Isolation of potential vertebrate limb-identity genes

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Summary

Forelimbs and hindlimbs of tetrapods have different morphological patterns. One plausible explanation for the difference is that the cells that give rise to the limbs differentially express genes which control their pattern of development. Amphibian limb regeneration is an excellent system to test this hypothesis, since the same ultimate morphology is attained in regeneration as through embryogenesis. Using a combination of homeobox probes and differential screening, I have isolated two newt genes which are differentially expressed in regenerating forelimbs and hindlimbs. One of these genes displays properties expected of a gene involved in controlling limb morphology, including expression in mesodermal tissue and constancy of expression upon transplantation. Based on sequence analysis, this gene appears to be homologous to a homeobox-containing gene previously isolated from frog and human libraries.

Key words: limb regeneration, pattern formation, homeobox.

Introduction

When an adult newt (Notophthalmus viridescens) loses a limb, it can regenerate a virtually perfect replacement from the remaining stump (Chalkey, 1954). Non-limb cells can also give rise to a new limb. For example, if a newt's sciatic nerve is deviated from its normal location in the hindlimb to a nearby region of the flank, flank cells are stimulated to produce an extra ('ectopic') hindlimb (Guyenot, 1927). If the same nerve is brought to certain other parts of the flank, different structures can result: forelimbs, tails, or dorsal crests (Guyenot & Schotte, 1926; Guyenot et al. 1948; Kiortsis, 1953). Thus, one region will give rise to forelimbs, another to hindlimbs, and still another to tails irrespective of the origin of the deviated nerve. The ectopic limbs are not attached to the normal underlying structures, such as the pelvic or shoulder girdles. The morphological form of an ectopically grown limb, therefore, is not directed by the structures on which it is built, but rather is determined by some property of the 'territory' of the flank where it develops.

The existence of specific limb territories each consisting of cells with determined developmental potential is reminiscent of the cell lineage compartments in Drosophila development that give rise to different body segments (García-Bellido, Ripoll and Morata, 1973; Crick and Lawrence, 1975; Struhl, 1981). Many of the genes whose expression is thought to delineate the Drosophila compartments share a characteristic DNA sequence, the homeobox (Scott & Carroll, 1987; Gehring, 1987). The homeobox motif has also been detected in vertebrate DNA (McGinnis, 1984). I wondered if, as in Drosophila, there might be specific genes whose differential expression either mark the boundaries of the newt limb territories or control the morphology of the structures within a territory; and if a subset of those genes might carry homeobox sequences.

Experimental procedures

Isolation of clones

Clones were isolated from a λgt10 forelimb blastema (regenerating tissue) cDNA library provided by Jeremy Brockes. Four Drosophila homeobox probes, from the ftz, ubx, ant, and scr loci, were provided by Gary Struhl. These were radioactively labeled by randomly primed DNA polymerization (Feinberg & Vogelstein, 1984), and used to screen the library. The hybridization and wash were carried out at low stringency: 43% formamide, 5 x SSCE, 5 x Denhardt's solution, 37 degree C hybridization; 2 x SSCE, 0-1 % SDS wash. The cDNA probes were made from RNA prepared from either regenerating forelimbs or hindlimbs (blastemas) and copied into αP-labeled, single stranded cDNA with a specific activity of 4x10⁸ cts min⁻¹ µg⁻¹ (Rebagliati et al. 1985). After a week's exposure plaques hybridizing with the forelimb-specific, but not the hindlimb-specific probe were identified and plaque purified.
DNA sequencing

The EcoRI insert of the FH-2 cDNA clone was digested with a number of restriction enzymes and the resulting fragments were subcloned into the M13 sequencing vectors mpl8 and mpl9. They were sequenced by the chain termination technique (Sanger et al. 1977).

Preparation and analysis of RNA

To prepare each sample, dissected tissue was immediately frozen in liquid nitrogen. To obtain 'denuded blastema' RNA, tissue was treated with 0.2% EDTA in Ca²⁺-free PBS for one hour to loosen the epidermis. The epidermis was then manually removed with number 5 fine point forceps, and the remaining mesenchymal blastema was frozen. RNA was purified from frozen tissue in guanidium thiocyanate (Chirgwin et al. 1979). Poly(A)⁺ RNA was selected by oligo-dT chromatography (Aviv & Leder, 1972). 10µg of each sample (except for Fig. 6, where 3-3 µg was used) was subjected to electrophoresis through a 1-2% agarose-formaldehyde gel, and bioted to Genescreen (Lehrach et al. 1977). Filters were then hybridized with probes prepared by randomly primed polymerization (Feinberg & Vogelstein, 1984). FH-1 and FH-2 probes were hybridized and washed at high stringency, and the Xenopus EF-1α probe was hybridized and washed at reduced stringency using conditions identical to those described above for screening the library. For all normalizations of RNA abundance reported in this study, autoradiographs were scanned on a densitometer; major peaks were manually cut from the graph paper, and the peaks were weighed to determine relative quantities.

Animal care, surgery, and injection

For amputation, newts (Notophthalmus viridescens from Charles Sullivan Inc.) were anesthetized with 0.1% Tricaine (Sigma). Limbs were cut with scissors, the bone trimmed back and then excess skin removed under a microscope. 0.5% Sulfamerazine (Sigma) was used postoperatively for 24h to reduce infection. Transplants were performed with midbud stage blastemas. Each operated animal was placed on its chest on damp kimwipes, with its limbs extended so that the amputated surfaces did not contact any surface. Forelimb blastemas were transplanted to hindlimb stumps on the same animal (to avoid possible immune reactions) and on the same side and dorsal-ventral orientation (to avoid possible induction of supernumerary limbs). After transplantation, animals were kept in individual crystalizing dishes. After other procedures, newts were kept in communal tanks. Retinoic acid (Sigma) was dissolved in DMSO at a concentration of 10 mM. 30 µl were injected intraperitoneally at the seventh day after amputation.

Results

Isolation of differentially expressed cDNA clones

Acting on the assumption that the limb territories might be marked by homeobox-containing genes, I screened a Newt regenerating- forelimb cDNA library (provided by Jeremy Brookes) with a mixture of homeobox probes: ftz, ubx, ant and scr (provided by Gary Struhl). Approximately 50 plaques were picked and replated. The secondary plaques were screened in duplicate with radioactive cDNA probes (Rebagliati et al. 1985) prepared either with regenerating forelimb or hindlimb RNA. Several clones were identified that hybridized to the forelimb-derived but not the hindlimb-derived probe.

Two of these, FH-1 and FH-2 (for Forelimb-Homebox) were subcloned into plasmids for further characterization. They have distinct restriction maps (Fig. 1). In Southern blots the two clones show no evidence of cross hybridization outside of the restriction fragments which were identified as containing the homeobox related sequences (data not shown).

Expression of FH-1 and FH-2 in the limb territories

Non-homeobox containing subfragments were used to probe RNA made from various surgically dissected regions of the newt (Fig. 2). Both FH-1 and FH-2 probes detect messages that are approximately 1.8 kb long. FH-1 transcripts are detected in the forelimb territory (including normal and regenerating forelimbs and the surrounding flank tissue). The FH-1 message is also present further posterior along the flank. It is not detected at all in the hindlimb territory (at least 16 fold lower level than in the forelimb region by densitometer tracing). The FH-2 message is found in all of the tissues tested. However, it is present at an approximately 4 fold higher level in tissues of the forelimb territory (forelimb, regenerating forelimb, and surrounding flank) than in RNA from more posterior regions (middle of the flank, hindlimb, regenerating hindlimb, and flank near the hindlimb). The amount of RNA loaded in each

Fig. 1. Restriction maps of FH-1 and FH-2. Regions shown by southern blot analysis to hybridize with a mixed Drosophila homeobox probe are indicated by an open line. Regions isolated and used as probes in Northern analysis are indicated by a heavy line.
Fig. 2. Expression of FH-1 and FH-2 in various regions of the newt. Northern analysis was done with poly(A)^+ mRNA from (A) whole forelimbs removed at the mid upper arm, (B) early-bud stage forelimb blastemas (regenerating cells) resulting from amputations through the middle of the humerus 15-18 days earlier, (C) flank tissue (including skin, connective tissue and muscle wall) just posterior to the forelimb, (D) flank tissue midway between the forelimb and the hindlimb, (E) whole hindlimbs removed at mid thigh, (F) early-bud stage hindlimb blastemas resulting from amputations through the femur 15-18 days earlier, and (G) flank tissue just posterior to the hindlimb. Filters were hybridized with 3^P-labeled transcripts were determined by comparison to RNA size markers purchased from BRL.

lane was normalized by reference to transcripts detected rehybridizing the filter with a probe (provided by Doug Melton) which would be expected to be equally expressed in cells of a given cell type from any region of the animal, EF1-α (Krieg & Melton, 1989).

Tissue specificity of FH-1 and FH-2 expression
Both FH-1 and FH-2 are thus differentially expressed in the forelimb and hindlimb, consistent with their having a role in controlling morphogenesis. To play such a role they would have to be expressed in the cell types that are involved in the determination of pattern during regeneration.

Amphibian limb regeneration does not take place by direct regrowth of stump tissues. Rather, a mass of undifferentiated cells, called blastema cells, first accumulates under the wound epidermis at the tip of the stump (Fitsch, 1911). These cells are of mesodermal origin, arising by morphological dedifferentiation of skeletal, myogenic and connective tissue cells of the stump (DiGiorgi, 1924). The blastema cells proliferate and then redifferentiate to form the structures of the regenerating limb. It has been shown by transplantation experiments that the information for producing limb pattern resides in the mesodermally derived 'blastema' cells rather than in the wound epidermis that covers them (Stocum & Dearlove, 1972). It was therefore important to ascertain whether FH-1 and FH-2 are expressed in the mesenchymal blastema cells and in the mesodermal tissue in the territory of the flank which can give rise to forelimbs.

It is possible to separate ('denude') the blastema cells from the wound epidermis by EDTA treatment followed by manual dissection (Stocum & Dearlove, 1972). Denuded forelimb blastema RNA was prepared and compared with total (non-denuded) forelimb blastema RNA by hybridizing to various probes (Fig. 3A). In addition RNA was prepared from mesodermal tissue dissected from the flank near the forelimb and similarly analyzed (Fig. 3B). Some mesodermal tissue directly under the skin was not included since it could not be cleanly separated from the epidermis. FH-1 hybridization is detected to the total blastema RNA, but not to denuded blastema RNA. This would imply that this gene is expressed in the wound epidermis, but not mesenchymal blastema tissue. FH-1 transcripts are also not detected in the mesoderm of the flank. Thus FH-1 is not expressed in the correct tissue type for it to be involved in controlling limb identity. This gene was therefore not characterized further. FH-2 hybridization, however, is observed to both denuded and total blastema RNAs. FH-2 is thus expressed in the mesenchymal blastema cells. In addition, FH-2 hybridization is detected RNA purified from mesodermal tissue at the base of the forelimb. This is consistent with FH-2 having a role in marking the limb territories.

Sequence of the FH-2 cDNA
The screen used in the isolation of the FH-2 clone was designed to identify any homeobox-containing gene which was differentially expressed in the forelimb territory. Having established that FH-2 is expressed in such a manner, I sequenced the clone to verify that it does, indeed, have a homeobox. The nucleotide sequence is shown (Fig. 4). Amino acid comparison (Fig. 5) shows extensive homology outside as well as within the homeobox which would indicate that FH-2 is the newt homologue of the frog homeobox-containing gene XHbox-1 (Cho et al. 1988) and the human gene c8 (Simeone et al. 1987).

Expression of FH-2 in limbs regenerated in heterologous territories
A regenerating blastema is autonomously self-organizing; in the sense that when transplanted to a heterologous location where there is no underlying limb stump to direct it, the blastema nonetheless regenerates a limb characteristic of its origin (Stocum, 1968b). This autonomous patterning is maintained even when the blastema is grafted onto a foreign limb territory. Thus, a forelimb-blastema transplanted to a freshly ampu-
tated hindlimb-stump on the same animal was reported to efficiently give rise to a regenerate of forelimb morphology (Stocum & Melton, 1977). Consequentially, if the expression level of FH-2 is involved in marking the forelimb and hindlimb territories it must also be unaffected by transplantation.

Animals were amputated near the shoulder. When blastemas were of transplantable size (mid-bud stage) they were transplanted to freshly amputated hindlimbs near the ankle of the same animal. This site on the hindlimb was chosen because the ankle amputation itself would only be able to give rise to the distal foot; and therefore any regenerates forming a full limb would have to be descended from the upper arm blastema.

In every case the transplanted blastema appeared to be at least partially reabsorbed before regeneration commenced. In general this resulted in the regeneration of only lower limb elements which could not, therefore, be verified as being of forelimb origin. However, out of two hundred transplants (from one hundred bilaterally amputated animals) 11 formed proximal-distally complete regenerates. The reason for the relatively high level of reabsorbtion as opposed to the results of others (Stocum & Melton, 1977) is not clear, but may have to do with differences in the age and size of the animals since adult transplants don’t revascularize as fast as in larva.

The 11 transplanted forelimb regenerates were then reamputated, and the reamputated transplants were allowed to develop through redifferentiation and morphogenesis before they were harvested. Ultimately 3.3 μg of poly(A)+ transplant-RNA was obtained. Non-transplanted hindlimbs and forelimbs were also amputated and allowed to develop through morphogenesis, and RNA was prepared from them for comparison. The transplanted and control RNAs were then hybridized to the FH-2 probe (Fig. 6). The abundance of FH-2 RNA

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**Fig. 3.** Expression of FH-1 and FH-2 in the mesodermal cells of regenerating blastema and flank tissue. A. Northern analysis was done on poly(A)+ mRNA from total forelimb blastemas (epidermis and mesenchyme) and denuded forelimb blastemas (mesenchyme only). The same filter was probed with the three probes FH-1, FH-2 and EF1-α. B. Northern analysis was done on poly(A)+ mRNA from mesodermal tissue dissected from the flank at the base of the forelimb. The same probes were used as in A.

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**Fig. 4.** Nucleotide sequence of FH-2. The homeobox sequence is shown in bold print. Coding region is printed in large case and in triplets. Non-coding 3′ untranslated sequence is shown in lower case.
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**Fig. 5.** Predicted amino acid sequence of FH-2. Partial amino acid sequence of two homologous genes are also shown. Ac-1 is a *Xenopus laevis* gene and c8 is a human gene. Stars (*) represent amino acids which are identical to those of FH-2. Percentage of amino acid identity between each pair of sequences are shown below the sequence.

The expression of FH-2 along the proximal-distal axis of the regenerating limb was examined by comparing RNA isolated from midbud stage blastemas derived from amputations near the shoulder and amputations midway between the elbow and the wrist (Fig. 8). A reproducibly three-fold higher level was observed in the RNA of proximal blastemas. This raised the possibility that FH-2 might be involved in specifying proximal-distal positional information to a regenerating limb as well as, or instead of, limb identity. That possibility could be tested, because exogenously added retinoic acid (RA) can alter the anterior-posterior position of a regenerating limb (Niazi & Saxena, 1978). In the presence of RA a distal blastema will give rise to limb structures that would normally be regenerated from a more proximal amputation site (an effect termed 'proximalization'). If FH-2 acts to specify proximal-distal position, its expression would, therefore, be expected to be altered by RA treatment; in particular it should be increased to a level appropriate for a more proximal position.

**Discussion**

**Control of limb territories**

The regenerative territories of a newt are operationally defined as regions which can be experimentally induced to produce a specific appendage. I isolated two genes, FH-1 and FH-2, which are differentially expressed between the forelimb and hindlimb territories. Thus, the patterns of expression of both FH-1 and FH-2 are consistent with their having roles in demarkation of cells of the newt limb territories. At minimum the expression of FH-1 and FH-2 are not sufficient to instruct a region of the newt to form a limb, as they are expressed at a low level in the middle of the flank, which can not be induced to form ectopic limbs. The fact that transcripts from both of these genes
are found at a reduced level in the inert territory is reminiscent of genes in other systems known to define developmental compartments that are expressed to different extents in adjacent territories (for example the *Drosophila* homeotic gene Ubx is expressed in a graded fashion across several compartments; White & Wilcox, 1984; Beachy et al. 1985). *Drosophila* homeotic genes are thought to act in a combinatorial fashion (Lewis, 1978) with complex regulatory and phenotypic interactions (Kaufman & Abbot, 1984; Struhl & White, 1985) in defining segmental compartments. It would seem likely that this would be true for the genes defining vertebrate limb territories as well, and that a number of other genes will ultimately be found to be part of territorial control.

To identify other genes potentially involved in the control of limb pattern, I have screened a regenerating-hindlimb cDNA library in a manner analogous to that reported here and have tentatively identified one clone with a hindlimb-biased expression pattern (Tabin, unpublished observation). Other control genes might not contain homeoboxes; a more general differential screen will be attempted in the hopes of uncovering them. Many control genes may be involved in specifying limb territories.

FH-1 is not expressed in the mesenchymal cells which are known to determine whether a blastema will form a hindlimb or a forelimb. FH-1 might be involved in delineating epidermal cells of the forelimb compartment, and in determining the presence or absence of territory-specific epidermal tissues such as the nuptial excrescences on the male hindlimbs. However, as FH-1 can not be involved in specifying the overall morphology of a regenerating structure, its function was not addressed further in these studies. FH-2, on the other hand, is expressed in the correct cell type as well as a differential pattern that would be expected of a gene involved in the specification of a limb territory. These results are consistent with the pattern of expression found for the homologous gene in mouse and frog embryonic limb buds where the expression is in both the mesenchyme and epidermis of the forelimb but only the epidermis of the hindlimb (Oliver et al. 1988). However, to determine whether the limits of FH-2 expression are truly congruent with the forelimb territory will require both exactly determining the extent of the territory in the newt and examining FH-2 expression at finer resolution by *in situ* hybridization or immunohistochemistry.

**Homeobox homology**

The scheme used to isolate FH-2 utilized a preliminary screen for clones containing homeobox-hybridizing sequences. This was done in the hope of increasing the likelihood of finding a developmentally significant, differentially expressed gene. Sequence analysis showed that, as expected, the FH-2 gene contains a homeobox. Others have isolated vertebrate homeobox-containing sequences in the faith that they might be tags for developmentally significant genes (McGinnis et al. 1984; Burglin, 1988). The isolation of such a gene in a particular developmental context adds credence to such an approach. In addition, the finding that FH-2 is the same gene as c8 and XHbox-1 implies that the function of FH-2 has been conserved in distantly related species, as would be expected for a gene playing as basic a role as controlling limb territory identity.

The sequence also revealed that FH-2 is identical to NvHbox-1 isolated in a similar study of newt limb regeneration by Jeremy Brockes and colleagues (Savard et al. 1988). The FH-2 clone is identical to their p158 clone, except for a 161 bp deletion in the 3' untranslated region of the FH-2 clone. Their analysis of the structure of the gene shows that there are at least two different FH-2 splicing patterns. These are regionally expressed; one being specific to the tail regenerative territory, a region not investigated here. For the splicing pattern of FH-2 to be altered in a particular territory requires the action of a second gene product in that territory, an example of the combinatorial action of developmental control genes discussed above.

**Transplantation invariance**

While nerve deviation experiments defined the territories that have the capacity to give rise to limbs, it is transplantation experiments that established the fact that the information specifying the character of the regenerate is inherited from the progenitor cells,
regardless of the territorial context to which they are moved (Michael & Farber, 1961; Stocum, 1984). Thus, any gene which is responsible for providing regional positional information in regeneration might be expected to be similarly expressed in regenerating cells and parental limb cells of the same territory. Both FH-2 and FH-1 fulfill this prediction. Moreover, the expression of genes which control limb territories cannot be altered by tissue transplantation since the identity of the limbs is not affected. This was tested by transplanting forelimb blastemas to the hindlimb territory, and the level of FH-2 was not down-regulated.

This experiment also is a molecular confirmation of the self-organizing ability of the blastema using FH-2 expression as a marker. A rigorous demonstration of this would require further transplantation experiments.

Retinoic acid

When a blastema is transplanted it retains proximal-distal positional information in addition to information about its regenerative territory. One might expect that different genes would be involved in specifying the two types of information, since the proximal-distal pattern is a continuous function while the different territories are discrete. Whether this proves to be true in general or not, FH-2 does not appear to be involved in specifying the proximal-distal position of a blastema, since its expression is unaffected by retinoic acid.

Conclusion

We expect the only difference between hindlimbs and forelimbs to be one of pattern, not of cell types. Thus, the finding of a difference in gene expression in the two limbs is strongly suggestive of that gene playing a role in controlling limb morphology. By every criterion tested here, the pattern of expression of FH-2 is consistent with it playing an important role in limb identity. This represents the first molecular inroad into the old question of what determines the difference between the morphologies of an arm and a leg. It also adds further support to the idea that the homeobox will be useful in providing a molecular handle on vertebrate pattern formation. Yet, as with all candidate pattern-control genes so far studied in vertebrates, definitive identification of the function of FH-2 must await direct manipulation of its activity in vivo. Cells from regenerating limbs can be grown in culture (Stocum, 1968b). Techniques are being developed that will allow efficient gene transfer into these cells, they can then be reimplanted into amputated limbs, allowing the function of these and other genes to be directly tested.

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References


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