
Molecular Approaches to Nerve Regeneration

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Molecular approaches to nerve regeneration

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SUMMARY

Current research into regeneration of the nervous system has focused on defining the molecular events that occur during regeneration. One well-characterized system for studying nerve regeneration is the sciatic nerve of rat. Numerous studies have characterized the sequence of events that occur after a crush injury to the sciatic nerve (Cajal 1928; Hall 1989). These events include axon and myelin breakdown, changes in the permeability of the blood vessels, proliferation of Schwann cells, invasion of macrophages, and the phagocytosis of myelin fragments by Schwann cells and macrophages. The distal segment of the injured sciatic nerve provides a supportive environment for the regeneration of the nerve fibres (Cajal 1928; David & Aguayo 1981). Within a period of weeks, the injured sciatic nerve is able to regrow and successfully reinnervate the appropriate targets.

Some of the molecules that provide trophic support for the regrowing nerve fibres have been identified, including nerve growth factor (NGF) (Heumann *et al.* 1987) and glial maturation factor beta (Bosch *et al.* 1989). Another class of molecules show changes in their rates of synthesis during regeneration, including both proteins (Skene & Shooter 1983; Muller *et al.* 1986) and mRNA species (Trapp *et al.* 1988; Meier *et al.* 1989).

To better understand nerve regeneration, we have taken two, parallel molecular approaches to study the events associated with regeneration. The first of these is to study in detail the mechanism of action of a molecule that has been implicated in the regeneration process, nerve growth factor. The second approach is to identify novel gene sequences which are regulated during regeneration. Once the genes are isolated, it will be possible to test for functional roles of the encoded proteins during regeneration. Results from the two approaches are presented in this paper.

STRUCTURE AND FUNCTION ANALYSIS OF THE NGF RECEPTOR

The role of NGF in the development and survival of peripheral sensory and sympathetic neurons has been well characterized (Levi-Montalcini 1987). Studies have also suggested a role for NGF in the development and maintenance of cholinergic neurons in the mammalian forebrain (Williams *et al.* 1986). With regard to regeneration, the levels of NGF mRNA and protein synthesized by the non-neuronal cells of the distal nerve increase markedly after nerve injury, and only decline when axonal regrowth to the muscle is complete (Heumann *et al.* 1987*b*). This local supply of NGF serves to rapidly re-establish the critical retrograde flow of NGF in the neurons (Korsching & Thoenen 1983) after injury, and, since the synthesis of the NGF receptor (NGFR) in neurons is regulated by NGF (Lindsay *et al.* 1990), to maintain the level of neuronal NGFR. Recent studies have also shown that Schwann cells in rat sciatic nerve express high levels of the low-affinity NGFR following nerve crush or transection (Taniuchi *et al.* 1986; Heumann *et al.* 1987). This

expression is repressed once contact is re-established between the Schwann cells and the regenerating axons. These low-affinity receptors may bind the NGF which is synthesized by non-neuronal cells distal to a peripheral nerve injury, and create a localized source of NGF on the surface of the Schwann cells over which the advancing neuronal growth cone migrates (Johnson *et al.* 1988).

(a) *The NGF receptor*

The first step in the mechanism of action of NGF is the interaction of NGF with its specific receptor on responsive cells or cell lines. There are two types or states of NGFR as determined by binding studies (Sutter *et al.* 1979). On sensory and sympathetic neurons, these receptors are described as high affinity (HNGFR, $K_d = 10$ pM), and low affinity (LNGFR, $K_d = 1$ nM), respectively. Although the NGFRs in PC12 cells differ less in their K_d 's, they share many features of the receptors on primary neurons, including a slower rate of dissociation of NGF from the HNGFR compared to the LNGFR (Vale & Shooter 1985). The biological response of NGF in neuronal cells correlates with occupancy of the HNGFR. Indirect evidence

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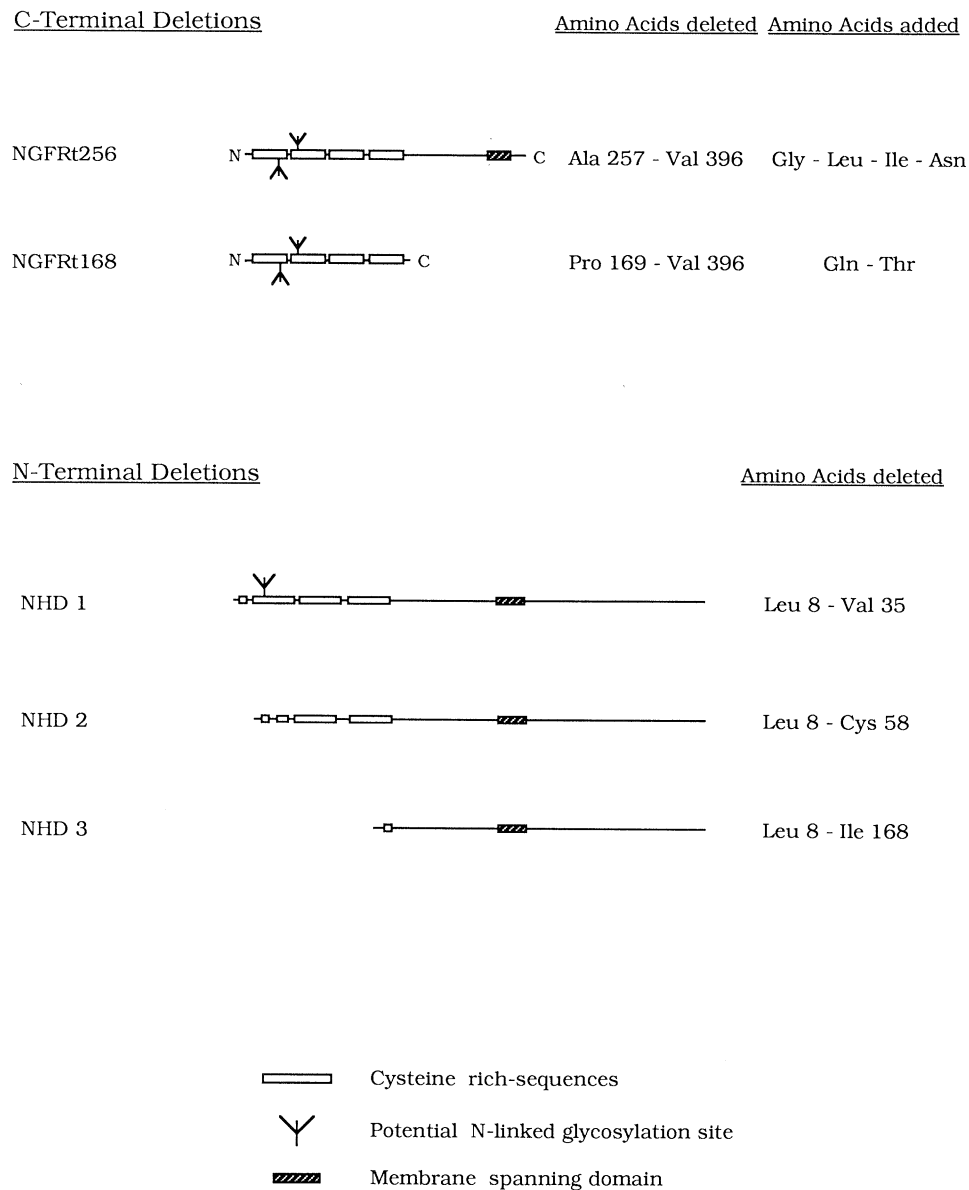


Figure 1. The structure of the nerve growth factor receptor (NGFR) mutants.

suggests that the HNGFR comprises the LNGFR complexed with an additional cytoplasmic or membrane-associated protein or proteins (Hosang & Shooter 1985; Hempstead *et al.* 1989). The LNGFR has been cloned in rat (Radeke *et al.* 1987), chicken (Large *et al.* 1989), and human (Johnson *et al.* 1986), and the three receptors share considerable homology.

Details of the mechanism of NGF binding to the LNGFR have been examined by studying the interaction of NGF with appropriate mutants of the receptor. In this approach, mutations are made in the cDNA encoding the NGF receptor, the mutant cDNAs are then expressed in cell lines that do not normally express the NGFR, and the effects of the mutations on the ability of NGF to bind to the receptor are determined. By using this approach, it should be possible to identify the amino-acid residues that are involved in NGF binding and subsequent signal transduction events. The importance of defining the NGF binding site is highlighted by the recent isolation of the genes for two related neurotrophic factors, brain-

derived neurotrophic factor (BDNF, Leibrock *et al.* 1989), and neurotrophin-3 (NT-3, Ernfors *et al.* 1990). Both of these factors have been shown to compete with NGF for binding to the LNGFR (Rodriguez-Tébar *et al.* 1990; Ernfors *et al.* 1990). Since these factors have different biological effects in different tissues (Davies 1988), it is essential that the binding of these factors to LNGFR is understood, to determine the specificity of action of these different neurotrophic factors.

The structure of NGFR protein can be divided into four domains. The first domain contains approximately 160 amino-acid residues, 24 of which are cysteine residues. The cysteine-rich sequence can be divided into four blocks each containing six cysteine residues. The blocks are numbered 1-4, with block 1 representing the cysteine-rich block closest to the amino terminus of the protein. Blocks one and two are 83% homologous between species, while blocks 3 and 4 are, respectively, 63% and 64% homologous between species. Another striking aspect of the cysteine-rich sequence is the large number of negatively charged amino acids. In the rat

cDNA, the net charge for blocks 1, 2, 3 and 4, are -1 , -6 , -5 , and -8 , respectively. The NGF protein is very basic with a pH of 9.2 (Greene *et al.* 1971). Therefore, the charge interactions between a positively charged NGF molecule and a negatively charged receptor molecule may play an important role in the binding process.

The next structural motif of the receptor is approximately 60 amino-acid residues in length, and separates the cysteine-rich sequence from the membrane-spanning region. This region's main characteristic is the presence of a number of serine and threonine amino acid residues, which are potential sites for O-linked glycosylation. After this region, comes an approximate 20 amino-acid residue region that contains the receptor's single membrane spanning domain. The last region is approximately 150 amino acid residues in length, and contains the intracellular domain which is presumably involved in signal transduction.

(b) The NGF binding domain of the LNGFR

The importance of the extracellular region of the NGFR for NGF binding was shown by the inability of NGF to bind to PC12 cells which had been treated with trypsin (Schechter & Bothwell 1981). Several studies suggest that the cysteine-rich sequences within the extracellular domain contribute to NGF binding. Sehgal and co-workers (1989) cotransfected genomic sequences, encoding the human LNGFR, into mouse fibroblasts and obtained expression of a deleted form of the receptor. The mutant receptors bound NGF, but at a reduced level compared to the wildtype receptor. S1 analysis suggested that the first 40 amino acids of the cysteine-rich sequence of the receptor were missing from the mutant. A truncated form of the low-affinity receptor is secreted into the extracellular media of cultured Schwann cells, PC12 cells and superior cervical ganglia neurons, as well as the plasma and urine of normal rats (DiStefano & Johnson 1988). Isolation and analysis of this truncated form revealed that it could bind NGF (Zupan *et al.* 1989). However, the carboxy terminus of the deleted receptor has not been determined. A soluble, recombinant form of the human NGF receptor, containing the cysteine-rich sequences and an additional 51 amino-acid residues, was expressed in the baculovirus expression system, and this truncated form of the receptor also bound NGF (Vissavajhala & Ross 1990). Taken together, these results suggest that the cysteine-rich sequences contribute to the binding of NGF.

To test whether the intracellular region of the LNGFR is important for NGF binding, for example, by stabilizing the extracellular domain, the first receptor mutation in this study was devised to remove the intracellular region. The mutant pNGFRt256 (figure 1) retained only 16 amino-acid residues of the intracellular domain. This construct was transfected into mouse fibroblasts. Cells that were transfected with the full-length receptor cDNA (wt NGFR) expressed a receptor protein with an apparent molecular weight of 81 000, while cells transfected with the truncated cDNA

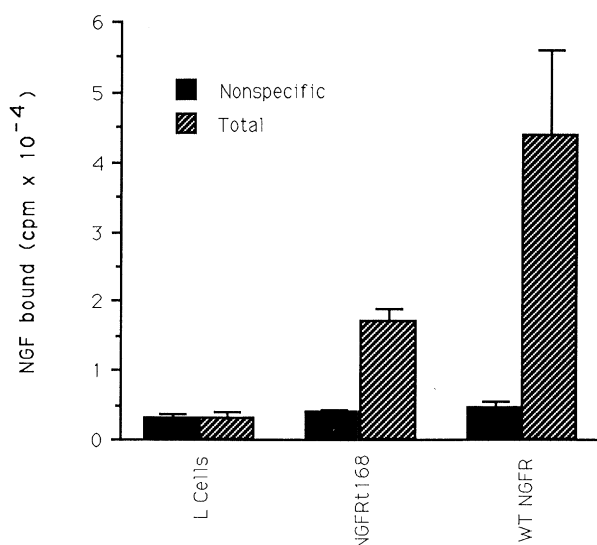


Figure 2. NGFRt168 binds NGF. Transfected fibroblast cell media was incubated with MC192 (anti-receptor antibody)-sepharose beads. Receptors bound to the beads were then incubated with [¹²⁵I]NGF. The data shows [¹²⁵I]NGF cpm bound in the presence (non-specific) of a 500-fold excess of unlabelled NGF or absence (total) of unlabelled NGF. (—) refers to supernatant from fibroblasts transfected with the NGFRt168 antisense construct. The other two sets of binding data are from the supernatants of fibroblasts transfected with the NGFRt168 or wt NGFR constructs. Difference between total cpm bound and non-specific cpm bound represents specific NGF binding.

clone expressed a receptor with an apparent molecular weight of 67 000. Not only did NGF bind to the truncated form of the receptor (*ca.* 45 000 receptors per cell), but it also had the same binding constant (*ca.* 1 nM) as the full-length receptor. Therefore, all, or at least most of the essential components for binding of NGF to the NGFR are contained within the first 256 amino-acid residues of the protein. Similar results have recently been reported for the human LNGFR (Reddy *et al.* 1990).

The second construct (NGFRt168) encoded the first 168 amino acid residues of the LNGFR, encompassing only the four cysteine-rich sequences (figure 1). When mouse fibroblast cells were transfected with this construct, a secreted form of the receptor, with an M_4 of 48 000 was found in the supernatant. Since this form of the receptor bound NGF (figure 2), it is clear that the four cysteine-rich blocks comprise a major part of the NGF binding site. Further mutations were made by removing sequentially, blocks, or parts of blocks from the amino terminus of the LNGFR (figure 1). All the mutant LNGFRs were expressed in COS cells at equivalent levels, as determined by immunoblotting using an antipeptide antibody which recognizes an intracellular epitope of the LNGFR (D. Shelton, personal communication). As judged by cross-linking of ¹²⁵I-NGF, the mutants lacking the first block (NHD 1) or the first and part of the second block (NHD 2) retained NGF binding activity, although at a much lower level than the wildtype receptor, whilst the mutant lacking all four blocks (NHD 3) failed to bind NGF. Although the cross-linking method of measuring

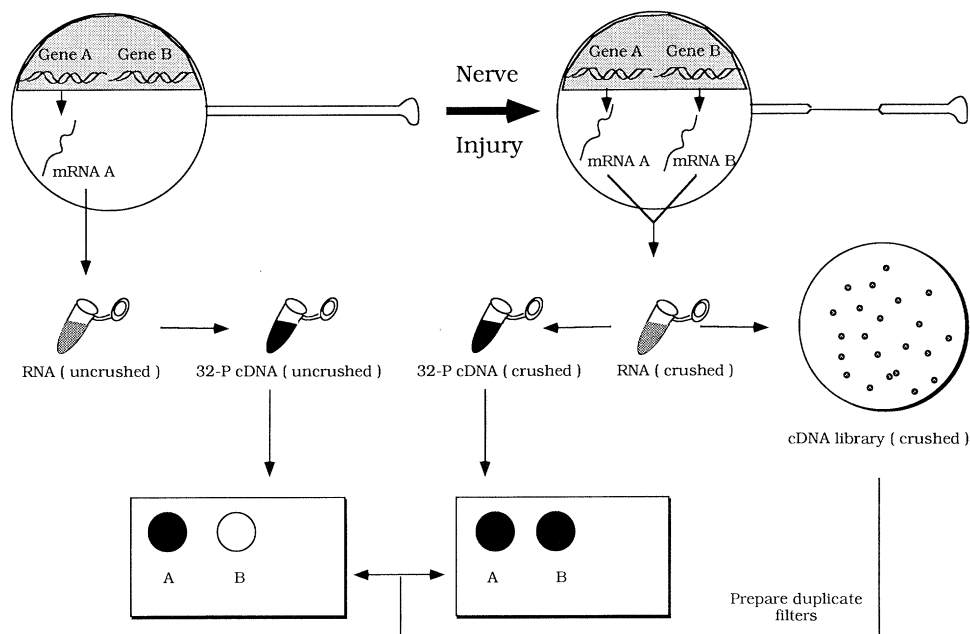


Figure 3. Outline of differential hybridization protocol to identify sequences whose expression is induced after sciatic nerve injury.

NGF binding is not quantitative and is subject to variability within the mutants, this data does suggest that multiple elements in the four cysteine-rich blocks create the NGF-binding site.

ISOLATION OF GENES WHOSE EXPRESSION IS REGULATED AFTER SCIATIC NERVE INJURY

It is clear that there are molecules other than NGF and its receptor involved in the process of nerve regeneration (Skene & Shooter 1983; Muller *et al.* 1986; Trapp *et al.* 1988; Meier *et al.* 1989). To extend these studies, we examined the extent of gene regulation during regeneration. This section describes the use of differential hybridization to identify genes whose expression is regulated during regeneration. It is likely that the proteins encoded by these genes will have some function during regeneration. By only limiting the screen to genes that are regulated, it was anticipated that the procedure would isolate both known and unknown genes.

The principal of differential hybridization as applied to nerve regeneration is diagrammed in figure 3. This example shows the isolation of induced sequences. The upper part shows the gene expression before and after nerve injury. After nerve injury, some mRNA species will be increased, e.g. mRNA B. RNA is isolated from injured and uninjured nerves, and a cDNA library made from the RNA from the injured nerve (middle right). Duplicate filters containing individual colonies are screened with ^{32}P -labelled cDNA made from RNA from uninjured or injured nerves (bottom part). Colonies containing sequences whose expression is induced during regeneration will hybridize more of the ^{32}P -labelled cDNA from the injured than from the uninjured nerve. The cloned sequences can then be

analysed to try to determine the function of the regulated, encoded proteins during regeneration.

Accordingly, two cDNA libraries from the sciatic nerve of rats were constructed and screened (De Leon *et al.* submitted manuscript). The first cDNA library was prepared by using RNA from the distal segment of the sciatic nerve, three days after a crush injury. This cDNA library is referred to as the distal library (DL), and it was constructed to identify sequences whose expression is induced during nerve regeneration. A second cDNA library was constructed by using RNA from the uninjured contralateral nerves of the same pool of animals used for the first library. This contralateral library (CL) served for the isolation of sequences whose expression is repressed during nerve regeneration.

Both libraries were screened by differential hybridization to identify clones containing sequences which were regulated during nerve regeneration. Figure 4 shows representative duplicate filters after the second round of screening. The particular filters shown were from the crushed nerve library, and were used to identify sequences whose expression was induced after sciatic nerve injury. Several examples of increased hybridization to colonies on the right filter (^{32}P -labelled crushed nerve cDNA), as compared to hybridization to colonies on the left filter (^{32}P -labelled contralateral nerve cDNA), can be seen. A total of 2000 colonies were screened from the DL, and 11 colonies were isolated that contained sequences induced during neuronal regeneration. A total of 5000 colonies of the CL library were also screened by differential hybridization. After two rounds of screening, 30 colonies contained sequences whose expression was repressed three days after nerve crush.

The cDNA inserts in these colonies were tested by cross-hybridization analysis to identify related, homologous sequences within all of the isolated sequences.

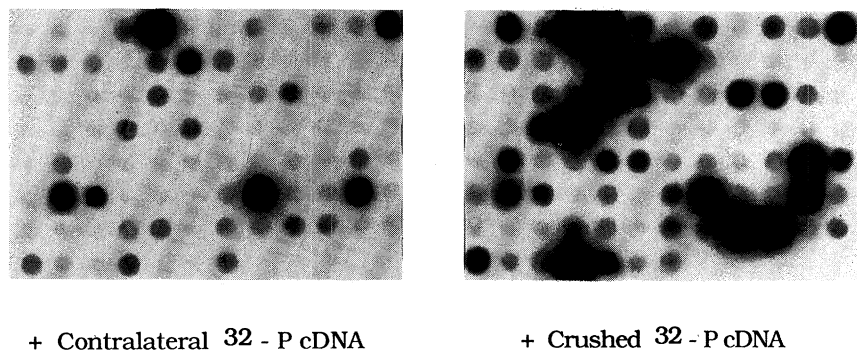


Figure 4. Isolation of induced sequences after sciatic nerve injury. Duplicate filters with colonies containing cDNAs from the mRNA of crushed sciatic nerve were screened with ^{32}P -labelled cDNA from the contralateral nerve (left filter) or ^{32}P -labelled cDNA from the crushed nerve (right filter). Colonies on the right filter showing stronger hybridization than the corresponding colonies on the left filter contain induced cDNA sequences.

Table 1. List of induced and repressed sequences isolated from the distal and contralateral cDNA libraries. Regulated cDNA sequences were isolated and partially sequenced. The sequences were compared to the GenBank and EMBL databases. Sequences were hybridized to each other to determine if they contained related sequences. Novel sequences showed no homology to DNA sequences listed in the databases

(Induced sequences isolated from the distal nerve library three days after nerve crush.)

clone	number of copies identified	induction level	identification
CD 2	4	++	Vimentin
CD 5	4	++	28S rRNA
CD 6	1	++	novel sequence
CD 8	1	++	18S rRNA
CD 12	1	++	novel sequence

(Repressed sequences isolated from the contralateral nerve library three days after nerve crush.)

clone	number of copies identified	repression index	identification
SR 7	1	++	novel sequence
SR 10	1	++	novel sequence
SR 13	7	+++	novel sequence
SR 17	12	+++	myelin P_0
SR 18	1	+++	alpha-globin
SR 22	1	++	novel sequence
SR 37	1	++	novel sequence
SR 39	1	+++	myelin basic protein
SR 43	3	+++	cytochrome oxidase sub. 1
SR 49	1	++	creatine kinase
SR 58	1	++	collagen type I

Unique clones from each group were partially sequenced, and the sequences were compared to the GenBank and EMBL DNA sequence databases. A summary of the identity of the induced and repressed sequences is shown in table 1. Four of the induced sequences were homologous to vimentin, five were

homologous to rRNA, and two were not homologous to any sequences in the database (novel sequences). The induction levels were 3–5-fold for all sequences mentioned in table 1.

Similarly, the identity of the repressed, isolated sequences are shown in table 1. Numerous known sequences were isolated including myelin P_0 , alpha-globin, myelin basic protein, cytochrome oxidase (subunit I), skeletal muscle creatine kinase (M creatine kinase), and collagen (type alpha-1). Also, five groups of novel sequences, as determined by cross-hybridization studies, were isolated. The extent of repression of these sequences ranged from 3-fold to more than 10-fold.

Next, the isolated sequences were tested by northern blot analysis to determine the regulation of their expression throughout the degeneration and regeneration process. The results can be summarized as follows. One group of sequences, such as vimentin, showed an increase in their expression on the crush side, compared to the contralateral side, during the first few days after nerve crush, after which expression gradually returned to a normal level after two or three weeks. The second group of sequences, such as myelin P_0 , showed a strong decrease in their expression on the crush side, as compared to the contralateral side, within a day after nerve injury, but eventually increased to normal levels, several weeks after the nerve crush. The last group of sequences, such as SR 7, showed complex regulation, including oscillating gene expression throughout the degeneration and regeneration process.

The induction or repression during neuronal regeneration of the specific sequences reported here was assessed by the comparison of the crushed and uninjured, contralateral nerves of the same animals. During the analysis, however, it became clear that there was a second level of regulation operating, when the expression was compared between operated and unoperated, naive animals. This effect can be small for genes such as myelin P_0 , or large, as observed for genes such as SR 7. It has been previously shown, that the sciatic nerve lesion produces changes in the contralateral, uninjured nerve (Rotshenker 1988). At present, it is not clear if the second level of regulation seen here is the same as the previously described contralateral

effect, or is due to systemic effects of the surgery. Whatever the explanation, it is of interest that the time course of the effects reported here parallel the timecourse of neuronal degeneration and regeneration, suggesting that these effects are specific to the injured and uninjured nerves.

The next step in this process is to identify the role these proteins may play during regeneration. Studies in other systems on some of the isolated, known genes suggest possible roles. For example, the expression of creatine kinase mRNA is regulated during the process of myogenesis. Creatine kinase RNA is not expressed in myoblasts at the time when they are undifferentiated and are still proliferating. However, both the RNA and protein level of creatine kinase M increase when myoblast fusion occurs (Trask *et al.* 1988). Studies with the BC3H1 muscle cell line showed that the expression of creatine kinase M mRNA increased several hundred fold in tissue culture conditions that stimulate differentiation and cessation of proliferation (Spizz *et al.* 1986). However, when these differentiated, quiescent cells were stimulated to divide with fibroblast growth factor (FGF) or media containing 20% serum, the expression of creatine kinase M was repressed. It is intriguing that during sciatic nerve regeneration, the repression of creatine kinase parallels the initial proliferation of Schwann cells, followed by their later differentiation. Schwann cells may use creatine kinase M during normal metabolism, but then switch to a different energy pathway during proliferation.

Finally, it is worth noting that 13 out of the 41 sequences examined in this work represent unknown sequences, corresponding to as yet unknown genes which may play a role in neuronal degeneration and regeneration. Clearly, the regeneration process is quite complex at the molecular level.

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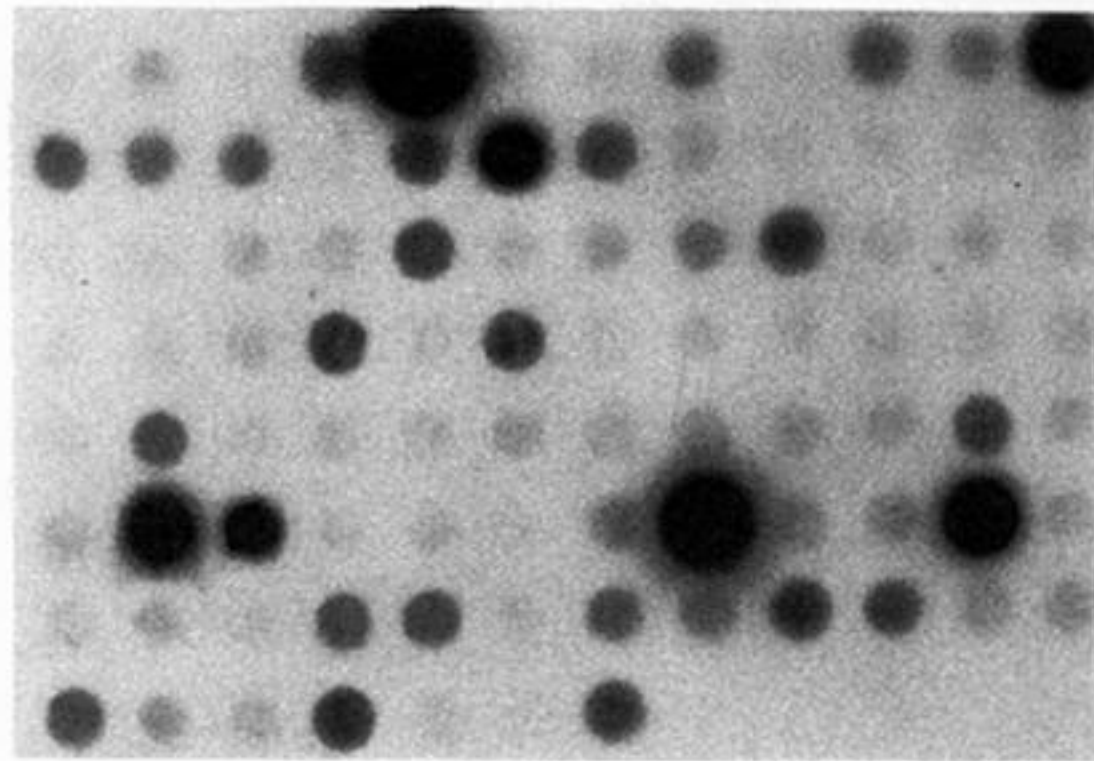
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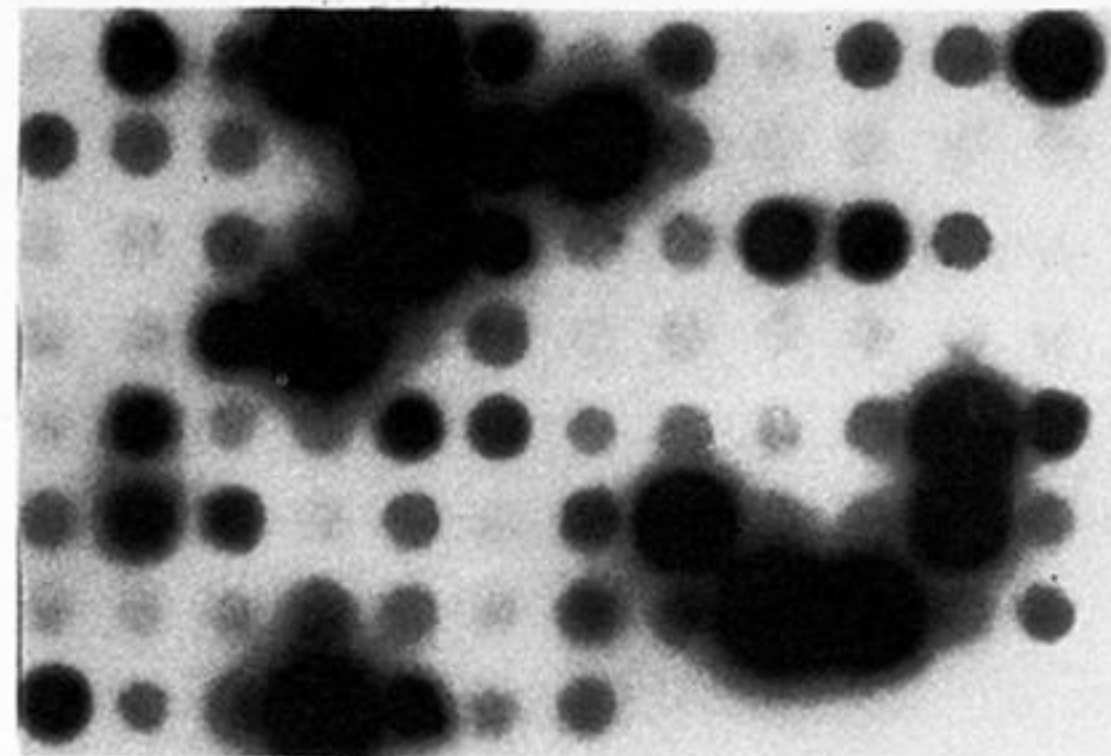
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+ Contralateral 32 -P cDNA



+ Crushed 32 -P cDNA

Figure 4. Isolation of induced sequences after sciatic nerve injury. Duplicate filters with colonies containing cDNAs from the mRNA of crushed sciatic nerve were screened with 32 P-labelled cDNA from the contralateral nerve (left filter) or 32 P-labelled cDNA from the crushed nerve (right filter). Colonies on the right filter showing stronger hybridization than the corresponding colonies on the left filter contain induced cDNA sequences.