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Protein separation techniques in the study of tissue regeneration

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Abstract

The present report reviews the use of protein separation by means of two-dimensional gel electrophoresis in the study of tissue regeneration. It is shown that such an approach can provide data on protein synthesis in different stages of limb regeneration or comparative data with other regenerative processes such as tail and lens regeneration. Such an approach is more realistic than other methods employing gene cloning or generation of antibodies and can lead to the actual identification and characterization of factors that are involved in these phenomena.

1. Introduction

Gene regulation underlying developmental events is a complex network involving many different factors. The characterization of these factors is essential in our quest for understanding the basic mechanisms of development. Several methods have been developed during the past 20 years to identify and characterize these factors. These methods included the development of monoclonal antibodies, gene cloning and protein electrophoresis. Especially with the development of two-dimensional (2D) analysis of proteins, scientists were provided with a method that was more economical and less time consuming than the other methods. The separation techniques based on the 2D gel electrophoresis led to the development of databases where the expression of proteins could be monitored. For example, if somebody wanted to analyze protein synthesis in different stages of development or during differentiation, protein databases from the different stages can be constructed. From them protein expression can be studied and specific proteins can be pinpointed with interesting expression patterns. These proteins can then be isolated from preparative gels and partial sequences obtained. The sequences could then be used to construct oligonucleotide probes and the encoding gene isolated. The advantage of 2D electrophoresis as a separation technique is that a particular protein can be in fact seen in the different gels. Virtually expression of hundreds of proteins can be at once known and compared from the 2D gels. Such a reliability is not provided with differential screening of libraries or monoclonal antibodies. In addition the development of protein separation by 2D gels circumvented the problems from the regular one-dimensional gel electrophoresis. In our laboratory we have employed this method to study gene expression during tissue regeneration in salamanders. This report aims to draw attention to the usefulness of this method in the field of

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regeneration and likewise in other developmental phenomena.

The remarkable regenerative processes in amphibia are most pronounced in the adult newt which is capable of regenerating the limbs, the tail and the lens. These processes are initiated by dedifferentiation of the terminally differentiated intact tissues. In the case of limb and tail the dedifferentiation leads to the formation of the blastema which upon redifferentiation rebuilds the missing parts [1]. After lentectomy the pigmented epithelium dedifferentiates and by transdifferentiation produces the new lens [2]. In all cases the process of dedifferentiation is the unique step. Tissue dedifferentiation is characterized by morphological changes and by matrix remodelling. Such a phenomenon is likely to involve specific synthesis or breakdown of extracellular matrix molecules. Support for such alterations has been provided in studies where specific monoclonal antibodies have been produced. For example the monoclonal antibody (mAb) ST1 cross-reacts with an antigen which disappears as blastema is formed [3]. The same is the case with another antigen which disappears during lens regeneration only in the dorsal iris but not in the ventral iris and in the limb blastema [4,5]. In fact, Eguchi [2] has shown that the disappearance of this antigen not only is necessary but it is sufficient to induce lens regeneration. In experiments where ventral iris was treated with the mAb lens differentiation occurred. The identification, therefore, of such antigens is imperative in order to generate tools to study and apply the phenomenon of regeneration.

In the past, a systematic study has been performed and a 2D gel protein database has been generated from the intact and regenerating limb of the adult newt [6,7]. Such a database can help pinpoint the different proteins that are specific for the different stages of regeneration. Since dedifferentiation is a unique process involved in all regenerative processes of the newt the studies were extended on protein synthesis in tail and lens regeneration. This could enable us to search for common patterns in all types of regeneration and to narrow down the proteins of importance. Since the extracellular matrix is involved in tissue remodelling during dedifferentiation and since the proteins of the matrix are usually abundant this experimental approach should be suitable in resolving such differences.

2. Experimental

2.1. Animals

Adult newts *Notophthalmus viridescens* used in this study were purchased from Amphibia of North America (C. Sullivan). In some experiments the animals were injected with [^{35}S]methionine and the proteins analyzed by autoradiography. This was the choice for generating the databases. In other series the proteins were isolated and run on preparative gels to enable us isolation and sequencing of the proteins.

2.2. Protein isolation

Tissues used in our studies were: intact limbs, tails, regenerating limb (1-week and 2-week blastema), regenerating tail (2-week blastema) and dorsal and ventral iris (six days after lentectomy). The tissues were placed in osmotic lysis buffer [10 m*M* Tris, pH 7.4 and 0.3% sodium dodecyl sulfate (SDS)]. The samples were freeze-thawed twice before 1/10 of 10 × nuclease solution was added (50 m*M* $MgCl_2$, 100 m*M* Tris pH 7.0, 500 μg/ml RNase from Sigma R5125 and 1000 μg/ml DNase from Sigma D4527). The nucleases were allowed to react for 15 min. Next an equal amount of SDS boiling buffer (5% SDS, 5% β-mercaptoethanol, 10% glycerol and 60 m*M* Tris pH 6.8) was added and the samples were boiled in a water bath for 15 min. The samples were then cooled, the undissolved material removed and stored in −70°C until use. All solutions were purchased from Kendrick Labs. (Madison, WI, USA). Protein determination was performed by taking an aliquot before the addition of the nuclease solution using the BCA total protein assay from Pierce.

2.3. 2D Electrophoresis

This was performed according to the method of O'Farrell [8] as follows. Glass tubes of 2.0 mm inside diameter were prefocused using 2.0% pH 4–8 ampholines (BDH, Poole, UK) for 1 h at 300 V and 2 h at 600 V with 30 μl of urea buffer. Equal amount of samples (150 μg) was loaded and isoelectric focusing (IEF) was carried out for 11.6 h at 700 V. A 1-μg amount of an IEF internal standard, tropomyosin, M_r 32 700 (relative molecular mass) and p*I* 5.2 was added to one of the samples (shown by arrows in Fig. 4A–D and F). The final tube gel pH gradient usually extended from pH 4.1 to 8.1 as measured by a surface pH electrode (Bio-Rad) and colored acetylated cytochrome p*I* markers (Calbiochem-Behring, La Jolla, CA, USA) run in an adjacent tube. After equilibration for 10 min in SDS sample (10% glycerol, 50 m*M* dithiothreitol, 2.3% SDS and 62.5 m*M* Tris pH 6.8) the tube gel was sealed to the top of a two day aged and prerun 12.5% acrylamide slab gel (0.75 mm thick). To the agarose that sealed the tube gel to the slab gel M_r standards (Sigma) were added. The slab gel had been prerun for about 2 h at 12.5 mA/gel with 0.07% thioglycolic acid in the upper chamber. After addition of the tube gels the slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel with 0.07% thioglycolic acid. The electrophoresis was stopped when the bromophenol blue dye front had reached the bottom of the slab gels. The gels were next placed in 0.1% Coomassie Brilliant Blue R250, 50% methanol for 10 min, then destained in two rinses of 50% methanol for 10 min and twice in distilled water for 5 min each, and they were air dried. When radioactivity was used the gels were processed at −70°C. The procedure for electrophoresis was virtually the same and according to Garrels [9]. The gels were digitized and compared with each other in order to quantitate expression of proteins. Quantification and statistical analyses were performed using the PDQUEST software (Protein Databases). According to this procedure, spot quantification and resolution of overlapping spots is performed by 2D 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 in each pattern can be matched and that fewer than 1% of the spots are matched inconsistently. The reproducibility of all aspects of this 2D analysis by using the QUEST program (that is the matching of the spots from different runs) has been treated by Garrels [9].

2.4. Blotting and microsequence analysis

For transfer to poly(vinylpyrrolidine difluoride) (PVDF) membranes, 150 μg of protein were used per gel. Transfer to the membranes was performed by the method of Towbin et al. [10]. 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 synthesis was obtained on an Applied Biosystems (ABI) pulsed liquid phase sequencer (477A) with on-line phenylthiohydantoin amino acid analysis provided by an ABI 120A analyzer. Internal sequences were obtained using the CNBr/*o*-phthalaldehyde (OPA) strategy at the Biotechnology Center, University of Wisconsin, Madison, WI, USA. After the initial sequencing run revealed no information, the PVDF membrane was treated with CNBr. Sequence information from the multiple CNBr fragments was obtained using 25% of the original sample. The remaining of the sample was used to obtain unique internal sequences.

3. Results and discussion

3.1. Comparative protein synthesis

About 800 proteins were resolved in experiments using protein samples from the intact and regenerating limb (Fig. 1). By matching the digitized images from gels representing protein synthesis we were able to compare synthesis in the intact limb in the 1-week limb, representing the dedifferentiation stage and in the 2-week

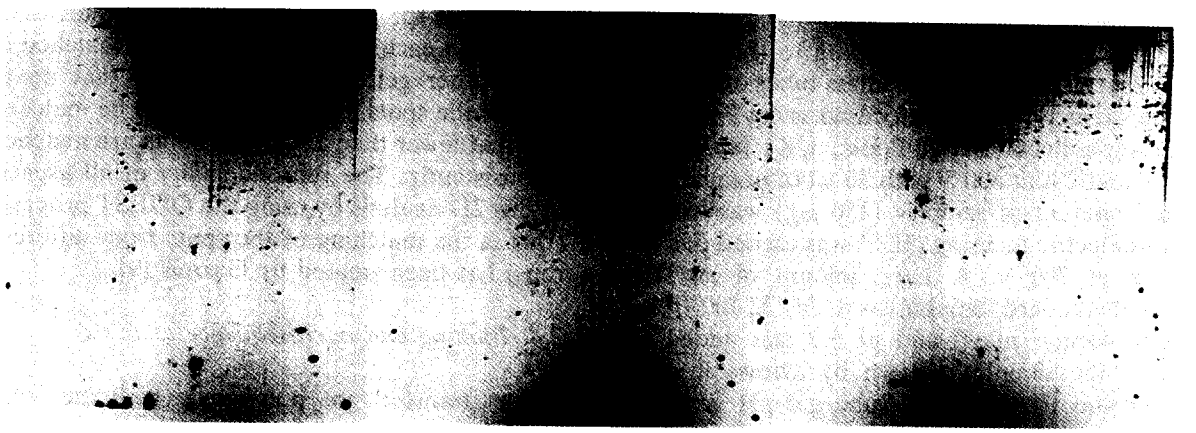

Fig. 1. 2D Gels (autoradiograms) of 2-week regenerating (left), 1-week regenerating (middle) and intact (right) limbs. The pH is linear from 4 on the left to 6.8 on the right.

limb, representing the accumulation of blastema. Fig. 2 shows these results for the different stages. As can be seen in Fig. 2 the dedifferentiation stage is marked by the disappearance or down-regulation of many proteins, while blastema formation is marked by the specific protein synthesis of more than a 100 proteins. In Fig. 3 a sample of the database is shown with a cutoff dpm value of 37. The proteins have been given a number which is then used in comparative studies.

In order to use this database and results for

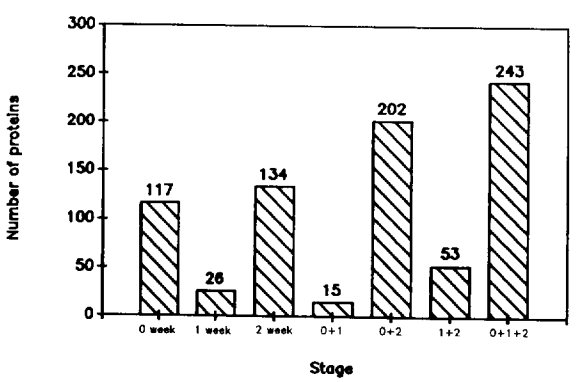

Fig. 2. Specific protein synthesis in different stages of limb regeneration. The numbers over the bars indicate the number of proteins found to be expressed only in the particular stage or stages. 0 is intact limb, 1 is 1-week regenerate, 2 is 2-week regenerate, 0 + 1, 0 + 2, 1 + 2 and 0 + 1 + 2 are combinations of the different stages.

meaningful applications in regeneration biochemistry we were interested to examine whether or not similar patterns can be observed during the other regenerative processes such as tail or lens regeneration. As can be seen in Fig. 4 there are patterns of protein synthesis common to the different regenerating tissues. Protein 1 shown in Fig. 4A, C and E by a small arrowhead is present in intact tail, intact limb and ventral iris (non-regenerating tissues) but is absent in all regenerating tissues (tail blastema, Fig. 4B; limb blastema, Fig. 4D; dorsal iris six days after lentectomy, Fig. 4F). Other common patterns can be seen in regenerating limb and tail. Proteins 2 and 3 shown by small arrowheads in Fig. 4A and C are present only in the intact tail and limb, but disappear from the regenerating blastema. On the other hand there are proteins that appear specifically in the regenerating tissues. Proteins 1, 2 and 3 shown by big arrowheads in Fig. 4B, D and F are specifically expressed during regeneration. Protein 1 appears in all, while proteins 2 and 3 appear in regenerating blastema of the tail and limb. As judged from the already established database for the regenerating limb [6,7], protein 1 is a keratin (see below). A group of low-molecular-mass acidic proteins present in the intact limb and tail disappear during regeneration (not shown). The nature of these proteins is unknown.

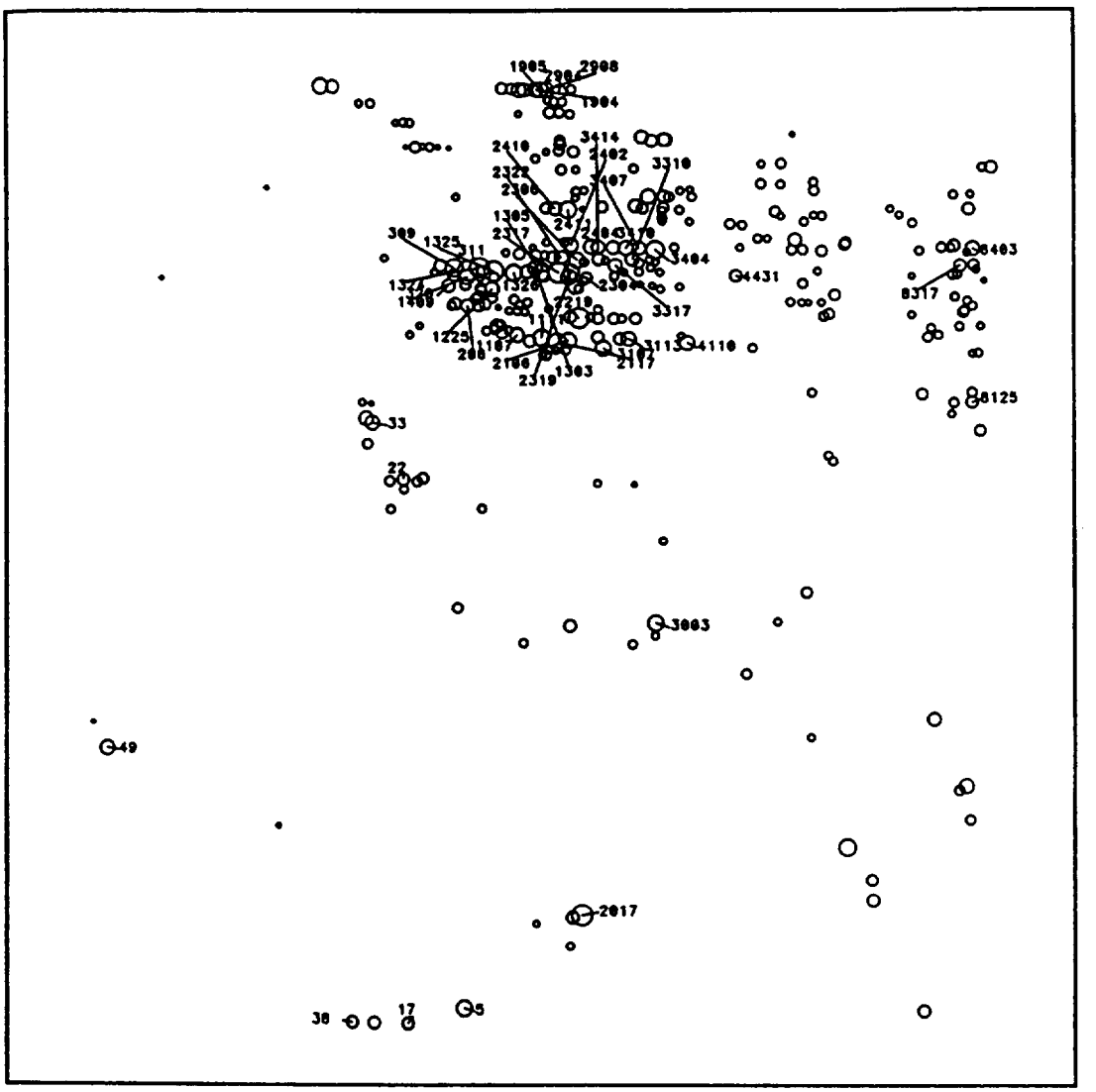

Fig. 3. Computer printout from digitized gels (from Fig. 1) with a dpm cutoff of 37 (dpm = disintegrations per minute, a value derived from the autoradiograms and serves as a quantitation). All spots with a value of 37 and less are not presented in the map; 300 polypeptides are presented. Numbers indicate the identification in the database.

3.2. Protein characterization

An analysis such as the one presented in the present paper pinpointed some proteins whose expression might be of vital importance to the field of regeneration. Since dedifferentiation involves remodelling of the extracellular matrix, disappearance and appearance of proteins is expected. If these proteins are known and their role studied they can prove valuable tools to probe regenerative processes. For example the 2NI-36 antibody recognizes an antigen that disappears from the dorsal iris and from the limb upon regeneration [4,5]. When ventral iris was treated with the antibody it was able to dedifferentiate and regenerate an additional lens [2]. Such experiment could open the way for experimental regeneration from tissues which

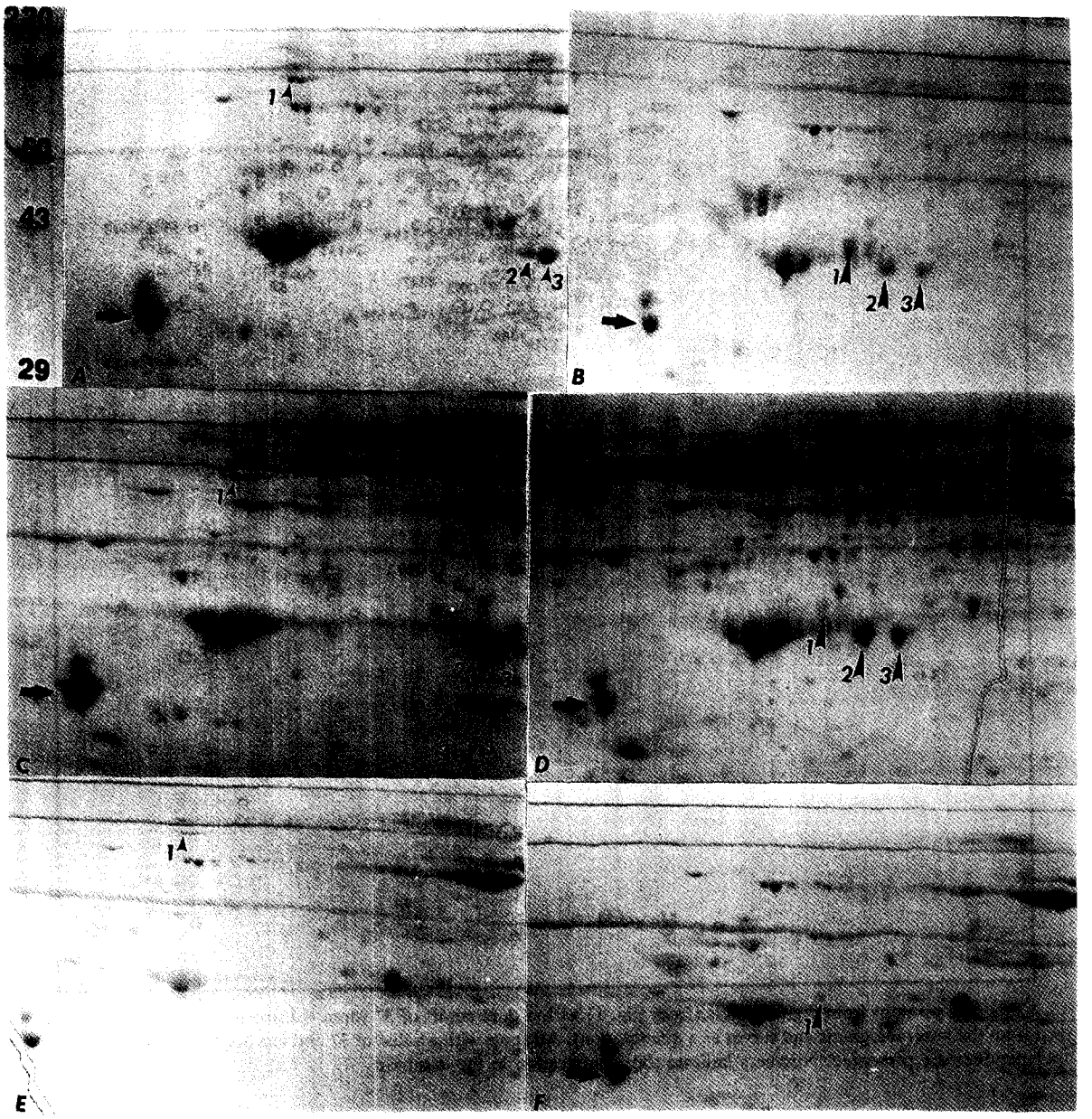

Fig. 4. 2D Gel electrophoresis analysis of protein synthesis in newts undergoing regeneration. (A, C, E) Protein synthesis in intact tail, intact limb and 6-day (post-lentectomy) ventral iris respectively; (B, D, F) protein synthesis in the 2-week tail blastema, 2-week limb blastema and 6-day (post-lentectomy) dorsal iris. Proteins marked with small arrowheads disappear during regeneration. Protein 1 disappears from all tissues and proteins 2 and 3 disappear from tail and limb blastema. Proteins marked by big arrowheads appear during regeneration. Protein 1 appears in all (this is a keratin), proteins 2 and 3 appear during tail and limb regeneration. The horizontal lines are relative molecular masses as indicated ($\times 10^{-3}$) in the upper left part of the panel. The following proteins (Sigma, St. Louis, MO, USA) were added as molecular mass standards to the agarose which sealed the tube gel to the slab gel: myosin (220 000), phosphorylase A (94 000), catalase (60 000), actin (43 000) and carbonic anhydrase (29 000). The arrow in A, B, C, D and F indicates an internal standard (tropomyosin, p*I* 5.2, M_r 32 700). The pH ranges from 4 to 8 left to right.

lost their capacity and this could have enormous applications in medicine. The identification, therefore, of these proteins is of paramount importance. We decided to proceed with sequencing of protein 1 (big arrowhead, Fig. 4B, D, F) which is upregulated during regeneration and with protein 1 (small arrow, Fig. 4A, C, E) which disappears from all tissues undergoing regeneration. The N-terminus of both proteins appeared to be blocked, therefore, we received internal sequences. The upregulated protein is a keratin of sequence: PAVDLGXIL(T)SDMR-A(T)QYXT which is highly homologous to Xenopus keratin B2 (or closest to human keratin 14, type I). Due to the little amount of the protein that is lost during regeneration we were able to receive a partial sequence of only 8 amino acids. The sequence was TEV-LLA(I)L(S,Y,G)V. This sequence is highly homologous to the sequence TEVLAAVI found in a human M_r 90 000 heparan sulfate proteoglycan [11]. Interestingly, protein 1 is also of $M_r \approx$ 90 000. This information implies that the protein that disappears from all regenerating tissues could be a proteoglycan, which is a component of the extracellular matrix. This in turn strengthens the importance of the extracellular matrix and its degradation during regeneration. In fact sequential disappearance of proteoglycans has been reported during lens regeneration [12]. This information can now be used to study proteoglycans in regenerating and intact tissues.

Separation techniques involving 2D gel electrophoresis have indicated that this is a reliable and accurate method for identifying factors that are involved in development and differentiation. Analysis of more proteins which were shown in the present study to be implicated in the different regenerative processes could provide the necessary molecular tools in order to probe the process of dedifferentiation and regeneration in newts.

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