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# Amphibian Limb Regeneration: Rebuilding a Complex Structure

Jeremy P. Brockes

**The ability to regenerate complex structures is widespread in metazoan phylogeny, but among vertebrates the urodele amphibians are exceptional. Adult urodeles can regenerate their limbs by local formation of a mesenchymal growth zone or blastema. The generation of blastemal cells depends not only on the local extracellular environment after amputation or wounding but also on the ability to reenter the cell cycle from the differentiated state. The blastema replaces structures appropriate to its proximodistal position. Axial identity is probably encoded as a graded property that controls cellular growth and movement through local cell interactions. The molecular basis is not understood, but proximodistal identity in newt blastemal cells may be respecified by signaling through a retinoic acid receptor isoform. The possibility of inducing a blastema on a mammalian limb cannot be discounted, although the molecular constraints are becoming clearer as we understand more about the mechanisms of urodele regeneration.**

Many larval and adult animals are able to regenerate large sections of their body plan after transection or amputation (*1*), and this usually restores the structures that were removed by the operation. In some invertebrates this occurs in a bidirectional fashion (Fig. 1). Thus, if a planarian worm is transected, the head fragment regenerates tail structures, whereas the tail fragment grows a new head. The importance of ani-

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mal regeneration as a topic for investigation was first recognized in the 18th century, when the work of Reaumur, Trembley, Spallanzani, Bonnet, and others provided anatomical descriptions of the process and identified some of the species in which it occurred (*2*). In addition, the origin of the regenerate became a focus of controversy between the advocates of epigenesis and the advocates of preformation. We now consider that regeneration of such complex structures is distinct from cellular turnover and from the regulation of embryonic structures at stages before commitment and differentiation. It remains a challenge, however, to understand precisely how the combination of tissue repair mechanisms with reactivation of embryonic programs can generate growth, pattern formation, and morphogenesis in an adult animal.

Although the ability to regenerate appears to be widespread throughout metazoans, many species within each phylum are unable to regenerate. The only adult vertebrates that are able to regenerate their limbs are the urodele amphibians, such as the newt and axolotl, as first reported in 1768 by Spallanzani (*2*). A newt can regenerate its tail and limbs, as well as its jaws and ocular tissues such as the lens (Fig. 2A). The regeneration of appendages proceeds by local formation of a growth zone or blastema at the plane of amputation—a mechanism shared by most of the examples shown in Fig. 1 and termed epimorphic regeneration by Morgan (*3*). After amputation of the limb at a position along the proximodistal axis (PD; from shoulder to fingertips), the wound surface is sealed in approximately 12 hours by the rapid migration of epithelial cells from the circumference. This forms the wound epidermis, a transient epithelium that is critical for subsequent outgrowth. Urodeles are able to effect local reversals in the differentiated state of cells in response to amputation or tissue removal, and the postmitotic cells of the limb mesenchyme beneath the wound epidermis reenter the cell cycle and lose their differentiated character. The resulting blastemal cells express several markers that are not expressed by differentiated mesenchymal cells of the normal limb nor by cells of the developing limb bud (*4*). The blastemal cells proliferate to produce a conical mound of cells (Fig. 2B), then progressively exit from the cell cycle and differentiate into the cartilage, connective tissue, and muscle of the regenerate. Although these aspects are characteristic of blastemal cells arising at all levels along the PD axis, Fig. 2B illustrates that axial identity is profoundly important for regeneration, in that the blastema only gives rise to structures that are distal to its level of origin—a wrist blastema gives a hand, whereas a shoulder blastema makes an entire arm. The blastema has considerable morphogenetic autonomy; this is shown if it is transplanted to a permissive location such as the tunnel of the dorsal fin or the anterior chamber of the eye, where it gives rise to a regenerate that is appropriate for its level of origin (*5*). The mechanisms underlying the plasticity of the differentiated state and the positional identity of the blastema are the focus of this article. Other important aspects of regeneration, in particular the role of the nerve supply, are not considered here but are discussed elsewhere (*6*).

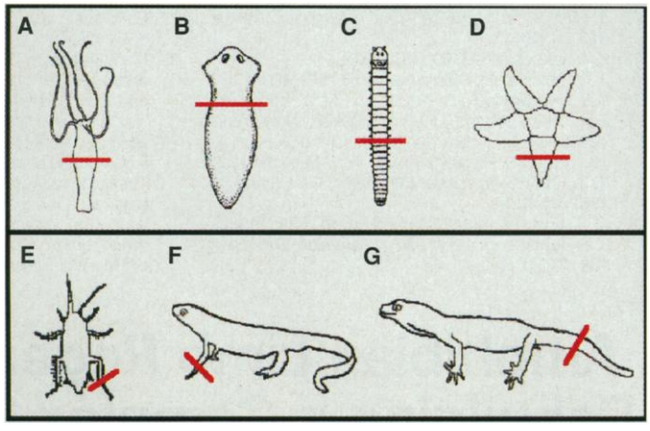


**Fig. 1.** Regeneration is widespread among metazoans. (**A** through **D**) Bidirectional regeneration. These animals can respond to transection by regeneration from both fragments. (A) *Hydra*, a cnidarian. (B) *Dugesia*, a planarian worm. (C) *Nereis*, an annelid worm. (D) *Linckia*, an echinoderm. (**E** through **G**) Unidirectional regeneration of appendages. (E) Cockroach limb. (F) Newt limb. (G) Lizard tail. The amputation or transection plane is represented by a red line.

## Reversal of the Differentiated State

The two most informative systems for analyzing the mechanisms of reversal of cell differentiation in urodeles are lens and limb regeneration. Regeneration of the lens proceeds without the complex aspects of pattern formation seen in the limb and has the advantage of occurring through the transitions of a single cell type: the pigmented epithelial cell (PEC) of the iris (*7*). After removal of a newt lens, the dorsal PECs reenter the cell cycle and lose their pigment granules. Some of the dedifferentiated cells subsequently transdifferentiate into lens cells; others reconstruct the local architecture of the iris epithelium. The conversion of newt iris PECs into lens cells was established by experiments with clonal cell culture of pigmented cells, in which at least 15% of the clonal colonies examined underwent definitive lens differentiation (*8*). Although newts are the only adult vertebrates that are able to regenerate the lens,

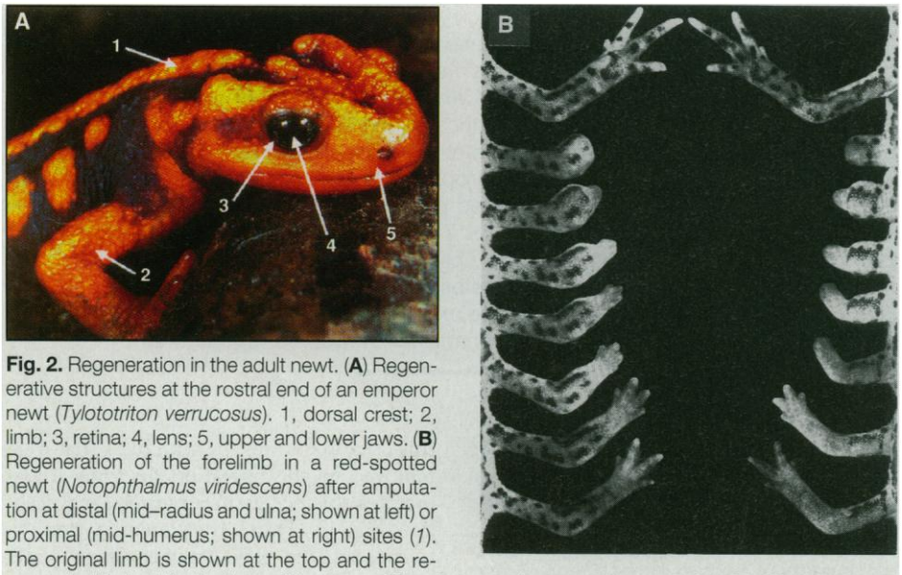


**Fig. 2.** Regeneration in the adult newt. (**A**) Regenerative structures at the rostral end of an emperor newt (*Tylototriton verrucosus*). 1, dorsal crest; 2, limb; 3, retina; 4, lens; 5, upper and lower jaws. (**B**) Regeneration of the forelimb in a red-spotted newt (*Notophthalmus viridescens*) after amputation at distal (mid–radius and ulna; shown at left) or proximal (mid-humerus; shown at right) sites (*1*). The original limb is shown at the top and the regenerated limb at the bottom of the image. The photographs were taken at 7, 21, 25, 28, 32, 42, and 70 days after amputation. Note that the blastema gives rise to structures distal to its level of origin.

the ability of cultured PECs of the iris or retina to dedifferentiate and transdifferentiate into lens cells is quite widespread under appropriate conditions in culture, where chick and even human PECs will form lens cells and express crystallins (9). Such conditions include the use of phenylthiourea to inhibit melanogenesis and the use of basic fibroblast growth factor (FGF) to promote dedifferentiation and transdifferentiation. These studies of ocular epithelial cells point to the importance of the local environment in evoking plasticity in the newt, rather than to a distinctive aspect of urodele cells.

The limb mesenchyme contains a number of cell types, particularly cartilage, muscle, and interstitial fibroblasts; and although the anatomical descriptions of early stages of regeneration are consistent with reversal of differentiation (*10*), it is necessary to introduce a cell marker or lineage tracer to follow the fate of the differentiated cells. If labeled cartilage is implanted beneath the wound epidermis, the label is found in mononucleate cells of the blastema and subsequently in connective tissue and cartilage of the regenerate; the evidence in this case is strong that the label in the implanted tissue is confined to chondrocytes (*11*). Muscle is an interesting case, first because there is considerable information about the molecular basis of muscle differentiation and its regulation, and second because muscle regeneration in higher vertebrates proceeds by mobilizing reserve or satellite cells that lie beneath the basal lamina, rather than by reversal of the differentiated state of the multinucleated myofiber. Reversal of multinucleate cells in regeneration has been established by studies on newt myotubes, which can be manipulated in culture and also implanted into a blastema. The experiments do not indicate that resident myofibers necessarily participate in blastema formation, but the cultured myotube has proved an excellent target cell for investigating the mechanisms of cell cycle reentry and reversal of differentiation.

Newt limb and blastemal cells can be propagated in culture without any evidence of crisis or senescence, and several isolates fuse to form myotubes when the serum concentration in the medium is lowered (*12*). The myotubes stably express characteristic markers of muscle differentiation such as myosin heavy chain (*13*) while down-regulating expression of blastemal cell markers. In order to follow their fate during regeneration, purified myotubes were selectively microinjected with a lineage tracer and implanted underneath the wound epidermis (*14*). Many labeled mononucleate cells were subsequently detected in the early blastema, indicating that at least 15 to 20% of the implanted nuclei undergo a reversal of the mononucleate-to-multinucleate transition; and after 3 to 4 weeks, labeled cells were found in muscle and, in a few cases, in cartilage (*14*). The environment of the blastema is thus able to destabilize the differentiated state of the implanted myotubes and to promote return to the cell cycle. The parallels between lens and limb regeneration in this respect are underlined by the striking observation that when newt iris tissue is transplanted to a control site such as the fin, brain, or normal limb, the epithelial cells retain their pigmented identity, whereas iris tissue transplanted into the blastema forms lens cells (*15*). Although these studies point to the importance of external signals in promoting cell cycle reentry and dedifferentiation, recent work on newt myotubes in culture has shown that there are also intrinsic differences in responsiveness between urodele and mammalian cells.

## Cell Cycle Reentry from the Differentiated State

When newt myotubes in culture are shifted into high serum concentrations, the myotube nuclei reenter the cell cycle (Fig. 3, A and B), traverse S phase, and arrest at $G_2$ phase without any evidence of cytopathology or cell death (*13*). These results reveal a significant difference between urodele muscle cells and those of other vertebrates. Avian and mammalian myotubes enter a state of postmitotic arrest after fusion, in which they are refractory to serum growth factors that act on their mononucleate precursors (*16*). They can be induced to enter S phase after transfection with viral oncogenes, such as T antigen, that are capable of sequestering the retinoblastoma (Rb) protein, although the subsequent events lead to widespread cell death (*17*). Serum-induced reentry has been reported in one circumstance in rodent myotubes (*18*). Myogenic cells derived from mice that are homozygous null for the Rb gene ($Rb^{-/-}$) fuse in culture to form myotubes expressing muscle markers, but after exposure to serum the myotubes enter S phase just as the newt cells do. The Rb protein regulates passage through the $G_1$-S restriction point by complexing with members of the E2F family of transcription factors, whose activity is required to accomplish this transition (*19*). The association between Rb and E2F is relieved by phosphorylation of Rb by cyclin-dependent kinase 4 (CDK4) or CDK6. This kinase activity is inhibited by the INK4 family, which includes p16 and p18, and also by p21 (*19*). It has recently been suggested that the p21 and p18 proteins are implicated in the differentiation and postmitotic arrest of mammalian myotubes (*20*).

Urodele myotubes are not $Rb^{-/-}$, but there is evidence that inactivation of Rb by phosphorylation is an end point of serum-induced reentry (*13*). In mammalian myotubes, Rb is maintained in the hypophosphorylated form, and this is consistent with the state of postmitotic arrest (*21*). In newt myotubes, however, elevated serum concentrations stimulate the formation of the hyperphosphorylated form (Fig. 3C), demonstrating a difference in Rb regulation that is suggestive regarding the plasticity of the urodele cells. This difference is critical, as shown by the expression of proteins that inhibit the Rb pathway, such as p16 or mutant Rb, and also inhibit S phase reentry (*13*). It is possible that the serum pathway acts by inactivating or down-regulating endogenous INK4 proteins and hence activating CDK4. It seems likely that the reentry response of the myotubes underlies their ability to give rise to mononucleate progeny after implantation (for example, by execution of mitosis with attendant cytokinesis), although this remains to be established. Additional signals may be required in culture to overcome the $G_2$-M arrest and possibly to promote further reversal of differentiation in addition to S phase reentry, although it is noteworthy that

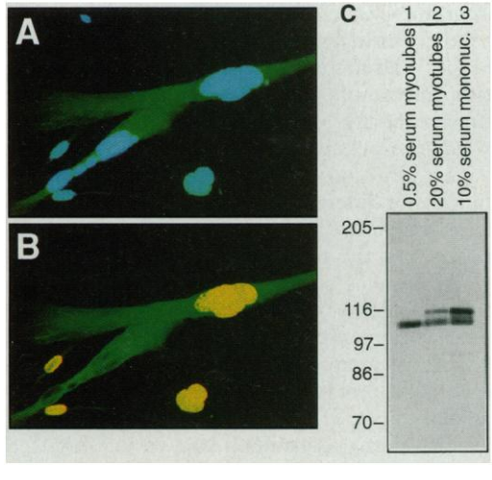


**Fig. 3.** Newt myotubes reenter the cell cycle after phosphorylation of the Rb protein. (**A**) The two myotubes are stained with antibody to muscle myosin (green) and Hoechst dye to stain DNA (blue). (**B**) The same myotubes showing myosin- and bromodeoxyuridine (BrdU)- positive nuclei (yellow) in the myotube on the right. It has entered the S phase after serum stimulation, whereas the myotube on the left is negative for BrdU. (**C**) Profile of Rb phosphorylation in purified myotubes maintained in low serum (lane 1), high serum (lane 2), and in serum from mononucleate cells (mononuc.) (lane 3). The lower band is the hypophosphorylated form, and the upper band is the hyperphosphorylated form. For details, see (*13*).

activation of CDK4 in mammalian myoblasts can also lead to inactivation of the myogenic regulator myoD (*22*). An analysis of several muscle genes has suggested that expression of the regulator myf-5 may continue in blastemal cells derived by reversal of differentiation during limb regeneration (*23*).

The identity of the signals that provoke reentry and reversal after amputation is not yet known, but amputation is not a prerequisite to initiate these events in urodeles. For example, flank wounds evoke the expression of several markers of blastemal cells, and in conjunction with nerve deflection can lead to formation of a limb (*24*). The cultured newt myotubes are refractory to a variety of mitogenic growth factors, such as platelet-derived growth factor, epidermal growth factor, or FGF, which are active on mononucleate precusors—this aspect of the postmitotic arrest is intact and is equivalent to that in mammalian myotubes (*13*). Urodele cells must normally maintain a stable differentiated state (this requirement is no different from that in other vertebrates) and only enter the cycle under the local circumstances of amputation or wounding. The identity of the serum activity is unknown, but it could be a signal related to wounding or clotting that acts on the myotubes and other differentiated cells. The response in culture is inhibited by contact with other cells, and it may be significant that blastema formation is associated with marked induction of proteolytic activity (*25*). For example, a matrix metalloprotease is expressed in the mesenchyme as early as 3 to 4 hours after amputation (*26*). As well as permitting cells to escape from their matrix and migrate into the blastema, these activities may also facilitate reversal by disrupting cell contacts and allowing responses to soluble mediators. In the jellyfish *Podocoryne* (*27*) and in the newt eye (*28*), such matrix degradation has also been implicated in events leading to transdifferentiation; and several examples of matrix remodeling have been noted in the limb blastema (*29*), although direct evidence for their functional importance is currently lacking.

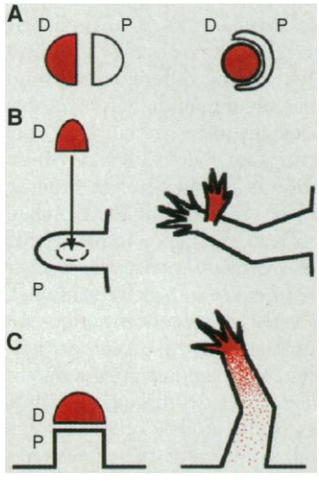


**Fig. 4.** Assays for PD identity in the urodele limb blastema. (**A**) When distal cells (D; red) are juxtaposed with proximal cells (P), the distal cells are engulfed by the proximal ones. (**B**) When a distal blastema is grafted to the dorsal surface of a proximal blastema so that the cells make contact, it translocates during regeneration to a position corresponding to its original level of origin. (**C**) When a distal blastema (wrist) is grafted onto a proximal stump (shoulder), a normal arm results, but the intercalated structures between the wrist and shoulder derive predominantly from the stump and the transplanted cells are predominantly found in the hand. Intercalation provides a cellular assay for positional identity based on the distribution in the regenerate.

## Regeneration and Cancer

There are several interesting but unresolved issues about the relationship between epimorphic regeneration and neoplasia. Two of the mechanisms thought to restrict tumor formation—cell death provoked by cell cycle reentry and finite proliferation due to replicative senescence—may be suspended during regeneration. Cell cycle reentry in general is subject to control points, and loss of Rb function in the context of differentiation leads not only to S phase entry but to apoptosis. For example, when Rb is removed by mutation in the mouse, lens cells that would normally withdraw from the cycle continue to synthesize DNA and die by apoptosis (*30*). The role of apoptosis in this context is often viewed as a defense against tumor formation arising from unscheduled proliferation in association with differentiation. Although urodele cells are able to engage in multiple reversible episodes of cell cycle reentry in regeneration, it has long been recognized that this is not associated with susceptibility but with marked resistance to tumor formation (*31*). For example, after application of chemical carcinogens to the blastema, the mesenchymal cells retain their ability to undergo differentiation and morphogenesis, and in some cases supernumerary regenerates are formed. It should be possible to test whether oncogene expression in urodele cells leads to reversal of differentiation, followed by differentiation in the blastema and incorporation into the regenerate.

All systems of epimorphic regeneration show the ability to sustain multiple cycles of regeneration with little change in time course, a feature possibly inconsistent with the finite proliferative potential of most animal cells (*32*). Indeed, newt limb blastemal cells can be maintained in culture for more than 200 generations without signs of crisis or senescence (*12*) and with retention of blastemal markers, whereas a variety of differentiated newt cell types have a short life-span in culture (*33*). Cellular senescence is a state with certain analogies to differentiation; for example, it entails withdrawal from the cell cycle and concomitant elevation of certain CDK inhibitors such as p16 or p21 (*34*). It is often regarded as a mechanism that prevents accumulation of multiple mutations required for tumor formation, because it restricts the total number of generations that a cell can undergo. Because blastemal cells have indefinite proliferative potential yet are resistant to tumorigenesis, they may use alternative mechanisms of cell cycle regulation to bypass or supersede this requirement for senescence. It may be possible in the future to understand the basis of the indefinite life-span of the newt cells at the molecular level. Although the potential may be indefinite, proliferation in the blastema is subject to regulation by the mechanisms of pattern formation.

## Positional Identity of the Limb Blastema

In most contexts of development and regeneration, it remains unclear how positional identity is encoded and is manifested in cellular properties that are important in tissue patterning, such as proliferation, adhesion, or migration. Epimorphic regeneration provides an accessible context in which to study these issues. Juxtaposition of blastemal cells from different axial levels suggests that these cells have distinctive cell surface properties (*35*). When two blastemal mesenchymes from different PD levels are juxtaposed in hanging-drop culture, the more proximal member engulfs the distal (Fig. 4A), whereas two from the same level maintain a stable boundary. This behavior is suggestive of graded differences in surface adhesivity along the axis. These differences are also indicated when a distal blastema is grafted to the dorsal surface of a proximal blastema in situ so that the mesenchymal cells are in contact (Fig. 4B). Under these conditions, it moves during regeneration to the distal level that is identical to its level of origin, as illustrated for the wrist blastema in Fig. 4B (*35*). In a third assay (Fig. 4C), a distal blastema, from the wrist level for example, is transplanted onto a shoulder stump so that wrist and shoulder cells are juxtaposed—a classic experiment referred to as intercalary regeneration. The result is a normal limb in which structures between the shoulder and wrist are generated predominantly by growth from the proximal partner, whereas most of the cells

from the wrist blastema give rise to the hand (*35*, *36*). Transplantation of a shoulder-level blastema to a shoulder stump (no disparity) does not mobilize the stump tissue but leads to a normal distal outgrowth from the shoulder blastema. The assays shown in Fig. 4 suggest that PD identity in urodele regeneration is encoded as a graded property, probably in part at the cell surface, and that cell behavior relevant to axial specification (growth, movement, and adhesion) is a function of the expression of this property relative to neighboring cells.

The way in which such positional confrontations arise and are resolved during regeneration is of considerable interest (*37*). It is not clear to what extent blastemal cells inherit a particular PD identity (from their differentiated precursors, for example) and to what extent they are subject to signals that induce the appropriate expression. Although the precise relationship between *Hox* gene expression and positional identity is not understood either for limb development or regeneration, the expression of HoxA9 and A13 in the mesenchyme is an important indication of local specification as early as 1 to 2 days after amputation (*38*). The early dedifferentiation stage is exposed to a variety of signals from the wound epidermis and from the general injury response; retinoic acid (RA) (*39*), Hedgehog protein (*40*), and FGF (*41*) are some possible candidates, although the evidence for axial variation in expression in each case is either incomplete or absent. Whatever the role of such signals in establishing positional identity, the most plausible models of pattern formation in limb regeneration stress the critical role of local interactions between blastemal cells, as indicated by the assays shown in Fig. 4 for the PD axis and by much other work for the transverse axes (*37*). For instance, in the early stages of regeneration, cells migrate into the center of the blastema and make contact, allowing such interactions to happen (*42*). It is possible that after amputation at a particular PD location, cells arise with the appropriate level-specific identity and then generate more distal identities by successive local interactions (*37*, *38*). This view can be distinguished from that invoking a role for the graded distribution of a morphogen signal within the blastema and seems more attractive for a mechanism that can operate on the scale of an adult urodele limb. This does not rule out the possibility of global signals for proliferation, such as diffusible growth factors, because it may be responsiveness that is locally regulated during intercalation. The somewhat hypothetical nature of this discussion underlines the fact that the most important unresolved issue is the molecular basis of positional identity as it is reflected in assays such as those shown in Fig. 4. It has not been possible to use these assays to guide purification of the molecules involved, and no invertebrate homolog has yet shed light on this issue. Some progress has been made through identification of the pathway activated by RA.

RA is known to respecify the PD axis during limb regeneration (*43*) and under certain circumstances to respecify the dorsoventral and anteroposterior axes (*44*); furthermore, it can switch the identity of a tail blastema in some anuran tadpoles so that it gives rise to limbs (*45*). Thus, if a distal limb blastema is exposed to RA for 48 hours, the blastemal cells are respecified to a more proximal level, resulting in the serial duplication of certain proximal structures (Fig. 5). Respecification is also detected with the displacement assay in Fig. 4B and the intercalation assay in Fig. 4C. The mechanism of proximalization has been clarified by experiments in which the RA response pathway was activated in individual distal blastemal cells rather than by global application of RA (*46*). The proximalizing activity is mediated by activation of RA receptors (RARs), which are ligand-dependent transcription factors of the nuclear receptor family. Five different newt RARs have been identified by cDNA cloning and shown to be expressed in cells of the limb and limb blastema (*47*). In order to activate single receptors, the ligand-binding domain of each RAR was replaced with the corresponding region of the thyroid hormone (T3) receptor (Fig. 6A) (*48*, *49*). The chimeric receptors were transfected into distal limb blastemal cells; and after activation with T3, one receptor—RARδ2—was necessary and sufficient to proximalize the distribution of transfected cells in intercalary regeneration (*46*). Activation of the other receptors, including the closely related RARδ1, gave a control distal distribution (Fig. 6, B and C). The effect of RA on PD identity is therefore mediated by RARδ2, the urodele equivalent of mammalian RARγ2 (*47*).

These results have several implications for our understanding of the mechanism of respecification and the nature of positional identity. Although less than 1% of the distal cells were transfected, these redistributed effectively after T3 treatment, which underlines the fact that migration or translocation is an important feature of positional identity and can act on a minor population of proximalized cells. It also indicates that RA acts directly on cells to respecify their identity, in contrast to its effect on limb development, in which local application to the bud is thought to induce a signaling center (the polarizing region) that acts locally with the epidermis to respecify the underlying mesenchyme. Furthermore, it seems unlikely that RA might act indirectly on PD identity by promoting dedifferentiation or inhibiting proliferation; the effect on proliferation is mediated by a different receptor, RARα1 (*48*). Finally, it shows that PD identity is an isoform-specific target of RARδ2 activation, an observation that encourages approaches to identify the target genes of δ2 in blastemal cells that are not common to activation of the closely related δ1.

## Some Prospects

The relationship between development and regeneration is a complex issue. The generation of progenitor cells by local reversal of differentiation and reentry to the cell cycle is a major difference between the two, reflecting the local origin of the regenerate from a substrate of differentiated tissue. On the other hand, the mechanisms for patterning the blastema and the limb bud must converge at some point. There is evidence from the formation of supernumerary limbs after juxtaposition of the axolotl blastema and limb bud that the basis of positional identity is related or identical in the two contexts (*50*). Much of the recent progress in our understanding of avian and mammalian limb development has underlined the role of diffusible signals exchanged between the epidermis and mesen-

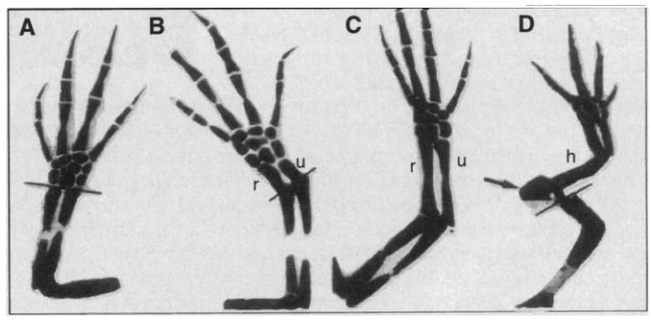


**Fig. 5.** RA treatment proximalizes a distal blastema during limb regeneration. (**A**) Control regenerate shows normal skeletal pattern of the hand after amputation at the wrist level (shown by a line segment). (**B** through **D**) Increasing doses of RA progressively proximalize the regenerate. In (D), a complete arm, including the shoulder girdle (arrow), is produced; r, radius; u, ulna.

chyme (*51*, *52*). One example is the importance of the polarizing region on the posterior margin as a local source of Sonic Hedgehog protein (*51*); this view has been largely confirmed by the phenotype of the Sonic Hedgehog knockout mouse, which shows deletion of distal limb structures (*53*). It has long been recognized that the regenerating limb does not have a polarizing region, but it is possible that the same molecular pathways could operate throughout the blastema. There may be other distinctions reflecting the fact that limb development occurs before regeneration and may leave imprints in the mechanism; for example, the suggestion that genes within the *HoxA* complex show a departure from temporal colinearity of expression and are expressed together in regeneration could be in this category (*38*). The comparison of signals and responses in the two contexts may help to identify what is most fundamental about limb morphogenesis; a satisfying account will eventually encompass limb regeneration and development.

It has often been asked whether epimorphic regeneration of a limb will ever be possible in mammals, and although this question cannot be answered at present, some implications arise from the issues discussed here. This is an area of potential clinical importance, and not only in relation to epimorphic regeneration. For example, although repair of cardiac lesions in mammals is limited to compensatory hypertrophy and fibrosis, newt ventricular cardiomyocytes can reenter the cell cycle and participate in the restoration of cardiac function (*54*). The blastema has considerable morphogenetic autonomy, and perhaps the most critical step in establishing regeneration is therefore the initial generation of blastema cells, corresponding to activation of the processes of reentry and reversal. If this depended solely on the operation of a particular signal transduction pathway in differentiated urodele cells, it is possible that it could be established in a mammal. There is evidence, however, that urodele cells are different with respect to aspects of the overall regulation of division, differentiation, senescence, and neoplasia, and this may be problematic to engineer in a mammalian context, even when it is understood more precisely. It is possible that selective pressures have led to the loss of regenerative ability (*55*), and it is only by comparing cellular regulation in urodeles and other vertebrates that this issue will be clarified.

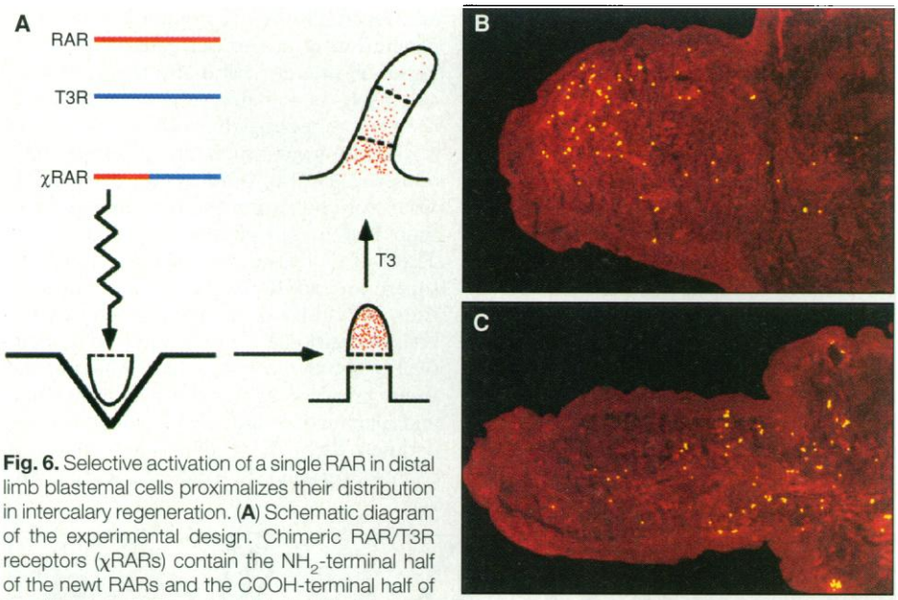


**Fig. 6.** Selective activation of a single RAR in distal limb blastemal cells proximalizes their distribution in intercalary regeneration. (**A**) Schematic diagram of the experimental design. Chimeric RAR/T3R receptors (χRARs) contain the $NH_2$-terminal half of the newt RARs and the COOH-terminal half of the *Xenopus* T3Rα, which binds T3. They are transfected into distal mesenchymal cells by particle bombardment of an inverted blastema, which is then grafted to a proximal stump in the configuration shown in Fig. 4C. After activation of the chimeras with T3, the distribution of the transfected cells (red dots) in the intercalary regenerate is determined with a semiautomated procedure using fluorescence-based detection of alkaline phosphatase (a cotransfected marker) with a laser scanning microscope, and subsequent image analysis. The boundaries of the intercalated region with the transplanted blastema and with the stump can be recognized in sections and are shown as dashed lines. The diagram shows a proximalized distribution. (**B**) Distribution of cells transfected with RARδ1 in a section of an intercalary regenerate. This is a false-color image in which transfected cells are shown as yellow dots. The distribution is weighted toward the distal tip. (**C**) Distribution of cells transfected with RARδ2. The distribution is weighted toward the proximal base of the regenerate. Details of the analysis are given in (*36*, *46*).

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32. If a nucleus in a differentiated mesenchymal cell is recruited into the blastema, it may undergo 5 to 10 generations before differentiating in the regenerate. Healthy adult newts can undergo at least 20 cycles of regeneration without significant change in timing, which suggests a considerable, possibly indefinite, proliferative potential within the epimorphic field.

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