0022-1554/86/$3.30
The Journal of Histochemistry and Cytochemistry

Vol. 34, No. 7, pp. 923–926, 1986
Printed in U.S.A.

Original Article

Specific Detection of Neuronal Cell Bodies: In Situ Hybridization With a Biotin-labeled Neurofilament cDNA Probe

PÄIVI LIESI, JEAN-PIERRE JULIEN,[1] PEKKA VILJA, FRANK GROSVELD, and LEENA RECHARDT[2]

Laboratory of Gene Structure and Expression (PL,J-PJ,FG) and Laboratory of Neurobiology (PL), National Institute for Medical Research, Mill Hill, London NW7 1AA, UK; Department of Biomedical Sciences, University of Tampere, 33101 Tampere, Finland (PV,LR).

Received for publication September 17, 1985 and in revised form December 27, 1985; accepted for publication January 8, 1986 (5A0549).

We have used a biotinylated, 300-nucleotide cDNA probe which encodes the 68,000 MW neurofilament protein to detect neurofilament-specific mRNA in situ. The neurofilament message specifically demonstrates the neuronal cell bodies, in contrast to the usual antibody staining which detects their neurites. The hybridization is detected only in neuronal structures. Consequently, detection of the biotinylated neurofilament DNA probe by silver-intensified streptavidin–gold can be specifically used to identify neuronal cell bodies.

KEY WORDS: In situ hybridization; Neurofilament cDNA probe; Neurofilament; Biotin-labeled probe.

Introduction

Neurofilament (NF) triplet proteins form intermediate filaments expressed exclusively by neurons (Lazarides, 1980). The 68,000 MW neurofilament protein appears early in the course of neuronal differentiation (Shaw and Weber, 1982; Cochard and Paulin, 1984) but, like the other triplet components, its role in neuron function is not presently understood. Recently, cDNA probes encoding for this protein have been cloned and used to detect neurofilament transcripts on RNA blots (Julien et al., 1985; Lewis and Cowan, 1985). The availability of these probes provides a new approach to investigate NF gene expression and its role in the central nervous system.

Materials and Methods

A 300-BP PstI mouse cDNA fragment was labeled with biotin, using a modification of the radiolabeling procedure described by Feinberg and Vogelstein (1983). The polymerization reaction that was primed by random hexadeoxyribonucleotides was carried out at 20°C for 2 hr in the presence of 2 µM dATP, dbGTP, dCTP, and biotinylated dUTP (Enzo Biochem. Inc., New York, NY). The reaction was stopped with 50 mM EDTA and the biotinylated DNA purified on a 1-ml Sephadex G-50 column.

Preparation of the Streptavidin–gold Complex. Colloidal gold (particle size about 15 nm for visualization under a light microscope) was prepared according to Frens (1973) by boiling tetrachloroauric acid (0.01%, 100 ml) with trisodium citrate (1%, 5 ml). The pH of the gold solution was adjusted to 6.6 with 0.2 M K_2CO_3. The protein concentration required for stable labeling of colloidal gold was determined by the salt flocculation assay of Geoghegan and Ackerman (1977). Streptavidin 70 µg (Bethesda Research Laboratories, Gaithersburg, MD) was used to stabilize 10 ml of colloidal gold as described (De Mey, 1983). In short, after 2 min of reaction, bovine serum albumin (BSA; 96–99% pure; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1%, and the crude preparation was centrifuged twice at 14,000 × g for 1 hr at 4°C. The resulting pellet (streptavidin–gold complex) was re-suspended to 1.5 ml of 20 mM Tris-buffered saline, pH 8.2, containing 1% BSA and 0.02% NaN_3, and used at a twofold dilution.

Preparation of Adult Brain Tissue for In Situ Hybridization. Brain and liver tissue was removed from anesthesized Sprague–Dawley rats and quickly frozen. Cryostat sections 10-µm were cut and placed on glass slides which had been pre-washed with ethanol and coated with poly-L-lysine (Sigma). The sections were then processed for in situ hybridization according to the following protocol:

1. Air-dry for 2 hr at room temperature (RT).
2. Fix in 2% paraformaldehyde in PBS for 30 min at RT.
3. Wash in PBS 3 × for 5 min.
4. Dehydrate through an ethanol gradient (sections can be stored for weeks at RT).
5. Incubate in 0.2 N HCl at RT for 20 min.
6. Rinse in distilled water for 5 min.
7. Incubate in 0.3 M NaCl–0.03 M Na citrate, pH 7.0, for 30 min at 70°C.

[1] Present address: Institut du Cancer de Montreal, Centre Hospitalien Notre-Dame, 1560 est, Sherbrooke, Montreal, Canada H21 4M1.

[2] Correspondence to: Dr. Leena Rechardt, Department of Biomedical Sciences, Univ. Tampere, Box 607, 33101 Tampere, Finland.

8. Rinse in distilled water at RT for 5 min.
9. Blot the sections dry.
10. Treat with predigested pronase (0.25 mg/ml; Sigma) in 50 mM Tris–HCl–5 mM EDTA, pH 7.5) 10 min at RT. (Predigestion of pronase: dissolve pronase in distilled water to a final concentration of 40 mg/ml. Incubate at 37°C for 4 hr.)
11. Quick-dry.
12. Immerse in glycine (2 mg/ml) in PBS.
13. Wash in PBS.
14. Fix in 4% paraformaldehyde in PBS for 20 min at RT.
15. Acetylate (Hayashi et al., 1978).
16. Wash in PBS for 5 min.
17. Dehydrate through an ethanol gradient.
18. Prepare the probe solution as follows: dissolve 20% dextran sulphate in deionized formamide (A). Use salmon sperm DNA (200 µg/ml dissolved in Denhardt's medium to prepare sol (B). Mix equal volumes of A and B with the biotinylated probe to give a final concentration of 2 ng/µl.
19. After brief denaturation, apply 100 µl of the probe solution to a brain section, and incubate in a moist chamber for 48 hr at 37°C in the dark.
20. Wash in 50% formamide in 0.6% NaCl–10 mM Tris–HCl (pH 7.5)–1 mM EDTA for 30 min at RT with vigorous shaking.
21. Wash in PBS 3 × for 5 min.
22. Incubate in streptavidin–gold solution diluted 1:2 in PBS for 2 hr at RT.
23. Wash in PBS and intensify with silver as described (Danscher and Nörgaard, 1983)

Results

In Situ Hybridization Method

Figure 1 shows a restriction cleavage map of our mouse NF cDNA clone (Julien et al., submitted). For in situ hybridization we used a 300-BP cDNA fragment corresponding to the α-helical region of the 68,000 MW NF protein. In our experiments, similar results were obtained with a tritiated (not shown) or biotinylated DNA probe. We found, however, the detection by streptavidin–gold more rapid and sensitive than autoradiography. Essentials for successful hybridization were a) use of poly-L-lysine to pre-coat the glass slides, b) freezing of the tissue before fixation, c) acetylation of the sections, and d) brief but vigorous washing after the hybridization and streptavidin–gold steps. In our case, acetylation of the glass slides and sealing of the sections during incubation with the probe (Haase et al., 1985) were not found necessary. The advantage of the biotin–streptavidin–gold hybridization method is its possible direct application to electron microscopy.

Localization of NF Transcript

The 68,000 MW neurofilament transcripts were localized in neuronal cell bodies leaving the nuclear and axon hillock areas unstained (Figure 2A). Neuronal cell layers in the locus coeruleus (Figure 2A) and the cortex (Figure 2D) were clearly demarcated. In cerebellum, the internal granular cell layer was positive and the Purkinje cells expressed the neurofilament message (not shown). Hybridization was not found in the glial elements, capillaries, ependymal cells, meningeal structures, or nerve fiber projections (Figures 2A and 2D).

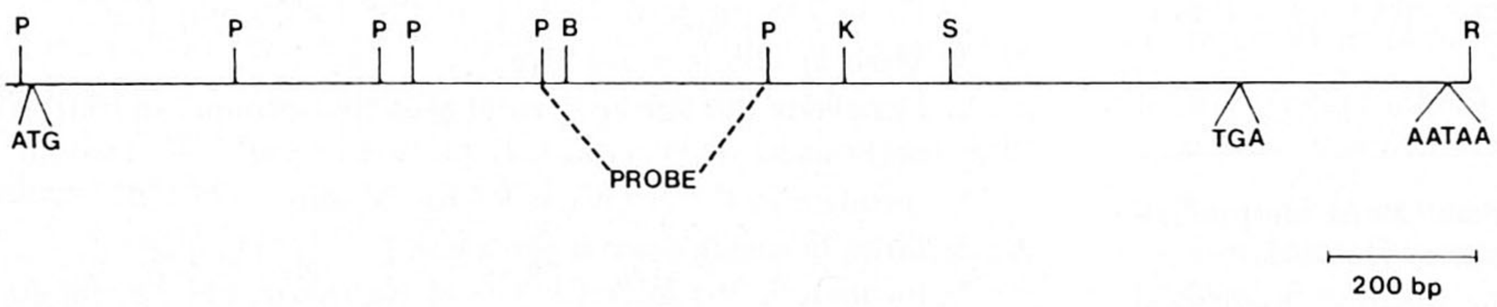
The liver control (Figure 2E) and a brain control treated with a probe derived from the plasmid pBR322 yielded no hybridization signal above background (Figure 2B). Treatment only with the silver intensification procedure did not stain the sections, nor did treatment with only the streptavidin–gold complex intensified with silver (not shown).

Discussion

The present report shows that in situ hybridization with NF cDNA probes serves as a marker for neuronal perikarya. Biotin-labeled cDNA probes have previously been applied for localization of DNA (Hutchison et al., 1982; Manuelidis et al., 1982) and RNA (Singer and Ward, 1982). The authors used as a detection system either avidin coupled to rhodamine or peroxidase (Singer and Ward, 1982), or biotin antibody followed by second antibody coupled to gold particles (Hutchison et al., 1982).

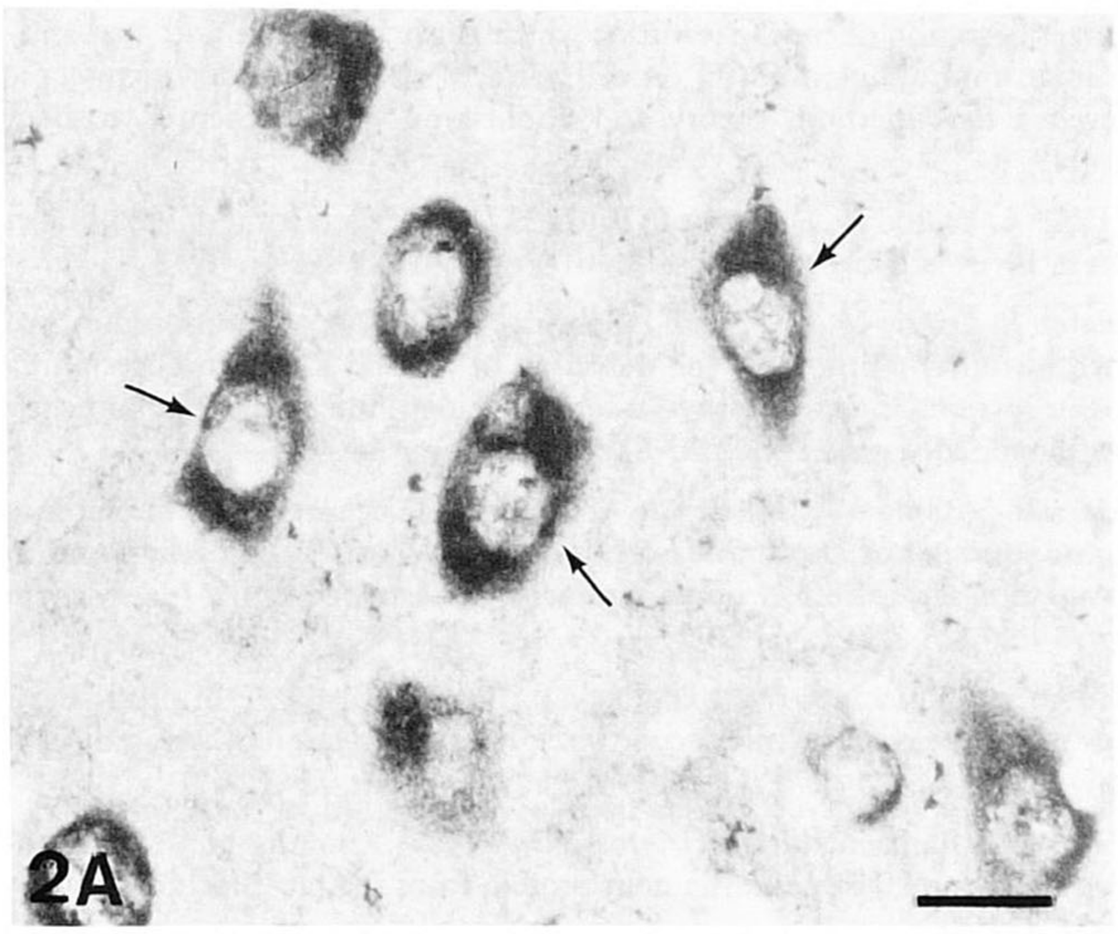
Our improved detection system for in situ hybridization of cellular mRNA is based on the use of a biotinylated, 300 nucleotide-long, 68,000 MW neurofilament-specific double-stranded cDNA probe and a combination of several published techniques. The application of the biotin-labeled probe is based on the strong affinity between avidin and biotin. To avoid possible nonspecific binding of avidin (Finn et al., 1980; Morris and Saelinger, 1984), we used streptavidin (Chaiet and Wolf, 1964). The streptavidin–gold complex, in combination with silver intensification, ensures high sensitivity of this non-isotopic detection system. Omission of several steps from the original approach of Haase et al. (1985) and use of streptavidin–gold detection instead of a non-isotopic double-antibody system (Hutchison et al., 1982) or autoradiography (Hafen et al., 1983) shortens the whole procedure.

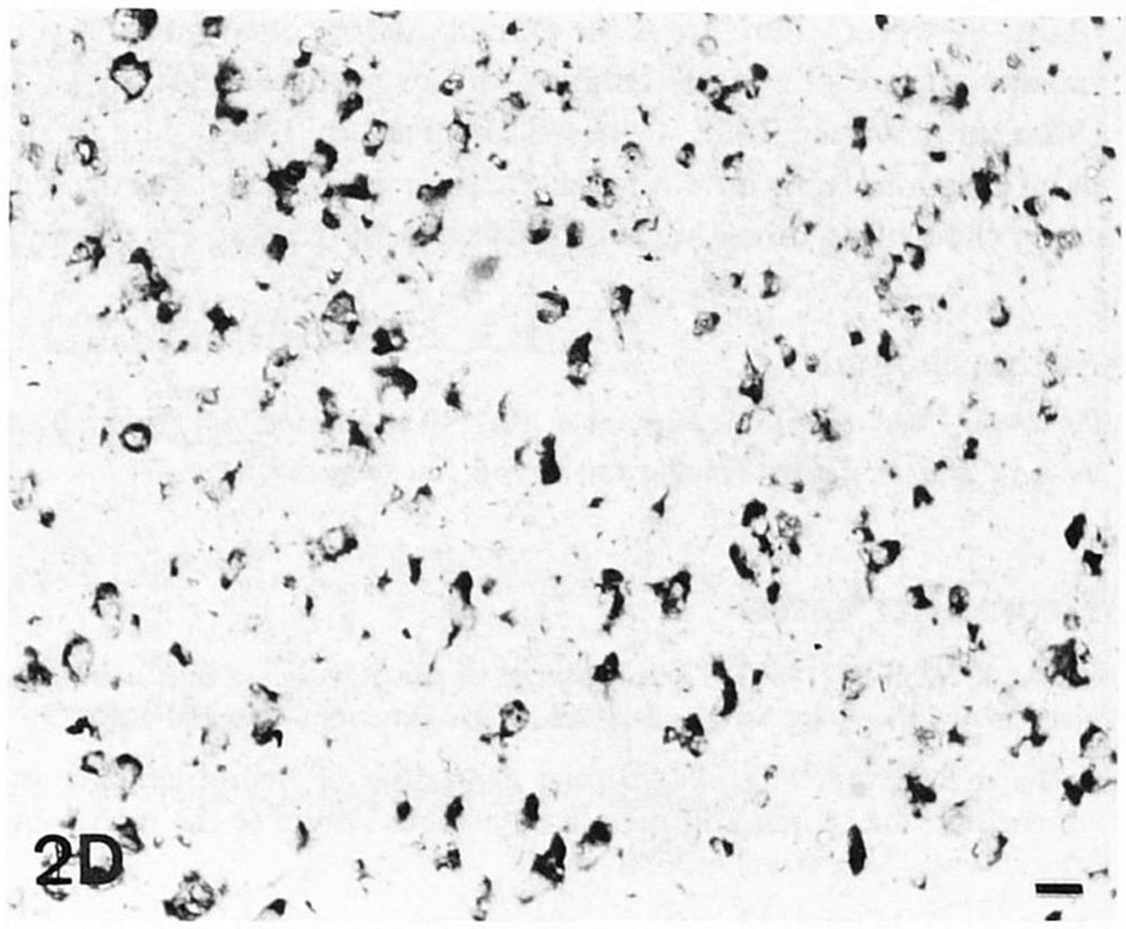
In conjunction with a highly specific neurofilament cDNA probe, this method makes possible distinct and rapid localization of neuronal cell bodies. Neurofilament antibodies have been widely used to detect nerve fibers in the developing and mature

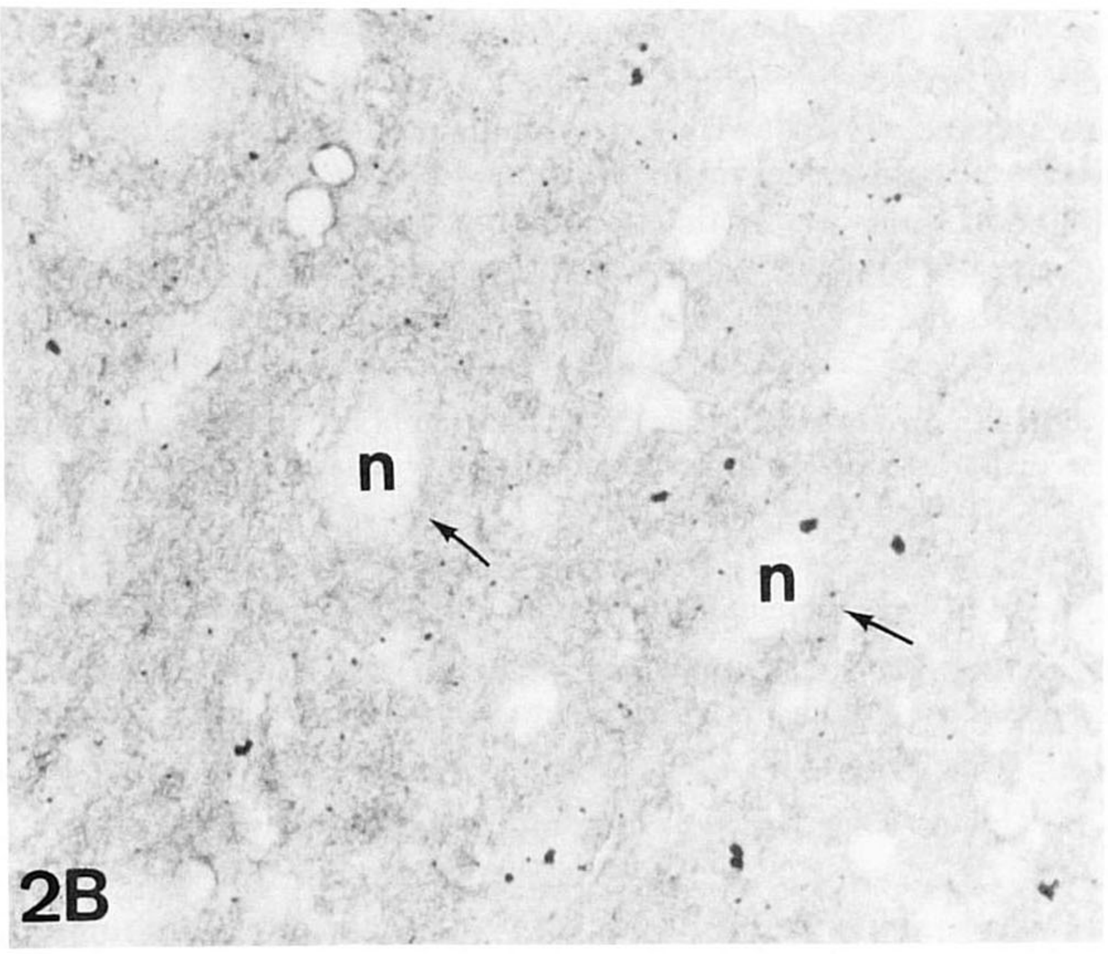


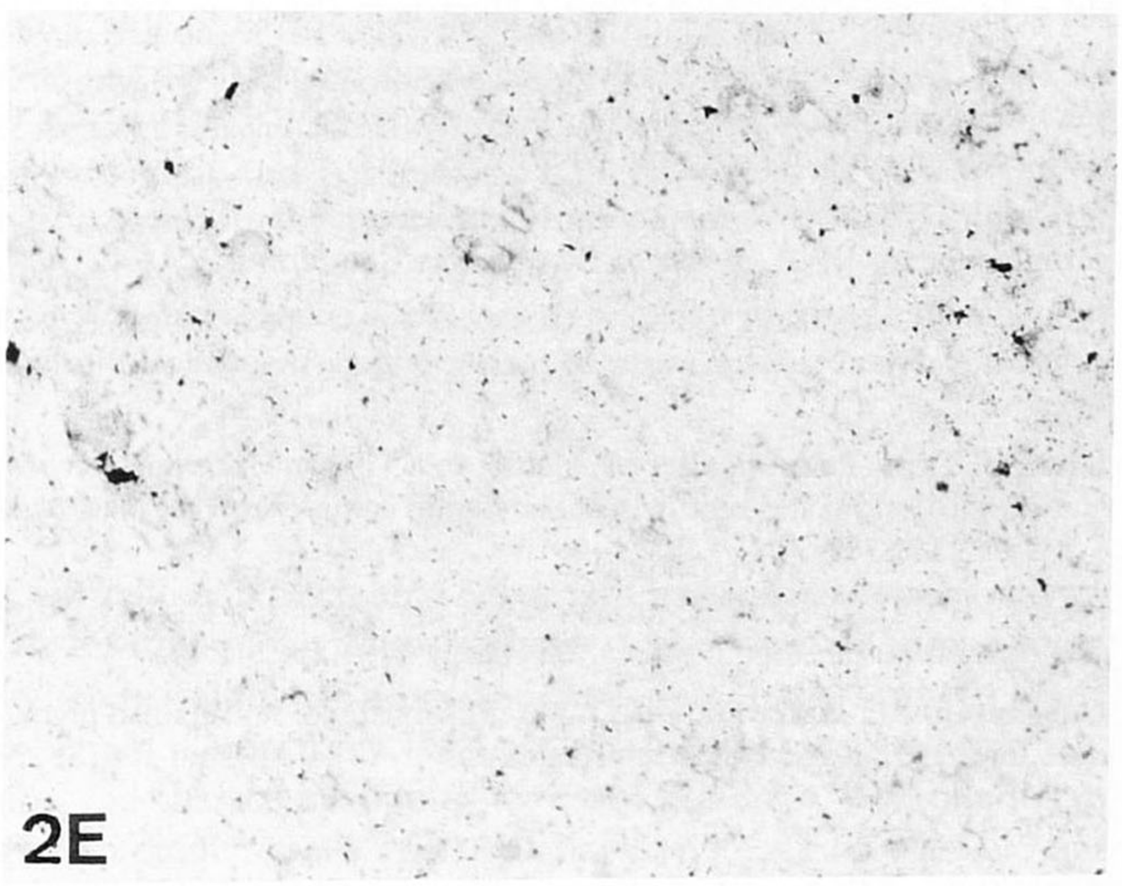
1

Figure 1. Restriction cleavage map of mouse cDNA encoding the 68,000 MW NF protein. The restriction map was established by single and double digestions with PstI (P), Bgl II (B), KpnI (K), and SacI (S) enzymes and by DNA sequence analysis. The locations of the initiating codon (ATG), stop codon (TGA), and a polyadenylation site (AATAA) are indicated. The cDNA fragment used as a probe corresponds to the α-helical region of the protein.









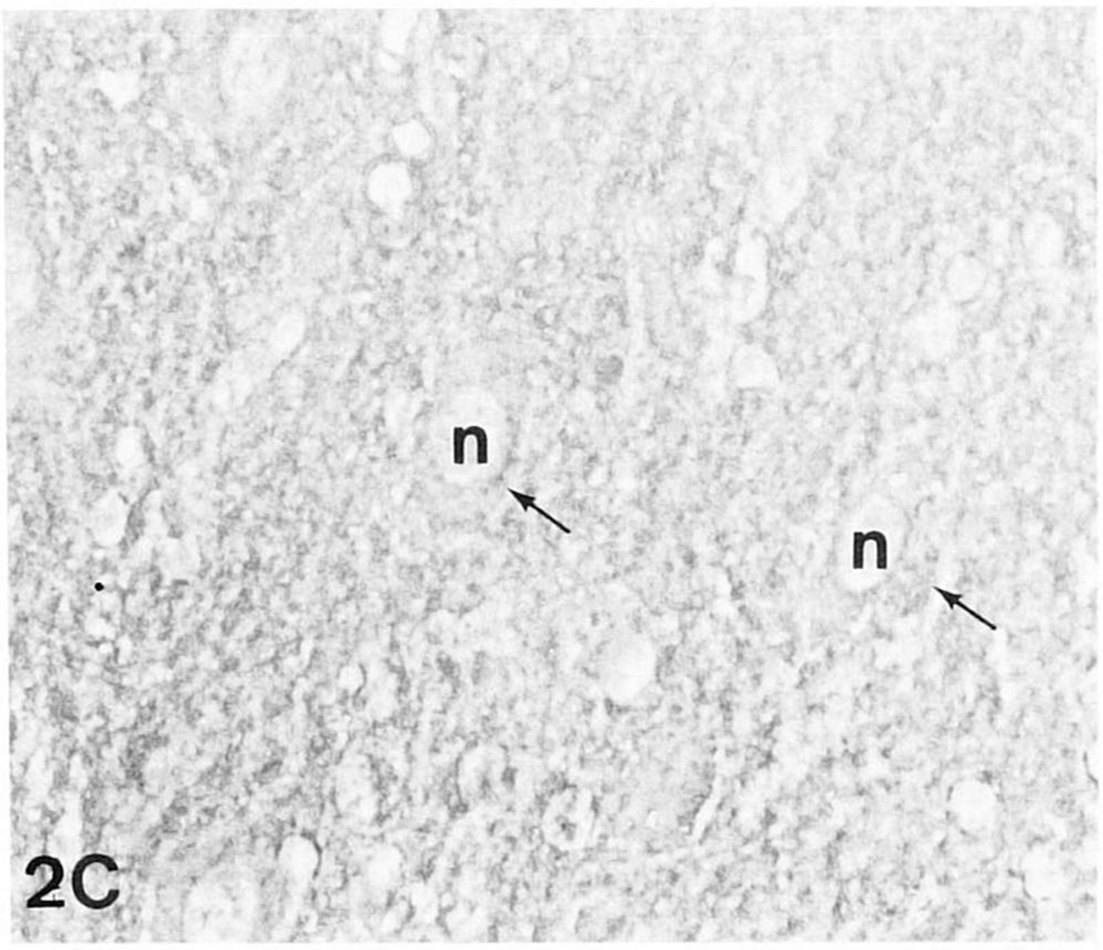


Figure 2. In situ localization of NF mRNAs in neuronal cell bodies of adult rat brain. A biotin-labeled cDNA probe encoding the 68,000 MW NF protein, hybridized as described in Materials and Methods, specifically detects neuronal cell bodies (arrows) in the nucleus locus coeruleus (**A**). In a control hybridization experiment with pBR322 plasmid DNA probe, no signal was obtained in neuronal cell bodies of the locus coeruleus (arrows). n = nucleus (**B**). (**C**) shows the phase-contrast picture of the cells (arrows) in (**B**). The low-magnification micrograph in (**D**) shows the specific detection of neuronal cell bodies in the cerebral cortex by the neurofilament cDNA probe. In a control experiment with adult rat liver, no hybridization signal was detected using the neurofilament cDNA probe (**E**). Bar = 10 μm.

brain. However, there are some discrepancies concerning the initial appearance of neurofilament proteins in the developing brain (Shaw and Weber, 1982; Cochard and Paulin, 1984). The in situ hybridization technique should provide a reliable approach to study the timing of the initial maturation of developing neurons.

Acknowledgments

We thank Miss Ursula Starega and Miss Minna Laiho for technical assistance and Mrs. Rita Findon for typing the manuscript.

Literature Cited

Chaiet L, Wolf FJ (1984): The properties of streptavidin, a biotin-binding protein produced by Streptomycetes. Arch Biochem Biophys 106:1

Cochard P, Paulin D (1984): Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. J Neurosci 4:2080

Danscher G, Nörgaard J (1983): Light microscopic visualization of colloidal gold on resin embedded tissue. J Histochem Cytochem 31:1394

De Mey J (1983): Colloidal gold probes in immunohistochemistry. In Polak JM, Van Noorden S, eds. Immunochemistry: Practical applications in pathology and biology. Bristol, Wright–PSG, 82

Denhardt DT (1966): A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641

Feinberg AP, Vogelstein (1983): A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6

Finn FM, Titus G, Montibeller JA, Hofmann K (1980): Hormone-receptor studies with avidin and biotinylinsulin. Avidin complexes. Proc Natl Acad Sci USA 255:5742

Frens G (1973): Controlled nucleation for the regulation of the particle size in monodisperse gold solutions. Nature Phys Sci 241:20

Geoghegan WD, Ackerman GA (1977): Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. J Histochem Cytochem 25:1187

Haase A, Brahic M, Stowring L, Blum H (1985): Detection of viral nuclei acids by in situ hybridization. Meth Virol VII:189

Hafen E, Levine M, Garber RL, Gehring WJ (1983): An improved in situ hybridization method for the detection of cellular RNAs in Drosophilia tissue sections and its application for localizing transcripts of the homeotic Antennapedia gene complex. EMBO J 2:617

Hayashi S, Gillam I, Delaney A, Tener G (1978): Acetylation of chromosome squashes of Drosophilia melanogaster decreases the background in autoradiographs from hybridization with ^{125}I-labelled RNA. J Histochem Cytochem 26:677

Hutchison N, Langer–Safer P, Ward D, Hamkalo B (1982): In situ hybridization at the electron microscope level: hybrid detection by autoradiography and colloidal gold. J Cell Biol 95:609

Julien J-P, Ramachandran K, Grosveld F (1985): Cloning of a cDNA encoding the smallest neurofilament protein from the rat. Biochim Biophys Acta 825:398

Lazarides E (1980): Intermediate filaments as mechanical integrators of cellular space. Nature 283:249

Lewis S, Cowan N (1985): Genetics, evolution and expression of the 68 000 mol wt neurofilament protein: isolation of a cloned cDNA probe. J Cell Biol 100:843

Manuelidis L, Langer–Safer PR, Ward DC (1982): High-resolution mapping of satellite DNA using biotin-labelled DNA probes. J Cell Biol 96:619

Morris RE, Saelinger CB (1984): Visualization of intracellular trafficking: use of biotinylated ligands in conjunction with avidin–gold colloids. J Histochem Cytochem 32:124

Shaw G, Weber K (1982): Differential expression of neurofilament triplet proteins in brain development. Nature 298:277

Singer RH, Ward D (1982): Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. Proc Natl Acad Sci USA 79:7331