A Nerve Growth Factor–regulated Messenger RNA Encodes a New Intermediate Filament Protein

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Abstract. Differential screening of a cDNA library from the PC12 rat pheochromocytoma cell line previously revealed a clone, clone 73, whose corresponding mRNA is induced by nerve growth factor (NGF). Induction parallels NGF-stimulated PC12 differentiation from a chromaffinlike phenotype to a sympathetic neuronlike phenotype. We report that DNA sequence analysis reveals that clone 73 mRNA encodes an intermediate filament (IF) protein whose predicted amino acid sequence is distinct from the known sequences of other members of the IF protein family. The sequence has highest homology with desmin and vimentin and includes the highly conserved central α-helical rod domain with the characteristic heptad repeat of hydrophobic residues, but has lower homology in the amino-terminal head and carboxyl-terminal tail domains. The head domain contains a large number of serine residues which are potential phosphorylation sites. The expression of clone 73 in vivo in the nervous system of the adult rat was investigated by in situ hybridization of clone 73 probes to tissue sections. The mRNA is expressed at high levels in ganglia of the peripheral nervous system, including the superior cervical ganglion (sympathetic), ciliary ganglion (parasympathetic), and dorsal root ganglion (sensory). In the central nervous system, motor nuclei of cranial nerves III, IV, V, VI, VII, X, and XII as well as ventral horn motor neurons and a restricted set of other central nervous system nuclei express the clone 73 mRNA. Tissues apart from those of the nervous system did not in general express the mRNA, with only very low levels detected in adrenal gland. We discuss the implications of these results for the mechanism of NGF-induced PC12 cell differentiation, the pathways of neuronal development in vivo, and the possible function of the clone 73 IF protein and its relationship to other IF proteins.

IT is well established that the peptide nerve growth factor (NGF)[1] is required for the differentiation and maintenance of sympathetic and sensory neurons in the peripheral nervous system (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968). However, the molecular mechanisms by which NGF acts to stimulate neuronal precursor cells to express differentiated functions are not well understood. Attempts to deduce these mechanisms in vivo are made difficult by the large number of cell types in the nervous system and the complexity of their interactions. The NGF-responsive PC12 pheochromocytoma cell line provides a convenient model system for the investigation of the process of neuronal differentiation (for review see Greene and Tischler, 1982). In the presence of NGF, PC12 cells differentiate from a chromaffinlike phenotype to a sympathetic neuronlike phenotype. Features of the response to NGF deduced with PC12 cells may be further examined in neuronal tissues to deduce their role in neuronal differentiation in vivo.

We have previously described a set of cDNA probes for mRNAs which undergo long-term increases or decreases during the PC12 cell differentiation process (Leonard et al., 1987). One of these probes (clone 73) recognizes a mRNA which is increased five- to sevenfold by NGF or fibroblast growth factor (FGF), another factor known to promote the neuronal differentiation of PC12 cells (Togari et al., 1983 and 1985; Rydel and Greene, in press). The kinetics of this induction by NGF are similar to those of the extension of neurites from PC12 cells. Dexamethasone also regulates the mRNA, however in a manner opposite to NGF, causing a two- to threefold decrease of its level. The opposing actions of NGF and glucocorticoids have been observed previously in the differentiation decisions of neural crest-derived precursor cells, which can give rise to chromaffin cells under the influence of glucocorticoids, or to sympathetic neurons in the presence of NGF (Bjerre and Bjorklund, 1973; Doupe et al., 1985a and b; Anderson and Axel, 1986). A variety of other factors and growth conditions that affect PC12 cells but do not induce their neuronal differentiation are without effect

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1. *Abbreviations used in this paper:* CNS, central nervous system; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; IF, intermediate filament; NF, neurofilament; NGF, nerve growth factor; SCG, superior cervical ganglion.

on the level of this mRNA. Northern blot analyses of the distribution of expression of clone 73 mRNA in rat tissues established that this mRNA is very abundant in the sympathetic superior cervical ganglion (SCG), is present at very low levels in the brain and adrenal gland, and is undetectable in all other tissues of the rat which we examined. Thus, clone 73 represented a potentially neuronal specific mRNA that is induced in a model system for neuronal differentiation. In light of these properties, further characterization studies were undertaken.

We report here the complete nucleotide sequence of clone 73 cDNA and an analysis of the predicted encoded peptide as well as the distribution of clone 73 mRNA in the central and peripheral nervous systems as determined by the method of in situ hybridization. We show that clone 73 mRNA encodes a new intermediate filament protein that is expressed in a particular subset of central and peripheral neurons. The narrow range of neuronal cell types which express clone 73 mRNA and the coincidence of its induction in PC12 cells with neurite outgrowth suggest this new intermediate filament protein serves a specialized function in the differentiated cell. These results also raise the possibility that there is a previously unrecognized developmental or functional relationship between the neurons that express the clone 73 gene.

Materials and Methods

Sequencing of Clone 73 cDNA

Sac I, Pst I, Stu I, and Pvu II fragments of clone 73 cDNA (Fig. 1) were purified from a 1% agarose mini-gel and cloned into bacteriophage M13 mp18 vector (Messing and Vieira, 1982). Single-stranded DNA templates were prepared as described previously (Sanger et al., 1980) and sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977; Sanger and Coulson, 1978) using [α-^{35}S]thio dATP, and fractionated on TBE buffer gradient polyacrylamide gels (Biggin et al., 1983). The se-
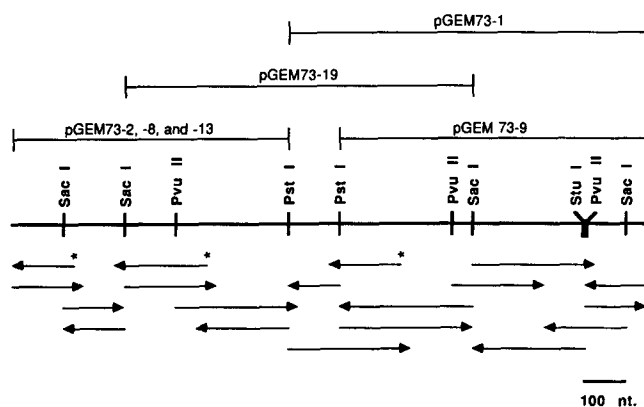

Figure 1. Physical map of clone 73 cDNA. The partial restriction map of clone 73 cDNA is shown at the middle of the figure. Below the restriction map are the fragments used for sequencing. Arrows indicate the direction and extent of sequence read from each fragment. DNA was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977; Sanger and Coulson, 1976). Asterisks indicate the positions of three oligonucleotide primers used in the final stages of the sequence determination. Above the restriction map are the restriction fragments subcloned into pGEM-1 and used for the synthesis of single-stranded, ^{35}S-labeled RNA probes for in situ hybridization to tissue sections.

quence analysis was completed by the use of three specific oligonucleotide primers, as indicated by the asterisks in Fig. 1.

In Situ Hybridization

Adult female rats were anesthetized by intraperitoneal injection of 100 mg Nembutal per kg body wt and fixed by cardiac perfusion with 4% paraformaldehyde in 100 mM Na phosphate buffer, pH 7.2. The tissue to be sectioned was removed, immersed in ice-cold fixative for 0.5–1 h on ice, and transferred to 15% sucrose in PBS for 1 h on ice (except for the brain and spinal cord which were incubated in 30% sucrose in PBS for 24 h at 4°C). The tissue was embedded and frozen in O.C.T. (Tissue Tek, Lab-Tek Products, Naperville, IL) at −30°C for 1 h. Sections were prepared at 8–10 μm thickness using a cryostat microtome (Hacker Instruments, Inc., Fairfield, NJ) and were transferred to acid-washed, poly-L-lysine-coated glass microscope slides (prepared as described by Cox et al., 1984). Generally, adjacent serial sections were placed on separate slides to generate two or three sets of serial sections. Then one set of sections was stained with cresyl violet, the adjacent sections in the second set of slides was hybridized with anti-sense probe, and the third was hybridized with control sense probe. The cresyl violet-stained set was used to better visualize the cellular morphology of the tissue, because the in situ hybridization procedure alters the staining properties of many cells. For the serial brain sections, 25-μm sections were cut for cresyl violet staining, alternating with 10-μm sections for in situ hybridization. For one serial brain study, parasaggital sections were taken every 80 μm through one lateral half of the rat brain, whereas for a second brain study, parasaggital sections were taken every 150 μm. Tissue sections were either used immediately or stored at 4°C for 1–2 d before in situ hybridization. The in situ hybridization procedure was performed as described by Cox et al. (1984) with modifications as described by Anderson and Axel (1985). Briefly, sections were pretreated as described, using a 15-min digestion with 0.1 μg/ml of proteinase K, followed by a postfixation step (4% paraformaldehyde in PBS, 10 min, room temperature). Pst I and Sac I restriction fragments of clone 73 cDNA (as illustrated in Fig. 1) were subcloned into the pGEM-1 vector (Promega Biotec, Madison, WI). Single-stranded RNA sense and anti-sense probes were prepared using the SP6-T7 Gemini system (Promega Biotec) and were labeled with [α-thio-^{35}S] UTP to a sp act of 7.8 × 10^7 dpm/μg. All sense and anti-sense probes were tested for specificity of hybridization by Northern blot analysis to PC12 cell RNA. Only the anti-sense probes hybridize specifically to a 1,800-nucleotide mRNA which is increased in relative abundance by NGF. However, the sense probes of PGEM73-2, 8, 13, and 19 hybridized strongly for the control in situ hybridization analysis. Once this high background for these control probes was observed, only the pGEM73-1 and 9 subclones were used for further in situ hybridization studies. The probe sizes were reduced to an average size of 150 nucleotides by alkaline hydrolysis which was verified by gel electrophoresis. Hybridization was performed as described by Cox et al. (1984) at 55°C under paraffin oil at a probe concentration of 0.5 μg/ml for 12–20 h. Slides were also washed and autoradiographed according to Cox et al. (1984), except that 50% formamide was included in the 2× SSC wash. The final wash was with 0.1× SSC at 50°C. All hybridization and wash buffers contained 10 mM dithiothreitol, except when used for treatment with RNase A. The NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) used to coat the sections for autoradiography was exposed for 1.5 to 14 d at 4°C. Tissue sections were stained in cresyl violet, dehydrated through xylenes, and coverslipped using Permount. An Olympus BH-2 microscope was used to examine the tissue sections; tissue morphology was best visualized with bright-field illumination, whereas the silver grains in the emulsion were best visualized with dark-field illumination. Photographs of sections of peripheral ganglia and high-power photographs of central nervous system (CNS) regions were kindly taken with an Olympus exposure control unit on Kodak Plus-X pan film. The low-power photographs of the CNS sections were taken by Dr. Robert Baker (Dept. of Physiology, New York University Medical Center) with a Leitz microscope and photomicroscopic unit (vario-Orthomat).

Results

Sequence Analysis of Clone 73

The complete DNA sequence of clone 73 and its translation are shown in Fig. 2. Both strands of clone 73 cDNA were fully sequenced by the subcloning and sequencing of overlapping restriction fragments (Fig. 1). The cDNA is 1,556

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Head                          10                              20                              30
leu arg ser ser ile ser ser thr ser tyr arg arg thr phe gly pro pro pro ser leu ser pro gly ala phe ser tyr ser ser ser
CTC CGG TCC AGC ATC AGC TCC ACC TCA TAC CGC CGG ACC TTC GGG CCA CCG CCC TCA CTG TCC CCC GGG GCT TTC TCC TAC TCA TCC AGC

                              40                              50                              60
ser arg phe ser ser ser arg leu leu gly ser gly ser pro ser ser ser ala arg leu gly ser phe arg ala pro arg ala gly ala
TCT CGC TTC TCC AGC AGC CGC CTG CTG GCC TCG GGG TCC CCG AGC TCC TCG GCG CGG CTG GGC AGC TTC CGT GCC CCT CGA GCG GGG GCC

    Rod                       70                              80                          1a
leu arg leu pro ser glu arg leu asp phe ser met ala glu ala leu asn gln glu phe leu ala thr arg ser asn glu lys gln │glu
CTG CGC TTG CCC TCG GAG CGC CTC GAC TTC TCC ATG GCC GAG GCC CTC AAC CAG GAG TTC CTG GCC ACT CGG AGC AAC GAG AAG CAA│GAG

                              100                             110                             120
leu gln glu leu asn asp arg phe ala asn phe ile glu lys val arg phe leu glu gln gln asn ala ala leu arg gly glu leu ser
CTA CAG GAG CTC AAC GAC CGC TTC GCC AAC TTC ATC GAG AAG GTC CGC TTC CTG GAG CAG CAG AAC GCA GCC CTG CGC GGG GAG CTG AGC

                              130                         1b      140                             150
gln ala│arg gly gln glu pro ala arg ala asp gln leu │cys gln gln glu leu arg glu leu arg arg glu leu glu leu leu gly arg
CAG GCG│CGG GGC CAG GAG CCG GCG CGC GCC GAC CAG CTG │TGC CAG CAG GAG CTG CGC GAG CTG CGG CGC GAA CTG GAG CTG CTG GGC CGG

                              160                             170                             180
ser ala tyr arg val gln val glu arg asp gly leu ala glu asp leu gly ala leu lys gln arg leu glu glu glu thr arg lys arg
AGC GCG TAT CGG GTG CAG GTC GAG CGC GAC GGG CTG GCG GAG GAC CTG GGG GCG CTC AAG CAG AGG TTA GAA GAA GAA ACT CGC AAG CGG

                              190                             200                             210
glu asp ala glu his asn leu val leu phe arg lys asp val asp asp ala thr leu ser arg leu glu leu glu arg lys ile glu ser
GAG GAT GCG GAG CAC AAC CTG GTG CTC TTC CGT AAG GAC GTG GAC GAC GCC ACG CTG TCC CGC CTA GAA CTA GAG CGC AAG ATC GAA TCT

                              220                         230     240
leu met asp glu ile glu phe leu lys lys leu his glu glu glu leu arg asp leu │gln val ser val glu ser gln gln val gln gln
CTG ATG GAC GAA ATT GAG TTC CTC AAG AAG CTA CAC GAA GAG GAG CTT CGA GAC CTG │CAG GTG AGC GTG GAG AGC CAG CAG GTG CAG CAG

                              250                         2       260                             270
val glu val glu ala thr val lys pro glu leu thr │ala ala leu arg asp ile arg ala gln tyr glu asn ile ala ala lys asn leu
GTG GAG GTA GAG GCA ACA GTG AAG CCA GAG CTG ACC │GCG GCG CTG AGG GAC ATC CGT GCA CAG TAT GAG AAC ATC GCG GCG AAG AAT CTG

                              280                             290                             300
gln glu ala glu glu trp tyr lys ser lys tyr ala asp lys ser asp ala ala asn arg asn his glu ala leu arg gln ala lys gln
CAG GAG GCA GAG GAG TGG TAT AAG TCA AAA TAC GCT GAC CTG TCC GAT GCT GCC AAC CGC AAC CAT GAG GCC CTA CGT CAG GCC AAG CAG

                              310                             320                             330
glu met asn glu ser arg arg gln ile gln ser leu thr cys glu val asp gly leu arg gly thr asn glu ala leu leu arg gln leu
GAG ATG AAC GAG TCC CGA CGT CAG ATC CAG AGT CTG ACG TGC GAA GTG GAC GGG CTT CGT GGC ACG AAC GAG GCA CTG CTC AGA CAG TTG

                              340                             350                             360
arg glu leu glu glu gln phe ala leu glu ala gly gly tyr gln ala gly ala ala arg leu glu glu glu leu arg gln leu lys glu
CGG GAG CTG GAG GAG CAG TTC GCC CTG GAG GCT GGA GGG TAC CAA GCA GGC GCA GCA CGG CTG GAG GAA GAG CTT CGA CAG CTG AAG GAA

                              370                             380                             390
glu met ala arg his leu arg glu tyr gln glu leu leu asn val lys met ala leu asp ile glu ile ala thr tyr arg lys leu leu
GAG ATG GCG AGG CAC CTG CGA GAG TAC CAG GAG CTC CTC AAC GTC AAG ATG GCC CTG GAC ATC GAG ATA GCC ACC TAC AGG AAG CTA CTG

                              400                     Tail    410                             420
glu gly glu glu ser arg ile ser │val pro val his ser phe │ala ser leu ser leu lys thr thr val pro glu val glu pro pro gln
GAA GGG GAA GAG AGC CGG ATC TCA │GTG CCC GTT CAT TCC TTT │GCT TCC CTA AGT TTA AAG ACG ACT GTG CCT GAG GTG GAG CCT CCC CAG

                              430                             440                             450
asp ser his ser arg lys met val lys ile arg thr ile glu thr arg asp gly glu lys val val thr glu ser gln lys glu gln his
GAC AGC CAC AGC CGG AAG ATG GTT CTG ATC AGG ACT ATC GAG ACT CGG GAT GGG GAG AAG GTG GTG ACA GAG TCT CAG AAG GAG CAG CAC

                              460
ser glu leu asp lys ser ser ile his ser tyr │  *
AGT GAA CTG GAC AAG TCT TCT ATT CAC AGC TAC│TGA GGC CTC AGC TGG ATC TCT GAG GCT GAC TGA AGC CCG TTC CTA AGC TCG AAC CCT

CCC TTG CAC CAG TCT GCA ACC AGT CCT GCT CAG CAT GGG CAC CCT CCT AGC CGA GCT CCA GCC CGA TCC TGA GGC TGG ACC TCT CCC TGA

TGC ATT GAT GTG ACC CAT AGC TTT CCT CGGAATTCC G
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Figure 2. The complete nucleotide and translated amino acid sequences of clone 73 cDNA. There are 90 nucleotides and 30 amino acids per line. Vertical bars indicate head, rod, and tail domains of the intermediate filament structure. Coils *1a*, *1b*, and *2* of the rod domain are enclosed in boxes. Linker nucleotide sequences at the termini are shown in bold type.

bp in length and has an open reading frame encoding 461 amino acids followed by a single stop codon (asterisks in Fig. 2). The cDNA does not appear to be full length because the translated amino acid sequence does not begin with a methionine.

Based on homology searches of the Dayhoff protein and translated GenBank data bases, the amino acid sequence encoded by clone 73 has homology with all the known intermediate filament (IF) proteins. IF proteins are characterized by a central α-helical rod domain that is highly conserved, and flanking, nonhelical amino- and carboxy-termini that are distinct for each IF protein (for example see Weber and Geis-
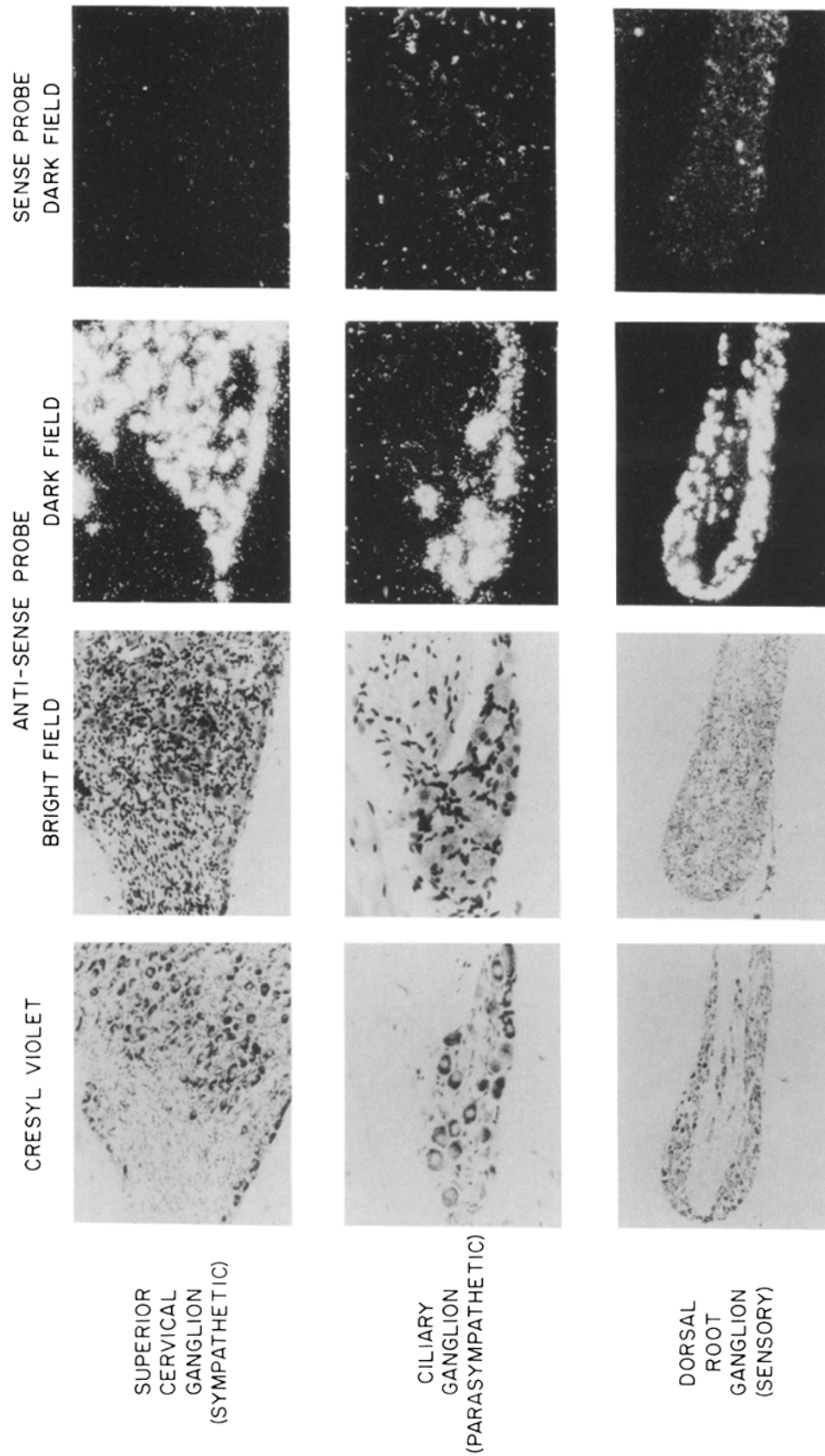
Figure 3. The distribution of clone 73 mRNA in the PNS. In situ hybridization analysis of clone 73 in the ganglia of the PNS of the rat is shown. Three adjacent sections from each of the ganglia (superior cervical ganglion, ciliary ganglion, and dorsal root ganglion) were treated in one of three ways: (*a*) stained with cresyl violet and photographed with bright-field illumination (column 1); (*b*) hybridized with an anti-sense probe of clone 73, exposed for one week, and photographed with bright-field illumination (column 2) and dark-field illumination (column 3); or (*c*) hybridized with the corresponding control, sense probe, exposed for 1 wk, and photographed with dark-field illumination (column 4). Silver grains in the emulsion appear as very small black dots with bright-field illumination and as white dots with dark-field illumination.

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ler, 1985). The rod region consists of three coils that have a heptad repeat (McLachlan and Stewart, 1975) with hydrophobic residues in the first and fourth amino acid positions of the seven–amino acid repeat unit. These hydrophobic residues are thought to form a hydrophobic backbone along the rod region that increases the association of two IF proteins to form a coiled coil, which is the basic subunit in 7–10 nm IF (for review see Steinert et al., 1985*b*). These characteristic IF protein domains are indicated in Fig. 2 including the head (amino-terminus), rod, and tail (carboxy-terminus) regions (delimited by vertical bars) and the three coils (*la*, *lb* and *2*) of the rod (enclosed in boxes). The three coils of the translated clone 73 have the hydrophobic heptad repeats, complete with the heptad reversal near the amino-end of coil 2, typical of all IF proteins (not specifically indicated in Fig. 2). Therefore we conclude that the protein encoded by mRNA clone 73 represents a new member of the IF protein family. We consider the homologies with other IF proteins below.

In Situ Hybridization Analysis of Clone 73 mRNA

Clone 73 mRNA was originally of particular interest because of its restricted tissue distribution (Leonard et al., 1987). The mRNA was detected in the SCG at levels comparable with those found in PC12 cells and at very low levels in the brain and adrenal gland and was undetectable in a variety of other tissues surveyed. We therefore used in situ hybridization analysis to determine the cell type(s) in the SCG that expresses the clone 73 mRNA, to localize the expression of this mRNA in the brain and adrenal gland, and to test for its expression in the spinal cord and parasympathetic and sensory ganglia. The in situ hybridization analysis was carried out according to the protocol of Cox et al. (1984), as modified by Anderson and Axel (1985). Single-stranded, ^{35}S-labeled RNA probes were synthesized by SP6 or T7 polymerases from restriction fragments of the clone 73 cDNA cloned into the pGEM-1 vector (Fig. 1). The anti-sense experimental probe, which was complementary to the clone 73 mRNA, was used to detect the mRNA in tissue sections; a sense control probe, which has the same sequence as the mRNA and therefore will not hybridize to the mRNA in the tissue sections, was used as a control.

The results for experiments with the sympathetic SCG, the parasympathetic ciliary ganglion, and the sensory dorsal root ganglia are shown in Fig. 3. An adjacent cresyl violet–stained section of each ganglion is also shown in Fig. 3. It is clear by comparison of the in situ hybridization-labeled and adjacent stained sections that clone 73 mRNA is localized to the neurons of all three types of peripheral ganglia.

A
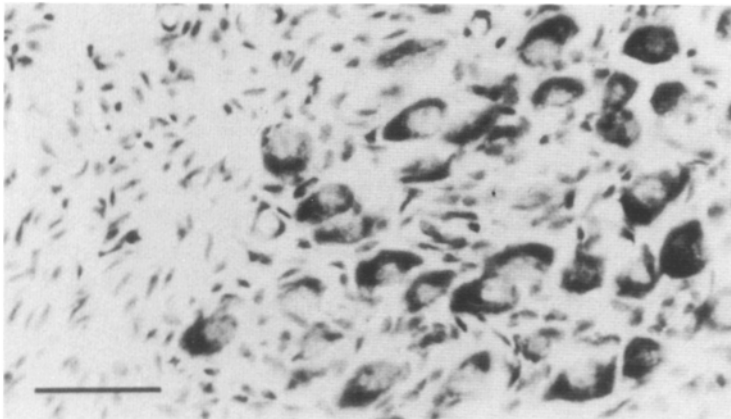
B
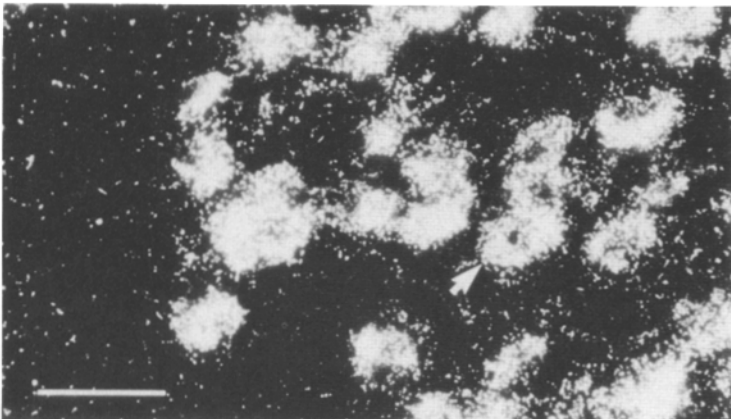
Figure 4. In situ hybridization of clone 73 in the superior cervical ganglion (SCG) at higher magnification. (*A*) Section of the SCG was stained with cresyl violet and photographed with bright-field illumination. (*B*) Section of SCG, adjacent to the section shown in *A*, was hybridized with the experimental anti-sense probe of clone 73, exposed for 1 wk, and photographed with dark-field illumination. Silver grains in the emulsion appear as white dots. Arrow indicates ringlike pattern of silver grains over a neuron. Bar, 50 μm.

The intensity of labeling of each neuronal type is approximately equivalent. Unlabeled neurons were never seen in any of the ganglia examined, indicating that all the neurons of these ganglia are expressing this IF-related mRNA. Labeling was not seen over the nerve roots entering and leaving these ganglia, nor was it observed over support cells in the ganglia or roots (Fig. 3; and SCG at higher magnification, Fig. 4). Also at higher magnification, a ringlike pattern of labeling over the cytoplasm with a paucity of silver grains over the nucleus is observed for some of the neurons (Fig. 4 b, arrow). The lack of axonal labeling and the ringlike labeling over the cell body indicate that the probe was hybridizing to a mRNA in the cytoplasm of the neuronal perikarya. Dorsal root ganglia from several vertebral levels were examined and all had this same pattern of hybridization. In addition, cells of the carotid artery included in sections of the SCG did not hybridize with clone 73 anti–sense probes, consistent with our earlier observations of the restricted tissue distribution of clone 73 mRNA (Leonard et al., 1987). As controls for probe specificity, neuronal labeling was not seen when the tissue sections were pretreated with RNase A (data not shown), or when the sense probe was used (Fig. 3, sense probe, column 4). In addition, probes from different regions of the cDNA (e.g., pGEM73-8 and pGEM73-9, Fig. 1) gave the same specific pattern of hybridization.

We also examined sections of the adrenal gland by in situ hybridization. The adrenal gland was studied because of the low level of clone 73 mRNA detected in this tissue by Northern blot analysis in a previous study (Leonard et al., 1987). No signal above background was detected in the sections of the adrenal gland that we examined (data not shown). These sections included both the adrenal cortex and the adrenal medulla. Whereas there are sympathetic ganglia in the adrenal gland, it is possible that none of these were included in the adrenal sections we used. None could be identified, especially after in situ hybridization analysis, and serial sections were not examined. Whereas we have determined that adrenal medullary chromaffin cells are not expressing clone 73 mRNA at levels detectable by in situ hybridization, we cannot presently differentiate between very low levels of expression in chromaffin cells or higher levels of expression from the less numerous sympathetic neurons present in the adrenal gland.

A detailed survey of the distribution of clone 73 mRNA in the CNS was accomplished by the in situ hybridization analysis of serial parasaggital sections of rat brain and attached cervical spinal cord (Table I and Figs. 5 and 6). Two representative brain sections (Figs. 5 A and 6 A) and line drawings of those sections (Figs. 5 B and 6 B) illustrate many of the CNS regions with positive signal hybridization to clone 73. In general, the neurons of the motor nuclei of cranial nerves III, IV, V, VI, X, and XII express clone 73 mRNA. Strong hybridization signal was also present in the ventral horn motor neurons of the spinal cord (Figs. 6 and 7). In addition, the mRNA is detected in the neurons of the dentate nucleus of the deep cerebellar nuclei (DN, Fig. 5), in the medial and lateral vestibular nucleus (VC, Fig. 6), in the red nucleus (RN, Fig. 6, A and B), in the tegmental nucleus of Gudden (TN, Fig. 7, A and B), and in scattered reticular neurons (RN, Fig. 6, A and B) (Table I). The identification of the rostral part of the vesticular complex is uncertain; it is also pos-

Table I. Central Nervous System Distribution of Clone 73 mRNA

CNS Region	Abbreviation
Oculomotor nucleus	Not shown
Trochlear nucleus	Not shown
Motor nucleus of the trigeminal	V
Abducens nucleus	Not shown
Facial nucleus	VII
Vagal motor nucleus	Not shown
Hypoglossal nucleus	XII
Ventral horn motor neurons	VH
Dentate nucleus	DN
Vestibular complex (medial [?] and lateral vestibular nuclei)	VC
Red nucleus	RN
Tegmental nucleus of Gudden	TN
Scattered reticular neurons	R
Nucleus ambiguus (?)	NA

All regions of the CNS that hybridized to the anti–sense clone 73 probes in a cellular pattern are listed with the abbreviations used for the regions when shown in Figs. 5 or 6.

sible that this labeled region is either the mesencephalic nucleus of the trigeminal nerve or the locus ceruleus. There is also diffuse labeling of the pyramidal layer of the hippocampus, which cannot be localized to any cell bodies and is much less intense than the signal from other regions of the CNS or PNS. None of these regions are labeled with the control sense probe (for example, see Fig. 6 C). The light appearance of the cerebellar granule cell layer in Fig. 6, A and C, does not represent silver grains and is a consequence of dark field illumination of the stained tissue. Labeling is never observed over axonal fiber tracts in the CNS. The regions of labeling generally correspond to collections of large neuronal perikarya in the adjacent cresyl violet-stained sections (Figs. 7 and 8). The ringlike pattern of silver grains noted for the neurons of the peripheral ganglia is also observed in the CNS (Fig 7, arrow). Therefore, we conclude that we are hybridizing to a mRNA in neuronal cell bodies and not in axons or nonneuronal cells. A comparison of the signal intensity observed in the PNS and CNS regions was made by sectioning the spinal cord to include a dorsal root ganglion in the same section as the ventral horn motor neurons. Although the signals were not quantitated, there was only slightly less labeling of the motor neurons relative to the sensory neurons (data not shown). Serial sections of the entire rat brain, including the midbrain, cortex, and forebrain (including the olfactory bulb) as well as the cervical spinal cord, were screened for expression of clone 73 mRNA. No signal was detected in any region except those listed in Table I.

Discussion

Clone 73 mRNA Encodes a New IF Protein

IF proteins are a group of closely related proteins which form 7-10-nm filaments in the cytoplasm of a variety of cell types (for review see Lazarides, 1982). These proteins have been divided into subclasses or types based on their biochemical and immunological properties, and their tissue dis-
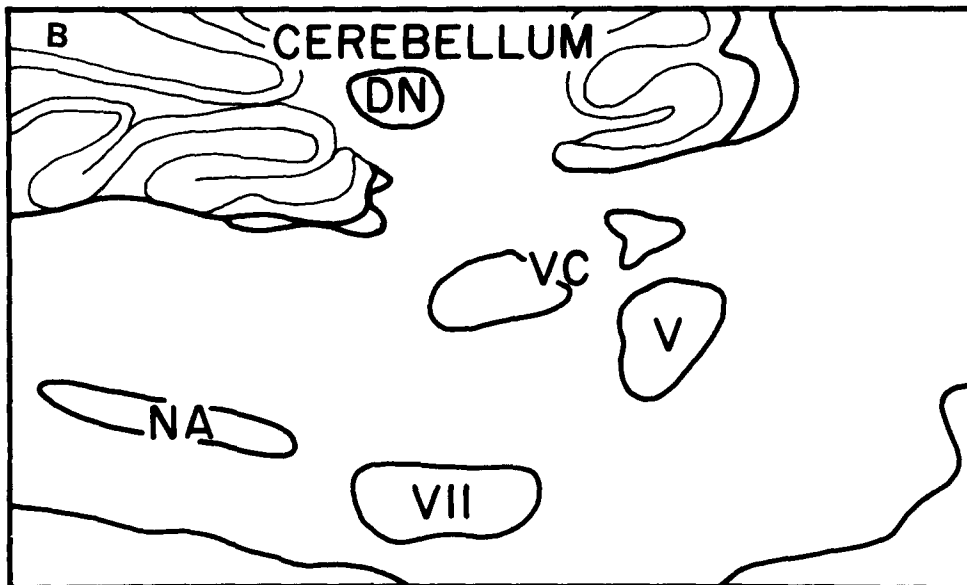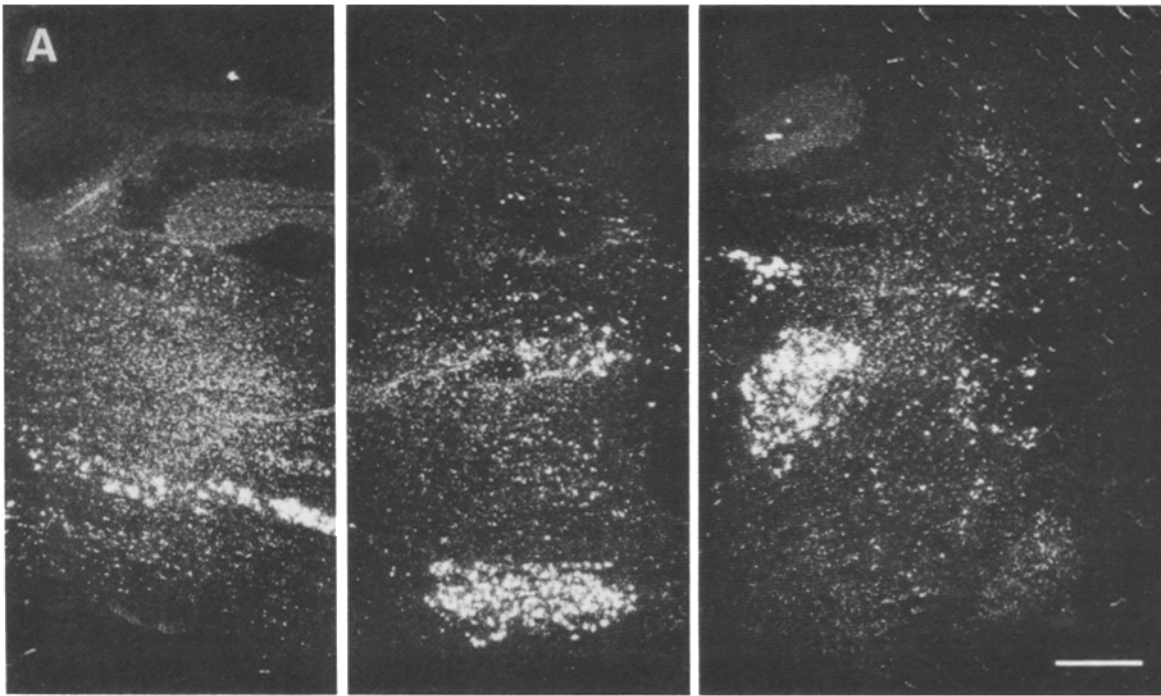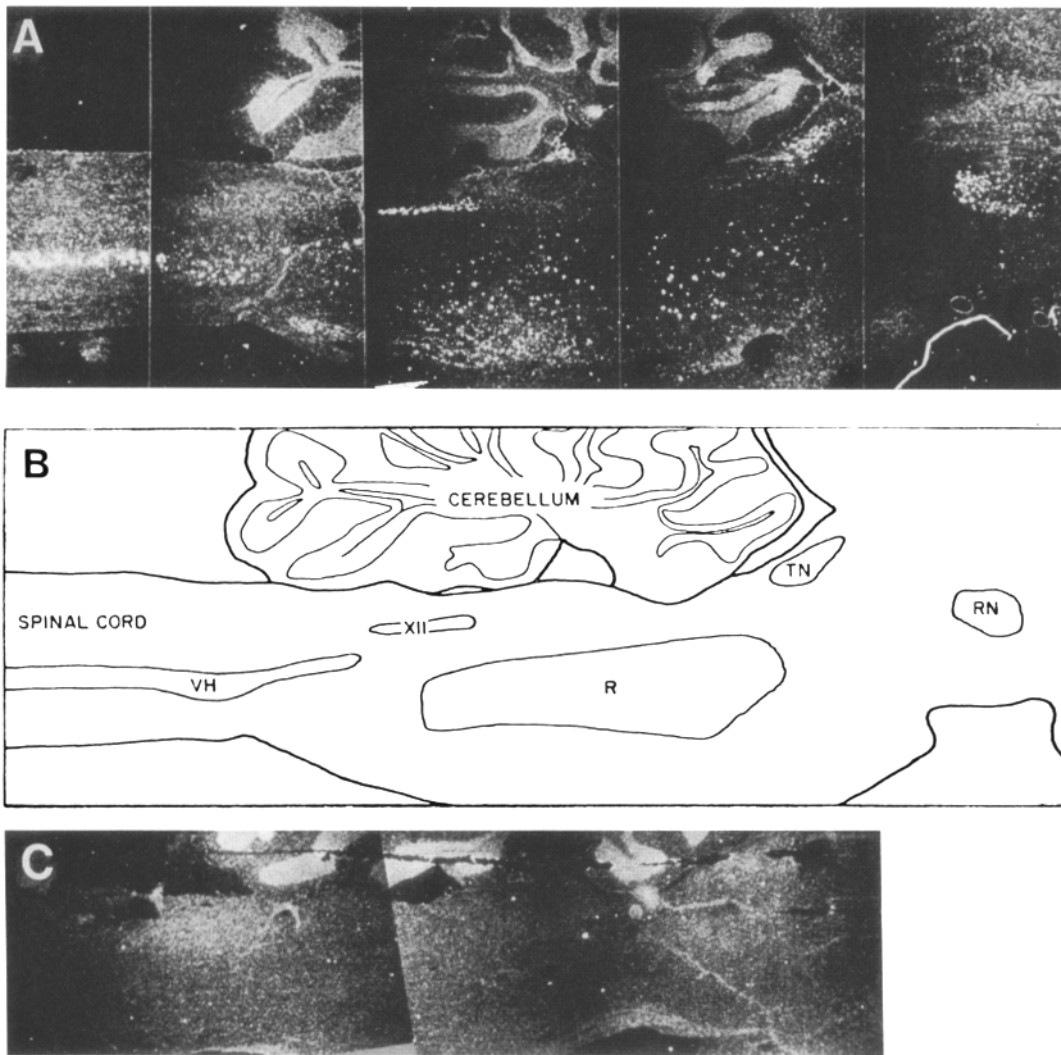
Figure 5. Distribution of clone 73 mRNA in the CNS I. (*A*) A parasaggital section of the rat brain ~1.7 mm from the midline was hybridized with an anti-sense probe of clone 73 and exposed for 2 wk. The section was photographed with dark-field illumination so that the silver grains appear white. (*B*) Line drawing of the rat brain section shown in *A*, outlining the regions with positive hybridization signal. *DN*, dentate nucleus of the deep cerebellar nuclei; *D*, deiter's neurons of the lateral vestibular nucleus; *NA*, possibly the nucleus ambiguus; V, the motor nucleus of the trigeminal; *VII*, facial nucleus; *VC*, vestibular complex, lateral (left) and medial (right, uncertain identification) vestibular nuclei. Bar, 500 μm.

tributions. The currently known IF proteins are desmin, vimentin, glial fibrillary acidic protein (GFAP), the three neurofilaments, the heterogeneous group of the cytokeratins, and, most recently, the nuclear lamins. The type I and II keratins are the type I and II IFs; desmin, vimentin and GFAP are all type III IFs; and the three neurofilament (NF) proteins, designated by size as high (NF-H; ~200 kD), medium (NF-M; ~150 kD), and low (NF-L; ~68 kD), are the type IV IFs.

One distinctive feature of most of these IF proteins is their particular tissue distributions. The keratins are found in all classes of epithelial and epithelial-derived cells. Desmin is only found in muscle cells. Vimentin is widely distributed in most mesenchymal cells. GFAP and the NFs are restricted to astrocytes and most, but not all, neurons, respectively (Trojanowski et al., 1986). The role(s) of these IF proteins in cellular architecture and function are not yet fully understood, nor is it clear that all types of IF proteins have yet been defined.

The present study describes the characterization of a cDNA clone originally identified as hybridizing to a mRNA that is increased in relative abundance by NGF in the PC12 pheochromocytoma cell line (Leonard et al., 1987). The amino acid sequence translated from the nucleotide se-

Figure 6. Distribution of clone 73 mRNA in the CNS II. (*A*) A parasaggital section of the rat brain ∼0.7 mm from midline hybridized with an anti–sense probe of clone 73 and exposed for 2 wk. (*B*) Line drawings of the section shown in *A*, outlining the regions with positive signal. *R*, scattered reticular neurons; *RN*, red nucleus; *TN*, tegmental nucleus of Gudden; *VH*, ventral horn motor neurons of the spinal cord; *XII*, hypoglossal nucleus. (*C*) Parasaggital section adjacent to the section shown in *A* but hybridized with the corresponding control, sense probe of clone 73, and exposed for 2 wk. The sections in *A* and *C* were photographed with dark-field illumination so that the silver grains appear white. In *A* and *C*, the light appearance of the cerebellar granule cell layer does not represent silver grains and is a consequence of dark-field illumination of the stained tissue.

quence of this cDNA reveals strong homology to all the known IF proteins, with strongest overall homology to the type III IFs. The degree of homology of the more highly conserved rod domain of the IF proteins can approach 70% when comparisons are made within an IF type and decrease when comparisons are made between the types. The homology of the clone 73 amino acid sequence to each of the presently available IF protein sequences was calculated as the percentage of identical amino acid matches obtained from a computer alignment of the sequences (Table II). In general the clone 73 amino acid sequence is most homologous to hamster desmin (59% overall; 69% for the rod) (Quax et al., 1985), followed by the other two members of the type III IF proteins, hamster vimentin (Quax et al., 1983) and mouse GFAP (Lewis et al., 1984) (55% and 54%, respectively, overall; 65% and 60%, respectively, for the rod). Mouse NF-L (Lewis and Cowan, 1986) and rat NF-M

(Napolitano et al., 1987) have approximately the same overall homology (41% and 37%, respectively) with the translated clone 73 sequence. Even less homologous are the mouse 59-kD type I (Krieg et al., 1985) and mouse 67-kD type II (Steinert et al., 1985a) keratins (23% and 27%, respectively). Least homologous are the nuclear lamins (20% overall), which have recently been reported to have the characteristic IF domains (McKeon et al., 1986; Fisher et al., 1986). Whereas the clone 73 amino acid sequence is most homologous to the carboxy-terminus and the rod domain of desmin, the clone 73 amino-terminal region is most homologous to the 68-kD NF-L protein (Table II).

Several features of the clone 73 amino acid sequence are apparent when it is aligned with the other type III IF protein sequences and NF-L (Fig. 9). The alignment demonstrates that the clone 73 amino acid sequence has all the domains characteristic of the IF proteins, as just described. The clone

A
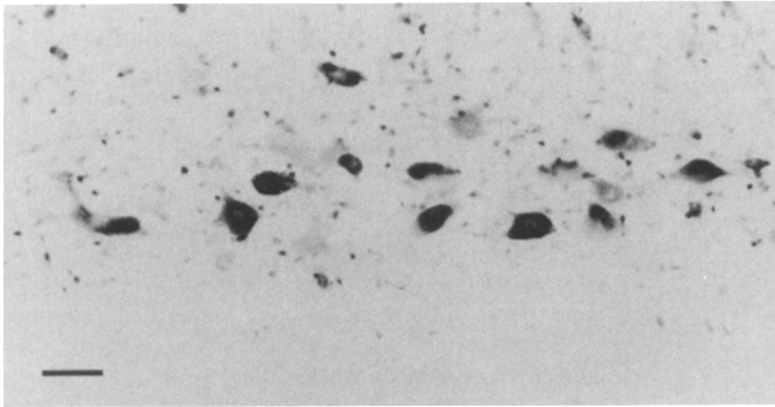
B
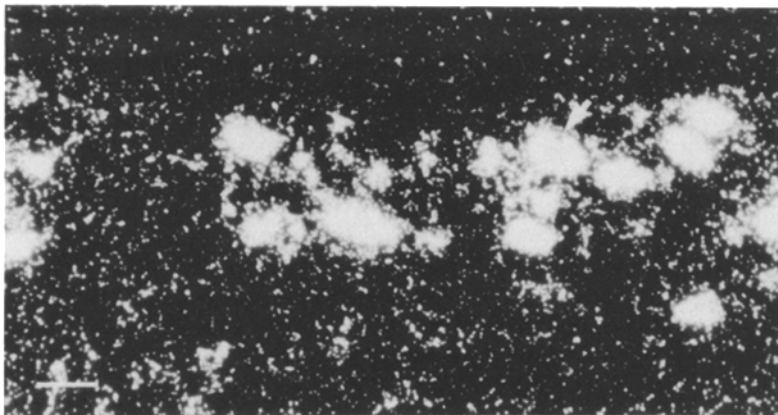
Figure 7. In situ hybridization of clone 73 to the ventral horn motor neurons. (*A*) Parasaggital section of the spinal cord was stained with cresyl violet and photographed with bright-field illumination. (*B*) Section of the spinal cord, adjacent to the section shown in *A*, was hybridized with the experimental anti–sense probe of clone 73, exposed for 2 wk, and photographed with dark-field illumination. Silver grains in the emulsion appear as white dots. Bar, 50 μm.

73 amino acid sequence also contains the two consensus-type sequences at the amino-terminal end of coil 1a and at the carboxy-terminal end of coil 2 (indicated by boxes in Fig. 9), which are found in all IF proteins (Osborn and Weber, 1986). By the homology analysis shown in Table II, the head region of clone 73 is slightly more homologous to NF-L than to the type III IF proteins, which have the greatest overall homology. There is a sequence in the head domain shared by GFAP, vimentin, desmin NF-L, and clone 73 (enclosed in boxes, Fig. 9). There is a second short region of homology between clone 73 and the type III IF proteins, but not NF-L, in the tail domain (enclosed in a box, Fig. 3). It is thought that the head and tail domains of the IF proteins provide the unique functional properties of each of the IF proteins. It is possible that these short homologous sequences represent functionally significant regions of these IF proteins. These are particularly noteworthy in light of the general lack of homology between the IF proteins in the head and tail domains. A final observation from the alignment of these sequences is that the amino acid sequence of clone 73, while not complete, is very close to the amino-end of the protein, assuming that clone 73 protein is approximately the same size as the other type III IF proteins.

Whereas the clone 73 amino acid sequence has all the characteristic features common to IF proteins, it is distinct from all the known IF proteins. One novel feature of this new IF protein is the high percentage of serine residues in the amino-terminus of the clone 73 amino acid sequence. These serine residues are potential phosphorylation sites and could play a role in the regulation or function of this protein in the cell. The final evidence that clone 73 represents a new IF protein is the unique tissue distribution of this mRNA in the PNS and CNS. Clone 73 mRNA is expressed in sympathetic, sensory, and ciliary ganglion neurons, in ventral horn motor neurons, in the neurons of the motor nuclei of the cranial nerves, and in several other hindbrain and midbrain regions. It is not detectable in a wide variety of nonneuronal tissues, in glia, or in most CNS neurons. This is different from the expression pattern of all previously known IF proteins.

The only other IF proteins with a purely neuronal distribution are the three NF proteins. However, these IF proteins have been detected by immunohistochemical methods in all identifiable PNS and CNS axons, in nearly all PNS perikarya, and in most CNS neuronal perikarya (Trojanowski, et al., 1986). The CNS neurons lacking detectable NF proteins were generally small. In addition, the NF proteins are also present in adrenal chromaffin cells and cells of the pars distalis and pars intermedia. The NF proteins are clearly detectable in the large neurons of the cerebral and cerebellar cortices, which do not express clone 73 mRNA. Though
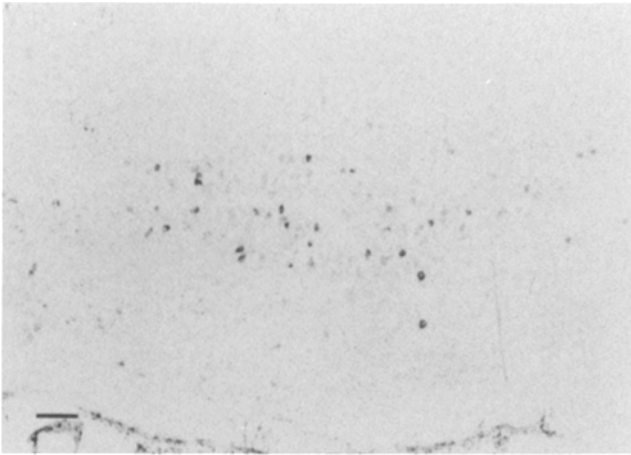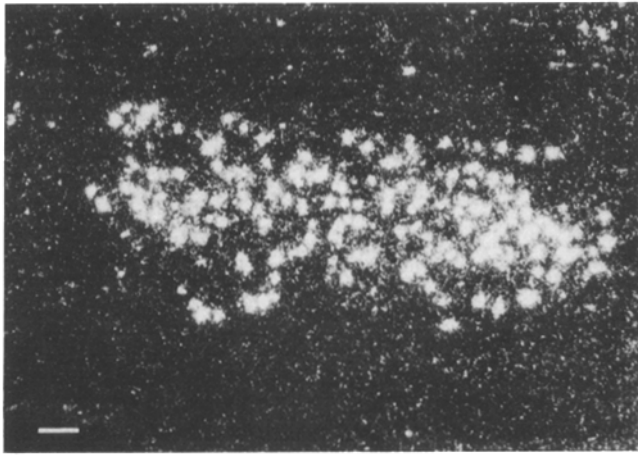
overlapping, the nervous system distributions of clone 73 mRNA and the neurofilaments are different. A final distinguishing feature between the NF proteins and the clone 73 amino acid sequence is that clone 73 does not have the highly acid carboxy-terminus characteristic of the NF proteins (Lewis and Cowan, 1986).

CNS Distribution of Clone 73 mRNA

The major feature of the CNS distribution of clone 73 mRNA is that most of the regions are related to motor functions (see Paxinos, 1985, and Bordal, 1981, for discussions of these CNS regions). The motor nuclei of the cranial nerves consist of motor neurons that directly innervate the extraocular muscles (III, IV, and VI), the superficial muscles of the head and neck (V), the muscles of mastication (VII), the laryngeal muscles (nucleus ambiguus), and the tongue (XII). The ventral horn motor neurons innervate skeletal muscles throughout the body. The red nucleus functions as a major relay center, receiving its input primarily from the cerebral cortex and cerebellum (at least in part from the dentate nucleus) and sending its major output to ventral horn motor neurons, to nuclei of the Vth and VIIth cranial nerves, to the inferior olivary nucleus, and to the vestibular complex. The medial and lateral vestibular nuclei receive afferents from the vestibular nerve and the cerebellum, and send efferent fibers to the ventral horn of the spinal cord and to the oculomotor nuclei. The dorsal motor nucleus of the vagus contains preganglion parasympathetic neurons that are part of the two-cell relay to innervate the glands and the smooth muscle of much of the abdominal viscera. Although we have not defined the specific reticular formation neurons that are expressing clone 73 mRNA, the reticular formation does modulate muscle activity via the reticulospinal tracts. Fi-

Figure 8. In situ hybridization of clone 73 to the facial nucleus. (*A*) Parasaggital section of the brain was stained with cresyl violet and photographed with bright-field illumination. (*B*) Section of the brain, adjacent to the section shown in *A*, was hybridized with the experimental anti-sense probe clone 73, exposed for 2 wk, and photographed with dark-field illumination. Silver grains in the emulsion appear as white dots. Arrow indicates a ringlike pattern of silver grains over a neuron. Bar, 93 μm.

Table II. Sequence Homologies of the Translated Clone 73 cDNA and IF Proteins

	NH₂-terminus	NH₂-terminal helix	Coil 1a	Linker 1	Coil 1b	Linker 1-2	Coil 2	COOH-terminus	Rod region	Termini	Overall
	%	%	%	%	%	%	%	%	%	%	%
Hamster desmin	22	75	79	45	58	48	77	44	69	32	59
Hamster vimentin	12	54	76	36	55	26	79	41	65	24	55
Mouse GFAP	13	50	73	27	53	39	69	27	60	24	54
Mouse NF-L	28	29	58	36	50	30	58	2	51	15	41
Rat NF-M	23	21	55	18	44	13	53	13	44	14	37
Mouse type II keratin	25	18	52	18	27	13	38	5	32	15	27
Mouse type I keratin	0	4	42	18	31	35	36	0	32	0	23
Human Lamin A	5	14	15	9	24	13	38	0	27	2	20

The NH₂-terminus is the head domain (60 aa). The NH₂-terminal helix is the region from the beginning of the rod domain to the beginning of coil 1a (28 aa). Linker 1 is the region between coil 1a and 1b (11 aa), and linker 1-2 extends between coil 1b and 2 (23 aa). Coils 1a (33 aa), 1b (96 aa), and 2 (146 aa) are as indicated in Figs. 1 and 9. The COOH-terminus is the tail domain (57 aa). The rod region (337 aa) is as indicated in Figs. 1 and 9, and the termini (123 aa) are the NH₂- and COOH-termini together. The homologies are calculated as the number of identical aa matches in a computer alignment of the sequences being compared out of the total number of aa in clone 73 for that region. The NH₂- and COOH-termini were calculated as the number of matches out of the number of aa in the shorter of the two sequence termini being compared. The sequences are: hamster desmin (Quax et al., 1985); hamster vimentin (Quax et al., 1983); mouse GFAP, glial fibrillary acid protein (Lewis et al., 1984); mouse NF-L, 68-kD neurofilament (Lewis and Cowan, 1986); rat NF-M, 150-kD neurofilament (Napolitano et al., 1987); mouse type II 67-kDa keratin (Steinert et al., 1985a); mouse type I 59-kD keratin (Krieg et al., 1985); and human lamin A (McKeon et al., 1986; Fisher et al., 1986).

Figure 9. Comparison of clone 73 amino acid sequence with the type III IF and NF-L proteins. The alignments were computer generated by comparison of each sequence with clone 73 amino acid sequence. Identical amino acid matches of clone 73 and one or more of the IF protein sequences are indicated by bold type. Head, rod, coil, linker, and tail region are delineated by vertical bars. L1 and L1-2 are the linker regions between coils 1a and 1b, and coils 1b and 2, respectively. The boxes indicate two consensus type homologies at the beginning of coil 1a and end of coil 2, and two small regions of homology between NF-L and clone 73 in the head region and between the type III IF proteins and clone 73 in the tail region. The sequences are NF-L, mouse 68 kD neurofilament (Lewis and Cowan, 1986); GFAP, mouse glial fibrillary acid protein (Lewis et al., 1984); hamster vimentin (Quax et al., 1983); and hamster desmin (Quax et al., 1985).

nally, we have found labeling in the ventral tegmental nucleus of Gudden, which is the one CNS region that is not clearly related to motor function. However, the connectivity of this nucleus is also the least well known of all the regions expressing clone 73 mRNA. This tegmental nucleus contributes fibers to the medial forebrain bundle that projects rostrally to many regions including the hypothalamus, olfactostriatum, and other forebrain structures. Perhaps the expression

of this mRNA has implications for a possible motor-related role for this tegmental nucleus.

Implication of the Distribution of Clone 73 mRNA

There is currently no functional or developmental theme that can unify the diverse regions of the PNS and CNS that express clone 73 mRNA. Functionally, the distribution includes motor, sensory, and interneurons, as well as peptidergic, cholinergic, and nonadrenergic transmitter systems (see Paxinos, 1985, and Brodal, 1981). Developmentally, the PNS is derived from the neural crest, whereas the CNS derives from the neural tube (Jacobson, 1978). Two characteristics are shared by many, but not all, of the neurons expressing clone 73 mRNA. Most of the neurons have axons that are located, at least in part, outside the CNS. Most have relatively long axons. Perhaps this new IF protein, encoded by the clone 73 mRNA, functions to stabilize these axons in a manner different from that of microtubules or the neurofilaments. In well-differentiated PC12 cell cultures, the neurites can remain extended for at least 24 h after essentially total disassembly of the microtubules by the drug nocodazole (Aletta and Greene, 1987). The stabilizing cytoarchitecture that remains can be seen by electron microscopy to possess numerous intermediate filaments. These could consist of the clone 73 IF protein, because there are only relatively low levels of the NF proteins present in PC12 cells (Franke et al., 1986).

A final very broad feature in common to the neuronal regions expressing clone 73 mRNA is that they are all evolutionarily old. The CNS distribution of this mRNA is predominantly in the hindbrain, and not in the more recently evolved forebrain structures. The significance of the expression of this mRNA, encoding a protein that is potentially cytoarchitecturally important, in such a diverse group of neurons remains to be understood. The existence of the clone 73 mRNA raises new questions about the possible common features of the cytoarchitecture, development, evolution, and functions of the diverse group of neurons that express the clone 73 intermediate filament protein.

Other Studies of Novel IF Proteins and Their Possible Relation to Clone 73

Liem et al. (1978) have described a 60-kD protein that was present in neurofilament preparations of spinal nerve roots but that did not correspond to one of the neurofilament triplet proteins. Portier et al. (1984a) electrophoretically characterized a triton-insoluble protein of apparent M_r 56 kD that is present in PC12 cells and murine neuroblastoma cells. The level of this species is induced in PC12 cells in response to NGF. This protein was also detected in sympathetic and sensory neurons, but not in a variety of nonneuronal cell types or in homogenates of whole brain or of several brain regions (Portier, 1984a and b) and was therefore designated as "peripherin." On the basis of its cross-reactivity with intermediate filament proteins, filamentous staining pattern upon immunostaining, and possession of a single tryptophan residue in the central part of the molecule, it was proposed that peripherin represents a new member of the IF protein family (Portier et al., 1984b).

Parysek and Goldman (1986 and 1987) have described a 57-kD IF protein which they have isolated from PC12 cells. By immunofluorescence staining with a specific antibody, this 57-kD IF protein appears to be abundant in the axons of spinal cord roots and in nerve bundles of sections of skin, tongue, and sciatic nerve. The distribution is distinct from that of vimentin and the 150-kD and 200-kD NF proteins by double-labeling experiments. Franke et al. (1986) have analyzed the IF proteins of PC12 cells and noted a 59-kD protein in two-dimensional gel analyses of PC12 cell cytoskeletal preparations. This protein was reported to be increased by NGF treatment of PC12 cells. It is quite possible that these studies have identified the same protein, and that this may be the same as the clone 73 IF protein. However, this identity remains to be established.

Regulation of Clone 73 mRNA by NGF

Although clone 73 mRNA is known to be increased by NGF in PC12 cells with a time course similar to that of neurite outgrowth (Leonard et al., 1987), the regulation of this mRNA and its encoded protein in vivo is unknown. NGF is necessary for the normal development of sympathetic and some sensory neurons and for the continued survival of sympathetic and some sensory neurons in vivo and in vitro (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968). Therefore, if the NGF-induced differentiation of PC12 cells does model the differentiation of sympathetic, and possibly sensory neurons, this protein may have a role in the in vivo differentiation of these neuronal types. More recent studies have demonstrated the presence of NGF and its receptor in many CNS regions (Korsching et al., 1985; Richardson et al., 1986), and there is some evidence for the NGF responsiveness of some cholinergic neurons of the forebrain (see for example, Honnegger and Lenoir, 1982; Mobley et al., 1985). There is evidence in the chick embryo that the high-affinity NGF receptor is transiently expressed in the ventral horn of the spinal cord and in muscle at the time of motoneuron synapse formation and elimination (Raivich et al., 1985). And, NGF has been shown to stimulate neurite outgrowth from dissociated parasympathetic neurons from the ciliary ganglion, although it has no effect on increasing the survival of these neurons in culture (Collins and Dawson, 1983). The role of NGF, if any, in the development of other areas of the nervous system, and the details of its action during the entire course of the developmental process are not fully known. Thus, it remains to be determined whether NGF regulates the clone 73 mRNA and its encoded IF protein during specific developmental windows, or throughout neuronal development, or under other special circumstances such as axonal regeneration, for some or all of the neurons expressing this mRNA in the adult. Alternatively, other neurotrophic factors could regulate clone 73 mRNA and protein in some or all of these neurons. Indeed, we have shown that the clone 73 mRNA is regulated by FGF as well as NGF (Leonard et al., 1987).

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