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SHORT COMMUNICATION

A color in situ hybridization method with improved sensitivity for the detection of low-abundance mRNAs

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ABSTRACT We have modified a nonradioactive (color) in situ hybridization method for the detection of mRNA populations, transcribed from the calmodulin (CaM) I gene, that exist in low abundances in tissues such as the white matter of the rat spinal cord. Our results indicate that increasing the pH of the hybridization solution from neutral to slightly alkaline (pH 8.0-8.5) drastically improves the detectable signal intensity of the digoxigenin-labeled CaM I gene-specific riboprobe while providing high spatial resolution. This method could be useful for the detection of other mRNA populations present in cells in low concentrations or in tissues where probe penetration might be impaired (*e.g.* a high lipid content).

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KEY WORDS

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Calmodulin (CaM), an intracellular calcium receptor protein, is present in all eukaryotic cells and is involved in the regulation of numerous calcium-mediated cellular processes (Clapham 1995). The protein is encoded by multiple CaM genes (CaM I, II and III) that produce at least 7 transcripts. However, in consequence of the highly conserved sequences of the coding regions of these genes, their protein products are identical. The nervous tissue displays a widespread and differential distribution of the CaM mRNA populations in the brain under both normal (Ni et al. 1992; Solà et al. 1996; Palfi et al. 1999) and experimental conditions (Solà et al. 1997; Palfi and Gulya 1999; Vizi et al. 2000; Palfi et al. 2001). The CaM mRNA populations not only show region- and neuron type-specific distributions in the adult rat brain, but are differentially targeted to intracellular compartments of the neurons (Palfi et al. 1999). While CaM can be detected in glial cells, white matter areas of the brain and the spinal cord display little or no CaM mRNA expression. This could be due to the relatively low CaM mRNA concentrations in glial cells residing in these areas. Basyuk et al. (2000) reported an improved sensitivity when alkaline fixation preceded the nonradioactive in situ hybridization. In our present study, we investigated the possibility of improving the sensitivity of our color in situ hybridization method by using a modified alkaline hybridization solution to detect the CaM I mRNA populations present in low abundances in the white matter of the spinal cord.

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Materials and Methods

The experimental procedures were carried out in strict compliance with the European Communities Council Directive (86/609/EEC), and followed the Hungarian legislation requirements (XXVIII/1998 and 243/1998) regarding the care and use of laboratory animals. Adult (200-220 g) male Sprague-Dawley rats were maintained under standard housing conditions. The animals were killed by decapitation, and their lumbar spinal cords were quickly removed, embedded in Cryomatrix embedding medium (Shandon Scientific Ltd., Pittsburgh, PA, USA) and frozen immediately at –70ºC. Serial coronal cryostat sections (15 μm) were cut onto 3-aminopropyltriethoxy silane-coated glass slides, air-dried and stored at –70ºC until further processing.

For the preparation of digoxigenin- (DIG)-labeled cRNA probes for in situ hybridization, the genomic sequence of the 3'-nonhomolog region of CaM I mRNA (Nojima and Sokabe 1987; Nojima 1989) was amplified by polymerase chain reactions (PCRs); sequence alignment was completed with the software BLASTN version 2.0.6 (Zhang and Madden 1997). PCRs were performed by employing EcoR I and BamH I restriction enzyme cleavage site-extended primers. The primer sequences complementary to rat genomic CaM I DNA were as follows: 5'-AGACCTACTTTCAACTACT, corresponding to the 30-48 bp sequence, and 5'-TGTAAAACTCATGTAGGGG, corresponding to the 237-255 bp sequence of exon 6 (Nojima and Sokabe 1987). Standard PCRs were run for 35 cycles (Palfi et al. 1998), and the resulting PCR product was cloned into a pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA) and sequenced (AB 373 DNA Sequencer, PE Applied Biosystems, Foster City, CA, USA) to confirm its identity. In vitro RNA syntheses

from the purified and linearized vector were carried out to prepare antisense and sense CaM I cRNA probes. The probes were synthesized with a DIG RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany). The complementary probe sequence was 225 bp long.

Coronal cryostat sections from the lumbar spinal cord of the adult rat were fixed for 5 min in 2x SSC containing 4% formaldehyde, washed twice in 2x SSC for 1 min, and then rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 5 min at room temperature (RT). The sections were washed in 2x SSC for 5 min, dehydrated, air-dried and hybridized in 50 µl hybridization solution (50% formamide, 4x SSPE, 1x Denhardt's reagent, 10% dextran sulfate, 100 mM DTT, 0.1% SDS, 100 µg/ml salmon sperm DNA, 100 µg/ml yeast tRNA; the pH of the solution was adjusted to 8.5) containing 200 fmol/ml DIG-labeled probe. Hybridization was performed under parafilm coverslips in a humidified chamber at 55ºC for 24 h. The sections were rinsed in 2x SSC at RT and 55ºC for 5 and 10 min, respectively, then treated with RNase A (16 µg/ml) at 37ºC for 30 min. The sections were washed in 2x SSC/50% formamide at 55ºC for 2 x 10 min, and in 2x SSC at 55ºC and RT for 10 min and 5 min, respectively. After posthybridization, the sections were washed in buffer B1 (100 mM Tris-HCl pH 7.5 and 150 mM NaCl) for 5 min, blocked in 5% heat-inactivated sheep serum in B1 for 2 h and incubated in sheep anti-DIG-alkaline phosphatase conjugate (Boehringer Mannheim; 1:1,000 dilution) in 5% sheep serum in B1 at 4ºC for 24 h. Sections were washed in B1 for 3 x 5 min, and then in buffer B2 (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) for 10 min, and were developed in B2 containing 340 µg/ml nitro blue tetrazolium (NBT) and 180 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 24 h under darkroom conditions. The color reaction was stopped by rinsing the sections in a mixture of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA for 5 min in RT, and the sections were then dehydrated and covered with Entellan (Merck, Darmstadt, Germany).

Results and Discussion

The conventional color in situ hybridization technique did not detect any cellular elements expressing CaM I mRNAs in the white matter area of the spinal cord (Fig. 1A). When our sensitive color in situ hybridization technique employing DIG-labeled riboprobe in a hybridization solution adjusted to slightly alkaline pH was used, widespread distribution of

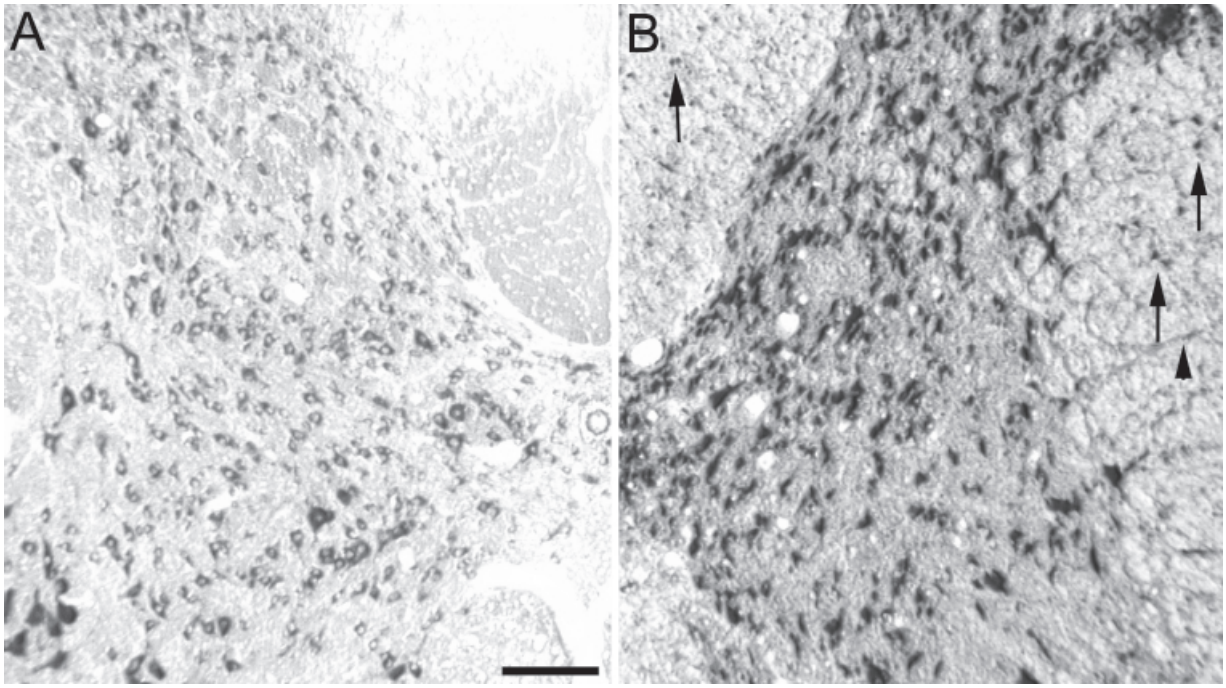

Figure 1. Nonradioactive in situ hybridization histochemistry of DIG-labeled CaM I-specific riboprobes in the rat lumbar spinal cord. **A.** Hybridization at neutral pH. CaM I mRNA is abundant only in the cells residing in the gray matter of the spinal cord, but completely absent in the white matter area. **B.** Hybridization at alkaline pH. Apart from the heavy labeling seen in the gray matter area, CaM I mRNA is widely expressed in small-to-medium-sized cells in the white matter area. Small cell somata (arrows) and sometimes a few processes (arrowhead) running radially to distances of 50-100 µm could be seen. Note that the neuropil of the gray matter is abundant in CaM I mRNA. Scale bar: 200 µm.

CaM I mRNA-expressing cells could be seen throughout the white matter area of the spinal cord (Fig. 1B). Many small and medium-sized cell bodies, probably glial cells, were heavily labeled in the dorsal, lateral and ventral funiculi of the white matter. A few of the labeled cells with fine processes could also be seen. The surrounding neuropil was also considerably labeled, especially in the dorsal column. DIG-labeled sense CaM I cRNA merely resulted in a level of labeling comparable to the background level (not shown).

The radioactive in situ hybridization technique, especially when combined with emulsion autoradiography (Palfi et al. 1999, 2000), provides a reasonably good spatial resolution, and is suitable, albeit cumbersome, method for the quantitative assessment of mRNA abundances. DIG-labeled riboprobes, however, are excellent choices for in situ hybridization histochemistries where high spatial resolution is needed, but where there is no option for quantitative measurements. Neither method provides an easy means of detecting mRNA populations present in low concentrations. To circumvent this problem, Basyuk et al. (2000) introduced alkaline fixation to in situ hybridization techniques in order to improve the sensitivity. Their studies indicated that alkaline formaldehyde dramatically increased (5- to 6-fold) the in situ hybridization signal with RNA probes. The signal increase was observed for the detection of both low and high-abundance messages. We adapted this trick to our color in situ hybridization protocols, and by using DIG-labeled riboprobes were able to detect CaM I mRNA populations in cells where their presence had previously escaped detection. Since alkaline fixation does not improve the retention of mRNA during in situ hybridization (Basyuk et al. 2000), but rather increases the accessibility of the target for the DIG-labeled cRNA probe, hybridization at alkaline pH is crucial for the increased sensitivity.

In summary, the protocol described here is easy to carry out and reliable, while retaining the high spatial resolution characteristic of the conventional DIG-based in situ hybridization technique, and can be applied to any mRNA class present in low quantities in a tissue.

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