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Original Article

Nerve-dependent expression of c-myc protein during forelimb regeneration of *Xenopus laevis* froglets

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ABSTRACT The consequences of denervation on the expression of c-myc protein have been analyzed on the regenerating forelimb of young froglets of *Xenopus laevis*. The level of c-myc expression, low in control limbs and enhanced in the regenerate, is transiently increased after a three-hour total denervation. For this protein, the level of expression is not a function of the quantity of nerve in the regenerate. Four days after denervation, c-myc signal is back to its base level observed in the regenerate. A different pattern of expression is obtained for an S phase marker (PCNA protein) taken as a control in the same experimental conditions. The data presented here show that the nervous system normally exerts a negative control on the expression of c-myc and PCNA proteins in the limb regenerate of *Xenopus*.

KEY WORDS: *c-myc protein, PCNA protein, forelimb regeneration, Xenopus*

Introduction

Forelimb amputation at the level of the radius-ulna (zeugopodium) in young post-metamorphic *Xenopus* froglets is followed by the formation of a pattern-deficient as digit-free elongated spike (Korneluk and Liversage, 1984). The overall growth of the «regenerate» is nevertheless controlled by the presence of an intact innervation which supplies to the cells «signal» molecules controlling their progress through the cell cycle. Indeed, one of the consequences of denervation in an epimorphic limb blastema is that it leads to a decrease in the radioactive thymidine labeling index and mitotic index after a transient increase. Others effects have been reported. In a limb, besides loss of sensitivity and paralysis, denervation provokes muscle atrophy and remodeling of the synaptic junction organization at the morphologic and molecular levels. In addition, during limb regeneration, cutting the axons prevents the further elongation of the regenerate, at least in *Xenopus* (Liversage *et al.*, 1987). In the newt, later stages of limb regeneration are not so drastically nerve-dependent. (Wallace, 1981). This nerve dependence of the limb regeneration phenomenon is not limited to Amphibians, since fish fins (Géraudie and Singer, 1979) present similar features. In invertebrates, regeneration of the caudal metameres of annelid worm or flatworm eyes depends on the neural network (Goss, 1969), and in coelenterates, a neuropeptide is implicated in the cell division of interstitial cells responsible for the regeneration of the ablated head.

The nervous tissue is the site of the synthesis of numerous mitogenic growth factors. In any system capable of regeneration, the molecular nature of the «growth» factor(s) delivered by the nerves has not yet been defined. Most of the studies have been carried out basically during urodele limb regeneration. Singer (review in 1978) demonstrated from his classical quantitative studies on nerve density in the forelimb that the growth of the early regenerate cells requires the presence of an adequate number of axons of motor or sensory origin which release a polypeptide, the «neurotrophic factor» or NTF. This NTF would affect «the rate of ongoing events in the cells and not the quality and kind of events». Singer considered the NTF as «one of the many «conversational» peptides, including nerve growth factor (NGF) and epidermal growth factor (EGF), which function to alter the absolute rate of ongoing cellular events». The neural peptide, substance P, is also a potent mitogen for the cultured newt blastemas (Globus *et al.*, 1983). Another possible candidate could be the Glial Growth Factor (GGF) or a related molecule as proposed by Brockes (1987), who accumulated evidence in favor of it (Brockes and Kintner, 1986) although the decisive experiment — the abolition of nerve-dependent cell proliferation of the blastema by an anti-GGF antibody — has not yet proved possible. Recently, a nonregulatory growth factor, transferrin, a plasma protein specialized in the delivery of iron to the cells for which it controls the cell cycle *in vitro*, has been found abundantly in axons and Schwann cells of regenerating nerves of limbs axolotls (Kiffmeyer *et al.*, 1991). The relevance of these last results to the influence of nerves during limb regeneration is still under study.

During limb regeneration, there are indications that these putative molecules of neural origin are axonally transported although GGF could be directly synthesized by the Schwann cells, released

Abbreviations used in this paper: PCNA, Proliferating Cell Nuclear Antigen.

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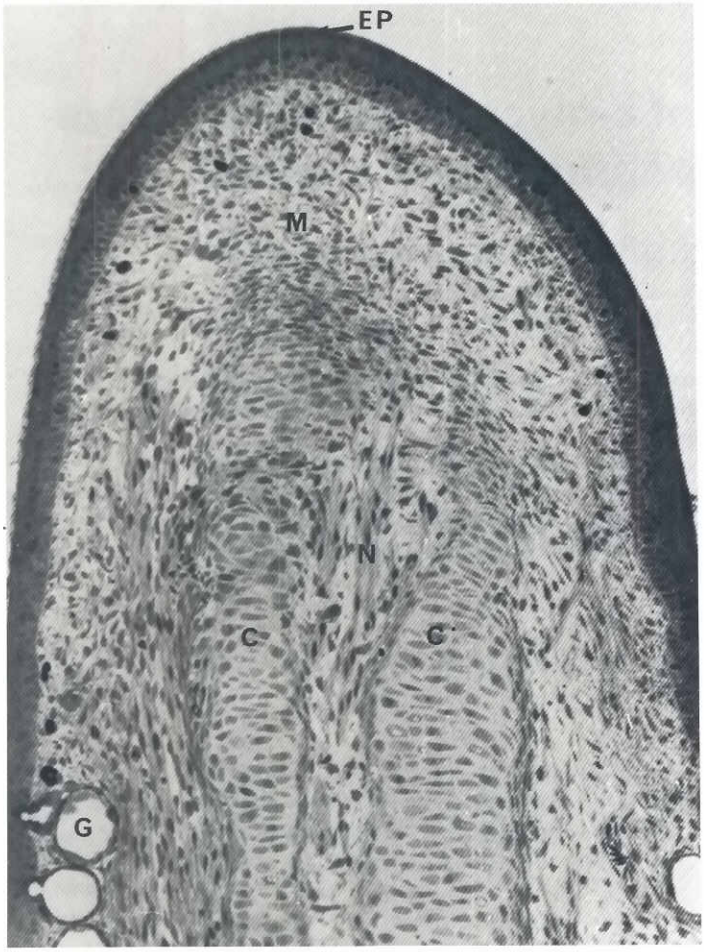

Fig. 1. Longitudinal section of the distal region of the Xenopus limb regenerate. *Note the two cartilaginous rods (C) featuring the regenerating zeugopodium skeleton beneath the apical mesenchyme (M) of the regenerate, the nerve bundles in their vicinity (N). The skin comprises the stratified wound epidermis (EP) and the glands in the process of differentiation into a proximo-distal direction (1 μm thick Epon section stained with toluidine blue. x200.*

within the regenerate where targets are the epidermal and blastemal cells as well as probably the axons themselves. From these data and others, it should be expected that more than one molecule of neural origin can act on the blastemal cells and their influence is above all most critical for limb regeneration. Molecules of non-neural origin have always been considered active on limb blastema. Factors such as hormones and the wound epidermis itself probably interact to control synergistically the fate of the regenerate cells (see Wallace, 1981).

As cell proliferation and differentiation in the limb regenerate involve a multifactorial cascade of molecular events, we were interested during limb regeneration in the consequences of denervation upon c-myc proto-oncogene expression. This proto-oncogene encodes a nuclear phosphoprotein involved in cell proliferation and cell differentiation of many cell types (review in Cole, 1986; Lüscher and Eisenman, 1990), events met simultaneously in the *Xenopus* limb regenerative outgrowth. Although the function of the c-myc protein is still elusive, its participation both as a transcription factor and a replication factor has been suggested (Iguchi-Ariga *et al.*, 1987; Kaddurah-Daouk *et al.*, 1987). The protein contains several sequence elements, such as a helix-loop-helix motif, a leucine zipper motif known to participate in protein-protein interactions and a basic motif which confers to it the ability to bind to a specific DNA sequence. The interaction with another helix-loop-helix protein termed c-Max has recently been demonstrated (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991), as well as the ability for c-myc protein to associate with the tumor suppressor protein Rb (Rustgi-Anil *et al.*, 1991).

We previously demonstrated in the forelimb regenerate that the proto-oncogene mRNA c-myc was overexpressed (Géraudie *et al.*, 1990), and in this present work we analyzed the expression of c-myc at the protein level during the process of forelimb regeneration in *Xenopus* in normal conditions of growth and after denervation in different experimental conditions. The expression of the proliferating cell nuclear antigen (PCNA) was studied in parallel as PCNA is considered to be a marker of cell proliferation. PCNA is a subunit of the DNA-polymerase δ which is involved in DNA replication. Its rate of mRNA and protein synthesis is known to increase during the S phase of the cell cycle (Bravo and Celis, 1980; Bravo and MacDonald-Bravo, 1985; Tan *et al.*, 1986; Almandral *et al.*, 1987).

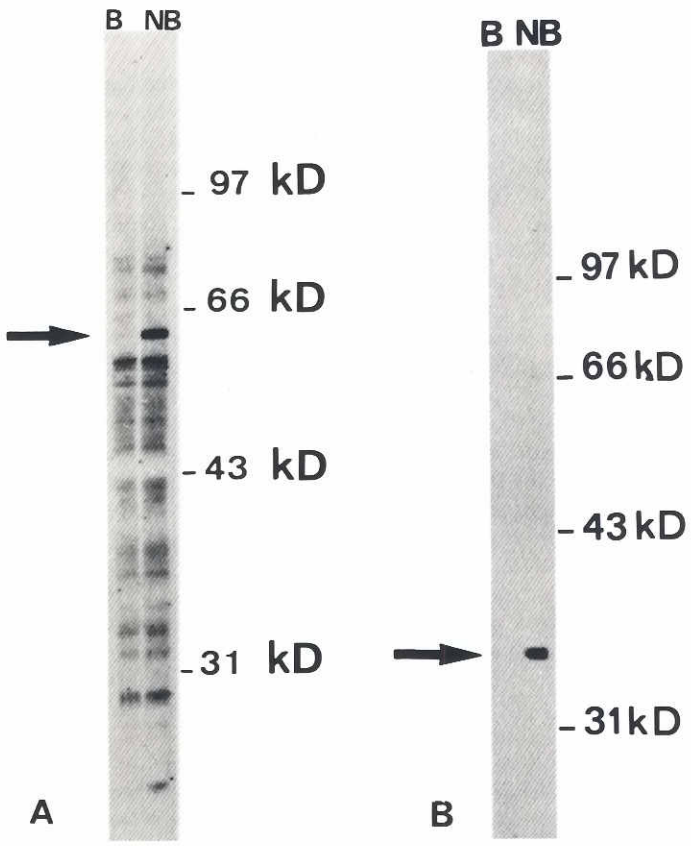

Fig. 2. Blockage experiments applied respectively to c-myc (A) and PCNA (B) proteins. (B) *Blockage by presence of an excess of protein. NB: no blockage = control lane.*

Results

Structure of the regenerate

At the time of harvest, the regenerate is composed of an axial skeletal rod made of hyaline cartilage surrounded by a thick layer of chondroblasts. A few regenerated myofibers are present only in the third proximal region of the spike (Fig. 1). Capillaries and regenerated bundles of axons are abundant throughout its length. Towards the apex, under the wound epidermis devoid of differentiated epidermal glands, there persists a population of undifferentiated mesenchymal cells forming an apical growth zone. Mitoses are occasionally observed in the epidermal cells as well as in the chondroblasts.

Expression of c-myc protein after axotomy

Verification of the specificity of the two antibodies used in this work was achieved by two blockage experiments depicted in Fig. 2A and B for anti c-myc antibody and anti-PCNA antibody, respectively.

We found that c-myc protein was expressed at a low level within the tissue of the control limb (lane 1 in Fig. 3A). This level of expression increased 5-fold in the regenerate (lane 2). A 3-hour total denervation (lane 3) yielded to a stronger signal than in its control regenerate (lane 2) and a 2-fold increase was further observed. Consequently, an accumulation of c-myc protein occurred after nerve resection. A partial denervation (lane 4) gave a signal similar to the control. The increase in c-myc protein level was observed transiently as the amount of c-myc protein fell down to its basal level 4 days post-denervation. Data are summarized in the histogram of Fig. 2B. It appears that the expression of c-myc protein is under the control of the peripheral nervous system in the forelimb regenerate.

Specific features of an S phase marker: PCNA protein

Results of PCNA protein expression in similar experimental conditions are depicted in Fig. 4A and B. The profile of PCNA expression in the same experimental conditions was significantly different from that of c-myc protein. The level of PCNA was low in the control limbs and the regenerates. After a 3 hour, total or partial denervation, the signal was enhanced in the same proportions as an 8-fold increase was observed in both cases, whatever the quantity of nerves present in the regenerate. This strong signal persisted for 4 days. These results suggest a different pattern of regulation of the expression of c-myc and PCNA proteins by the nervous system.

The results of the expression of alpha-enolase protein taken as a «housekeeping» protein in the same experimental conditions as for c-myc and PCNA are depicted in Fig. 5A and B. The high signal, observed especially in lane 1 indicates that the signals of low intensity observed for c-myc and PCNA proteins are not related to a lack of deposition of proteins in these lanes. This data is in agreement with the homogeneous staining of equivalent protein band levels on the blot used here and stained with Ponceau Red (not shown). The level of expression of this enzyme is also under the control of the nervous system in the regenerate cells (lanes 3 and 5). This suggests alterations of the glycolytic metabolism in the denervated regenerate cells, as foreseen by Schmidt (1968). As in the case of PCNA protein, only total nerve resection leads to an accumulation of this protein. The accumulation of alpha-enolase in the denervated regenerate increases with time. Four days after total denervation, it reaches the same level of expression as for normal control non-amputated limbs.

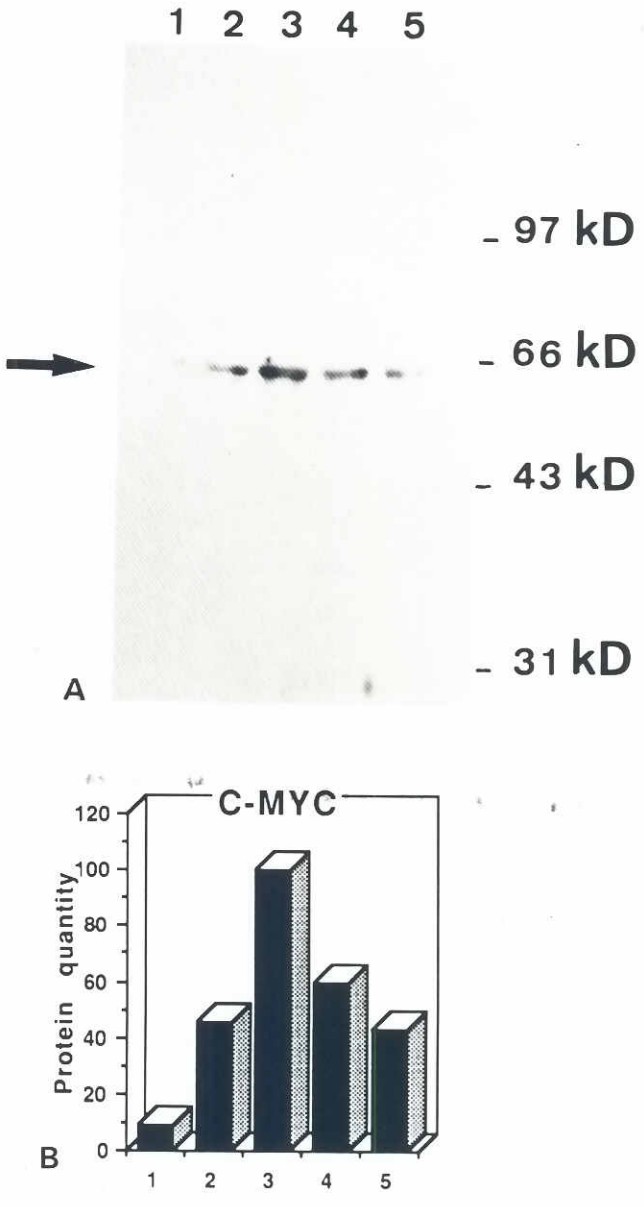

Fig. 3. Western blot (A) and histogram (B) showin the expression of c-myc protein different experimental conditions. (A) *Western blot. Lane 1: control limb. Lane 2: one month-old regenerate. Lane 3: three h total denervation of the 1-month-old regenerate. Lane 4: three h partial denervation. Lane 5: Four-day total denervation. MW markers are indicated. C-myc protein is a 60 kDa protein.* **(B)** *Histogram drawn from densitometry results. Note the transient accumulation of c-myc protein 3 h after the total denervation of the regenerate at the level of the stump. A partial denervation is not considered to yield to any protein accumulation.*

Discussion

The present work shows that the denervation of the limb regenerative outgrowth interacts with the metabolism of the three proteins studied. Within a mere 3 hours following the disturbance of the distribution of the «factors» of neural origin by axotomy, these

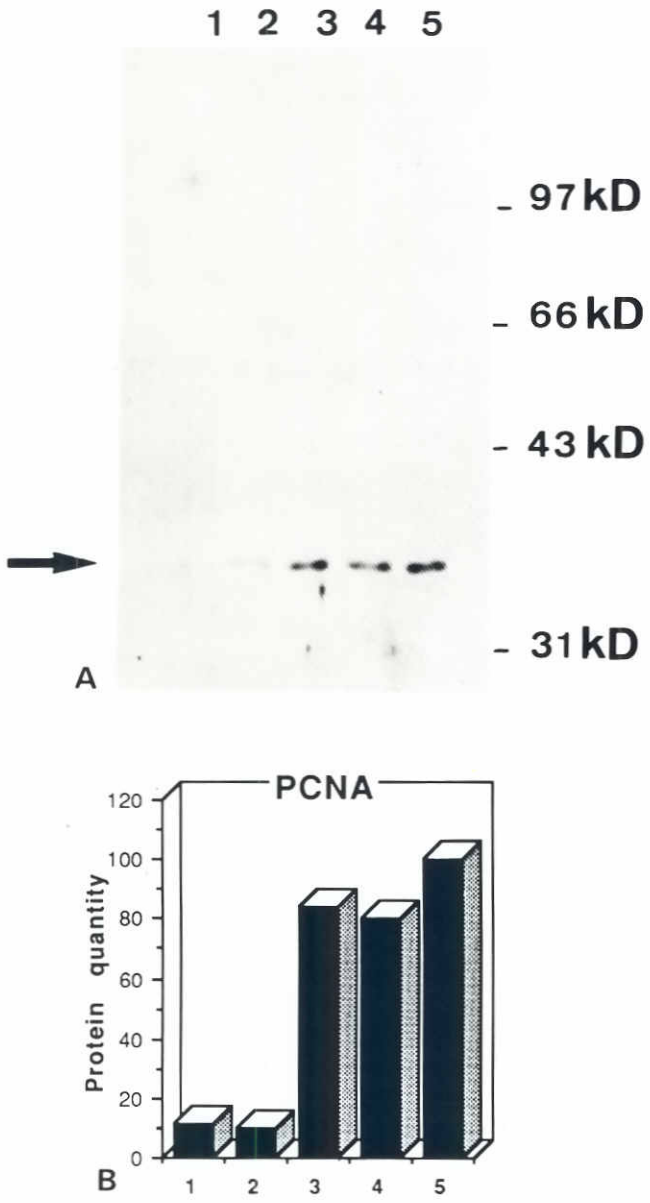

Fig. 4. Same procedure as above applied to PCNA protein (MW: 36 kDa). *Note that, contrary to c-myc protein, PCNA accumulates in the totally or partially denervated regenerate for 3 h within the same range. This accumulation persists for 4 days with a slight increase.*

three proteins accumulate in the tissues of the regenerate. Indeed, after a short period of denervation, their level is always higher in the denervated regenerate than in the control non-denervated one. Four days after denervation, the patterns of protein accumulation varied according to the specific proteins studied. This indicates that the neurotrophic molecule(s) interact differently according to their target molecules, which have all their specific control mechanisms.

The accumulation of the three proteins studied after a 3 hour denervation is not in proportion to the quantity of axons present in the regenerate or presumably to the release of molecules of neural origin. The resection of only one nerve branch of the two present in the forelimb does not induce an intermediate accumulation of the proteins within the regenerate cells.

Nevertheless it is obvious that axotomy induces in the regenerate a deregulation of the mechanisms of protein synthesis and/or degradation. Our experiments demonstrate that a short-period denervation yields an accumulation of c-myc, PCNA and alpha-enolase proteins in the limb regenerate, although they are not necessarily newly synthesized. Indeed, the factor(s) of neural origin could interact according to different pathways: either at a transcriptional or translational level; however, they could also act on the stability of the gene products: mRNA or protein. Previous data dealing with the study of the protein turnover in the newt regenerate showed that it was the absolute rate of protein synthesis that was affected by denervation (Singer and Ilan, 1977). In the early blastema of the forelimb of the newt *Notophthalmus* (Singer and Caston, 1972; Géraudie and Singer, 1978), total denervation of the regenerating limb is followed by a transient increase of aminoacid incorporation in newly synthesized proteins. This initial outburst is followed within 48 hours by a decline to a basal level estimated at 60% of its initial value. In late-stage regenerates, where cell differentiation is taking place and thus resembles the *Xenopus* regenerative outgrowth, Dresden (1969) observed a decline in aminoacid incorporation after denervation. Similar data assessing the fate of aminoacids in the sum of proteins neo-synthesized after denervation are not available on *Xenopus* forelimb outgrowth, but this conclusion may apply to some proteins present in the *Xenopus* regenerate or it may only be valid for the early newt limb blastema. In any case, it should be kept in mind that a limb regenerate is composed of different cell populations that may not react in the same fashion to the neural influence. In *Xenopus* forelimb outgrowth, cell proliferation coexists with cell differentiation. All cell types may not be affected by the effects of axotomy. Besides, target cells may very well behave differently under the influence of the neurotrophic factor(s) and secondarily influence the metabolism of other cells. Consequently, here, as in the early newt limb blastema where subpopulations of cells have been uncovered (review in Fekete *et al.*, 1987), we are dealing with with an imbricated model. Its growth nevertheless has the advantage of being controlled by an identified source which is the peripheral nervous tissue components: axons and glial cells.

The level of c-myc protein in the regenerate is higher than in the control limb. This data confirms the enhanced expression of c-myc gene we described at the level of mRNA (Géraudie *et al.*, 1990). *In situ* hybridization showed that the majority of c-myc transcripts in the regenerate were localized to the wound epidermis and some of the mesenchymal cells of the apex. A total denervation of the regenerate made at the level of its stump for 3 hours yields an important transient signal increase of c-myc protein which can be considered as a consequence of an accumulation of c-myc either in all the cells of the regenerate or only in a subpopulation of cells. This transient accumulation of c-myc protein could be due to a stabilization of the c-myc mRNA. This possibility has been proposed during regeneration of the kidney in the mouse where an increased expression of c-myc was correlated with a control of mRNA stability (in Spencer and Groudine, 1991). Preliminary studies of c-myc mRNA expression by *in situ hybridization* do not favor the possibility of the persistence of c-myc mRNA in the denervated regenerates, as the quantity of c-myc transcripts present in the denervated regenerates is very low compared to the controls (Hourdry, unpub-

lished results). So the hypothesis that the neural molecule(s) would act on the stability of c-myc protein, probably on post-translational mechanisms, can be proposed. It is to be noted that a disparity between the level of expression of c-myc RNA and protein has already been reported in the case of mouse erythroleukemia cells (in Spencer and Groudine, 1991). It is accepted that high levels of c-myc protein are not compatible with cell survival (Pallavicini *et al.*, 1990; Evan *et al.*, 1992). We observed that the level of c-myc protein which increases transiently as a consequence of axotomy soon returns to its base level, compatible with cell life found in the regenerate control. We also demonstrated that there is no direct relation between the quantity of nerves present in the regenerate and the accumulation of the c-myc protein. The reduced quantity of molecules of neural origin available in the regenerative outgrowth and delivered by the *radialis* nerve as we resected only the *ulnearis* branch of the *brachialis* nerve is apparently sufficient to maintain a basal level of c-myc protein. On the other hand, in the early stages of growth of the newt limb blastema, the incorporation of radioactive aminoacids in the bulk of newly synthesized proteins was in direct proportion with the quantity of innervation in the early regenerate (Lebowitz and Singer, 1970).

The metabolism of PCNA protein is most sensitive to neural influence. Whatever the amount of nerves left in the regenerate, the normal mechanism of PCNA regulation maintaining a low level of PCNA in the limbs is disturbed so that the post-denervation signal observed is similarly and rapidly high. Besides, it persists for at least 4 days. This is not the case for c-myc and alpha-enolase proteins, where only a total denervation induces their accumulation. Consequently, an «all-or-none event» response is observed for PCNA only. A 3 hour total or partial denervation of the forelimb regenerate induces a full response.

For PCNA and alpha-enolase proteins, the accumulation observed is not transient, since in the regenerate deprived of nerves for 4 days, the level of the signals is much higher than in the control non-denervated regenerate. Knowing the accepted function of PCNA as a cell marker of the S phase, its accumulation consecutive to the disturbance of the neural network could indicate that in the regenerate a cell population has entered in the cell cycle as a consequence of denervation.

This interpretation is consistent with the results of c-myc expression because a high number of the gene products has been correlated with the G0-G1 transition of the cell cycle. Four days after nerve resection, the lack of decrease in the quantity of PCNA observed 3 hours post denervation could indicate that the cells involved would reach the G2 phase but be blocked at this stage. Indeed, as the PCNA protein quantity is increased 2- or 3-fold during the transition G1-S, a decrease is observed in the same ratio during the S-G2 transition (Bravo and Celis, 1980; Morris and Mathews, 1989). It is to be noted that in the case of the denervated blastema of urodele epimorphic limb regenerate, such a determination of the phase of the cell cycle in which cells are arrested as a consequence of denervation was not really conclusive (Tassava *et al.*, 1987).

In conclusion, the data collected here show that, in the regenerative forelimb outgrowth of *Xenopus*, the putative factor(s) delivered by the nervous system exerts a *negative control* on the expression of the proteins studied here. It probably acts at the *translational level*, especially for c-myc, and rather like a signal as the accumulation of the protein studied 3 hours after denervation is not in proportion to the quantity of axons present in the regenerate. We can finally add that the pattern of c-myc and PCNA proteins observed 3 hours post-denervation indicates that certainly a subpopulation of cells, present in the regenerate and blocked in a G0 state, are induced to pass throughout the G1 and S phases indicating that the putative neurotrophic factor(s) could be responsible for the blockage of a subpopulation of cells in a G0 state.

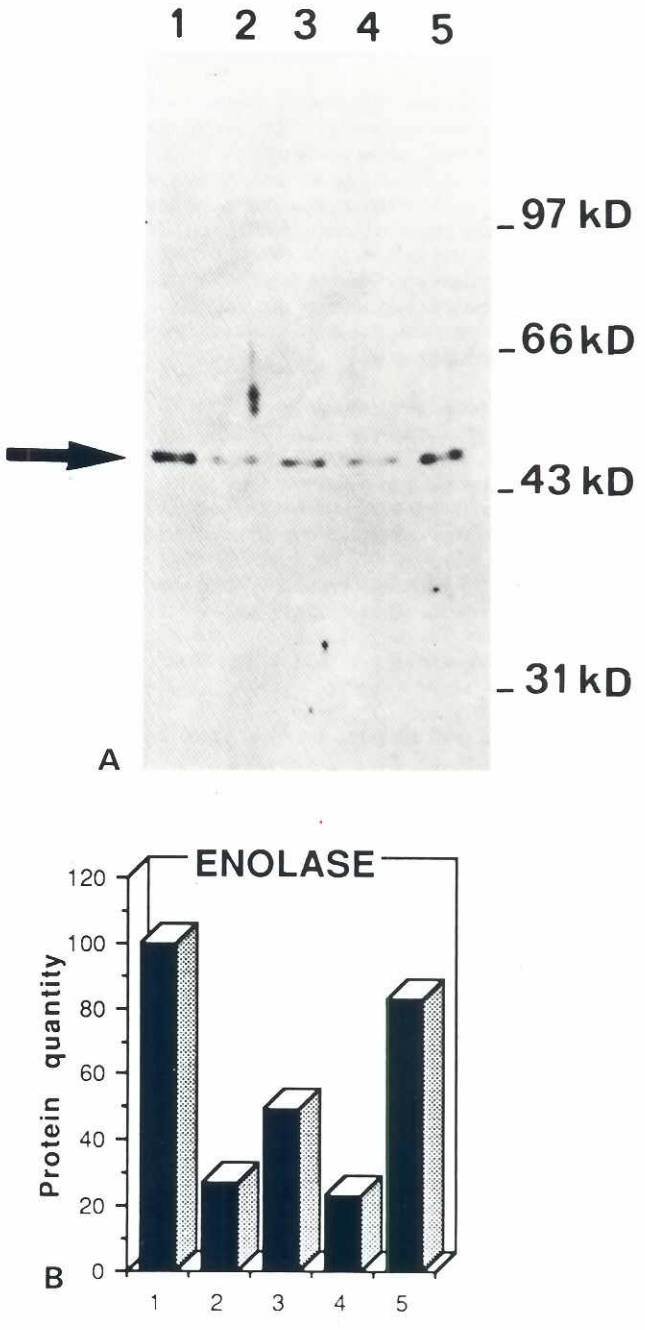

Fig. 5. Same procedure as above applied to alpha-enolase protein (MW: 49kDa). *The pattern of expression of this protein is different from the two others studied here and shows the diversity of the control exerted by the peripheral nervous system on different proteins expression in the limb regenerate.*

Material and Methods

Regenerates

About 3-month old post-metamorphic *Xenopus laevis* froglets were bilaterally amputated under anesthesia (MS 222-0.3%, Sigma) at the proximal level of the radius-ulna. Control limbs were frozen in liquid nitrogen and stored at -70°C until use. About 4 weeks later, the right limb was either totally denervated at the level of the stump (humerus) by sectioning the two branches of the *brachialis* nerve or partially denervated by resection of the only *ulnaris* nerve which runs ventrally, directly underneath the skin and is readily accessible. Care was taken to avoid blood loss.

Harvest of denervated regenerates and non-denervated controls were done 3 hours and 4 days later. Pooled samples were frozen in liquid nitrogen. A total of 16 froglets was used.

Limb protein extraction and analysis

Samples were ground with an Ultra-Turrax homogenizer in a modified Laemmli buffer (L.M.: 0.5M Tris/HCL pH 6.8 and 2.5 % SDS). After sonication, each sample was boiled and their A280 measured. Dilutions were made appropriately in order to deposit equivalent amounts of proteins for electrophoresis. Then, each volume of extract was adjusted to a Laemmli 1X buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 10% glycerol; 5% Mercaptoethanol; 0.01% Bromophenol Blue) for loading on 10% SDS-PAGE (Laemmli, 1970). Nitrocellulose Hybond C-extra (Amersham) was used for transfer in Trans-Blot (Biorad) at 400 mA for 4 hours at 4°C. Proteins were stained by 0.2% Ponceau Red in 0.3% TCA. The blot was then saturated with PBS containing 5% non-fat milk, 0.05% Tween 20 for 1 hour before storage at -20°C until use.

A6 cell proteins were extracted directly in Laemmli buffer.

Obtention of polyclonal anti c-myc protein

The coding region of *Xenopus laevis* c-myc cDNA clone was inserted into pERAT 308 expression vector in frame with protein A. *E. coli* strain (pop 2135) containing the recombinant plasmid was induced at 42°C to express the fusion protein. The expressed fusion protein A/c-myc was purified by affinity on a human IgG column (Pharmacia). Approximately 100 µg of purified fusion protein was mixed with Freund complete adjuvant and injected into a year-old New Zealand White rabbit, previously bled to provide preimmune serum. Booster injections of 50 µg of fusion protein were done every 4 weeks for 5 months. Immune serum was taken by ear bleeding 10 days after each boost. The antiserum was affinity-purified on a c-myc protein column attached to agarose BrCN beads.

Western blotting

Two duplicate gels, with similar quantities of proteins deposited in each lane, were always run and treated in parallel.

Polyclonal anti-c-myc protein

The anti c-myc polyclonal antibody (pAb) was applied overnight at 4°C, at the appropriate dilution (1/400). Then, after several washes at room temperature, the second antibody Garpox (Goat-anti-rabbit peroxidase, Miles) was applied at a 1/2000 dilution for 1 hour at room temperature in the same buffer as for the first antibody. Several washes preceded the final detection with ECL Luminol kit (Amersham) used according to manufacturer's instructions. The intensity of the signal was measured by densitometry (see below).

Polyclonal anti-PCNA protein

The anti-PCNA polyclonal antibody (1/500) was applied overnight, on the same blot as for anti-c-myc after its dehybridization in PBS containing 5% Tween 20. Results were revealed in the same conditions as for c-myc (Luminol kit) and densitometry applied.

Polyclonal anti-alpha enolase

The second blot was hybridized with a polyclonal alpha-human enolase antibody (1/1000) taken as a housekeeping protein in the limbs and used as such as a control. The antibody was directed against this enzyme involved in the glycolytic pathway. Then, the blot was dehybridized in PBS containing 5% Tween 20 and the anti-PCNA polyclonal protein (1/500) applied as above for a control.

Blockage experiments

The *Xenopus laevis* kidney cell line A6 was used as a control for antibody specificity. Cells were cultured at 20-22°C in 75% L15 Leibovitz Medium (Gibco) supplemented with gentamycin and 10% heat-inactivated fetal calf serum. Cells were harvested in their exponential phase of division and proteins extracted. Blockage experiments were carried out for c-myc and PCNA by using a 500-fold excess of peptides. The results are presented in Fig. 2A and B, respectively.

Densitometry

The intensity of the signals generated on the blot was measured on a Schimadzu CS-930 Dual-wavelength Thin-Layer Chromato Scanner from which the data presented on the histograms were collected.

Acknowledgments

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