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Up-Regulated Uridine Kinase Gene Identified by RLCS in the Ventral Horn after Crush Injury to Rat Sciatic Nerves

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Rat sciatic nerve crush injury is one of the models commonly employed for studying the mechanisms of nerve regeneration. In this study, we analyzed the temporal change of gene expression after injury in this model, to elucidate the molecular mechanisms involved in nerve regeneration. First, a cDNA analysis method, Restriction Landmark cDNA Scanning (RLCS), was applied to cells in the ventral horn of the spinal cord during a 7-day period after the crush injury. A total of 1991 cDNA species were detected as spots on gels, and 37 of these were shown to change after the injury. Temporally changed patterns were classified into three categories: the continuously up-regulated type (10 species), the transiently up-regulated type (22 species), and the down-regulated type (5 species). These complex patterns of gene expression demonstrated after the injury suggest that precise regulation in molecular pathways is required for accomplishing nerve regeneration. Secondly, the rat homologue of uridine kinase gene was identified as one of the up-regulated genes. Northern blot analysis on rat ventral horn tissue and brain revealed that the UK gene had three transcripts with different sizes (4.3, 1.4, and 1.35 kb, respectively). All of the transcripts, especially the 4.3 kb one, were up-regulated mainly in a bimodal fashion during the 28-day period after the injury. The RLCS method that we employed in the present study shows promise as a means to fully analyze molecular changes in nerve regeneration in detail.

Injury to the axons of the peripheral nerve induces various cellular and molecular events, which finally lead to nerve regeneration. Within several hours, axons start to sprout from the severed axonal tips and advance distally to the target organs with the help of cues provided by Schwann cells and target organs (1, 2). These molecular and cellular events are known to involve not only neurons but also other cells, including Schwann cells, macrophages and fibroblasts. Although much attention has heretofore been paid to extraneuronal elements that are operative in nerve regeneration, the neuron itself nevertheless plays a critical role in the regenerative process (3, 4).

Axonal damage in several types of regenerable neurons prompts the cell to synthesize *de novo* both RNAs and proteins. For instance, it has been suggested that axonal sprouting and elongation depend on the macromolecules synthesized in neurons, and that this synthesis is regulated in a variety of ways during the regeneration (5). In fact, crush injury to the axons of rat sciatic nerve up-regulates RNA-transcriptional activity in motor neurons located in the ventral horn(VH) of the spinal cord and also in sensory neurons present in the dorsal root ganglion (DRG). This activity was shown in a bimodal fashion: the first peak of the activity was detected on the third post-operative day (POD), while the second peak was seen on the eleventh POD (6). After the first peak of the RNA synthesis, protein synthesis reached a peak at 7 POD (7). Concordant with the protein synthesis, the rate of axonal outgrowth gradually increased, reaching a maximal value at 10–14 POD (8). It is worth mentioning that the administration of a protein synthesis inhibitor, applied at the injury site, reduced the rate of axonal elongation (9), suggesting that those proteins which were newly synthesized after the injury included those essential for nerve regeneration. Therefore, identification and characterization of these proteins would potentially be helpful in understanding the molecular mechanisms of

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession No. AB030700.

the regeneration. The large numbers of studies conducted so far have demonstrated that nerve growth factors (10, 11), cytoskeletal proteins (12), neuropeptides (13, 14), growth-associated protein (15) and transcription factors (16–19) are the molecules synthesized *de novo* after the nerve injury. Some of these studies also identified novel genes that are closely related to the regeneration (20, 21). However, to date, and to the best of our knowledge, no studies have been conducted on macromolecular synthesis in VH neurons after nerve injury. The identification of any gene products essential for nerve regeneration is also imperative to clarify the molecular mechanisms involved in nerve regeneration.

This is our first report on a series of experiments using a rat sciatic nerve crush injury model to elucidate the temporal changes of gene expression in cells from the VH of the spinal cord. For this purpose, we employed restriction landmark cDNA scanning (RLCS), a novel cDNA-analytic method by which more than 1000 genes can be simultaneously and quantitatively displayed as two-dimensional gel spots (22, 23). The temporal patterns of gene expression during a 7-day period after the injury have successfully been classified with the RLCS method. One of the genes cloned from the RLCS gel was identified as a rat uridine kinase gene, which showed an expression in VH cells with a peak at 7 POD.

MATERIALS AND METHODS

Surgical procedures and tissue preparation. We used male Sprague-Dawley rats weighing 200–250 g, 8 weeks in age. They were purchased from a local breeder. Under anesthetized with diethyl ether, their bilateral sciatic nerves were exposed at the mid-thigh level and were crushed by holding for 30 s with a fine forceps. The lumbosacral portion of the spinal cord (from L3 to L5) was resected at 1, 3, 5, 7, 11, 14, 21 and 28 days after the crush, and the ventral horns were carefully dissected out under the microscope (n = 8 on each day). Eight ventral horn tissues for each time point were pooled and kept frozen in liquid nitrogen until use. All the animals were treated in accordance with the guidelines governing the care and use of laboratory animals at Kyoto Prefectural University of Medicine.

RLCS. Total RNAs were isolated from pooled ventral horn tissue by the AGPC method (24). Poly $(A)^+$ RNA was purified from total RNA with Dynabeads-Oligo $(dT)_{25}$ (Dynal,Inc., Norway). RLCS analysis was performed as described by Suzuki *et al.* and Yaoi *et al.* (22, 23). Briefly, double stranded cDNA was synthesized with the Superscript Plasmid system (Life Technologies Inc., USA) and 5′-biotinylated oligo$(dT)_{15}$ anchor primers, including the *Not*I restriction enzyme site prior to the dT stretch. The cDNA corresponding to 1μg poly $(A)^+$ RNA was digested with a restriction enzyme *Eco*RI or *Hin*dIII (1-D restriction enzyme). The cohesive ends of the digested cDNA fragments were then labeled with Sequenase (Ver.2.0) and 50 μCi[α-^{32}P] dATP or dGTP (6000Ci/mmol) (Amersham, USA), respectively. Among those labeled, the biotinylated cDNA fragments were magnetically separated with Dynabeads M-280 streptavidin (Dynal, Inc., Norway) and recovered. The recovered radiolabelled cDNA fragments were then released from the magnetic beads by *Not*I digestion. These fragments were fractionated in 1% 1-D agarose gel electrophoresis. The gel strip was incubated in a reaction mixture containing a restriction enzyme *Hin*fI (2-D restriction enzyme), then subjected to 2-D 6% polyacrylamide gel electrophoresis. After the electrophoresis, the gel was dried and exposed to X-OMAT film (Kodak, USA). The densitometric analysis of spots on the autoradiogram was done with the image analysis software NIH Image Ver.1.60 (National Institutes of Health, USA). Cloning of the cDNA fragments corresponding to the RLCS spots were subcloned into pBluescript II plasmid (Stratagene, La Jolla, CA) using the PCR-mediated method as described by Suzuki *et al.* (25) and Yaoi *et al.* (23).

Sequencing. The cloned spot cDNA sequences were determined by using AutoRead Sequencing Kit (Pharmacia Biotech, Tokyo, Japan). The homological search for the spot cDNA sequences was done with the software BLAST Ver.2.0 (the National Center for Biotechnology Information), checking against the following database: dbEST, GenBank, EMBL, DDBJ, PDB, and SwissProt.

Northern blot. Poly$(A)^+$ RNAs denatured with formaldehyde were fractionated by 1% agarose gel electrophoresis, and then transferred to Hybond-N^+ nylon membranes (Amersham, USA). This blot was probed with ^{32}P-labelled DNA probe in Quick-Hyb hybridization solution (Stratagene, La Jolla, CA).

RESULTS

RLCS profiles obtained before and after the sciatic nerve injury from rat VH tissue. Using two kinds of 1-D restriction enzymes (*Hin*dIII and *Eco*RI), RLCS profiles were obtained from cells of the VH, both from the side where the sciatic nerve crush was performed as well as from the contralateral side where no crush was applied as the control. Typical RLCS patterns from the control VH are shown in Fig. 1A. 910 and 1081 spots (a total of 1,991 spots) were detected on the profile of the control by using *Hin*dIII and *Eco*RI restriction enzymes, respectively. Our previous studies (22) revealed that one mRNA species can principally be displayed as a single spot on a profile, and that the expression level of a gene corresponds well to the signal intensity of the spot. In this study, therefore, we surveyed the temporal change of the expression of these 1991 species of mRNA in VH, both before and after the injury.

We compared the profile from the control with five profiles obtained from the injured VH on the first, the third, the fifth, and the seventh post-operative day (POD), respectively. We could easily detect those spots showing changing patterns of expression with time after the injury, and these were counted. Figure 1B shows a representative pattern of changed spots. Densitometric analysis has facilitated the identification of changed spots, and spots whose intensities increased or decreased more than 1.5-fold could be counted as changed spots. We observed that spots corresponding to the same genes appeared in more than two profiles obtained at different time-points, and we classified these into 23 categories according to the patterns of their temporal changes, as shown in Fig. 2. Some of these spots were shown to be induced rapidly after the injury but with a transient expression, while others had a persistent expression. Some were slowly induced, whereas others were down-regulated. The number of

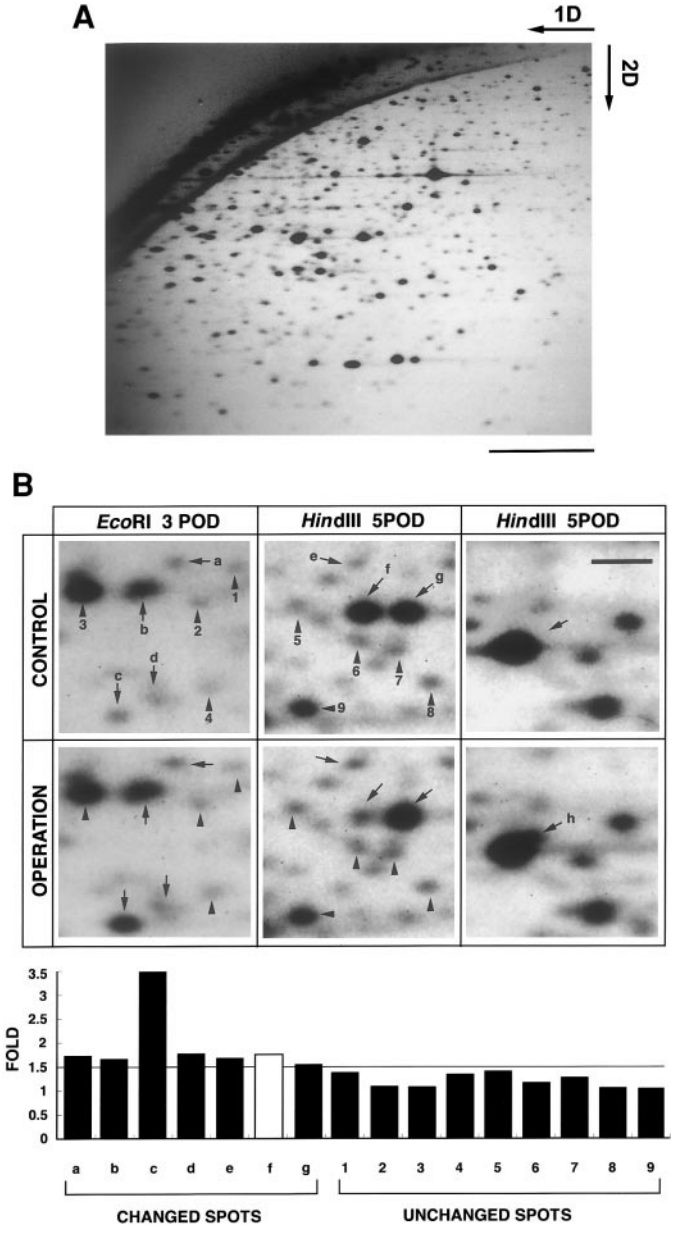


FIG. 1. (A) A typical rat ventral horn RLCS profile obtained using *Hin*dIII and *Hin*fI, as a 1-D restriction enzyme and a 2-D one, respectively. A horizontal bar indicates 6.5 cm. (B) Comparison in the signal intensity of the corresponding spots between after the crush and the control. Arrows indicate changed spots (spots a–h), and arrowheads alone indicate unchanged spots (spots 1–9). Another horizontal bar indicates 1 cm. The results of the densitometric analysis for the spots except spot h are shown below. Among up-regulated spots (black bars), each bar indicates a ratio of the intensity of a spot in the operation group to that of the corresponding spot in the control. White bar indicates a down-regulated spot, in which the spot intensity in the control was divided by that of the operation group. The ratio of changed spots exceeded 1.5, although that of apparently unchanged spots was 1–1.5.

spots in each category was counted and summarized in Fig. 2. Thus, 37 spots of the total 1991 spots were identified as changed spots: 32 of these spots were up-regulated and 5 spots were down-regulated: 1.6% and 0.3% of the surveyed genes were shared as an up-regulated and down-regulated gene, respectively. Thus, RLCS revealed that the gene expression in VH cells during the 7-day period after the crush injury was temporally regulated in a complex way, and that the number of species of up-regulated genes was 6.4 times that of down-regulated genes.

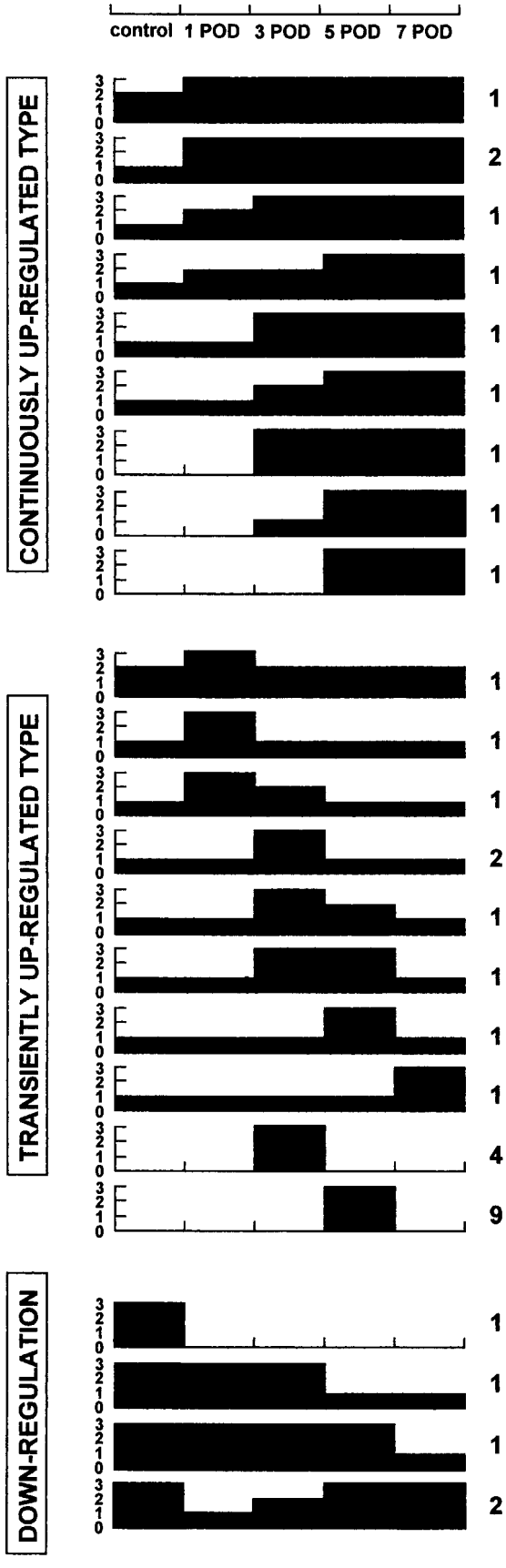
The rat uridine kinase gene as an up-regulated gene after injury. We have now begun to identify the genes corresponding to the changed spots. Although this task has not yet been completed, the rat homologue of uridine kinase (UK) gene has been identified. The spot DNA fragment was cloned, sequenced and then used as a probe for the northern blot analysis of poly $(A)^+$ RNA from L3, L4, and L5 of the ventral horn before and after the injury. The spot sequence (314 nucleotides) has shown 83% and 87% identity to a part of mouse and human uridine kinase cDNA sequences, respectively. Northern blot analysis revealed that the rat UK gene had three transcripts with different sizes (4.3, 1.4, and 1.35 kb, respectively) in VH and brain cells. All transcripts, mainly the 4.3 kb one, was up-regulated in a bimodal fashion through the 28-day period after the crush injury (Fig. 3). The gene expression first increased at 7 POD, decreased to the basal level at 11 POD, then increased gradually again, and reached the maximum level at 28 POD.

DISCUSSION

In order to clarify the molecular mechanisms involved in rat sciatic nerve regeneration, most of the studies conducted so far have been focused on the changes in the distal segment of the nerve or in DRG neurons (26–28). Genes identified through these studies include X16/SRp20, chargerin II, cytochrome c oxidase subunit I, fatty acid binding protein, SGP-1 and others, and their products are operative in RNA processing and translation, energy metabolism, lipid metabolism, iron metabolism. Among them cytochrome c oxidase subunit I, fatty acid binding protein and SGP-1 show transient up-regulation after injury.

Although motor neurons in VH, i.e., the proximal component of the sciatic nerve, are supposed to play important roles in nerve regeneration, few studies have been aimed at the VH. Therefore, we attempted to show the temporal changes in terms of gene expression in rat VH cells after injury using a RLCS method and demonstrated that there are three different patterns of gene expression in VH cells after the injury. In addition, we were able to demonstrate the first identification of the UK gene as one of the up-regulated genes in VH cells after the injury to rat sciatic nerves.

Identification of UK as an injury-induced gene. We have successfully identified UK as one of the injury-



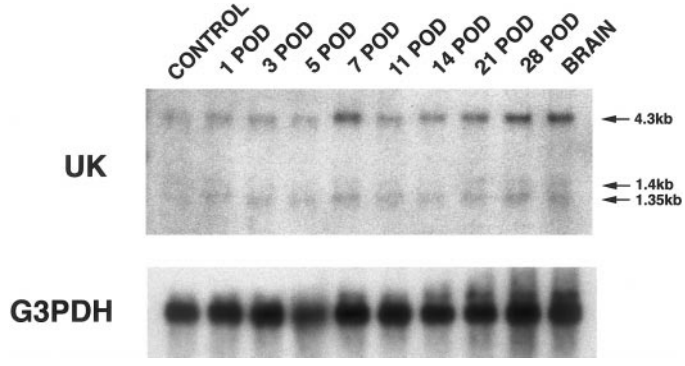


FIG. 3. Northern blot analysis of rat ventral horn tissue and brain. One μg of poly(A)$^+$RNAs from rat injured ventricular horn at indicated PODs and adult rat brain was loaded in each lane. After hybridization with rat uridine kinase (UK) or rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe, stringency washes were done in 2× SSC/0.1% SDS at 25°C twice, followed by 0.1× SSC/0.1% SDS at 65°C twice.

induced genes in VH cells after rat sciatic nerve crush injury. UK is known to be the rate-limiting enzyme of the salvage pathway in pyrimidine synthesis, since UK converts uridine into uridine-5′-mono-phosphate in the presence of ATP (29). Although this enzyme is produced by a number of normal tissue types and cells, UK is enriched in fetal as well as neoplastic tissue and cells (30).

The relationship between UK and nerve regeneration was first studied in the crushed optic nerve of goldfish (31), though the regeneration process proceeds more slowly in the goldfish than in the rat sciatic nerve. In the goldfish optic nerve, injured axons begin to sprout within a few days, and then reached the optic tectum at about 21 days after the injury (9). Both models share the following common features in the nerve regeneration process. First, axonal outgrowth in both of these nerves requires the support of glial cells; oligodendrocytes and astrocytes play roles in the goldfish optic nerve regeneration, whereas this role is played by Schwann cells in the sciatic nerve (32). While the oligodendrocytes in the goldfish optic nerve have the same characteristics as those in CNS, these oligodendrocytes are known to support regenerating axons, functioning like mammalian Schwann cells (33). Second, the axonal damage in both systems prompts injured neurons to synthesize *de novo* RNAs. This response is regulated in a bimodal fashion, being substantially similar in both systems (34).

FIG. 2. Classification of the patterns of temporally changed spots after the crush injury in rat sciatic nerve in the ventral horn. Each box represents the relative intensity of spots: When the maximum intensity of each spot is 100%, 3, 2, 1 and 0 indicate intensities of 100–67%, 67–33%, 33–0%, and 0%, respectively. The number of spots belonging to each class is also shown on the right side.

Kohsaka *et al.* reported a temporal change in UK activity in the goldfish model (31), showing that the axonal damage promoted the salvage pathway of pyrimidine synthesis. The UK activity after the injury reached a peak by 4 POD, and then returned gradually to the basal level by 21 POD. They suggested the possibility that this increase in UK activity may depend on the *de novo* synthesis of UK protein. However, the protein change was not measured. On the contrary, we demonstrated the *de novo* synthesis of UK mRNA by northern blot analysis, even though the UK activity in rat sciatic nerve injury remains unclarified. The temporal pattern of UK mRNA expression shown here in our model is compatible with the report on the change in UK activity in the goldfish optic nerve model. Therefore, the regulation of UK gene expression and/or of uridine metabolism may underlie the nerve regeneration process over the species. Moreover, it is worth mentioning that our northern blot analysis revealed that rat UK mRNA expression was up-regulated again at 14 POD, when axons were assumed to have reached the target organ and the maturation process and recovery of function have started (35, 36). This suggested that either the uridine metabolism and/or RNA *de novo* synthesis may play some role in both nerve regeneration and maturation. In this context, adenine phosphoribosyltransferase (APRT) and hypoxanthine guanine phosphoribosyltransferase (HGPRT), both of which are involved in the salvage pathway for purine nucleotide synthesis, may also be up-regulated in a similar pattern to UK. Further studies are required to prove this hypothesis.

The temporal changes in the expression pattern in VH cells after the sciatic nerve crush injury. There was a technical limitation in our study in that the cell type-specificity in the gene expression of the changed spots remains to be clarified. This limitation occurred because we could not discriminate completely between motor neurons and glial cells. Our VH samples, however, were dissected from the gray matter of the spinal cord where neurons are abundant. We supposed, therefore, that our profiles obtained with RLCS mainly reflect the gene expression of motor neurons. Temporally changed expression patterns in the 37 genes were classified into 23 patterns. This indicated that the gene expression is regulated in a complicated fashion in the VH cells after the injury. The expression of 22 genes out of 37 genes, however, was up-regulated transiently at different post-operative days. This transient and precise regulation in terms of gene expression suggests that there are a number of different molecular events proceeding in the VH in the course of axonal outgrowth after the injury.

Jacob *et al.* reported that, in motor neurons, acceleration of axonal outgrowth reached a peak at 9-12 days after injury (8). The *de novo* synthesis of the protein reached a peak at 7 POD (7). The transcription activity of motor neurons in VH is known to reach a peak at 3 POD (6). In addition, our present study showed that a small number of induced mRNA species occurred at 3 and 5 POD, which may be responsible for the acceleration of the axonal outgrowth of motor neurons.

Intensive studies to identify the genes associated with crush injury in rat sciatic nerves have been conducted employing the differential hybridization method, although the targets of these studies were mainly focused on the area from the nerve segment distal to the site of injury or DRG neurons. In the distal segment of the sciatic nerve, more than half of the genes were identified as the induced type (27), whereas in the DRG all of the identified genes were the induced type (28). In regard to injury-associated genes, our study showed that the percentage of gene induction in the VH is 86.5%. It may thus be concluded that the induced type of genes outnumber the repressed type of genes during the nerve regeneration process throughout the VH, DRG and the distal segments of sciatic nerves. Nevertheless, we could argue that the changing patterns of gene expression in VH differ from those reported in the distal segment or in DRG, since the number of the transiently up-regulated genes could be detected in the VH. In the distal segment of the sciatic nerve or in DRG, continuously up-regulated genes were predominant (27, 28).

The observation that the number of transiently expressed genes was higher in the VH than in the distal segment or in DRG may indicate the advantage of our RLCS technique over others, such as the differential and subtraction hybridization method, because the RLCS method, without hybridization reactions, enabled us to screen more than 1000 cDNA species simultaneously and quantitatively in a one-path electrophoresis process and to compare the expression patterns at several time points. On the contrary, differential screening of a subtraction cDNA library at a certain POD alone may result in the failure to detect transiently expressed type genes at other time points. In order to circumvent this problem, many subtracted libraries are required for screening. RLCS could thus become a powerful analytical tool to identify transiently expressed genes in the distal segment of the sciatic nerve as well as in DRG.

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