353,** 443–445.