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Molecular mechanisms in the control of limb regeneration: the role of homeobox genes

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ABSTRACT Axolotls are unique among vertebrates in their ability to regenerate lost appendages as adults. They provide the opportunity to study the mechanism of regeneration in vertebrates and are an inspiration to pursue the goal of appendage regeneration in humans. In this article, we review data on the role of homeobox-containing genes in the regulation of limb regeneration. As a group, these genes are important in pattern formation in the primary body axis, developing limbs and regenerating limbs. To date, a total of 22 homeobox genes have been identified as being expressed in regenerating limbs. Nearly all of these are also expressed during limb regeneration, further supporting the view that limb development and regeneration involve similar regulatory mechanisms. Our recent results on the expression of *HoxA* genes demonstrate that once a blastema has formed, subsequent outgrowth and pattern formation are similar to those of limb development. In contrast to developing limbs, reexpression of the *HoxA* genes in regeneration occurs by a non-colinear mechanism that likely is related to the necessity of mature limb cells to undergo dedifferentiation in order to give rise to the blastema. These studies also indicate that the pattern is respecified by a distal-first mechanism during regeneration in contrast to the apparent proximal-to-distal sequence observed in developing limbs. Expression of the *HoxA* genes is altered coordinately in response to retinoic acid in a manner consistent with the transformation of a distal blastema to a proximal blastema. Given the recent increase in studies of the molecules involved in regeneration, it is likely that many of the functionally important regeneration genes will be identified and characterized in the near future.

KEY WORDS: *homeobox, regeneration, limbs, pattern formation, retinoic acid*

Why is the urodele limb regeneration paradigm so significant?

Throughout the history of developmental biology, certain organisms have achieved celebrity status because they exemplify a property or principle of development that is difficult or impossible to study in other organisms. Urodele amphibians have earned such an honored position in part because, among vertebrates, they alone are able to regenerate lost appendages as adults. They provide not only the opportunity to study the mechanisms of regeneration, but even more importantly, they explicitly demonstrate to us that vertebrate limbs can regenerate. We thus are challenged to discover the regenerative potential in humans that is so easily activated in urodeles.

Although only urodeles regenerate limbs as adults, other vertebrates can do so during early stages of limb development. Anuran tadpoles can regenerate entire limbs at early limb bud stages, but as development continues, regenerative ability is lost beginning at proximal levels and progressing to more distal levels (Dent, 1962). This loss of regenerative ability is not a consequence of the altered hormonal environment at metamorphosis, but is an intrinsic, developmental property of limb cells (Sessions and Bryant, 1988). A similar proximal to distal loss of regenerative ability also occurs during mouse limb development, where late staged embryos can regenerate nearly entire digits (Wanek *et al.*, 1989), while in newborns, regeneration is restricted to only the distal tips of digits (Borgens, 1982).

Chick limbs allow us to distinguish between regenerative potential and regenerative ability. Chick limbs are not able to regenerate at any stage of development; however, studies of both developing and regenerating limbs have demonstrated that the presence of a permissive epidermis is required in order for limb outgrowth to occur. In chicks, removal of the limb bud epidermis, or even just the thickened apical ectodermal ridge (AER), is equivalent to amputation since the distal parts of the limb fail to develop (Saunders, 1948). Similar results are observed in

Abbreviations used in this paper: AER, apical ectodermal ridge; ZPA, zone of polarizing activity; RA, retinoic acid; ECM, extracellular matrix.

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amphibians by removing the epidermis of either developing (Tschumi, 1957) or regenerating (Stocum and Dearlove, 1972) limbs. Urodele limbs are able to regenerate in part because the outgrowth-permitting epidermis is reformed after amputation. In contrast, amputated chick limb buds are unable to reform a permissive epidermis, thereby inhibiting regeneration. If a permissive epidermis is grafted onto an amputated chick limb bud, it is able to regenerate a complete limb pattern (Hayamizu *et al.*, 1994; Rubin and Saunders, 1972), thus revealing its regenerative potential.

Although early stage chick limb buds are able to regenerate when supplied with a permissive epidermis, later stage limb buds are not. As with developing anuran limb buds, the regenerative ability of developing chick limb buds, albeit assisted by the graft of a permissive epidermis, progressively declines from proximal to distal and eventually disappears. Results from such experiments lead us to conclude that during development, limb cells exist in at least three states (Fig. 1).

State A: State A cells are localized in the apical mesenchyme, are actively involved in pattern formation, and are able to participate in regeneration. These cells correspond to the "progress zone" of the developing chick limb bud (Summerbell *et al.*, 1973), and are dependent on the apical epidermis in order to remain in State A.

State B: State B cells are located in the region proximal to the State A cells. They are no longer involved in pattern formation, but have not yet differentiated or made an irreversible commitment to differentiation. Cells in this state can become active in pattern formation and will regenerate distal regions when provided with a permissive epidermis.

State C: State C cells are found in the most proximal region of the limb bud, where cells are differentiating to form mature limb tissues, and are not involved in pattern formation. Except in urodeles, these cells have not been observed to become active in pattern formation, and consequently these cells do not participate in regeneration, even in the presence of a permissive epidermis (Hayamizu *et al.*, 1994) . As development proceeds, all cells of the limb progress to a state where pattern formation and differentiation are complete (State C).

Urodeles provide the unique opportunity to study the mechanisms by which cells of mature, differentiated tissues (State C) are converted into pattern-formation competent cells (State A), capable of recapitulating limb development. The challenge is to discover how mature limb cells become developmentally reactivated and how they acquire competence for pattern formation. Much is already known about the phenomenology of regeneration. Epidermal wound healing is rapid and complete within a few hours. During the next few days, differentiated cells from the mesenchyme are released from the surrounding extracellular matrix (ECM), and migrate towards the center of the wound epidermis (Gardiner *et al.*, 1986). These cells aggregate to form a blastema from which the new parts of the limb pattern are derived through growth and pattern formation. These events require the presence of a permissive epidermis (wound epidermis) (Stocum and Dearlove, 1972) and an adequate number of nerve fibers (Singer, 1978). As the early blastema begins to form, interactions between cells from different regions of the limb control the patterning and growth of the regenerate (Bryant and Gardiner, 1992). *We propose that it is the ability to undergo the early events leading to the formation of a blastema that distinguishes regenerating from non-regenerating species.*

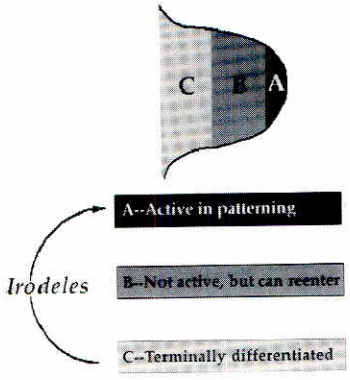

Fig. 1. Developmental states of limb bud cells. *The three states are designated A, B and C and the approximate regions they occupy within the limb bud are indicated. In urodeles, cells can move from State C to State A during regeneration, as indicated by the arrow.*

We are now in a position to use the wealth of information derived from decades of experimental embryological studies to guide the use of modern molecular techniques. We are at the threshold of being able to understand and manipulate the molecules controlling regeneration and thus to achieve the long desired goal of human regeneration. In this article, we summarize what has been learned recently about the role of one important group of molecules, the *Hox* complex genes, in regeneration, and how they are involved in the initiation of the regeneration cascade.

Hox complex genes function in pattern formation and growth

The molecular mechanisms involved in the subdivision of embryos into differently specified regions, and in controlling differentiation and growth, are remarkably conserved among animals. The first molecules to be recognized both for their role in pattern formation and their conserved homology across diverse phyla were the homeobox-containing genes. These genes were first identified as members of the homeotic complex *(HOM-C)* of *Drosophila* (McGinnis *et al.*, 1984; Scott and Weiner, 1984) and soon afterwards, homologs were isolated from vertebrates (Carrasco *et al.*, 1984). Not only do the vertebrate homologs share sequence identity with their *Drosophila* counterparts, they also share organizational structure along the chromosome. A major difference that has arisen during the evolution of vertebrates is that the *HOM* cluster has duplicated twice, leading to four separate *Hox* clusters in fish and tetrapods (Kappen *et al.*, 1993). Nevertheless, the function of *Hox* genes in the specification of segment identity has been conserved. This functional conservation is most directly evident from gene swap experiments between mouse and *Drosophila*, in which mouse *Hox* genes expressed in flies result in similar phenotypes to those observed when the homologous fly genes are similarly expressed (McGinnis *et al.*, 1990).

Several additional lines of evidence support the hypothesis that homeobox genes function in pattern formation in vertebrate embryos. Among this evidence is the colinearity between the position along the rostro-caudal axis at which a particular *Hox* gene is expressed and the physical location of that *Hox* gene within the complex (Duboule and Morata, 1994). Hence, 3' genes

are expressed rostrally and early, whereas more 5' genes are expressed more caudally and later. The domains of *Hox* gene expression overlap, leading to characteristic combinations of *Hox* gene products in particular segments of the body. Support for the idea that combinations of *Hox* genes specify positional identity comes from experiments in transgenic mice. When *Hox* gene expression is forced in ectopic locations or eliminated by gene knockout, mice often develop with predictable transformations in segment identity (Krumlauf, 1994).

In tetrapods, the limbs as well as the main body axis have a segmental organization, and *Hox* genes are also involved in pattern formation in developing limbs. Among the *Hox* genes, the *Abd-B* homologs (paralogous groups 9-13) at the 5' ends of the *A* and *D* complexes are expressed in limbs in a nested and overlapping pattern. Expression patterns of the *HoxD* genes suggests a role in pattern formation across the anterior-posterior limb axis (Dollé *et al.*, 1989; Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991), whereas expression of the *HoxA* genes correlates with segmentation along the proximal-distal axis (Yokouchi *et al.*, 1991; Haack and Gruss, 1993). Though the *HoxC* cluster contains *Abd-B*-related genes, they do not extend in a colinear fashion out into appendages, and different *HoxC* genes are expressed in different appendages (Oliver *et al.*, 1988; Tabin and Laufer, 1993). The *HoxB* cluster does not contain paralogs of groups 10,11,12 or 13, and expression of 3' genes is restricted to the base of the limb bud (Charité *et al.*, 1994).

Results from targeted gene alteration experiments confirm a role for Hox genes in limb pattern formation

When *HoxD11* is overexpressed in developing chick leg buds, the first digit sometimes develops with the morphology of a second digit, presumably because of a change in the *Hox* code in the position of the first digit (Morgan *et al.*, 1992). More dramatic effects, including mirror-image duplications of limbs, have been obtained by ectopic expression of *HoxB8* at the proximal/anterior boundary of the mouse limb bud (Charité *et al.*, 1994). Regardless of the primary effect of altered *HoxB8* expression, it induces a secondary pattern-regulation response equivalent to that resulting from grafting posterior limb bud cells (zone of polarizing activity, ZPA) to the anterior boundary of the limb bud (Tickle *et al.*, 1975).

Loss of function experiments have also yielded a range of phenotypes. A dramatic response is observed in many *HoxD13* knockout mice in which posterior cells that normally do not form a digit are induced to form an extra digit, a characteristic of cells located in a more anterior position (Dollé *et al.*, 1993). Other *Hox* knockout experiments have resulted in more subtle pattern alterations, possibly reflecting functional redundancy between paralogs. Double knockouts of paralogous *Hox* genes yield phenotypes that are more dramatic than predicted from the single knockouts, again suggesting overlapping function of paralogs (Condie and Capecchi, 1994). Functional redundancy would not be unique to *Hox* genes, as it occurs in other developmental pathways, such as those involving retinoic acid receptors (Mendelsohn *et al.*, 1994). In the *Hox* mutant mice with less dramatic phenotypes, the location of the defects coincides with the pattern of *Hox* expression and is as predicted by the rule of posterior prevalence (Johnson *et al.*, 1994). Hence, in mice lacking *HoxA11* or *HoxD11* function, defects are observed in the forearm and wrist, which is between the proximal boundary of normal expression and the proximal boundary of the next most 5' gene (Small and Potter, 1993; Davis and Capecchi, 1994). In *HoxD13* knockout mice, defects are observed in the hand/foot regions, where this gene is normally expressed (Dollé *et al.*, 1993). Although *HoxD* and *HoxA* genes were originally thought to have separate functions in the limb (Yokouchi *et al.*, 1991), paralogs share considerable overlap in expression domains, and overlap in function seems likely (Graham, 1994).

The phenotypes of lack-of-function mutants, including double knockout mutants affecting the neck region (Condie and Capecchi, 1994), have been interpreted as evidence of a role for *Hox* genes in controlling proliferation of precursor cell populations and/or the relative timing of developmental events (see Duboule, 1994a). The mechanisms by which *Hox* genes could influence cell proliferation or timing of gene expression, remain to be discovered. However, growth and pattern formation are known to be coordinately regulated in development and regeneration (Bryant *et al.*, 1981; French *et al.*, 1976), and *Hox* genes may provide a key to understanding the functional relationship between these two key developmental processes.

Many homeobox genes are expressed during regeneration

Researchers from a number of laboratories have investigated the role of homeobox genes in limb regeneration. Screens of cDNA libraries from regenerating newt limbs resulted in the identification of ten homeobox genes expressed during limb regeneration: *HoxA11* and *Hox* B3 (Beauchemin and Savard, 1993); *HoxC6* (Savard *et al.*, 1988; Tabin, 1989); *HoxC10* and *HoxD10* (Simon and Tabin, 1993); *HoxD11* (Brown and Brockes, 1991); *Dlx1*, *Dlx3* and *Emx2* (Beauchemin and Savard, 1992, 1993); *Msx1* (Crews *et al.*, 1995; Simon *et al.*, 1995). More recently, we isolated and identified a total of 18 different axolotl homeobox genes expressed during limb regeneration (Gardiner *et al.*, 1995; Table 1). Six of these axolotl genes are homologous to the newt genes. Hence, at present, a total of 22 different homeobox genes are known to be expressed during limb regeneration. It is now apparent that there is not a single gene, or even just a few, but many homeobox genes involved in regulating growth and pattern formation during limb regeneration. In addition, several of these genes are expressed as multiple transcripts with spatially distinct expression patterns (Savard *et al.*, 1988; Beauchemin and Savard, 1993; Torok, Gardiner and Bryant, unpublished), indicating an even more complex role in regeneration.

Most of the homeobox genes expressed in regenerating limb blastemas are also expressed in developing limb buds of other vertebrates (Izpisúa-Belmonte and Duboule, 1992; Duboule, 1994b). Only two genes expressed in blastemas, *em/msx* and *Hlx*, have not been reported to be expressed in developing limb buds. This broad overlap of gene expression between limb development and regeneration is supportive of the view that these two processes involve common mechanisms of growth regulation and pattern formation (see Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992). At the same time, it may turn out that some homeobox genes are expressed only in developing or regenerating limbs. For those genes that are expressed in both

developing and regenerating limbs, we are discovering differences in the way that their expression is regulated. These differences are most apparent during the initiation stages of regeneration, which is when limb regeneration and development are expected to differ the most (see Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992).

The obvious difference between regenerating and developing limbs is that regeneration begins with mature tissues, rather than with embryonic tissues. Consequently, the initiation of regeneration requires cells from mature tissues to give rise to a population of pattern formation-competent blastema cells, a process referred to as dedifferentiation. During dedifferentiation, patterns of homeobox gene expression differ for different genes (Table 1). Some are not expressed or are expressed below detectable levels in mature limbs, and are reexpressed during regeneration. Other genes are expressed in mature limbs, and are upregulated during regeneration, whereas others are downregulated during regeneration. Determining the order in which changes in gene expression occur during the initiation of regeneration is an important step towards unraveling cause and effect relationships among the various genes involved.

A striking result of our screen is the relative abundance and complexity of members of the *HoxA* complex expressed during limb regeneration. Studies of developing mouse and chick limbs indicate that the 5' *HoxA* genes function in specifying the proximal-distal limb pattern (Yokouchi *et al.*, 1991; Haack and Gruss, 1993). One difference between limb development and limb regeneration is that several steps in limb development are bypassed in limb regeneration. Regenerating limbs do not have to become specified as fore or hind limb, nor do they have to establish transverse axes. After loss of the distal part of the limb, regeneration occurs from a base of differentiated cells (hind limb or forelimb) derived from all points around the limb circumference (Gardiner *et al.*, 1986). These cells become mobilized and reenter a developmental pathway as they are released from their tissues during dedifferentiation. These stump derived cells carry a memory of their former position within the transverse axes into the blastema (Gardiner and Bryant, 1989). Hence in regeneration, limb pattern formation involves primarily the activation of cells for reentry into development and the reformation of the proximal-distal pattern, which may account for why the *HoxA* genes are so abundantly expressed.

TABLE 1

EXPRESSION OF HOMEOBOX GENES IN REGENERATING URODELE LIMBS

Expression not yet analyzed	Not expressed in mature tissues; reexpressed in regeneration	Expressed in mature tissues; upregulated in regeneration	Expressed in mature tissues; no change in regeneration	Expressed in mature tissues; downregulated in regeneration
HoxA4 (1)	*HoxA9* (1)	*HoxA11* (1, 8)	*Dlx-1* (8, 12)	*HoxD8* (1, 4)
HoxA5 (1)	*HoxA13* (1)	*HoxB3* (1, 8)	*Msx-1* (2)	
HoxA7 (1)	*HoxD10* (1, 4, 5)	*HoxC6* (9, 10)	*HoxC13* (1, 11)	
HoxA10 (1)	*HoxD11* (1, 4, 6)	*HoxC10* (5, 11)		
HoxB6 (1)	*Msx-2* (1, 7)	*Msx-1* (3)		
	em/msx (1, 7)	*Emx-2* (8)		
Hlx (1)		*Dlx-3* (1, 8, 12, 13)		

References in parentheses are as follows: (1) Gardiner *et al.*, 1995; (2) Crews *et al.*, 1995; (3) Simon *et al.*, 1995; (4) Torok, Gardiner and Bryant, unpublished; (5) Simon and Tabin, 1993; (6) Brown and Brockes, 1991; (7) Carlson, Gardiner and Bryant, unpublished; (8) Beauchemin and Savard, 1993; (9) Savard *et al.*, 1988; (10) Tabin, 1989; (11) Komine, Gardiner and Bryant, in preparation; (12) Beauchemin and Savard, 1992); (13) Mullen, Bryant and Gardiner, submitted.

Early expression of *HoxA* genes in regeneration is not colinear

Since urodeles both develop and regenerate limbs, it is possible to compare gene expression in developing limb buds with limb buds of other tetrapods as well as with regeneration blastemas. We have conducted such an analysis in axolotls for *HoxA9* and *HoxA13* using whole mount *in situ* hybridization. Axolotls are particularly appropriate for whole-mount studies because of the availability of pigment mutants (albino and white). In addition, since animals can be bred easily in the laboratory, it is possible to examine appropriate stages of developing limb buds.

The expression of *HoxA* genes in developing axolotl limbs follows the principles of temporal and spatial colinearity proposed by Duboule (Duboule and Morata, 1994), and in this respect is the same as developing mouse and chick limbs (Fig. 2). Expression of the more 5' gene, *HoxA13* is first detected at a later stage than the more 3' gene, *HoxA9* , and its expression domain is more distally restricted and is nested within that of *HoxA9*. Analysis of expression at later stages when differentiation begins indicates that the proximal boundary of *HoxA9* expression is within the distal third of the humerus, whereas the proximal boundary of *HoxA13* expression is at the wrist.

In contrast to developing limbs, the principles of temporal and spatial colinearity are abrogated during regeneration (Fig. 3). Neither gene is expressed in mature limbs, but expression of both is detected within one to two days after amputation in a stripe of mesenchymal cells immediately beneath the wound epidermis. At all time points examined, whenever *HoxA9* expression was detected in one limb, *HoxA13* expression was detected in the contralateral limb. Thus reexpression of *HoxA9* and *HoxA13* is synchronous rather than colinear. The temporal and spatial characteristics of the expression pattern are the same regardless of the proximal-distal level of the amputation, and expression continues to be colocalized until the stages of blastema cell accumulation. We are currently investigating genes in the other *Hox* complexes to see if non-colinearity is a general feature of the initiation stages of regeneration.

Spatially distinct domains of expression of *HoxA9* and *HoxA13* become established at later stages of regeneration (beginning at medium bud) as a consequence of blastema cell proliferation. During growth of the blastema, *HoxA13* expression is confined to a distal subset of the *HoxA9* expressing cells. During later stages of regeneration, *HoxA13* expression is correlated with regeneration of the hand, and *HoxA9* with regeneration of the distal humerus, lower arm and hand (Fig. 3). Hence, expression of the *HoxA* genes differs dramatically between regenerating and developing limbs at the early stages, which is when the two processes are most different (Bryant and Gardiner, 1992; Muneoka and Sassoon, 1992). In contrast, expression is similar during the blastemal stages of regeneration when the blastema and limb bud are comparable in appearance and function.

The function of *HoxA* genes in the regeneration cascade

One conceivable interpretation of the results described above is that there is a general gene activation step early in the regeneration cascade. Although only a few genes have been studied thus far, it is already clear that this is not the case. Preliminary data indicate that members of the Msx-class (Carlson, Gardiner and Bryant, unpublished), and the Dlx-class (Mullen, Bryant and Gardiner, unpublished) of homeobox genes are reexpressed or upregulated at later stages of regeneration than are the *HoxA* genes. Msx genes are implicated in maintaining cells in an undifferentiated and proliferative state (Song *et al.*, 1992), and Dlx genes are possibly related to the function of the epidermis in supporting limb outgrowth (Dollé *et al.*, 1992). Both of these activities are necessary for growth of the blastema after it has formed. In contrast, *HoxA* genes are reexpressed at the beginning of the hierarchy of regeneration genes, prior to or coincident with dedifferentiation, and prior to blastema formation.

The early expression of *HoxA* genes makes them candidate molecules for having a functional role in the process referred to as "dedifferentiation," in which cells of the mature limb give rise to blastema cells. In spite of the obvious importance of this process, little, other than basic phenomenology, is known about it, and it is essentially a black box with respect to molecular mechanisms. Among the changes necessary for mature limb cells to become pattern-formation competent blastema cells is the reexpression of molecules encoding positional identity. *Hox* genes are obvious candidates for positional identity genes because of their expression patterns and the effects on limb pattern of altering their expression experimentally (discussed earlier). Given that *HoxA* genes are implicated in the specification of the proximal-distal axis and that *HoxA9* and *HoxA13* are co-expressed in the most distal part of the limb pattern (hand/foot), their synchronous reexpression in mesenchyme at all amputation levels indicates that the distal-most positional identity is regenerated first. This finding raises the issue of the proximal-distal sequence of pattern specification in regenerating limbs, which is discussed further below.

We do not know yet about the relationship between *HoxA* reexpression and either the wound epidermis or nerves, both of which are required for regeneration. Based on the observation that expression of *HoxD13* (the paralog of *HoxA13*) in developing chick limbs is dependent on the AER (Hayamizu *et al.*, 1994), we suggest that a similar relationship exists between distally expressed *HoxA* genes and the wound epidermis of blastemas. It is also possible that the initiation of expression of *HoxA* genes is dependent on the wound epidermis. The timing of onset of expression is consistent with such a relationship since the limb stump is covered by the epidermis within 8-12 hours after amputation, and *HoxA* genes are reexpressed 12-24 h later. As in developing chick limbs, the function of the permissive epidermis is likely mediated by FGF2/*FGF4* (Fallon *et al.*, 1994; Taylor *et al.*, 1994; Niswander *et al.*, 1993). Evidence indicating the importance of FGF in limb regeneration includes the demonstration that FGF affects blastema cell proliferation (Mescher and Loh, 1981), that FGF is present in both blastemas and the apical epidermis (Boilly *et al.*, 1991), and that FGF receptors are expressed in blastemas (Poulin *et al.*, 1993). Future investigations of the relationship between *HoxA* genes and FGF will no doubt provide important insights as to the molecular mechanisms of dedifferentiation.

An additional, noteworthy characteristic of dedifferentiating limb cells is that they secrete large quantities of matrix-degrading enzymes (Yang and Bryant, 1994), a property they share with invasive cells such as cancer cells and trophoblast cells. In the case of dedifferentiating limb cells, these enzymes allow for the preblastema cells to escape from the mature ECM and begin their migration into the blastema (Gardiner *et al.*, 1986). During this process, matrix-bound growth factors would be released and thus could function in initiating the regeneration cascade. Although little is known at present about the expression and role of growth factors in regeneration, they do function in the control of growth and differentiation in other developing systems, and likely have a similar, conserved function in regeneration.

As mentioned earlier, numerous other genes that have important functional roles in limb development are also now known to be expressed during regeneration, although little is known about their regulation at present. These include homologs of several *HoxD* genes (Torok, Gardiner and Bryant, unpublished), *HoxC* genes (Komine, Gardiner and Bryant, in preparation) and *wnt* genes (Gardiner and Seguin, unpublished). Given the high degree of conservation of sequence and function of these genes, and the conserved developmental pathways within which they function, it is likely that many of the functionally important regeneration genes will be identified and characterized in the near future.

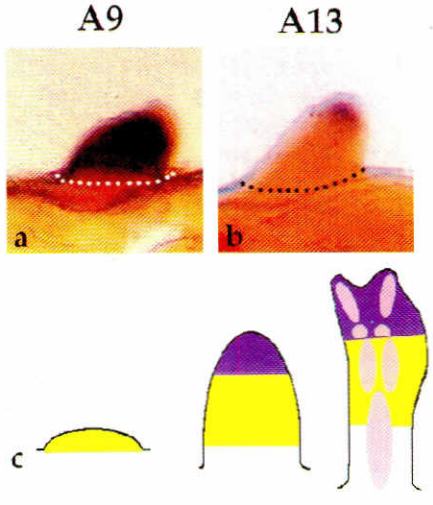

Fig. 2. Expression of *HoxA9* and *HoxA13* genes during forelimb development in the axolotl. (a) *Expression of HoxA9.* **(b)** *Expression of HoxA13. Anterior is to the left and the dotted line demarks the base of the limb bud.* **(c)** *Diagram summarizing the relative patterns of expression in early, medium and late stages of limb bud development. Regions expressing only HoxA9 are indicated in yellow. Regions expressing both HoxA9 and HoxA13 are indicated in purple. The pattern of differentiating skeletal elements is indicated in pink. The middle stage limb bud is comparable to the limb buds in* **(a and b)**.

Hox genes and retinoic acid

The effects of retinoids on developing and regenerating limbs has been well characterized at the level of cell biology, whereas effects on gene expression are less well understood, especially for regenerating limbs. Retinoids cause a distal blastema to regenerate as if it had been transformed to a proximal blastema, leading to the formation of duplicated pattern along the proximal-distal axis (Maden, 1982; Stocum, 1991). It is presumed that the changes in positional information are a consequence of retinoid-induced changes in gene expression, and especially in expression of genes such as the *HoxA* genes which appear to be involved in the specification of proximal-distal segment identity (Yokouchi *et al.*, 1991; Gardiner *et al.*, 1995).

We have been studying the effects of retinoids on *HoxA* gene expression in regenerating axolotl limbs (Gardiner *et al.*, 1995), and have found that while *HoxA9* expression in the blastema is not noticeably affected by retinoic acid (RA), *HoxA13* expression is strongly downregulated. Downregulation of *HoxA13* expression also occurs when regenerating axolotls are placed in a retinol palmitate solution, a treatment that also induces proximal-distal pattern duplications. The finding that proximalization of the blastema by retinoids is associated with the downregulation of *HoxA13* expression, but not of *HoxA9* expression, is consistent with the idea that the *Hox* code of the treated cells is changed to that of a more proximal limb segment. However, because blastemas at all limb levels *initially* express a distal *Hox* code, a retinoid-treated blastema, with a proximal *Hox* code, is not the same as a blastema arising at a proximal limb level. The steps leading from a retinoid-proximalized blastema to the final duplicated limb pattern have not yet been studied. *Despite this gap in our knowledge, the coincidence between altered HoxA13 expression and altered pattern provides further evidence of the importance of HoxA genes in limb pattern formation.*

The response of *HoxA9* and *HoxA13* to retinoid treatment of regenerating limbs *in vivo* is comparable to that of teratocarcinoma cells *in vitro* (Simeone *et al.*, 1991). Genes at the 3' end of the *Hox* complexes are activated by RA, genes located in the middle of the complex do not react strongly to RA, and genes at the 5' end are either not affected, are inhibited, or are strongly downregulated. In blastemas *HoxA9*, which is in the middle of the complex, is relatively unresponsive to retinoids, whereas *HoxA13*, at the 5' most end of the complex, is inhibited. Another 5' *Hox* gene, *HoxD13*, is also inhibited by RA *in vivo* during chick limb development (Hayamizu and Bryant, 1994). The coordinated upregulation of 3' *Hox* genes and downregulation of 5' *Hox* genes would cause positional identity to be shifted to a more rostral position along the rostro-caudal axis, and to a more proximal position along the limb axis.

These results argue against a role for endogenous retinoids in pattern formation during either limb development or limb regeneration. Since the most 5' *Hox* genes are involved in distal limb pattern formation and expression of these genes is inhibited by retinoids, it appears that retinoids are actually antagonistic to pattern formation in the distal regions of limbs. Since these genes are expressed at the distal tip of the limb bud where pattern formation occurs, it is unlikely that endogenous retinoids can be controlling gene expression in this region. Furthermore, both

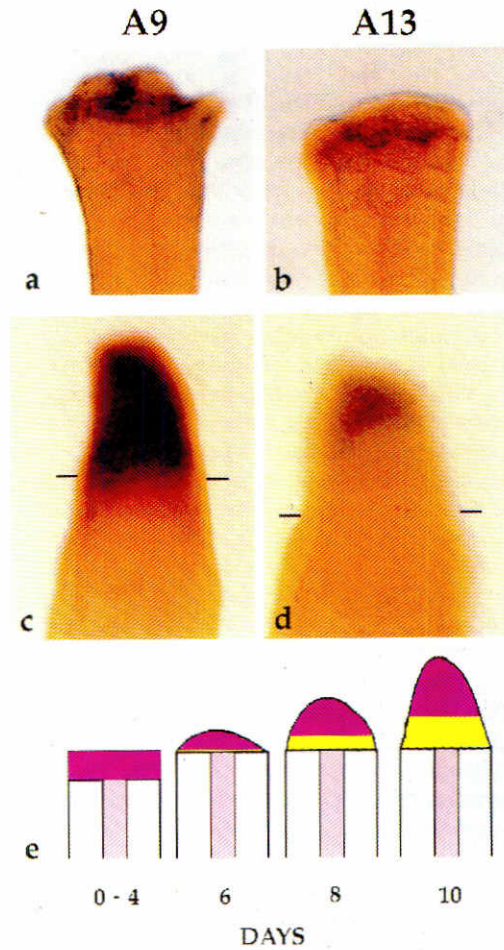

Fig. 3. Expression of *HoxA9* and *HoxA13* genes during forelimb regeneration in the axolotl. (a and b) *Initiation of expression of HoxA9* **(a)** *and HoxA13* **(b)** *in stump tissues two days after amputation through the hand region.* **(c and d)** *. Expression of HoxA9* **(c)** *and HoxA13* **(d)** *in late bud blastemas resulting from amputation through the middle of the upper arm. Amputation plane is indicated by hash marks.* **(e)** *Diagram summarizing the relative patterns of expression during the first ten days of regeneration from an amputation through the middle of the upper arm. Regions expressing only HoxA9 are indicated in yellow. Regions expressing both HoxA9 and HoxA13 are indicated in purple. The humerus is indicated in pink. At later stages when skeletal elements differentiate, the relative expression patterns are as illustrated in late stage limb bud in Figure 2c.*

introduced and endogenous reporter genes containing RA response elements fail to report the presence of RA in the distal limb bud (Mendelsohn *et al.*, 1991; Noji *et al.*, 1991; Rossant *et al.*, 1991; Hayamizu and Bryant, 1994). It is likely, as suggested previously (Bryant and Gardiner, 1992), that retinoids are involved in pattern formation at the limb base, where they could function in the initiation of outgrowth of the developing limb. Such a function would be consistent with the observation that expression of more 3' *Hox* genes, associated with proximal positional identity, is induced by RA and with recent data suggesting that *shh* expression is induced by RA, and is induced endogenously when the limb bud begins to grow out (Niswander *et al.*, 1994). A role for retinoic acid in the initiation of regeneration seems unlikely since

HoxA13, which is inhibited by RA, is expressed in cells of the stump at all proximal-distal levels when regeneration is initiated.

A possible role for retinoids in regeneration could involve the changes that occur in the wound epidermis, rendering it permissive for outgrowth. Evidence in support of this idea includes the observation that epidermal cells generally are dependent on retinoids for normal functioning, that one of the early effects of RA beads in chick limb buds is the induction of *FGF4* expression in the epidermis (Niswander *et al.*, 1994), and that expression of an antigen characteristic of the wound epidermis in newts is induced by RA (Tassava, 1992). Since there is no evidence that RA *in vivo* leaves the cells in which it is synthesized in order to travel to a new location, we propose that if RA is involved in regulating differentiation of the wound epidermis during regeneration, it functions within the cells that synthesize it.

Insights and conclusions from studies of limb regeneration

Studies in developing and regenerating axolotl limbs have revealed that the regulation of *Hox* gene expression is more complex than previously had been appreciated. During embryogenesis and limb development, *Hox* genes are activated in a strictly colinear sequence, both in space and time, with 3' genes activated earlier with a more anterior (or proximal) border than each successive 5' gene. This apparently universal colinearity of expression with gene order on the chromosome has been considered not only an essential aspect of *Hox* gene function, but also a manifestation of the way that *Hox* gene expression is controlled (see Duboule, 1994a). *The regenerating axolotl limb demonstrates that in addition Hox gene expression can be regulated by a non-colinear mechanism, and, therefore, colinear Hox expression is neither universal nor essential for function in all situations.*

Although *HoxA* reexpresssion in regeneration initially is neither spatially nor temporally colinear, it eventually becomes spatially colinear as a consequence of growth of the blastema. Thus the final, spatial colinear expression pattern is conserved even though the mechanism by which it is achieved is not. In both developing and regenerating limbs, the future hand/foot region expresses the most 5' *Hox* gene, as well as more 3' genes, whereas more proximal segments are characterized by expression of 3' genes and the absence of expression of more 5' genes. This conserved spatial colinearity further supports the idea that *Hox* genes, and in particular combinations of *Hox* genes both within and between the *Hox* complexes, form a code that specifies segmental identity (Kessel and Gruss, 1991).

From the pattern of activation of *HoxA* genes in regeneration, we have learned that, during dedifferentiation, stump cells do not simply reexpress the *HoxA* genes that they expressed prior to differentiation. During development, upper arm, lower arm and hand cells express different combinations of *HoxA* genes, and only hand cells express both *HoxA9* and *HoxA13*. When they dedifferentiate at the amputation plane, the cells express both *HoxA9* and *HoxA13* regardless of their origin along the proximal-distal axis. Thus the initial events in regeneration are the same at all limb levels, regardless of the different pattern of structures that eventually will be regenerated from each level.

The observation that dedifferentiating limb cells initially express the most distal limb *Hox* code leads us to conclude that the distal part of the pattern is specified first in regeneration. This conclusion is contrary to the prevailing view that the limb pattern is specified in a proximal-to-distal sequence during both development and regeneration, although there was an earlier time when alternative views were actively debated (see Wallace, 1981 for discussion). Evidence supporting the proximal-to-distal view includes the proximal-to-distal sequence of differentiation, the existence of a distal growth zone, and the lack of distal structures when outgrowth is terminated by removal of the AER/wound epidermis. None of these pieces of evidence tests directly the specification of different regions of the early outgrowth. The most compelling of the three pieces of evidence has been the AER/wound epidermis experiments; however, the strength of this evidence is questioned by the finding that expression of some progress zone genes, including 5' *Hox* genes, is inhibited by removal of the AER (Ros *et al.*, 1992; Hayamizu *et al.*, 1994). Inhibition of 5' *Hox* genes specifying distal positional identities is equivalent to amputation of distal values, and thus one cannot conclude whether or not those distal values had already been specified at the time of AER removal. The long and uncontroversial history of this topic notwithstanding, experimental data regarding the expression of genes that specify positional identity will provide direct evidence related to the issue of pattern specification.

The distal-first sequence for pattern specification aligns vertebrate limb regeneration with regeneration in many different groups of animals (Slack, 1980). During hydra regeneration, the head and foot regions form first and the middle regions develop later. Planaria regenerate the eye and brain first, regardless of the level of amputation, and the pharynx later. Annelids regenerate the end segments before the middle segments, and hemimetabolous insects regenerate the distal claws before more proximal leg segments. Finally mouse embryo limb buds regenerate structurally complete toe pads and distal segments although more proximal segments are incomplete (Wanek *et al.*, 1989).

Another aspect of pattern formation that can best be appreciated from studies of regenerating limbs is that *while Hox codes may specify segmental identity, the mechanism of specification of proximal-distal positional information within a segment is still a mystery*. There is no doubt that such a mechanism exists because when urodele limbs are amputated, they regenerate a perfect replacement for the piece removed, whether the amputation occurs between segments or within a segment. This issue raises questions about the lineage relationship of cells within different *Hox* domains of the early limb bud or the blastema, and how the different expression domains reach their appropriate dimensions if growth is primarily apical as has been proposed (see Wallace, 1981). Studies correlating details of cell lineage, patterns of growth and patterns of gene expression are needed to begin addressing these issues.

Aside from their functional roles in regeneration, the *HoxA* genes allow us to experimentally access the regeneration cascade very near its beginning. It is possible to investigate upstream regulatory events by using activation of *HoxA* expression as an experimental assay. In so doing, it will be possible to identify and experimentally test the role of candidate molecules involved in the initiation of regeneration and in the role of nerves and the wound epidermis in this process. In addition, experimental manipulations of *HoxA* expression will lead to further understanding the downstream events of regeneration such as dedifferentiation, cell migration and proliferation.

Studies of homeobox genes also will further our knowledge about pattern formation and the re-establishment of the proximal-distal pattern during outgrowth of the blastema. As has so often proven to be the case, comparative studies are necessary to observe the full range of developmental potential; investigating only one "ideal model" system is self-limiting if that system is derived, specialized or developmentally restricted. Urodeles have long demonstrated the full potential of vertebrate limbs, not only for regeneration of mature limbs, but also for pattern regulation during development, unlike other vertebrates such as chicks, in which the regulative ability of the limbs is restricted to only a limited subset of responses. In the case of molecular mechanisms, urodeles again are providing a more expansive view of both *Hox* gene regulation (the existence of a non-colinear mechanism for *Hox* gene activation) and pattern formation (the occurrence of a distal-first mechanism of pattern specification in vertebrate limbs). Future studies of the molecular mechanisms in the control of regeneration will contribute to further expanding our views of many important developmental processes.

We reemphasize the importance of urodeles in that they explicitly demonstrate to us that vertebrate limbs can regenerate. Results from earlier, embryological studies have emphasized the similarity of mechanisms controlling limb development and regeneration (see Bryant and Gardiner, 1992). Likewise, our recent molecular studies have demonstrated that once a blastema is formed, the same *Hox* genes are expressed and are regulated in the same manner as when the limb first developed. The implication of these results is that if a limb develops then it has the potential to regenerate. The corollary is that "non-regenerating" limbs do not regenerate because they arrest at one or more stages in the regeneration cascade; for example, because amputated chick limb buds do not reform an AER, they arrest at an AER-dependent stage in regeneration. Studies of the activation of *Hox* genes and their role in the control of dedifferentiation will certainly aid in the identification of the stages of regenerative arrest. By studying the molecular mechanisms controlling these stages, we will systematically advance toward our ultimate goal of human limb regeneration.

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