Rapid Nonradioactive *In-Situ* Hybridization for mRNA Detection of Calcineurin in Rat Brain Using PCR DNA Probe

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Received October 12, 1999; accepted March 24, 2000

A simple method of *in-situ* hybridization (ISH) without using any bacteriological or recombinant procedures was exemplified by examining the localization of the two isoforms of calcineurin catalytic subunits, A α and A β , in rat brain tissue. Total RNA was extracted from rat brain tissue and utilized as a template to perform probe labeling during reverse transcription and subsequent polymerase chain reaction steps (RT-PCR) by incorporating digoxigenin-11-dUTP. These PCR probes were employed for ISH. To confirm the validity of this method, the expression of mRNAs of alpha-fetoprotein and albumin in

rat liver tissue was also examined using the same procedure. The distribution of calcineurin A α and A β mRNAs was clearly detected in brain tissue. The entire procedure took less than a few days from starting the preparation of the probes to the light microscopic observation of the specimens. Moreover, the sensitivity of the ISH using these probes was higher than that of the end-labeled probe. This method has significant advantages as it allows laboratories without any recombinant facilities to accomplish ISH, and has the potential for widespread application.

Key words: *In-situ* hybridization, Polymerase chain reaction (PCR), DNA probe, Digoxigenin, Calcineurin, Alpha-fetoprotein (AFP), Albumin

I. Introduction

In-situ hybridization (ISH) has a widespread application for the detection of nucleic acid in basic science and diagnostic clinical research. Moreover, the advent of non-radioactive nucleic acid probes has made ISH more dramatically convenient. However, there is still room for improvement in the labeling of probes and the detection methods. Originally, probes had been labeled with radioactive isotopes, for example ³²P, ³³P and ³⁵S, and detected by autoradiography [11]. Such techniques, however, have limited actions due to the short half-times of radioisotopes, the health and environmental hazards associated with the handling and disposal of the material, and the long period of exposure required for detection [5].

To overcome these limitations, non-radioactive labeling and a detection method for DNA and RNA have been developed [5, 28]. Non-radioactively labeled probes are not hazardous to manipulate, are stable for months to years, do not require special laboratory facilities and can be detected by short immunohistochemical procedures. At present, one of the most successful labeling and detection systems is based on the utilization of a hapten digoxigenin (DIG) [9, 22]. This system has various methods of probe labeling, random primed DNA labeling [4], nick translation labeling [20], RNA labeling [3], and PCR labeling [13, 17], which have been examined employing DIG. On the other hand, recent advances in nucleotide and peptide technology have enabled us to gain oligonucleotide probes commercially for ISH [6, 19].

In this communication, we report a simple method using PCR probes as an alternative approach to probe labeling for ISH. PCR is a sensitive and powerful tool for the analysis of gene structure by amplifying extremely small amounts of genes without using phage and plasmid [21]. To assess this method, the localization of calcineurin (CaN) in the rat brain was examined. CaN is known as

Selected by "The 38th Annual Meeting of the Japan Society of Histochemistry and Cytochemistry" held in Mitaka, Japan on Oct. 6–7, 1997.

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a Ca^{2+} /calmodulin-dependent protein phosphatase, which consists of two subunits, A (or catalytic) subunit and B (or regulatory) subunit [14]. Three isoforms of the CaN subunit A have been demonstrated by molecular cloning as mammalian genes designated as $\text{A}\alpha$, $\text{A}\beta$, and $\text{A}\gamma$. The $\text{A}\alpha$ and $\text{A}\beta$ isoforms are mainly expressed in the mammalian brain [7]. After total RNA is extracted from a rat brain, this RNA is utilized as a template to prepare the probes of CaN $\text{A}\alpha$ and $\text{A}\beta$ isoforms incorporating DIG-11-dUTP during PCR, and then used in ISH to detect localization in the rat brain. Moreover, to confirm the specificity and sensitivity of these PCR probes, the following studies are performed: excess non-labeled probe for CaN $\text{A}\alpha$ added to ISH buffer is used for the competitive control study; the expression of mRNAs of alpha-fetoprotein (AFP) and albumin in rat liver, which has different expression in fetal and adult liver [23, 26], is also examined using the same procedure; A custom made DIG end-labeled oligonucleotide of CaN $\text{A}\beta$ is used for ISH to be compared with PCR probes; and dot spot hybridization is performed with both PCR probes and oligonucleotide probes.

II. Materials and Methods

cDNA by Reverse transcription

Total RNA was isolated from 100 mg male Wistar rat brain homogenates by 1 ml TRYSol® Reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions [2]. The resulting pellet was diluted in 100 μl of DEPC-treated water. The Reverse transcription (RT) reactions were performed in a GeneAmp® PCR system 2400 thermocycler (PE Applied Biosystems, Foster City, CA, USA) utilizing thin-walled MicroAmp® reaction tubes (PE Applied Biosystems). One μl of the isolated total RNA was added to a reaction mixture containing 2 μl of 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 4 μl 25 mM MgCl_2 , 2 μl dNTPs (10 mM each), 1 μl of downstream primer (40 pmol/ μl ; CaN- $\text{A}\alpha$: Cal-AR, CaN- $\text{A}\beta$: Cal-BR), 0.5 μl ribonuclease inhibitor (40 U/ μl ; Promega Corporation, Madison, WI, USA), 2.5 U AMV Reverse Transcriptase XL® (Life Science, St. Petersburg, FL, USA) and distilled de-ionized water to a final volume of 20 μl . The mixture was incubated at 50°C for 30 min, followed by heating to 99°C for 5 min to denature, and stored at 4°C.

PCR amplification of the CaN gene

The subsequent PCR was performed on the total volume of RT products by the addition of 8 μl of 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 6 μl 25 mM MgCl_2 , 1 μl of upstream primer (40 pg/ μl ; $\text{A}\alpha$: Cal-AF, $\text{A}\beta$: Cal-BF), 1 U of AmpliTaq Gold® DNA Polymerase (PE Applied Biosystems) and distilled de-ionized water to a final volume of 100 μl . Each oligonucleotide primer of CaN $\text{A}\alpha$ and $\text{A}\beta$ ($\text{A}\alpha$: Cal-AF and Cal-AR, $\text{A}\beta$: Cal-BF and Cal-BR) was synthesized to flank the conserved region of a 164 bp fragment ($\text{A}\alpha$) and a

211 bp ($\text{A}\beta$) by DNA synthesizer model 394® (PE Applied Biosystems) [10, 16].

The sequences of the oligonucleotide primers were:

Cal-AF (upstream): 5'-AAGCCAAACAACCTGGCTT-CC-3'
 Cal-AR (downstream): 5'-CCATGAAATTTGGGAGCC-AG-3'
 Cal-BF (upstream): 5'-TGATGTAGGTTCA-GCTGCAG-3'
 Cal-BR (downstream): 5'-GCCTCAATAGCCTCAACT-G-3'.

PCR amplification proceeded to preincubate at 94°C for 10 min, followed by 35 cycles at 55°C (in $\text{A}\alpha$) or 50°C (in $\text{A}\beta$) for 30 sec and at 72°C for 1 min and a final extension step of 7 min at 72°C. PCR products were purified to remove non-reactive primer by microcon 50® (Amicon, Beverly, MA, USA), resuspended in 100 μl Tris-EDTA (pH 8.0) buffer. Ten μl aliquots of the PCR products were run on a 2% agarose gel in 1 $\times$ Tris-borate-EDTA (TBE) buffer and stained with ethidium bromide to confirm the PCR products. Nucleotide sequences were analyzed by the method of dideoxynucleotide termination reaction using the Applied Biosystems ABI 310 Genetic Analyzer® (PE Applied Biosystems) for the PCR products. These products were stored to generate PCR probes.

Preparation of the DIG-labeled probe

The DIG-labeled Probes were also generated by using the PCR reaction. This second PCR was performed on 1 μl aliquot of the first PCR products by the addition of 10 μl of 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 6 μl of 25 mM MgCl_2 , 1 μl of 1 mM DIG-11-dUTP (Boehringer Mannheim, Mannheim, FRG, Germany), 1 μl of upstream primer (40 pg/ μl ; Cal-AF and Cal-BF, respectively), 1 μl of downstream primer (40 pg/ μl ; Cal-AR and Cal-BR, respectively), 1 U Taq polymerase and distilled de-ionized water to a final volume of 100 μl . The 35 PCR cycles were performed as described above. The second PCR products were also purified by microcon 50® (Amicon).

In-situ hybridization

Tissues of male Wistar rats (200 g) were fixed by perfusion under deep anesthesia with pentobarbiturate with a fixation consisting of 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4 (1 $\times$ PB) for 15 min, followed by immersion for 24 hr at 4°C. After dehydration with a graded ethanol series, they were embedded in paraffin. Paraffin sections with a thickness of 5 μm were deparaffinized in the series of xylene and graded ethanol and rehydrated. They were permeabilized with a solution containing 5 $\mu\text{g}/\text{ml}$ proteinase K (PE Applied Biosystems), 50 mM Tris (pH 7.5) and 5 mM EDTA at 37°C for 15 min. And the proteinase K reaction was stopped with 0.2% glycine in 10 mM phosphate buffer saline (1 $\times$ PBS) for 15 min. After treatment with 4% formaldehyde for 10 min, they were treated with 0.1 M HCl for inactivation

of internal alkaline phosphatase, and were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min to reduce the background reactivity [8], and prehybridized with a solution containing $2 \times$ SSPE ($1 \times$ SSPE is 180 mM NaCl, 10 mM NaPO₄, 1 mM EDTA, pH 7.7), $1 \times$ Denhardt's solution, 0.25 mg/ml yeast tRNA (Boehringer Mannheim), 50% formamide, 10% dextran sulfate (Amersham Pharmacia Biotech, Uppsala Sweden) and 0.5 mg/ml denatured herring sperm DNA (Boehringer Mannheim), for 1 hr at room temperature. Hybridization solution (500 ng/ml of heat denatured DIG-labeled probe in prehybridization buffer) was applied and incubated at 50°C (PCR probe) or 37°C (synthesized oligonucleotide probes) for 16 hr in a moisture chamber. After hybridization, they were gently washed in a series of $5 \times$ SSPE at 50°C, $2 \times$ SSPE at 50°C for 30 min, and two changes of $0.2 \times$ SSPE at 50°C for 20 min. Hybridized DIG-labeled probes were detected by the Nucleic Acid Detection Kit® (Boehringer Mannheim) according to the manufacturer's instructions. After a color reaction, the slides were rinsed with 10 mM Tris-HCl pH 8.0, 1 mM EDTA and coverslipped with glycerol gelatin (Merck, Darmstadt, Germany), and examined microscopically.

Negative and positive controls of the staining

To validate the specificity of the generated DIG PCR probe, a control study for CaN A α was examined by the same procedure, using a ISH solution which contained 20 times non-labeled CaN A α probe to act competitively [15]. Moreover, as another control study, the expression of AFP and Albumin mRNAs in embryonic (at day 18) and adult rat liver were also examined by the same procedure in case of CaN. The primer sets for AFP and albumin were selected to amplify the same range at the same cDNA position to assess the specificity of PCR probes strictly [12]. The sequences of the primers were:

AFP-F (upstream: NP 220-240): 5'-AACACGTTTCAT-CTACGATGTG-3'
 AFP-R (downstream: NP 445-464): 5'-ATGATGAGTA-CAGCCTGGAG-3'
 albumin (upstream: NP 218-238): 5'-TGGGACACTA-TTTGCATGAAG-3'
 albumin (downstream: NP 444-463): 5'-CTACTGCCCA-GGCTTTGAAG-3'

Each hybridization was also done at 50°C.

End-labeled oligonucleotide probe

For comparison of the sensitivity of the staining, the antisense oligonucleotide for CaN A β end-labeled on the 5' flanking region was synthesized by DNA synthesizer model 394® (PE Applied Biosystems). The sequence was: 5'-CCTACATCAAACCTGGTCTTCCCCTTCTGTC-3'.

The sense oligonucleotide labeled with DIG for CaN A β was also synthesized to perform a negative control study. After 500 ng of these probes were added per 1 ml of the ISH solution, ISH was performed at 37°C.

Dot spot hybridization

To test the specificity and sensitivity of the PCR DNA probes, the denatured non-labeled cDNAs were serially diluted and spotted on nylon membranes (Hybond N+; Amersham, Arlington Heights, Ill., USA), and then crosslinked by UV (254 nm) irradiation for 2 min. The membranes were hybridized developed under the same conditions as for ISH by using DIG Nucleic Acid Detection Kit® (Boehringer Mannheim).

III. Results

PCR probes

The agarose gel electrophoresis of the probes of CaN A α and A β with and without DIG-11-dUTP produced by PCR showed discrete bands, indicating successful amplification of the target mRNA sequence. Each size of the probes was consistent with a 164 bp (CaN A α) and a 211 bp (CaN A β) respectively (Fig. 1). The probes labeled with DIG showed lower mobility compared to the unlabeled probes (Fig. 1). The sequences unlabeled PCR products were analyzed by the method of dideoxynucleotide termination reaction and proved to be completely consistent with those of mRNA of CaN A α , CaN A β , AFP and albumin (data not shown).

In-situ hybridization of CaN mRNA using PCR probes

Figure 2 shows the result of ISH for CaN A α (Fig.

Fig. 1. Agarose gel electrophoresis of PCR probes for CaN A α and A β with or without labeling with DIG. lane 1, size marker; lane 2, CaN A α with DIG-11-dUTP labeling; lane 3, CaN A α without labeling; lane 4, CaN A β with labeling; lane 5, CaN A β without labeling.

Fig. 2. Rat brain tissue was stained by ISH for CaN A α and A β with DIG-labeled PCR probe. **A:** DIG-labeled PCR probe for CaN A α was employed for cerebral cortex. **B:** CaN A α probe for hippocampus. **C:** CaN A α probe for medulla oblongata. **D:** CaN A α probe for cerebellum cortex. **E:** CaN A β for cerebral cortex. **F:** CaN A β for hippocampus. **G:** CaN A β for medulla oblongata. **H:** CaN A β for cerebellum cortex. **I:** CaN A α probe and an excess amount of non-labeled CaN A α probe were simultaneously applied for negative control study. $\times 200$. Bar = 100 μ m.

2A–D) and CaN A β (Fig. 2E–H). The staining for CaN A α was positive in the nerve cell bodies throughout the brain tissue. In contrast, glial cells were consistently negative, as exemplified by the cerebral cortex (Fig. 2A), hippocampus (Fig. 2B), medulla oblongata (Fig. 2C) and cerebellum cortex (Fig. 2D). The mRNA distribution of CaN A α and CaN A β in the rat brain was similar (Fig. 2E–G). In the control study, when 20 times the amount of non-labeled probe for CaN A α was added in the ISH

solution, the staining of the nerve cells became negative (Fig. 2I).

In-situ hybridization of AFP and albumin mRNA using PCR probes

The expression of AFP and Albumin mRNAs in embryonic rat at day 18 and adult rat liver was also examined by the same procedure for CaN in order to confirm the specificity of the PCR probes. Figure 3 shows the results

Fig. 3. Rat liver tissue was stained by ISH for AFP (A and C) and albumin (B and D) by using DIG-labeled PCR probe in embryonic rat at day 18 (A and B) and adult rat liver (C and D). $\times 75$. Bar = 200 μm .

of ISH for AFP (Fig. 3A, C) and albumin (Fig. 3B, D). The staining for both AFP and albumin was positive for fetal liver tissue (Fig. 3A, B). In contrast, adult liver tissue was positive for albumin, but negative for AFP (Fig. 3C, D). In adult rat liver, gradation of the staining from the central vein to interlobular vein was observed (Fig. 3D).

In-situ hybridization of CaN $A\beta$ using oligonucleotide probe

To compare the sensitivity of PCR probes and oligonucleotide probes, expression of CaN $A\beta$ mRNA was examined using an oligonucleotide probe for CaN $A\beta$. The ISH was successful, however, it failed to show a more significant signal than ISH using a PCR probe for CaN $A\beta$ (Fig. 4A). In the negative control study using the sense oligonucleotide probe for $A\beta$, the nerve cells were negative (Fig. 4B).

Dot spot hybridization

The dot spot hybridization study with the serially diluted cDNA showed that the PCR probes of CaN $A\beta$ were more sensitive detecting as little as 1 pg DNA. The sensitivity of PCR DNA probe was higher than that of the oligonucleotide probe (Fig. 5). Each probe hybridized specifically to the corresponding spotted cDNA of CaN $A\beta$, but was not hybridized to the cDNA of CaN $A\alpha$. The

PCR probe whose concentration ratio of dTTP: DIG-11-dUTP was 10 : 1 (20 mM : 1 mM in the PCR solution) was more sensitive than when the ratio was 20 : 1.

IV. Discussion

The PCR is a rapid procedure for *in vitro* enzymatic amplification of a specific sequence. In a typical reaction, one molecule of the target DNA may be amplified one billion times in only a few hours without any kind of bacterial technique and instrument [25]. This study shows that ISH using PCR DIG probes generated by this PCR technique is a speedy procedure, and has a high specificity and sensitivity of staining as well. The total procedure, which contained several series of examinations using several kinds of probes, took less than 3 days from specimen preparation to microscopic analysis because the preparation of the probes is performed semi-automatically by PCR. Moreover, in the process of generating the probes, we can predict in advance whether a tissue has positive cells for a probe because the probe is generated using RT-PCR technique from the tissue which is to be examined on the expression of mRNA. This means that the preparation of probes using RT-PCR is to test the yield of mRNA in tissue. These probes are also easy to handle due to the absence of radioisotopes and may be stored for many months at -80°C without loss of activity.

Fig. 4. Rat brain tissue was stained by ISH for CaN A β by using DIG end-labeled oligonucleotide probe. **A:** DIG-labeled antisense oligonucleotide probe. **B:** DIG-labeled sense oligonucleotide probe for negative control study. $\times 200$. Bar = 100 μ m.

Fig. 5. Dot spot hybridization. Between 1 and 250 pg of serially diluted cDNA of A β and A α [negative control (NC)] were spotted on the nylon membrane. Both PCR probes (dTTP: DIG = 10 : 1, 20 : 1) and oligonucleotide probe (Oligoprobe) for A β were used for hybridization at the concentration of 500 ng/ml, respectively.

By using these PCR DIG probes, the mRNA distribution of CaN A α and A β was successfully visualized. The localization of CaN here is consistent with the localization shown in previous reports, which demonstrated by ISH the localization of CaN mRNA using isotope-labeled probes [24], and which proved by immunohistochemical study

that CaN was widely distributed in the rat brain but restricted to nerve cells [27]. In addition, the competitive experiment using the mixture of DIG-labeled CaN A α probe and the excess amount of non-labeled CaN A α probe assured the specificity of the PCR probe. The results of analyzing the sequences of PCR probes further

guaranteed the probes to be tissue specific.

Moreover, the specificity of PCR probes was confirmed by determining the expression of AFP and Albumin mRNAs in embryonic rat liver at day 18 and adult rat liver which was examined using the PCR probes. AFP is the most abundant plasma protein of the mammalian fetus. On the other hand, production of serum albumin increases severalfold after birth to become the predominant protein in the adult [1]. Both AFP and albumin mRNAs in liver increase before birth, but AFP mRNA dominantly decreases after birth [26]. Our results of ISH for AFP and albumin in the fetal and adult rat liver tissues were consistent with those stained by ISH using probes labeled with radioisotope [18].

In terms of sensitivity, the PCR probe for CaN A β was more densely stained than the oligonucleotide probe. These findings were also confirmed by dot spot hybridization study. Through recent advances in nucleotide and peptide technology, DIG-labeled oligonucleotide probes are widely used for ISH. These are easy to obtain commercially, can be designed to agree with the target cDNA completely, have such small nucleotide size as to easily penetrate into tissue, and are single stranded nucleotides. However, in our study, the oligonucleotide probe for CaN A β had lower sensitivity and specificity than PCR probes. One possible reason is that because the prepared end-labeled oligonucleotide probe had only one molecule of DIG to the one molecule of oligonucleotide, the sensitivity is lower than that of the PCR probe which has several molecules of DIG into the probe. Actually, dot spot hybridization study shows that DIG PCR probe can detect as little as 1 pg DNA.

In the present study, DIG-labeled probes were prepared by PCR reactions. For the preparation, bacterial techniques and instruments were not necessary. All the processes could be accomplished with some biochemical techniques. By using these DIG-labeled PCR probes, ISH was performed to study visualized tissue specific or cell specific localization, whose specificity was also ascertained with negative and positive control experiments. Moreover, several kinds of probes can be handled at once. The speediness of the procedure, low cost of the experiment for laboratories without biohazard systems, and high specificity and sensitivity of the staining are the advantages of the procedure examined in our study.

V. Acknowledgments

We would like to thank Dr. Kohei Jokura (Shinshu University, Matsumoto, Japan) for help with the tissue preparation and Dr. Katsutoshi Nakayama (Tohoku University, Sendai, Japan) for his technical support and suggestion.

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