

# Protein synthesis in the newt regenerating limb

## Comparative two-dimensional PAGE, computer analysis and protein sequencing

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Protein synthesis has been studied by two-dimensional PAGE during the early limb regeneration in the adult newt. Quantitative and statistical analyses have provided unique information on overall patterns of protein synthesis as well as on specific protein synthesis during formation of the blastema. Furthermore, from the patterns in the two-dimensional gels and their quantification, a particular protein has been selected and sequenced. Partial sequencing revealed sequence similarities to *Xenopus* type I keratin B2. Expression of this keratin is 10-fold greater in the blastema than in the intact limb. The implications of keratin expression by the blastema cells are discussed.

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### INTRODUCTION

The remarkable phenomenon of limb regeneration is restricted to some amphibian species. Among amphibians, the urodeles show good regenerative abilities even during their adult life. After amputation of the limb the remaining tissues of the stump dedifferentiate and produce a cell population called the blastema. The stump is covered by the wound epithelium whose presence and integrity are essential for the process of regeneration to take place. Blastema cell accumulation starts within 7 days after amputation. The blastema cells proliferate for about 2 weeks and form the regeneration cone. After that period the blastema cells redifferentiate into the tissues that constitute the limb, thus reforming the lost part (Wallace, 1982; Tsonis, 1990). The very faithful distal transformation (i.e. regeneration of only the missing structures distal to the amputation) can be disrupted by retinoic acid. This provides the blastema with a more proximal 'memory', so that more proximal structures can be regenerated as a result of the treatment (Maden, 1982).

Identification and characterization of factors involved during the formation of the blastema should be a very important step in understanding the mechanisms underlying the regeneration phenomenon. Molecular biology and biochemical techniques are now being applied which aim to isolate such factors. For example, homoeo-box genes and retinoic acid receptors have been isolated, and studies on their function during specifications of patterns have been published (Savard *et al.*, 1989; Tabin, 1989; Giguere *et al.*, 1989; Ragsdale *et al.*, 1989).

We have initiated studies to identify and characterize factors that are involved during limb regeneration. In order to do so, a detailed analysis of protein synthesis during blastema formation is needed. Therefore we have decided to map all proteins that are specific to the regenerating limb. From this pattern, we can select proteins that are specific to the process, carry out partial sequencing in order to characterize them, and generate probes for subsequent use to study limb regeneration. Such a study was performed by two-dimensional (2-D) PAGE, computer analysis and quantitative comparison of the protein synthesis patterns. In addition, based on the patterns of protein synthesis, we have microsequenced one protein which we found to have a similar sequence to that of *Xenopus* embryonic keratin B2, a type I acidic keratin.

### MATERIALS AND METHODS

#### Animals

Adult newts (*Notophthalmus viridescens*) purchased by Amphibian of North America, C. Sullivan, TN, U.S.A., were used in this study. Forelimbs were amputated bilaterally at the level of the elbow.

#### Protein isolation and 2-D PAGE

[<sup>35</sup>S]Met (1 mCi) was injected intraperitoneally 2 days before collection of the tissues. The tissues were then removed and rinsed several times with cold buffered saline, pH 7.5. The tissues collected were intact forelimbs, 1-week blastemas and 2-week blastemas. Since we are interested in all factors, even the ones produced specifically by the wound epithelium, the whole regenerates were removed. Tissues were crushed in liquid nitrogen and solubilized by adding hot SDSBME solution [0.1% SDS, 20 mM-Tris, pH 8.0, 5% 2-mercaptoethanol (Protein Databases Inc., Huntington Station, NY, U.S.A.)]. The tubes containing the samples were then cooled and 0.1 vol. of a solution containing DNAase (1 mg/ml) and RNAase (500 µg/ml) was added, and the tubes were incubated for 2 min on ice. The samples were then frozen in liquid nitrogen, ready to be analysed on 2-D PAGE. Gel electrophoresis was carried out by the method of Garrels (1983). For isoelectric focusing, 2% ampholyte (pH 4–8) was used (BDH Chemicals, Poole, Dorset, U.K.). The second-dimension slab gels were 12.5% acrylamide gels. At the end of electrophoresis, the gel were fixed in 25% methanol/15% acetic acid for 1 h and processed for autoradiography at –70 °C for different periods of time. Quantification and statistical analyses of the protein patterns as shown in autoradiographs were performed using the PD QUEST software (Protein Databases Inc.) (Garrels, 1989). This software can provide information on the quantification of each protein at different stages and can also compare overall protein synthesis patterns in two different samples. According to the procedure, spot quantification and resolution of overlapping spots is performed by 2-D Gaussian fitting. The matching of the patterns revealed by the autoradiograms is carried out for groups of gels, called matchsets, and within each matchset every gel is matched to every other gel. Tests have shown that up to 97% of the spots

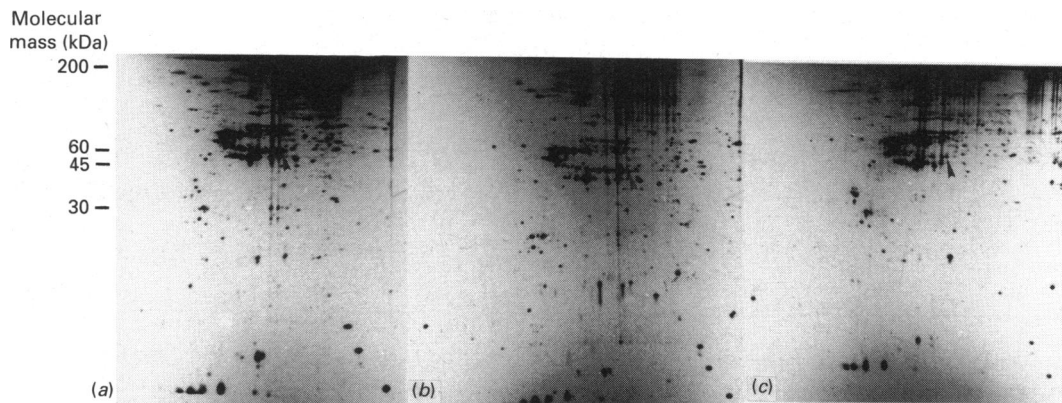


Fig. 1. 2-D gels of 2-week regenerating (a), 1-week regenerating (b) and intact (c) limbs

The pH is linear from 4 on the left to 6.8 on the right. The arrowhead in (c) indicates the protein that was sequenced (55 kDa; see Fig. 3).

in each pattern can be matched and that fewer than 1% of the spots are matched inconsistently.

#### Blotting and microsequencing analysis

For transfer to poly(vinylpyrrolidone difluoride) (PVDF) membranes, 150  $\mu$ g of protein was used per gel. Transfer to membranes was performed by the methods of Towbin *et al.* (1979). The membranes were stained with Coomassie Blue to ensure that there was enough protein to sequence, and the desired spot was cut out for further sequence analysis. Protein sequencing was obtained on an Applied Biosystems (ABI) Pulsed-Liquid Phase Sequencer (477A) with on-line phenylthiohydantoin amino acid analysis provided by an ABI 120A analyser. Internal sequences were obtained using the CNBr/o-phthaldehyde (OPA) strategy (Dr. S. Wadsworth, University of Wisconsin Biotechnology Center, Madison, WI, U.S.A.). Briefly, after the initial sequencing run revealed no information, the PVDF was treated with CNBr. Sequence information from the multiple CNBr fragments was obtained using 25% of the original sample. A proline was located at residue 4. The remaining 75% of the sample was used to obtain unique internal sequences, with OPA delivered to the proline at residue 4.

#### Indirect immunofluorescence

Blastema cells from 2-week blastema explants were grown in tissue culture. We used the culture conditions described by Ferretti & Brockes (1988). The cells were maintained in 60% minimal essential medium/10% fetal bovine serum at 25 °C. Cells were fixed in cold acid ethanol and were stained with the anti-(type I keratin) polyclonal rabbit antiserum 1920 (kindly provided by Dr. T. Sargent and Dr. I. Dawid, NIH, Bethesda, MD, U.S.A.). This antibody was made against a peptide from the C-terminus of the *Xenopus* XK81A type I keratin. However, the possibility of cross-reactivity to other keratin type I molecules due to sequence similarities should not be ruled out. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. The reactions were performed at 37 °C for 60 min. The slides were washed with phosphate-buffered saline for 3  $\times$  10 min.

## RESULTS

#### Specific protein synthesis in the blastema and statistical analysis

Fig. 1 shows the autoradiographs of the 2-D gel analysis of the proteins from the intact limb, 1-week regenerating limb and 2-week regenerating limb. A 1-week regenerating limb represents basically the dedifferentiation process and the onset of blastema

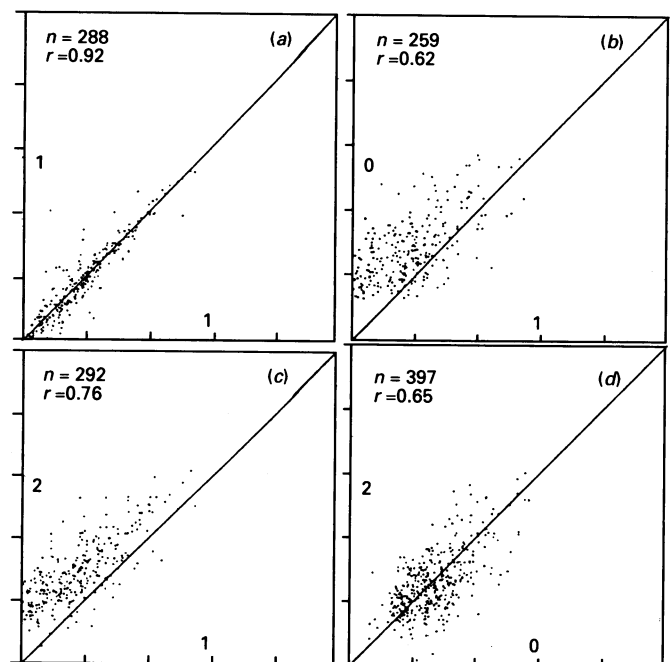


Fig. 2. Statistical comparisons of gels from intact (0), 1-week regenerating (1) and 2-week regenerating (2) limbs

$N$ , numbers of matching spots;  $r$ , correlation coefficient.

differentiation. The 2-week regenerating limb represents a well-formed blastema without apparent redifferentiation. The proteins which are synthesized differed considerably in the three different samples. Even though differences can be seen in the three autoradiographs they can only be appreciated to a certain extent; small differences cannot be revealed by visual inspection. We therefore decided to perform a detailed analysis using powerful computer software that can analyse the patterns in different gels and compare each protein in all samples (Garrels, 1989). Such an analysis can in fact map all proteins that are specific to the different stages and can give detailed information on the pattern of expression during the formation of the blastema in the early regenerating limb. In Fig. 2, for example, different runs from different samples are compared with each other. The more similar protein patterns are on a 2-D gel, the closer the matching spots (of number  $n$ ) (representing the common proteins) are to a diagonal line and the correlation coefficient ( $r$ ) is closer to 1. In

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      T   T
PAVDLGXILSDmRAQYXT 55 kDa protein
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***** ***** *  *
PGIDLNKILSDMRADYET  Xenopus keratin B2

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Fig. 3. Alignment of partial internal sequences revealed by microsequencing of the 55 kDa protein with the *Xenopus* keratin B2 sequence

In positions where two amino acids were shown in the sequencing signals, we present two letters. At position 9, T (Thr) and L (Leu) had similar signals, whereas at position 14 the sequence signal was: A (Ala) > T (Thr). m indicates methionine, but is shown in lower case to indicate weak signal. Identities are indicated by \*\* and structurally similar amino acids by \*. X is an unidentified amino acid.

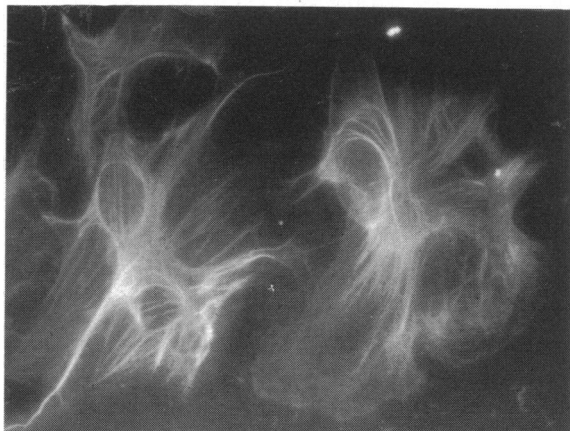


Fig. 4. Indirect immunofluorescence of blastema cells grown in tissue culture stained with antibody against type 1 keratin.

Fig. 2(a) comparison of two different runs of the same sample (here, 1-week regenerating limb) indicates a correlation coefficient of 0.92, which in turn means very similar, if not identical, patterns. However, when runs from intact limb (0), 1-week regenerating limb (1) or 2-week regenerating limb (2) are compared with each other, it can be observed that protein synthesis patterns are not similar. The correlation coefficient for comparing 0 and 1 is 0.62 (Fig. 2b), for 2 and 1 is 0.76 (Fig. 2c) and for 2 and 0 is 0.65 (Fig. 2d). This statistical analysis presents data to indicate that there are considerable differences in protein synthesis between 1-week and 2-week regenerating limbs. The reproducibility of all aspects of this 2-D gel analysis by using the QUEST program has been treated by Garrels (1989). It has been shown that in 10 replicate gels from the same lysates, the average coefficient of variation is 26.5%, which is significantly above the modal value due to the presence of spots with poor reproducibility. In addition, this analysis shows that in 1-week regenerating limbs newly synthesized proteins are rather few (as seen in Figs. 2b and 2c, bottom right, corresponding to 1 week). This analysis prompted us to examine each protein for its specific synthesis during each stage. By specific protein synthesis we mean that the proteins are synthesized only during one particular stage. Related to this, we found that 117 proteins were synthesized only in the intact limb, 26 proteins only in the 1-week blastema and 134 proteins only in the 2-week blastema. This analysis shows that blastema formation (2 weeks) is characterized by synthesis of many more proteins than during the dedifferentiation stage (1 week). These proteins are likely to be involved in the process of regeneration. Synthesis of other proteins was found not to be unique to one stage but common to any two or all three stages. In this case, 15 proteins were found to be common to the

intact limb and the 1-week blastema, 202 proteins common to the intact limb and the 2-week blastema, 53 proteins common to the 1-week and to 2-week blastema and 243 proteins common to all three stages. Taken together, these data present a detailed analysis of the newly synthesized proteins during the early regeneration of the limb in the newt. Such an analysis is very helpful in selecting a particular protein for further study. From the above patterns we can isolate protein(s) and microsequence them in order to identify factors that are involved during the process of regeneration.

#### Identification of a keratin expressed by the blastema cells

Having mapped all of the newly synthesized proteins, we decided to proceed with identification of one or them by microsequencing. The criteria for selection of a particular protein were the following. (1) The protein should be rather abundant, so that enough material could be transferred on to nylon membranes. This was ensured by staining the membrane with Coomassie Blue. If the desired spot was not well stained, it was not further pursued. (2) The protein should be specific to or highly expressed in the 2-week regenerating limb. In our initial test a number of proteins fulfilled these criteria, but we decided to work with the 55 kDa protein shown by an arrowhead in Fig. 1(c). The signal for this protein in the 2-week regenerating limb was 10 times greater than in the intact or 1-week limb, as judged from our quantification analysis. The N-terminus of this protein was blocked and we proceeded with internal sequencing (see the Materials and methods section). The partial sequences obtained are presented in Fig. 3. Homology searches of the PIR showed homologies with the *Xenopus* B2 keratin, an acidic type I keratin which has the closest sequence similarity to human keratin 14 (Miyatani *et al.*, 1986). As an additional step, we decided to examine the expression of type I keratins in the blastema by employing a polyclonal antibody directed against type I keratins of *Xenopus*. Indeed, blastema cells grown in tissue culture exhibited filamentous staining in the cytoplasm (Fig. 4).

#### DISCUSSION

Previous studies have reported 1-D analysis of protein synthesis in regenerative limbs, but resolution of 1-D gels cannot enable us to conclude differences (Donaldson *et al.*, 1974; Maden, 1988). 2-D PAGE protein analysis during formation of the early blastema has been the subject of limited work. The only detailed study was reported by Slack (1982) using the axolotl. Even though these studies were quite detailed in establishing differences in patterns between anterior, posterior and different stages of regeneration, comparison between intact and regenerating limbs was not included. Thus from these previous studies one cannot identify proteins specific to the regenerating limbs. Furthermore, quantitative and statistical analyses have not been presented in the past. We feel that the kind of analysis reported in the present paper is a prerequisite for further identification of factors involved in limb regeneration using the 2-D gel method. The statistical analysis presented here reveals general and specific details concerning patterns of gene expression during blastema formation. For example, specific protein synthesis is very limited during the first week of blastema formation. The patterns of protein synthesis in the newt are similar but not identical to the ones reported for the axolotl (Slack, 1982).

The type of analysis reported here could lead to identification of factors that are involved during regeneration. Such an analysis could lead to the isolation of structural proteins (which are rather abundant) or regulatory proteins, which are usually present in low amounts. As an initial step, we decided to sequence one of the proteins which showed interesting patterns

of expression and which was rather abundant (indicating a structural protein) in the 2-week regenerating limb. The identification of a keratin as a transiently expressed protein in the blastema is of particular interest. It has been reported previously that several keratins are expressed in the blastema. Ferretti *et al.* (1989) showed, using a panel of monoclonal antibodies to several type I and type II human keratins (different, however, from the one reported here), that some of them are expressed by the regenerating limb, but not by the developing limb, of the newt. Brockes (1989) has also suggested that keratins might be good markers of the action of the morphogen retinoic acid. Recently Ferretti *et al.* (1991) have cloned a type II keratin (NKII) from a *Notophthalmus viridescens* blastema cDNA library and have shown that its expression can be down-regulated by retinoic acid. In addition, the NKII mRNA shows greater expression in the distal blastema and less in the proximal blastema. Such results give much support to the idea that retinoic acid is a morphogen, and indicates that keratins could be good molecular markers of retinoic acid action. Such markers could play an important role in understanding the molecular mechanisms involved during limb regeneration. The identification, therefore, of a keratin homologous to *Xenopus* B2 reported in the present paper could provide additional means with which to study the action of retinoic acid.

This work was supported by a grant from the Research Council, Research Institute of University of Dayton.

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Received 30 May 1991/2 September 1991; accepted 10 September 1991

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