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Evidence for dedifferentiation and metaplasia in amphibian limb regeneration from inheritance of DNA methylation

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Summary

Amphibian limb regeneration is a process in which it has been suggested that cells of one differentiated type may dedifferentiate and give rise to cells of another type in the regenerate. We have used two tissue-specific hypomethylations in the newt cardioskeletal myosin heavy chain gene as lineage markers to follow the fate of cells during limb regeneration. Analysis of genomic DNA from different muscle cell populations allowed the assignment of one marker to the muscle (Hypo A) lineage and the other, more tentatively, to the 'connective tissue' (Hypo B) component of muscle. The contribution to regenerated limb cartilage and limb blastemal tissue by cells carrying these markers was estimated by quantitative analysis of Southern blot hybridizations using DNA from regenerate tissues. The results are consistent with a contribution of cells from both muscle and connective tissue lineages to cartilage in regenerated limbs. In addition, removal of the humerus at the time of amputation (eliminating any contribution from pre-existing cartilage), has provided evidence for an increased representation of cells carrying the connective tissue marker in regenerate cartilage but did not affect the representation of cells carrying the muscle cell marker.

Key words: regeneration, blastema, metaplasia, *N. viridescens*, dedifferentiation, DNA methylation.

Introduction

Cellular differentiation is a fundamental process of biology, yet the molecular mechanisms underlying this process remain poorly understood. One basic uncertainty relates to the stability of the differentiated state. That is, to what extent a cell committed to, or expressing, one specialized phenotype can revert to an undifferentiated state (dedifferentiation) and thence give rise to cells of an alternative specialized type, a process referred to as metaplasia.

Evidence that differentiation is not, in principle, an irreversible process has come from the classic nuclear transplantation experiments of Gurdon (Gurdon & Uehlinger, 1966), who demonstrated that a complete organism could be regenerated from a single differentiated nucleus. A number of differentiated cell types have subsequently been shown to have pluripotent nuclei (DiBeradino *et al.* 1984). Evidence for cellular transdifferentiation includes Wolffian lens regeneration in amphibians (Yamada, 1982), the *in vitro* conversion of chick muscle into cartilage (Nathanson, 1986), and multipotency of muscle in the jellyfish (Schmid & Alder, 1984).

One biological process where it has been postulated that metaplastic transitions take place is that of amphibian limb regeneration (for reviews see Wallace, 1981; Sicard, 1986). The urodele (tailed) amphibians have the ability, unique amongst adult vertebrates, to regenerate amputated limbs. Following amputation, regeneration proceeds by wound healing and migration of the epidermis. A population of mesenchymatous, morphologically identical, undifferentiated cells then arises at the stump end, forming a structure known as a blastema. It is from the blastema that all the internal tissues of the regenerate subsequently develop (Wallace, 1981). Despite their apparent morphological homogeneity

(Salpeter & Singer, 1960; Hay, 1962), an underlying heterogeneity has been revealed, more recently, by the use of monoclonal antibodies that identify specific blastemal subpopulations (Kintner & Brockes, 1985; Tassava, 1986). It is of major significance, therefore, to determine not only the origins of the cells making up the blastema but also to ascertain their eventual fates.

The traditional approach to the problems of dedifferentiation and metaplasia in limb regeneration has been to follow the fates of individually marked cells when grafted or transplanted into an unmarked host background (Steen, 1968; Namenwirth, 1974; Muneoka *et al.* 1982). Common methods of marking cells have been by radioactive labelling or the use of nucleolar number to identify cells of transplanted triploid tissue. These techniques have some attendant disadvantages relating to the small numbers of cells that can be analysed, the often limited extent of regeneration and difficulties in scoring cells for the marker. Despite this, evidence from such experiments has suggested that cartilage (an essentially homogeneous tissue) displays a stable phenotype, only giving rise to cartilage in the regenerate. Conversely, with grafts of labelled muscle (Steen, 1968), graft-derived cells were observed in many tissues, including regenerate cartilage; it remains unresolved, however, whether these cells were derived by metaplasia of muscle cells proper or originated in the connective tissue component of muscle.

To circumvent these problems and to enable a quantitative assessment of the results, we have developed an alternative and complementary approach. This has been to identify molecular markers in the DNA and to use these to follow cell fates during limb regeneration.

Because hypomethylation at specific sites in DNA has been shown to be tissue-specific, stable, heritable and associated with, but not obligatorily linked to, gene expression (for review see Razin *et al.* 1984), it was felt that these would make good candidates for lineage markers. As we were concerned with the fate of muscle cells, we investigated the methylation status of restriction sites in the cardioskeletal myosin gene of the newt, *Notophthalmus viridescens* (Casimir *et al.* 1988). Two tissue-specific sites of hypomethylation, identified in the last intron of this gene, were found suitable for use as markers.

Quantitative analysis of Southern blot hybridizations of DNA from regenerated cartilage and from blastemal tissue have provided evidence for a contribution from cells of both the muscle and connective tissue lineages. The latter cell type also gave evidence of an increased recruitment into regenerated cartilage in animals from which pre-existing limb cartilage had been removed.

Materials and methods

Animals

Adult newts *Notophthalmus viridescens* were obtained from Xenopus Ltd Care, feeding and surgery were all as described previously (Kintner & Brockes, 1985). Limbs were amputated, from animals anaesthetized by immersion for 10–15 min in 0·1 % Tricane (Sigma), just proximal to the elbow and trimmed to give a flat stump. The humerus was removed, if required, by freeing the protruding end of the bone from muscle and connective tissue, gripping it tightly with forceps, and pulling with steadily increasing force until the bone was just exarticulated. The humerus could then be gently pulled free from the limb stump. This procedure causes the least damage to the remaining tissues. The stump soft tissues were then trimmed as usual to provide a flat surface for regeneration.

Cloning

The genomic clone λNVM5, containing the 3′-terminal sequences of the newt cardioskeletal myosin gene, was isolated from a newt genomic library as described previously (Casimir *et al.* 1988), using a 112 bp cDNA fragment containing the 3′-terminal coding sequences and 26 bp of the untranslated region as a probe. Results obtained with this probe were compared with those obtained using a 5′-end-labelled (Maniatis *et al.* 1982), 18-mer oligonucleotide probe corresponding to the six carboxy-terminal amino acids of the protein. Oligonucleotide hybridizations were performed for 16 h at 50 °C (five degrees below dissociation temperature, T_D) in 6 × SSC, 5 × Denhardt's, 0·5 % SDS. Washes were twice for 15 min at hybridization temperature, followed by a 2 min wash at T_D, both in 6 × SSC, 0·1 % SDS. The single phage clone isolated was identified in a screening of 10^7 phage plaques. The identity of the cloned genomic sequences was confirmed by determination of DNA sequence in the last exon, (the region hybridizing with the probes) and by comparison with the previously sequenced cDNA (Casimir *et al.* 1988).

Cell culture

Amphibian cells were grown in MEM (adjusted to amphibian plasma osmolarity) supplemented with 10 % fetal calf serum, as described in Ferretti & Brockes (1988), until the cultures reached confluence. They were then allowed, with frequent changes of medium, to fuse into multinucleate myotubes. The cells were harvested by scraping in medium and were pelleted at 1000 revs min^{-1} for 10 min at 20 °C in a Beckman TJ-6 table-top centrifuge. The cell pellet was washed twice by resuspension in PBS (adjusted to amphibian plasma osmolarity) and repelleted as before. The final cell pellet was resuspended in ten volumes of lysis buffer (0·3 M-NaCl, 10 mM-Tris pH 7·4, 1 mM-EDTA, 0·5 % SDS) and DNA extracted as described previously (Casimir *et al.* 1988) for tissue samples.

Southern blotting

Nucleic acid preparation, restriction enzyme digestion, gel electrophoresis, transfer to nitrocellulose, hybridization

and washing of blots were all performed exactly as described previously (Casimir *et al.* 1988).

RNAse protection

RNAse protection experiments were performed as previously described (Casimir *et al.* 1988), using the same DNA fragment described for the library screening cloned into the 'Bluescribe' vector (Stratagene) to derive the RNA probe. The satellite 2 DNA probe pSP6-D6 was a single tandem repeat of the sequence (Epstein & Gall, 1987).

Quantification of hybridization data

Autoradiograms were scanned using a Joyce–Loebl scanning densitometer and peaks were quantified by weighing. Repetition of selected scans revealed no significant variations. Statistical significance of the differences between sample means of the data were tested using Student's *t*-test.

Results

The experimental system we have adopted is depicted in Fig. 1. This shows a schematic description of one of the classic observations of regeneration biology (Thornton, 1938). If, at the time of amputation, the skeletal elements lying proximal to the plane of amputation are removed (in this case, the humerus), a normal blastema forms on the stump, giving rise to a normal distal regenerate. No regeneration of the missing skeleton occurs in the proximal part of the limb. As the distal skeletal cartilage forms quite normally in the absence of any cartilage remaining in the stump, this raises the question of the origin of the cells that make up the newly formed (regenerate) cartilage. Our experiments were designed to examine whether cells originating in muscle could contribute cells to the blastema, and by metaplasia give rise to cartilage, in both standard regenerates and those in which the humerus had previously been removed.

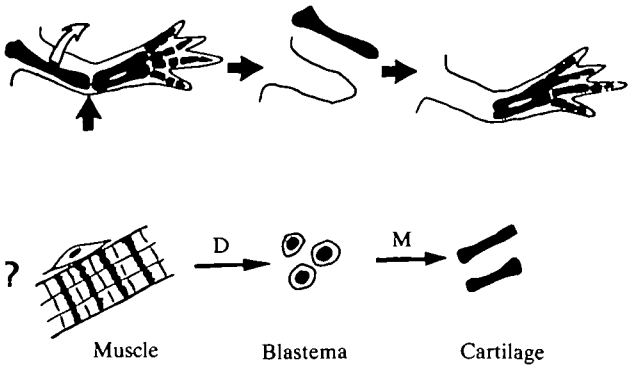


Fig. 1. Schematic diagram of experimental design. The diagram illustrates the finding that removal of the humerus at the time of amputation does not affect the process of distal regeneration. As there is no pre-existing source of cartilage cells in these regenerates, it provokes the question as to the identity of the cell populations in the stump which give rise to cartilage in the regenerate. One available source, as indicated, could be cells from within muscle. Our experiments were designed to test this possibility. D, dedifferentiation; M, metaplasia. The arrow in the first illustration indicates the plane of amputation.

Identification of molecular lineage markers

In order to trace the fate of cells from within muscle during the regeneration process, we identified two tissue-specific sites of DNA hypomethylation in the last intron of the cardioskeletal myosin gene of the newt (*Notophthalmus viridescens*). This gene encodes a myosin heavy chain isoform of essentially cardiac type but which is also expressed in the 'slow' fibres of skeletal muscle (Casimir *et al.* 1988); this being a mixture of 'slow' and 'fast' fibres. The location of these sites is indicated (by stars) in the restriction map in Fig. 2. The first of these (Hypo A) is an *Ava*I cleavage and marks muscle cells, probably of the 'fast' fibre type. The second (Hypo B) is an *Hpa*II site lying approximately 300 bp to the 3'-side of Hypo A but, interestingly, this site does not mark muscle cells *per se* but appears to identify cells within skeletal muscle that do not belong to the muscle lineage (for the basis of the marker assignments see below). A third hypomethylated site (Hypo C) with a tissue distribution similar to Hypo A was also found, but its relationship to Hypo B (see Fig. 2) and a lack of suitable flanking restriction sites made independent analysis of this site problematical. Because of this it was not considered further, except in its contribution to the total DNA hybridizing with the probe.

The rationale for assigning the markers to these different cell types (summarized in Table 1) comes from the results of Southern blotting experiments on DNA isolated from three different muscle cell populations and from normal cartilage (Figs 3 and 4). The three muscle populations are skeletal muscle, heart and cultured newt cells. These cultured cells (derived from limb muscle explants) have been shown to be highly myogenic in culture and at elevated densities undergo fusion to produce large numbers of myotubes (for a fuller characterization, see Ferretti & Brockes, 1988).

The Hypo B marker was assigned to connective tissue in the following way: hypomethylation at the marker *Hpa*II site is detected by the presence of a 2 kb hybridizing *Kpn*/*Hpa*II fragment (Fig. 3, right-hand panel). The presence of such a fragment is clearly detected in skeletal muscle DNA (lane M) but is only just visible in cartilage DNA (lane C). The presence of this band can therefore be used as a marker for cells within skeletal muscle. Results of hybridization to heart muscle and to tissue culture cells were used to ascertain what cell type in muscle was carrying the marker. Heart muscle is composed

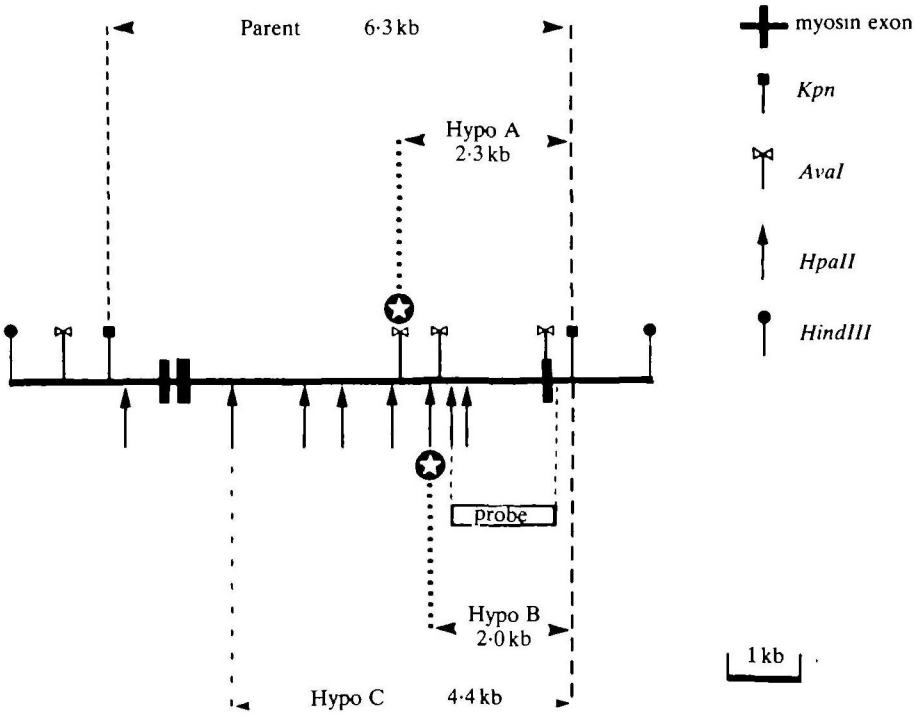


Fig. 2. Restriction map showing location of lineage markers. The figure shows the C-terminal three exons (39, 40, 41) of the newt cardioskeletal myosin gene (in 5′→3′ orientation) and the cutting sites of the enzymes used in this study. The enzymes *Hpa*II *and Ava*1 are both sensitive to methylation of the internal C residue in their respective recognition sites. The hypomethylated sites used as markers are indicated by stars and the sizes of the resulting restriction fragments are shown. The probe fragment is a 1·4 kb *Sac*1 to *Bam*H1 fragment, spanning the area indicated.

Table 1. *Allocation of marker sites to different cell types*

Tissue	Gene expression	Muscle fibre type	Level of marker A*	Level of marker B*
Skeletal muscle	yes	mixed	++	+++
Heart muscle	yes	slow	+	+
Cultured cells	no	fast	+++++	–
Normal cartilage	no	N/A	+	+

Conclusions: Marker A associated with muscle of fast fibre type. Marker B associated with 'connective tissue' cells.
*Note: for Marker A +≈10% hypomethylation, for Marker B +≈5% hypomethylation.

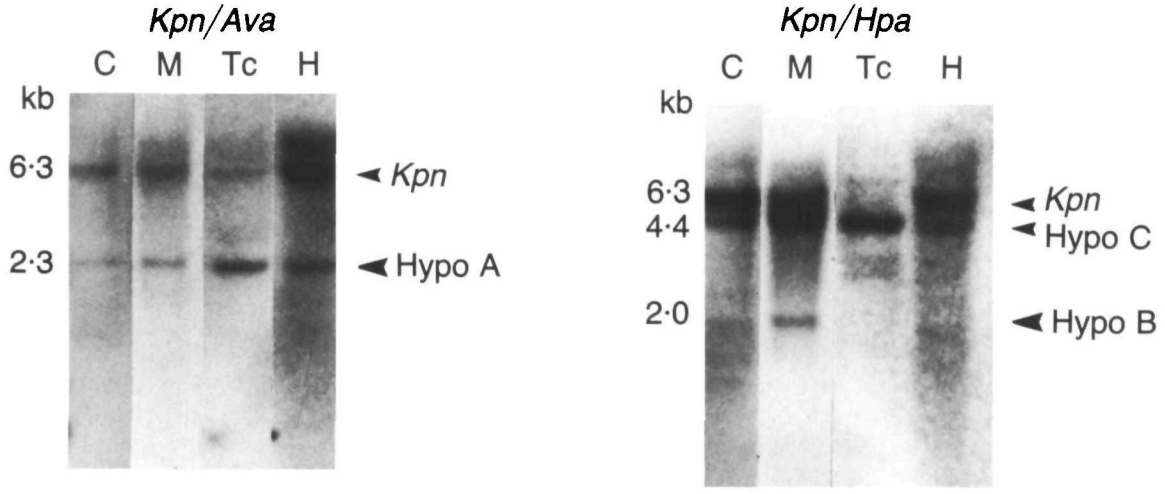


Fig. 3. Hybridizational analysis of lineage marker hypomethylations. Genomic DNA was digested with *Kpn*1 and *Ava*1 (left-hand panel) or *Kpn*1 and *Hpa*II (right-hand panel) and separated on 1% agarose gels. The DNA was transferred to nitrocellulose and hybridized with a radiolabelled DNA fragment containing myosin sequences (see Fig. 2). The cellular origin of the genomic DNA is indicated above the lanes. C, normal cartilage: M, skeletal muscle; Tc, cultured newt muscle cells; H, heart. The parent hybridizing fragment is shown (*Kpn*). Hypomethylation at the marker sites is indicated by the presence of the 2·3 kb *Kpn/Ava* (Hypo A) and 2·0 kb *Kpn/Hpa* (hypo B) hybridizing bands.

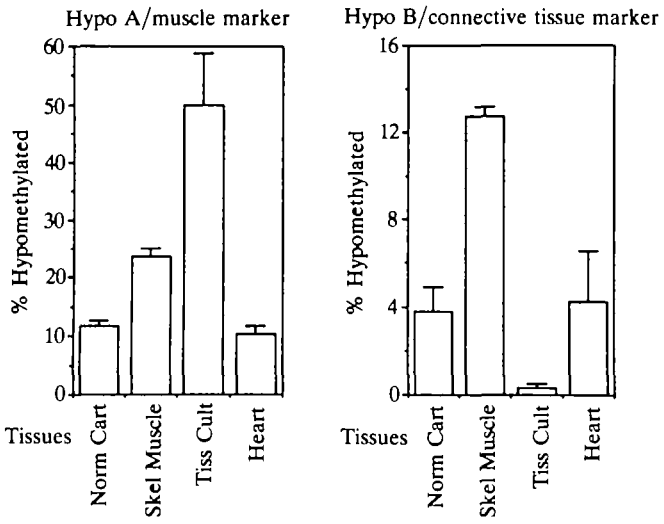


Fig. 4. Quantification of marker hypomethylations. Histographic representation of the proportion (%) of DNA (estimated from densitometric analysis of blot hybridizations) hypomethylated at the marker sites in different newt tissues. The results were derived from a minimum of two independently isolated DNA preparations. Norm Cart, normal cartilage; Skel Muscle, skeletal muscle; Tiss Cult, cultured newt muscle cells; Heart.

almost exclusively of 'slow' type muscle fibres, which express the cardioskeletal myosin (Mahdavi *et al.* 1982; Casimir *et al.* 1988). The marker was, however, quite poorly detected in heart (lane H). We took this to indicate a lack of correlation of the marker with 'slow', or expressing, muscle fibres. Conversely, the tissue culture cells (lane Tc) showed no 2 kb hypomethylated band. Based on monoclonal antibody staining, *in situ* hybridization and myosin RNA expression (Ferretti & Brockes, 1988; D. Fekete & C. Casimir, unpublished data) the cultured cells have characteristics of 'fast' type muscle fibres. This result indicated a lack of correlation also with 'fast' or nonexpressing fibres. From these data, we concluded that the most likely candidates for cells carrying the marker were those of a nonmuscle type. For this reason, we have termed Hypo B a 'connective tissue' marker, though clearly other assignments remain quite feasible. For example, this marker could be carried by Schwann cells, or a minor muscle type not represented in our control populations (this is considered further in the Discussion). As the magnitude of the difference between muscle and cartilage was not great, we have quantified these differences by densitometric scanning of the autoradiograms and integration of the respective peaks (see below).

The Hypo A marker was assigned to fast fibre type

Table 2. *Proportion of total hybridizing DNA detected in hypomethylated bands in quantitative analysis of blot hybridization*†

	Experiment						
Tissue	I	II	III	IV	V	Mean ± S.E.M.	*P**
(A) *Hypo A/muscle marker*							
Liver	72·2	63·5	52·0	nd	–	62·6 ± 5·8	
Brain	8·5	12·8	13·4	9·3	–	11·0 ± 1·2	
Norm Cart	14·5	11·2	9·1	11·7	–	11·6 ± 1·1	
Regen Cart	22·2	16·2	19·5	18·0	–	19·0 ± 1·3	<0·02
Hux Cart	nd	19·1	18·8	14·0	–	17·3 ± 1·7	<0·05
Blastema	17·1	16·4	16·2	17·6	–	16·8 ± 0·3	<0·005
Skel Muscle	22·0	22·2	nd	26·4	–	23·5 ± 1·4	
Tiss Cult	58·0	59·4	32·7	nd	–	50·0 ± 8·7	
Heart	6·5	12·0	10·5	12·3	–	10·3 ± 1·3	
(B) *Hypo B/connective tissue marker*							
Liver	9·2	9·3	9·3	nd	nd	9·3 ± 0·03	
Brain	2·3	3·9	4·7	4·7	4·2	4·0 ± 0·45	
Norm Cart	3·3	2·5	1·3	7·6	4·6	3·9 ± 1·1	
Regen Cart	7·3	7·7	7·5	nd	nd	7·5 ± 0·13	<0·05
Hux Cart	nd	9·7	20·2	13·0	8·2	12·8 ± 2·7	<0·02
Blastema	11·4	10·8	10·2	7·1	8·1	9·5 ± 0·82	<0·02
Skel Muscle	13·4	12·4	14·2	11·8	13·0	13·0 ± 0·41	
Tiss Cult	0·4	0·2	0·7	nd	0·0	0·3 ± 0·15	
Heart	2·6	0·4	11·0	nd	3·0	4·3 ± 2·3	

† Values are percentage of total hybridizing DNA represented by hypomethylated band.

* P values related to differences in means between normal cartilage and regenerate tissues and were calculated using Student's *t*-test.

S.E.M. = standard error of mean; nd = not determined.

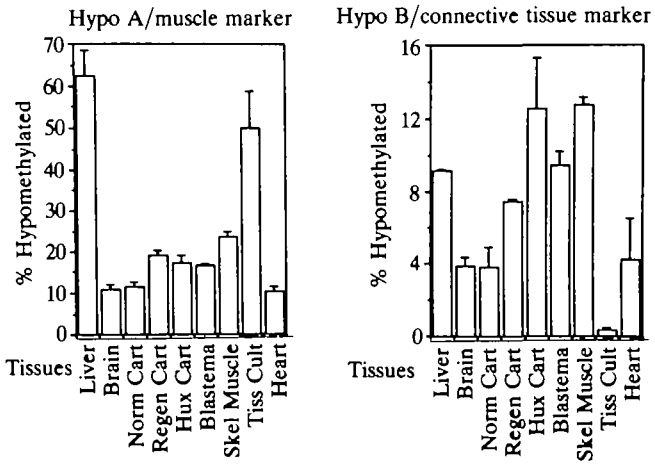


Fig. 5. Behaviour of marker hypomethylations in regeneration. Histograms showing the level of hypomethylation (mean ± S.E.) at the marker sites Hypo A and Hypo B (indicated in the figure). The results are derived from the data shown in Table 2, and used a minimum of two independently isolated DNA preparations of each tissue type. Hypomethylation was quantified in a number of different regenerate and normal newt tissue DNAs. Liver; Brain; Norm Cart, normal cartilage; Regen Cart, cartilage from regenerated limbs; Hux Cart, cartilage from regenerate limbs from which the humerus was removed at the time of amputation; Blastema, 'cone' stage blastemas; Skel Muscle, skeletal muscle; Tiss Cult, cultured newt muscle cells; Heart. Note the increased representation of the markers in the regenerate tissues relative to normal cartilage.

muscle cells by applying a similar type of logic. This hypomethylation marker is identified by cleavage at an *Ava*I site which gives rise to a 2·3 kb hybridizing *Kpn/Ava* fragment (Fig. 3, left-hand panel). Though difficult to determine reliably by eye, quantification of this fragment revealed a higher representation in skeletal muscle DNA than in cartilage DNA. Additionally, relative to the level observed in skeletal muscle DNA, Hypo A represented a higher proportion of tissue culture cell DNA but a lower proportion of heart DNA. This indicated a correlation with 'fast' muscle and therefore a 'nonexpressing' phenotype.

The results of the densitometric analysis of the hybridizations, estimated from a number of repetitions of this experiment, are depicted as histograms (Fig. 4) and also contained within Table 2. DNA present in the hypomethylated band was expressed as a fraction (%) of the total hybridizing DNA (the hypomethylated band plus the *Kpn* parent band) to quantify the representation of the respective markers.

Like others (Cedar, 1984; Yisraeli & Syf, 1984), we have taken the partial conversion of parent bands into hypomethylated bands to be reflective of the fact that tissues are made up from a variety of cell types; the extent of hypomethylation therefore reflects the fraction of the tissue that is made up by the marked cell type. This view would be consistent with the behaviour of the cultured cells, which being a much more homogeneous population also show greater degrees of conversion of parent into hypomethylated bands.

Although consistent differences in the levels of hypomethylation at the marker sites were observed, it was important to confirm that these differences did not derive from incomplete digestion by restriction enzymes. To control for this, we performed two types of experiment. The first was to add to the genomic DNA preparations a cloned DNA fragment containing an appropriate (*Hpa*II or *Ava*I) restriction site and monitor its digestion. As cloned DNA contains no methylated sites it should be cut quantitatively. Regardless of whether cutting of the exogenous DNA was monitored by hybridization, or by use of radioactively labelled DNA, no partial digestion products were observed (data not shown).

In addition, as the experiment described above only controls for the presence of soluble inhibitors, we also performed a control on all the genomic DNAs used to generate the methylation data. This was to rehybridize the Southern transfers used for the marker analysis with a probe homologous to a newt satellite DNA (Epstein *et al.* 1986; Epstein & Gall, 1987). With this probe, hybridization of genomic DNA digested by the methyl-sensitive enzymes (*Hpa*II or *Ava*I) produces a ladder of bands owing to its multiple representation in the genome. No differences between tissues attributable to partial digestion could be detected (data not shown, but see Fig. 7).

Behaviour of hypomethylation lineage markers during regeneration

The quantitative Southern blot analysis was repeated on DNA from a number of different cell populations (see Table 2 and Fig. 5). In particular, DNA from three types of regenerate tissue was investigated. These were regenerate cartilage, regenerate cartilage from humerus-removed animals and limb blastema. When compared with normal cartilage, both markers showed statistically significant (Table 2; $0{\cdot}005 < P < 0{\cdot}05$) increases in the level of the marker band in all regenerating tissues, suggesting that cells carrying the markers can contribute to the blastema and to the cartilage of the regenerate. This was most clearly visible in the case of the 'connective tissue' marker (Hypo B; Fig. 5, right-hand panel), where the difference between muscle and the control (cartilage) was greater. Nevertheless, despite the narrower margin between muscle and cartilage for the muscle marker, Hypo A (Fig. 5, left-hand panel), a reproducible

difference (consistent across at least two independent DNA preparations) between normal and regenerate tissues was observed. This result is consistent with the idea that cells of the muscle lineage can dedifferentiate, contribute to the blastema and undergo metaplasia into cartilage.

An interesting difference in the behaviour of the two markers appeared in their response to the effects of humerus removal. In the case of cells carrying Hypo B, humerus removal increased their contribution to regenerate cartilage (Fig. 5, right-hand panel). No such difference was observed, however, for the muscle marker, Hypo A (Fig. 5, left-hand panel). The effect of humerus removal on cells carrying the Hypo B marker was reproducible (humerus removal always gave rise to an increase) but the magnitude of the difference proved somewhat variable for different samples of humerus-removed regenerate cartilage DNA (see Table 2) and was therefore of marginal statistical significance. Because of this, the effect of humerus removal was further investigated by experiments in which animals were subjected to bilateral amputation but to humerus removal only unilaterally. Comparison of regenerated cartilage within this internally standardized population should control for any procedural variations or differences in rates of regeneration between batches of animals. The results (not shown) corroborated the findings described above that humerus removal led to an increase in the representation of Hypo B not observed for Hypo A. This increased contribution of Hypo-B-marked cells to regenerate cartilage though, did not appear to be reflected by a similarly increased representation in the blastemas of these animals (data not shown).

We infer from these data that humerus removal probably does lead to some selective recruitment from cells of the 'connective tissue' lineage into

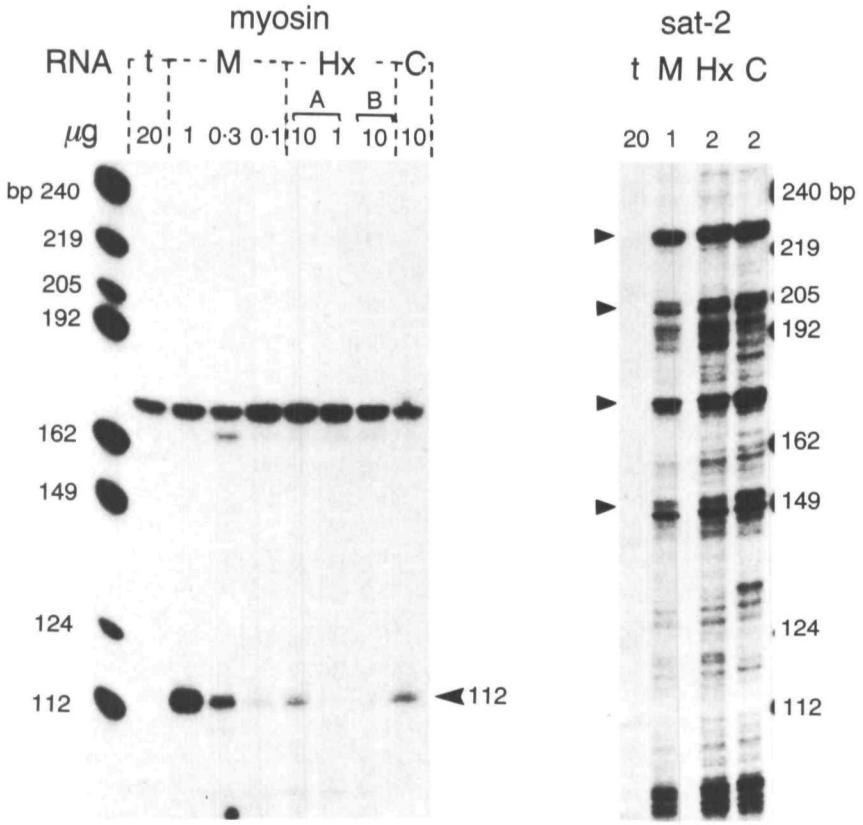


Fig. 6. Contamination of regenerate and normal cartilage by skeletal muscle tissue. RNAse protection experiments. The concentration of myosin RNA in the various tissue RNA preparations (left-hand panel) was assayed by the presence of a 112 bp protected RNA species. The quantity of input RNA is shown over the respective lanes. The source of the RNA is indicated as follows: t, yeast t-RNA; M, skeletal muscle; Hx, RNA from regenerate cartilage of humerus removed animals, two different preparations A and B; C, normal cartilage. The integrity and concentration of the different RNA preparations was assayed using a ubiquitously transcribed newt satellite DNA sequence (right-hand panel). The major protection products, corresponding to the cloned variant of the satellite sequence are arrowed. Source of the RNA is indicated as for the myosin experiment; the Hx sample used was sample B. The level of muscle contamination was estimated from the ratio of the quantities of muscle and cartilage or Hx RNA that gave rise to equivalent protection signals.

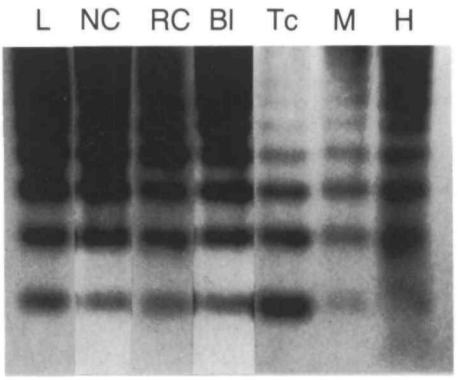


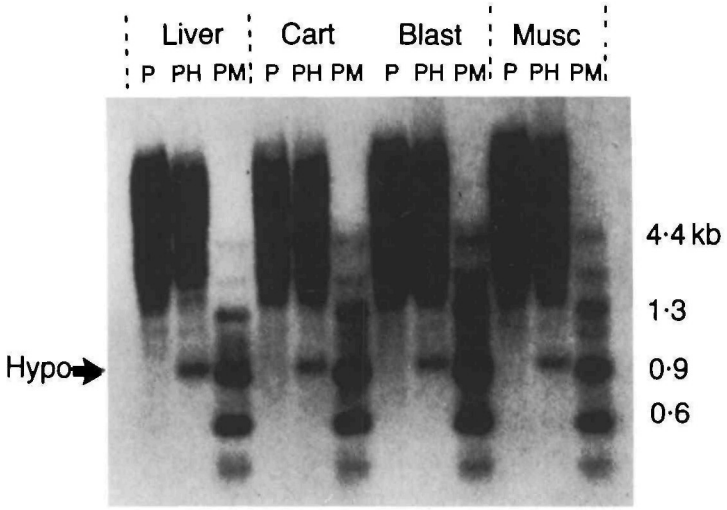
Fig. 7. Stability of methylation patterns in regeneration. The Southern transfers used to generate the data for the lineage-dependent hypomethylations were rehybridized with the satellite DNA probe. The resulting 'ladder' of hybridizing bands was dependent on digestion by the methylation-sensitive enzymes. Note the similarity between normal and regenerate tissues and the bias towards monomer-sized fragments seen with the cultured cells. The source of the DNA is indicated as follows: L, liver; NC, normal cartilage; RC, regenerate cartilage; Bl, blastema; Tc, cultured muscle cells; M, skeletal muscle; H, heart muscle.

cartilage in the regenerate. This seems to result from an increased conversion of connective tissue cells, or a faster rate of their proliferation in the blastemal population, rather than from an increased contribution of this cell type to the blastema.

Do increased levels of hypomethylation in regenerate tissues actually reflect changes in cellular origin?

The results presented above indicated that regenerate cartilage DNA could be distinguished from the DNA of normal cartilage. We have interpreted this as reflecting a difference in the origin of the cells that give rise to regenerate and normal cartilage. Alternative explanations for these data remain and we have tested two of these: (1) contamination of regenerated cartilage with muscle tissue; (2) instability in the level of DNA hypomethylation induced by the regeneration process.

To address the problem of contamination we first calculated, using the methylation data shown in Table 2, how much muscle contamination of regenerate cartilage would be necessary to account for these results. Such a calculation revealed that a contamination level of >50% would be required to explain even the results obtained with Hypo A. To demonstrate the validity of this calculation, a reconstruction experiment was performed. Normal cartilage DNA and muscle DNA were mixed in the appropriate ratio (determined by the calculation) and the representation of Hypo A compared with that obtained with regenerate tissue (in this case blastema) DNA. The



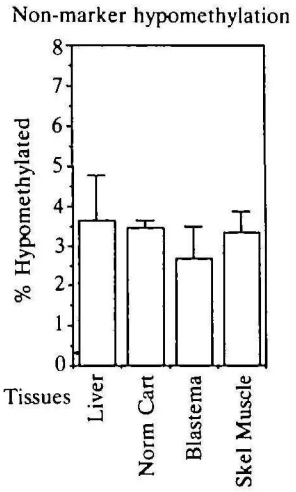


Fig. 8. Stability of a non-marker hypomethylation in regeneration. A specific site of DNA hypomethylation in an upstream region of the newt cardioskeletal myosin gene was assayed and its representation quantified exactly as described for the lineage markers Hypo A and Hypo B (Figs 3 and 4). (Top panel) DNA from the tissues indicated over the respective lanes was digested with *Pst*I (P), *Pst*I and *Hpa*II (PH), or *Pst*I and *Msp* (PM). Hypomethylation of the site is indicated by the presence of a 0·9 kb hybridizing band which is identified in the figure (Hypo→). (Bottom panel) Histogram showing quantification (mean ± S.E.) of hypomethylation at this site in normal and regenerate newt tissue DNAs, derived from three independent experiments. Note that this hypomethylation showed no specificity for muscle and its representation did not change in DNA of regenerate tissue.

reconstruction approached very closely the results obtained with blastema DNA (data not shown).

To compare with the predicted level of contamination required, a direct measure of the actual level

of muscle contamination in regenerate cartilage was obtained by assaying for the presence of myosin transcripts in RNA prepared from the same tissue samples used to prepare DNA. This was done using an RNAse protection assay (Melton *et al.* 1984). Myosin mRNA was detected by the presence of a 112 bp protection product. Fig. 6 shows a titration with three levels of *bona fide* muscle RNA, used as a standard and the results obtained with two preparations of humerus-removed regenerate cartilage RNA (Hux) and one preparation of normal cartilage RNA (NC). As a check on the concentration and integrity of the various RNA preparations, RNAse protection experiments were also performed using the satellite sequence probe (Fig. 6, sat 2 panel), which is ubiquitously expressed. Owing to the repetitive nature of this sequence, multiple protected species were detected. The levels of muscle contamination observed in Hux RNA were at least one order of magnitude lower than necessary (approximately 0·2 %–2·0 % of Hux RNA) to account for the DNA hypomethylation results. Moreover, similar levels of contamination (2·0 %) were also observed in preparations of normal cartilage.

The second class of controls tested whether general changes in levels of hypomethylation occur during regeneration, leading to increases in the degree of hypomethylation at the marker sites that are unrelated to lineage derivation. A number of observations militate against this. First, rehybridizing the Southern blots with the satellite DNA probe gave no evidence of differences between normal and regenerate tissues. A slightly greater overall degree of hypomethylation was, however, detected for the tissue culture cells (Fig. 7). Other cultured cells also seem to show lower than average levels of methylation (Razin, 1984). Second, no overall changes in the levels of hypomethylation during regeneration could account for the differential response of the two markers to humerus removal (see Fig. 5). Lastly, we have monitored the behaviour in regeneration of a previously identified site of hypomethylation in the cardioskeletal myosin gene (Casimir *et al.* 1988) located in a region upstream of exon 34. Hypomethylation at this site shows no tissue specificity for muscle compared with cartilage. Quantitative analyses of Southern blot hybridizations (Fig. 8) like those performed for the markers Hypo A and B have revealed no statistically significant increases in the degree of hypomethylation at this site in DNA from regenerate tissue.

We conclude from the above that the behaviour of the hypomethylated sites Hypo A and B is consistent with that of lineage markers and that an increased representation of these markers detected in the DNA of regenerate cartilage is most likely reflective of altered cell fates during amphibian limb regeneration.

Discussion

DNA hypomethylations as molecular lineage markers

This paper describes evidence for dedifferentiation and metaplasia in amphibian limb regeneration derived from the inheritance of tissue-specific sites of DNA hypomethylation. As sites of DNA hypomethylation have been most-commonly associated with gene activity (see Razin *et al.* 1983), it may seem strange that the two marker sites we have identified in the newt cardioskeletal myosin gene are correlated with a nonexpressing muscle type on the one hand and a nonmuscle cell type on the other. Precedents for both types of correlation already exist, however, in the case of the vitellogenin gene (Burch & Weintraub, 1983), where a site specific to oviduct was found (the gene is capable of binding hormone in this tissue, but is not expressed) and in the myosin *light chain* and α-actin genes (Shani *et al.* 1984), where sites of hypomethylation were found in skeletal muscle but not in a muscle cell line which could be induced to express the gene. Nonetheless, as has been true for other cell or lineage markers, a lack of precision in our understanding of the biochemical significance of DNA hypomethylations in no way precludes their use in tracing cell fates.

We have described earlier some of the potential advantages of employing molecular markers of lineage, the technique also has some limitations when compared to conventional cell marking techniques. Most importantly, it lacks resolution at the individual cell level, so whilst a marked population can be identified, the actual cells in that population carrying it cannot. Connected with this is the inability to assess anything other than the overall behaviour of the marked population. This means we cannot exclude the possibility that the (Hypo A) marked muscle cells are some kind of unusual reserve cell or bipotential precursor. What can be said is that the marked cells exhibit a defined commitment to the muscle lineage and it is in this sense that the observations in this paper are a 'molecular' definition of dedifferentiation as opposed to a cellular one. It remains possible, therefore, that the cells carrying Hypo A into the regenerate are from the 'post-satellite' (putative reserve cell) population identified in the newt (Cameron *et al.* 1986). It is unlikely, however, that these are the only cells carrying the marker, as its representation in the differentiated muscle cell population is relatively high ($\approx$20 % min.). Similarly, we cannot rule out the possibility that Hypo B marks a

minor muscle fibre type population, not represented in either heart or the cultured cells. Whilst the proportion of marked cells in muscle and its apparent sensitivity to humerus removal (not seen for the other marker) makes a correlation with a nonmuscle phenotype more likely, association with a minor muscle population would add materially to our evidence (from Hypo A) in favour of metaplasia of muscle cells into cartilage.

Does inheritance of DNA hypomethylation indicate metaplasia?

Whilst DNA methylation patterns are essentially stable (Razin, 1984; Cedar, 1984), the fact that tissue-specific differences exist demonstrates that changes must occur during development. Two periods of *de novo* methylation activity have been identified in birds and mammals. The first is during early embryonic development (Jäner & Jaenisch, 1984) and the second is during sperm maturation (Groudine & Conklin, 1985). These are both periods of major developmental change affecting gene expression, and it is not unreasonable to postulate that similar events could occur during regeneration. If major changes in methylation patterns do occur during regeneration, then one of our control experiments would have been expected to give some evidence for this. It is still formally possible that instability in the level of methylation could occur specifically at our marker sites during regeneration. The selective response of Hypo B to humerus removal, however, given that this does not affect formation of the blastema or the overall progression of regeneration, militates against the likelihood of such a proposal.

Another type of explanation that cannot be totally excluded is that, although we are detecting muscle-derived cells in regenerate cartilage, these cells had not actually been converted into cartilage, but were 'lost' muscle cells that had become engulfed. *In situ* hybridization experiments (D. Fekete & C. Casimir, unpublished observations), as well as the RNAse protection data presented here, have provided no evidence for myosin gene expression in regenerate cartilage. If this process does occur, then engulfed muscle cells must lose their differentiated phenotype. Our methylation results would also imply that this process must occur at an unreasonably high frequency (≈10–20 % of regenerate cartilage).

Consequences of dedifferentiation and metaplasia for regeneration

Evidence has been presented here consistent with contributions from connective tissue cells and of muscle cells (by metaplasia) to regenerate cartilage. Quantitative analysis has enabled us to estimate the contributions of these two cell types to approximately 10 % in each case. Previous investigators, using traditional grafting methods on axolotls, have arrived at broadly similar conclusions (Steen, 1968; Namenwirth, 1974), though it was not possible for them to separate contributions from the two different sources within muscle. Steen did not attempt to quantify his findings, but Namenwirth, who used irradiated host animals, arrived at values similar to those presented here, within the errors inherent in sampling small numbers of grafted cells.

The simplest interpretation of our data on Hypo A, the muscle marker, would be in agreement with the classical descriptions of regeneration (Hay & Fischman, 1961; Salpeter & Singer, 1960; Hay, 1969), that mature muscle fibres contribute to the blastema by dedifferentiation. For the reasons outlined above, it is not possible to exclude other interpretations based on the existence of various types of reserve cell populations. Nevertheless, it is important to note that these cells show a defined commitment to the muscle lineage, involving changes in a muscle-specific gene that is only expressed in terminally differentiated cells.

Alternative views on the origin of blastemal cells have emphasized the importance of dermal fibroblasts in contributing cells to the blastema (Dunis & Namenwirth, 1977; Muneoka *et al.* 1986). It is interesting that neither of the populations we have followed is the major contributor of cells to cartilage. Certainly, if the Hypo B marker does identify connective tissue, either it marks a defined subset of such cells found only in muscle, or it does not support models based on the idea that connective tissue fibroblasts are virtually the sole source of cells for the blastema. Although the connective tissue of muscle could differ from other sources within the limb, some continuity has been demonstrated; the monoclonal antibody marker 22/31 (Kintner & Brockes, 1985), for example, labels all such cells. It is also conceivable that Hypo-B-marked cells are Schwann cells. Other lines of evidence have pointed to a Schwann cell contribution to the blastema (Kintner & Brockes, 1985; Maden, 1977). Indeed, under special circumstances (so-called 'paradoxical' regeneration), it has been proposed that the blastema is wholly derived from Schwann cells (Maden, 1977). It would perhaps be of interest for the future, therefore, to follow Hypo B marked cells during 'paradoxical' regeneration.

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