Technical Note

Autoradiographic Visualization of $^{35}$S-labeled cRNA Probes Combined with Immunoperoxidase Detection of Choleragenoid: A Double-labeling Light Microscopic Method for In Situ Hybridization and Retrograde Tract Tracing

OLA HERMANSON, HANS ERICSON, GRACIELA SANCHEZ-WATTS, ALAN G. WATTS, and ANDERS BLOMQVIST

Department of Cell Biology, Faculty of Health Sciences, University of Linköping, Linköping, Sweden (OH, AB); Department of Neurology and Neuroscience, Cornell University Medical College, New York, New York (HE); and Department of Biological Sciences, University of Southern California, Los Angeles, California (GS-WAGW).

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We describe a protocol for simultaneous light microscopic visualization of a neuron's efferent projections and its expression of mRNA. We have combined immunohistochemical visualization of the retrograde marker cholera toxin subunit B (CTb) with autoradiographic visualization of $^{35}$S-labeled cRNA probes. Injections of CTb were made into rat brain. Immunoreactivity for CTb was demonstrated by modification of the peroxidase-anti-peroxidase immunohistochemical technique, with DAB and nickel ammonium sulfate or cobalt acetate as chromogen. On the same sections, in situ hybridization was performed with a $^{35}$S-labeled RNA probe complementary to preproenkephalin mRNA or tyrosine hydroxylase mRNA. Many double-labeled neurons were detected. These neurons contained peroxidase reaction product and were covered by an accumulation of silver grains in the overlaying emulsion layer. The present method has several advantages over double-labeling methods using the combination of fluorescent tracers and oligonucleotide probes. Both reaction products are permanent and can be visualized simultaneously by light microscopy. Furthermore, both CTb and cRNA probes are very sensitive markers. In addition, the sections can be counterstained. (J Histochem Cytochem 42:827–831, 1994)

KEY WORDS: Immunohistochemistry; Preproenkephalin mRNA; Tyrosine hydroxylase mRNA; Cholera toxin subunit B; Autoradiography; Rat.

Introduction

During the last decade, in situ hybridization histochemistry has become a powerful tool in neuroscience. Combined with retrograde tract tracing, the in situ hybridization method allows detection of mRNA in neurons with known projection. The availability of such combined techniques will benefit the study of gene expression and regulation in select neuron populations (1,14). Thus far, most double-labeling approaches have utilized oligonucleotide probes and retrograde transport of fluorescent markers (5,14). However, long cRNA probes also have been used in combination with fluorescent retrograde tracers, thereby increasing the sensitivity in the detection of mRNA (4,9). Fluorescent tracers are very versatile, but the fluorescence fades during analysis and with conventional optics it can be difficult to visualize simultaneously with the radiolabeled probes (20). Furthermore, the tissue cannot be counterstained with conventional histological stains, which makes the anatomic analysis more difficult.

We describe a double-labeling method that combines autoradiographic visualization of highly sensitive long cRNA probes with immunohistochemical detection of a sensitive retrograde tracer, cholera toxin subunit B (CTb) (6). We show the potential of this method in two systems: catecholaminergic nigrostriatal neurons, and enkephalinergic bulbobulbo and bulbo-spinal neurons. Our method yields reaction products that are permanent and can be visualized simultaneously. In addition, the sections can be counterstained.

Materials and Methods

Surgery and Tissue Preparation. Adult male Sprague-Dawley rats (200–400 g) (B&K Universal, Sollentuna, Sweden) were anesthetized with
6% chloral hydrate (0.5 ml/100 g) and placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA). Pressure injections of 1% CTb (Sigma; St Louis, MO) dissolved in 0.5 M Tris-HCl (pH 7.5) containing 2 M NaCl, 0.3 M Na\textsubscript{3}N\textsubscript{4}, and 0.1 M EDTA were made with a glass micropipette (8 = 40 μm) attached to a Hamilton syringe into the caudate putamen (0.05 μl), the thoracic spinal cord (0.45 μl), or the ventrolateral medulla (0.20 μl). After 24 h to 20 days, the animals were reanesthetized with sodium pentobarbital (100 mg/kg) (Apoeteksbolaget; Umeå, Sweden) and perfused transcardially with 100 ml of 0.9% NaCl followed by 500 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brain and spinal cord were removed and placed in 4% paraformaldehyde and 20% sucrose in 1 M PBS overnight at 4°C and then cut at 20 μm on a freezing microtome. The sections were collected and washed in sterile PBS before immunohistochemical processing. One series of sections was used for double labeling and adjacent series were used for single labeling with immunohistochemistry or in situ hybridization. All experimental procedures were approved by the Animal Care and Use Committees at all appropriate institutions.

Immunohistochemical Processing. The sections were incubated free-floating with the supernatant from two mouse hybridomas (anti-CTb2 and anti-CTb5; a gift from M. Wikström, Department of Medical Microbiology and Immunology, University of Göteborg, Sweden) (12); polyclonal antibodies to CTb are commercially available; see, e.g., ref. 13) diluted 1:50 each in sterile PBS containing bovine serum albumin (1%, Cohn fraction V; Sigma), heparin (500 IE/ml) (Kabi Pharmacia; Upssala, Sweden), and RNAsin (20 U/ml) (Promega; Madison, WI) for 72 hr at 4°C under constant agitation. Thereafter, they were washed six times in sterile PBS followed by an incubation in secondary antibody (1:30, rabbit anti-mouse) (Dakoport; Älvsjö, Sweden) diluted in sterile PBS with BSA (1%) and heparin (500 IE/ml) for 1 hr at room temperature (RT). The sections were washed three times in sterile PBS before incubation with peroxidase-anti-peroxidase antibody (1:100; PAP mouse monoclonal, Dakopatts) diluted in sterile PBS with BSA (1%) and heparin (500 IE/ml). After two rinses in sterile PBS and two rinses in 0.1 M sodium acetate (NaAc, pH 6) the sections were processed for 1–2 min in 0.5% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.02% H\textsubscript{2}O\textsubscript{2} added just before use to 0.1 M NaAc with 2.5% nickel ammonium sulfate (8). Alternatively, to nickel ammonium sulfate enhancement of the reaction product, the sections were pre-soaked with 1% cobalt acetate in 0.1 M NaAc for 10 min, then washed five times in NaAc before processing in DAB (0.05%), ammonium chloride (0.04%), glucose (0.2%), and glucose oxidase (0.07 U/ml; Sigma) in 0.1 M NaAc overnight at 4°C. After the DAB processing, the sections were rinsed in NaAc and sterile PBS, mounted on poly-L-lysine-coated slides in sterile PBS, and vacuum-dried. The slides were stored in boxes with desiccant at −20°C until use.

In Situ Hybridization. The slides were treated with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min, followed by a short (less than 1 min) wash in buffer (0.1 M Tris, 0.05 M EDTA, pH 8) and then incubated with 0.001% protease K (Boehringer–Mannheim; Mannheim, Germany) in the same buffer for 30 min at 37°C. They were dehydrated with ethanol (30%, 70%, 95%, 100%, and 100% for 3 min each) and vacuum-dried. Probes complementary to preproenkephalin mRNA or tyrosine hydroxylase mRNA were used. The preproenkephalin probe (24) was prepared from a cDNA insert (970 bp) in a pSEAY1 plasmid linearized with Sacl (17). The tyrosine hydroxylase probe (11) was prepared from a 280 BP cDNA insert subcloned into a pGEM-3 transcription vector and linearized with Hind III (21). Previous studies using these sequences (21,22) have shown for both probes that hybridization with sense strand cRNA probes, or pre-treatment with RNase and subsequent hybridization with anti-sense cRNA probes, did not reveal any hybridization signal. Probe synthesis and labeling were performed by using the protocol of Riboprobe Gemini System II (Promega) and 3\textsuperscript{5}S-labeled uridine 5'-[α-32P]-triphosphate (NEG-059H, Du Pont Scandinavia, Stockholm, Sweden). The probe was purified on a Sephadex G-50 column (Kabi Pharmacia) and the transcription yield was evaluated with a scintillation counter.

For 10 ml of hybridization solution, 2 ml of the probe mixture [composed of 500 μl tRNA (10 mg/ml; Boehringer–Mannheim), 1 ml diethiothreitol (DTT; Sigma, 1 M dissolved in 10 mM sodium acetate buffer), probe (1 × 10\textsuperscript{6} cpm/ml) and water up to 2 ml] was mixed with 8 ml hybridization buffer. The final concentration of the probe in the hybridization solution was 5 × 10\textsuperscript{6} cpm/ml and in addition it contained formamide (30%) (Fluka; Buchs, Switzerland), dextran sulfate (10%; Sigma), Denhardt’s solution (2%; Sigma), NaCl (0.3 M), Tris (10 mM), and EDTA (1 mM) (19). The hybridization solution was stored at −20°C for no more than 10 days. Before hybridization, the hybridization solution was heated for 10 min at 65°C and then centrifuged for 10 min at 4000 rpm. The hybridization was started by applying the hybridization solution on coverslips (80 μl for 60 × 22-mm glasses), which were placed on the slides in immediate contact with the sections (the edges of the coverslips were sealed with DPX (BDH; Poole, UK)). Hybridization was performed at 56–59°C for 48 hr (23).

After hybridization, the slides were placed in 4 × standard saline citrate (SSC) buffer for 30 min under constant agitation to remove the coverslips. The sections were rinsed in 4 × SSC (four times for 5 min) before incubation in 0.002% RNase A (Boehringer–Mannheim) in 0.5 M NaCl, 10 mM Tris, and 1 mM EDTA for 30 min at 37°C. After incubation, they were rinsed in the following series of SSC buffers: 2 × SSC (RT, 5 min), 1 × SSC (RT, 10 min), 0.5 × SSC (RT, 10 min), 0.1 × SSC (70–80°C, 30 min), 0.1 × SSC (RT, 5 min) (1 mM DTT was added to all buffers). The sections were finally dehydrated with graded ethanol (50%, 70% (both containing 0.1 × SSC and 1 mM DTT), 95%, 100%, and 100%, 3 min each) and were air-dried.

Before autoradiography, the sections were again dehydrated in ethanol (95%, 100%, 100%, 100%, 5 min each), deflated in xylene (twice for 15 min), and finally dipped twice in 100% alcohol (5 min each) and vacuum-dried. The slides were dipped in photographic emulsion [Kodak NTB2 (KEBO; Spånga, Sweden) diluted 1:1 in distilled water] and were stored at −20°C for 6–10 days before the emulsion was developed and fixed with Kodak D-19 and Agefik (Agfa; Leverkusen, Germany). Finally, the sections were rinsed for 30–60 min in running tapwater, air-dried, and coverslipped. Some sections were counterstained with thionin before being coverslipped. All chemicals were from Merck (Darmstadt, Germany) if not otherwise stated.

Results

Single- and double-labeled neurons in sections processed both for immunohistochemistry and in situ hybridization are shown in Figure 1. After CTb injection into the spinal cord, retrogradely labeled neurons were present throughout the reticular formation (Figures 1a–1c). Injections into the ventrolateral medulla resulted in retrograde labeling in the Kölliker-Fuse region on the side ipsilateral to the injection site. An injection into the caudate putamen (Figure 1d) resulted in retrograde labeling in the substantia nigra (Figures 1e and 1f). In single-labeled sections, the immunoperoxidase reaction product was dark blue after nickel ammonium sulfate enhancement and dark brown after cobalt acetate/glucose oxidase enhancement (data not shown). In sections processed also for in situ hybridization, the immunoperoxidase reaction product appeared light or dark brown, respectively (Figure 1). It filled the neuron cell body and was easily detected by light microscopy. Cell counts on the number of detectable retrogradely labeled cell bodies in adjacent single- and double-labeled sections through the Kölliker-Fuse region and the substantia nigra showed no significant difference between the sections. Therefore, all cell types and cell groups
Figure 1. Single- and double-labeled neurons after retrograde tracing with CTb and subsequent in situ hybridization with radiolabeled cRNA probes. (a–c) Labeled neurons in (a) the nucleus subcoeruleus, (b) the paralemniscal reticular nucleus, and (c) the gigantocellular reticular nucleus after CTb injection into the spinal cord and in situ hybridization for ppENK. Nickel intensification procedure. The retrogradely labeled neurons display a gold-brown reaction product in the cytoplasm. ppENK-positive neurons are covered by an accumulation of silver grains. Arrowheads indicate retrogradely labeled neurons; arrows indicate ppENK-positive neurons; and open arrows indicate double-labeled neurons. (d) Small CTb injection into the caudate putamen. LS, lateral septal nucleus; LV, lateral ventricle. (e,f) Retrograde labeling in the substantia nigra after the injection shown in d and tyrosine hydroxylase in situ hybridization. Cobalt acetate–glucose oxidase procedure and thionin counterstaining. Retrogradely labeled neurons were stained dark brown. Photomicrographs were taken at the periphery of the retrogradely labeled cell group in the substantia nigra to display both single- and double-labeled neurons. Symbols as in a–c. Bars: a–c,e,f = 50 μm; d = 500 μm.
that were immunolabeled in the single-labeled section were also immunolabeled in the double-labeled section.

Neurons expressing preproenkephalin mRNA (ppENK) or tyrosine hydroxylase mRNA (TH) were characterized by a grain accumulation in the emulsion layer over the cell body. They also displayed a "halo" of silver grains extending outside the borders of the cell body, an observation explained by the range of β-particles from 35S in the emulsion layer (2). After ppENK hybridization on sections processed for the immunoperoxidase reaction, large numbers of neurons displaying a dense accumulation of silver grains were present throughout the brainstem, and included both neurons that were single labeled and neurons that also contained peroxidase reaction product but did not display an accumulation of silver grains in the emulsion layer (Figures 1b and 1c), and ventrolateral medulla. After TH hybridization, radiolabeled neurons were present in substantia nigra, and part of these were also immunoperoxidase labeled (Figures 1e–1f). Neuron cell bodies labeled with a brown reaction product and covered by an accumulation of grains (more than five times the background level) (cf. 3,4) centered over the cell body were considered as double labeled. Many neurons that were retrogradely labeled only (i.e., they contained peroxidase reaction product but did not display an accumulation of silver grains in the emulsion layer) were present among the ppENK-radiolabeled neurons in the reticular formation (Figures 1b and 1c), and occasionally also among the TH radiolabeled neurons in the substantia nigra (Figure 1f), demonstrating the selectivity of the in situ hybridization procedure.

To test that the retrograde transport of CTb had not interfered with the mRNA expression, the numbers of radiolabeled cells were counted in several cell groups on either side of the brainstem. Although many of the cell groups, such as the Kölliker–Fuse nucleus after a CTb injection into the ventrolateral medulla and the substantia nigra after injection into the caudate putamen, were retrogradely labeled preferentially on one side of the brainstem, the number of radiolabeled cells was equal on both sides irrespective of post-injection survival time. Cell counts were also made on sections that had not been stained immunohistochemically. No significant differences in the number or pattern of radiolabeled cells were found between the single- and double-labeled sections.

Discussion

This study demonstrates that in situ hybridization with long cRNA probes can be successfully combined with immunoperoxidase detection of retrogradely transported CTb. Both reaction products (i.e., the silver grains and the peroxidase reaction product) are permanent, and double-labeled neurons are easily visualized simultaneously by light microscopy, contrary to the case when fluorescent tracers are used. An additional advantage is that the sections can be counterstained with conventional histological stains, facilitating the anatomic localization of labeled neurons.

The specificity and specific activity of radiolabeled cRNA probes (every uridine is radioactive) are higher than for oligonucleotide cDNA probes (7, 18), resulting in a superior sensitivity that permits detection of lesser amounts of mRNA. However, with increasing length of the probes, higher-stringency washing procedures are needed to decrease the background. With high temperatures the demand on the stability of the immunoperoxidase reaction product is increased. In addition, the hybridization solution may attenuate the reaction product (23). Since the antigen proteins will be destroyed by the proteinase K treatment in the hybridization procedure (G. Sanchez–Watts and A. Watts, unpublished observations), it is necessary to perform the immunohistochemistry before the in situ hybridization. In this study we show that a high signal and a low background of the in situ hybridization procedure, obtained by using a high-stringency, low-salt wash at high temperature (70–80°C), are compatible with the immunoperoxidase detection of retrogradely transported CTb. Nickel intensification of the peroxidase reaction was sufficient for retaining the reaction product and the labeled neurons were also readily visible after the hybridization, but the reaction product was somewhat attenuated (the color shifted from dark blue to light brown). Intensification of the peroxidase reaction with glucose oxidase and cobalt acetate (10, 16) produced a reaction product that appeared unaffected by the hybridization procedure. However, a disadvantage with the cobalt intensification compared with nickel intensification may be that the dark reaction product obscures the silver grains in the emulsion layer, making photographic documentation of double-labeled neurons more difficult (cf. Figures 1a–1c, 1e, and 1f).

Normal serum, which is regularly used in immunohistochemistry to block nonspecific binding of the primary antibody, cannot be used with in situ hybridization, since its RNAse activity will reduce mRNA expression (23). Instead, we successfully used high concentrations of BSA (16%, Cohn fraction V; Sigma).

Retrograde tracing with choleragenoid is a very sensitive method (6). Choleragenoid binds to the monosialoganglioside GM1 on the cell surface (15) and is actively taken up by neurons. It has been demonstrated that very small and restricted injections of choleragenoid also result in heavy retrograde labeling (6). In this study a small injection into the caudate putamen (Figure 1d) resulted in heavily labeled neurons in the substantia nigra (Figures 1e and 1f). The reaction product filled the cell body, facilitating detection and interpretation. The survival time for the animal can range from 1–30 days, with little or no loss in immunoreactivity (6).

A potential problem with combining retrograde tracing with in situ hybridization involves the possibility that the tracer substance could interfere with cell metabolism and thereby change the mRNA expression. It is also conceivable that the mRNA could be degraded during immunohistochemical processing or that the immunoperoxidase reaction product could interfere with in situ hybridization. However, no such effects were seen in the present study. Therefore, our observations demonstrated that neither the retrograde transport of choleragenoid, the immunohistochemical procedure, nor the immunoperoxidase reaction product interfered with in situ hybridization.

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