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A Novel Amplification Method of Nonradioactive *In Situ* Hybridization Signal for Specific RNA with Biotinylated Tyramine

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For a better performance of nonradioactive *in situ* hybridization for specific RNA sequences, signal amplification is sometimes required especially in the use of clinical specimens processed under suboptimal conditions and in the analysis of gene expression with very low reiteration. In the present study, we examined the usefulness of catalyzed signal amplification (CSA) or catalyzed reporter deposition system with biotinylated tyramine to amplify colorimetric *in situ* hybridization signals. Our CSA protocol included the use of biotinylated tyramine after the reaction of thymine-thymine (T-T) dimerized DNA with horseradish peroxidase (HRP) labeled anti-(T-T dimer) antibody, followed by the reaction with HRP-labeled streptavidin. When thymine-thymine dimerized λ phage DNA was spotted onto a nitrocellulose filter at 1 pg-10 ng and then detected by the direct immunoperoxidase method, the indirect immunoperoxidase method or the CSA method, the CSA method gave the highest sensitivity with about 100-fold increase compared to that of the direct method. Next, in the frozen sections of rat brain, which was fixed with 4% paraformaldehyde, 28S rRNA staining by *in situ* hybridization with only 10 ng/ml of T-T dimerized oligonucleotide probe complementary to rat 28S rRNA was compared among the direct method, the HRP-labeled avidin-biotin complex method and the CSA method. Again, intense 28S rRNA signal was obtained only with the CSA method. Therefore, we confirmed the usefulness of the catalyzed deposition system with biotinylated tyramine in nonradioactive colorimetric *in situ* hybridization for specific RNA sequences in tissue sections.

Key words: *In situ* hybridization, 28S rRNA, Oligonucleotide, Biotinylated tyramine, Rat brain

I. Introduction

Over the past decade, considerable efforts have been made to establish nonradioactive *in situ* hybridization, which is now essential to understand the states of specific gene expression at cellular and /or subcellular levels [10, 12]. Recent advances in this field have included introduction of various haptens such as digoxigenin [6, 11, 19] and thymine-thymine (T-T) dimer [10, 12, 13] to label probe nucleic acids, use of synthetic oligonucleotides as probe nucleic acids [8, 14, 16], and development of an evaluation system of hybridizable RNA retention using 28S rRNA as reference [7, 22, 23]. The resultant improvements in the protocol of nonradioactive *in situ* hybridization lead to a greater detection sensitivity. However, people sometimes felt that the sensitivity was still not enough.

To visualize specific RNA signals by nonradioactive *in situ* hybridization, activities of enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase, which are labeled to antibodies, have been very widely utilized. We currently favor the use of HRP, because of the stability and the limited diffusion of the final colored deposit. In the case of nonradioactive colorimetric *in situ* hybridization protocol using an HRP-labeled antibody, we can amplify the signal by various methods; the simple use of nickel and cobalt ions together with 3,3'-diaminobenzidine (DAB) and hydrogen peroxide resulted in about tenfold increase in sensitivity compared to that in the absence of both ions [1, 10]. Silver enhancement [18] and colloidal gold-silver enhancement [21] were reported to

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be useful to enhance HRP-based signals, while a high background staining was often encountered. The triple-layer method, where the reaction with the first antibody is followed by the second antibody and the third HRP-labeled antibody, gave a little more intense signal than that of the HRP-labeled avidin-biotin complex (ABC) method [17, 20]. Recently, Kerstens *et al.* [9] reported the successful use of biotinylated tyramine as a system of catalyzed reporter deposition in chromosomal *in situ* hybridization for specific DNA sequences. More recently, De Haas *et al.* [5] have found the effectiveness of the system with biotinylated tyramine in fluorescence *in situ* hybridization for specific RNA sequences in cultured cells.

In the present study, we attempted to assess the usefulness of catalyzed signal amplification (CSA) or catalyzed reporter deposition with biotinylated tyramine in nonradioactive colorimetric *in situ* hybridization for specific RNA sequences in tissue sections. First, to evaluate the sensitivity with biotinylated tyramine, we performed immunodetection of T-T dimerized DNA spotted onto a nitrocellulose filter with monoclonal anti-(T-T dimer) antibody, where the direct immunoperoxidase method with HRP-anti-(T-T dimer), the indirect immunoperoxidase method with the first anti-(T-T dimer) and the second HRP-anti-mouse IgG and the CSA method were compared. Second, using the frozen sections of rat brain as a model system, 28S rRNA was hybridized *in situ* with a T-T dimerized oligonucleotide probe complementary to rat 28S rRNA [23], and the signal was detected by the direct method, the ABC method and the CSA method. Finally, we confirmed that the signal amplification with biotinylated tyramine is a very powerful technique to amplify the HRP-based signal.

II. Materials and Methods

Reagents

Following reagents were purchased; formamide from Nacarai Chemical Co., Japan, DAB/4 HCl from Dojindo Chemical Co., Japan, proteinase K from Wako Pure Chemical Industries, Japan, the λ phage DNA from Toyobo Co., Japan, OCT compound from Tissue-Tek (Miles Inc.), USA, bovine serum albumin (BSA) (98–99% pure), salmon testis DNA (phenol-chloroform extract), yeast tRNA (type X-S), dextran sulfate (Mr, 500,000), Brij 35, Triton X-100, heparin, normal mouse IgG and normal goat IgG from Sigma Chemical Co., USA. Biotinylated tyramine and HRP-labeled streptavidin solutions were obtained as parts of the CSA kit from Dako Japan Co., Japan. Nitrocellulose filters were purchased from Schleicher and Schuell, USA. All chemicals and biochemicals used were of analytical and molecular biological grade.

Antibodies

Purified HRP-labeled and non-labeled monoclonal antibody against T-T dimer was provided by Kyowa Medex, Japan. Biotinylated rabbit anti-mouse IgG+IgA+IgM and HRP-labeled streptavidin were purchased as a SAB kit from Nichirei Co., Japan. HRP-labeled goat anti-mouse IgG was from Medical Biological Laboratory, Japan.

Tissue specimens

Male Wistar rats weighing 150–170 g were used. In this study, all procedures were done at room temperature (RT), unless otherwise specified. The brains were briefly perfused with 4% paraformaldehyde in PBS (pH 7.4) and removed. After the tissues were cut into small pieces, they were further fixed in the same fixative overnight at RT. Then the specimens were immersed in 30% sucrose at 4°C till they sunk to the bottom, embedded in OCT compound, and frozen, as detailed previously [13]. Five μm sections were cut, placed onto silane-coated glass slides, baked at 45°C for 2 hr and stored at −80°C until to be used.

Immunohistochemical detection of T-T dimerized DNA

Immunohistochemical detection of T-T dimerized DNA, which was fixed onto a nitrocellulose filter, was described in the previous paper [10, 14]. Briefly, a 2 μl-aliquot of T-T dimerized λ phage DNA solution, which was irradiated with ultraviolet-light at 10 KJ/m^2, was applied onto a 20 X SSC (SSC; 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) treated nitrocellulose filter at 0.1 pg – 10 ng per spot. After baking at 80°C for 2 hr, the filters were reacted with 5% BSA in PBS. The detection of T-T dimerized DNA was done by three methods; the direct immunoperoxidase method, the indirect immunoperoxidase method and the CSA method. In the direct method, the filters were reacted for 1 hr with HRP-anti-(T-T dimer) (1 : 80) dissolved in PBS containing 5% BSA, 100 μg/ml salmon testis DNA, 100 μg/ml yeast tRNA, 0.3 M NaCl, and then washed 4 times with 0.075% Brij 35 [11] in PBS for 15 min each. Some of filters were further reacted for the CSA method with the biotinylated tyramine solution for 15 min and washed three times with 0.075% Brij 35 in PBS for 5 min each. After reaction with the HRP-streptavidin solution for 15 min, the filters were washed again similarly. The HRP sites were visualized with DAB and hydrogen peroxide in the presence of both nickel and cobalt ions [1]. In the indirect method, the filter was incubated for 1 hr with anti-(T-T dimer) antibody (1 : 200) dissolved in PBS containing 5% BSA, 100 μg/ml salmon testis DNA, 100 μg/ml yeast tRNA, 0.3 M NaCl, and then washed 4 times with 0.075% Brij 35 in PBS for 15 min each. After reaction with HRP-anti-mouse IgG (1 : 100) in PBS containing 5% BSA, 100 μg/ml salmon testis DNA and 100 μg/ml yeast tRNA, the signal was detected in the same way to that of the direct method.

In situ *localization of 28S rRNA*

A 34-nucleotides sequence complementary to the part of rat 28S rRNA was selected and synthesized together with ATT repeats at both 3′ and 5′ ends [12, 23]. After

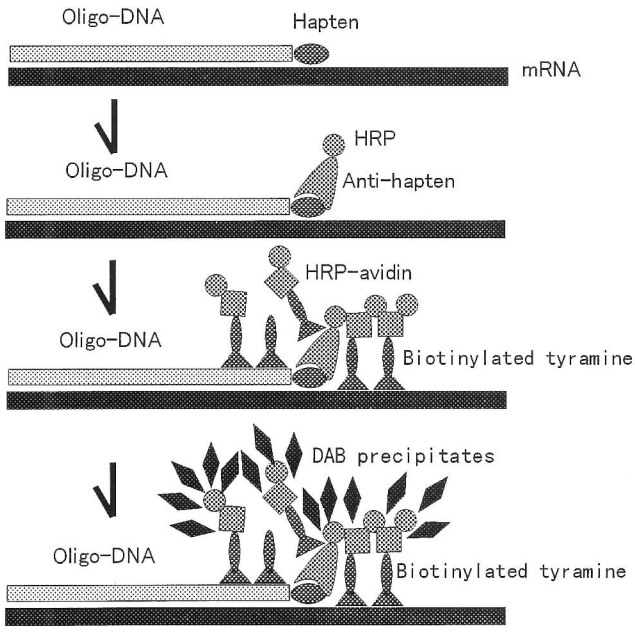

Fig. 1. Principle of nonradioactive *in situ* hybridization method combined with catalyzed signal amplification using biotinylated tyramine. Firstly, target RNA is hybridized with a haptenized oligonucleotide (oligo-DNA) probe. Next, HRP-labeled anti-hapten antibody is reacted with the hapten molecules of the probe. Then, biotinylated tyramine is allowed to be precipitated around the sites of HRP in the presence of hydrogen peroxide. After successive reaction with HRP-labeled streptavidin, the sites of HRP are visualized by precipitation of chromogen compounds.

ultraviolet-light irradiation to form T-T dimers, the oligonucleotide was used as a probe.

The protocol for nonradioactive *in situ* hybridization was detailed previously [12, 15]. Briefly, the frozen sections were treated with 0.2 N HCl, Triton X-100 and proteinase K. After these pretreatments, the sections were hybridized at 37°C for 15 hr with only 10 ng/ml of T-T dimerized 28S rRNA probe dissolved in 10 mM Tris/HCl buffer (pH 7.4) containing 0.6 M NaCl, 1 mM EDTA, 1 X Denhardt's solution, 125 μg/ml salmon testis DNA, 250 μg/ml yeast tRNA, 40% deionized formamide, 10% dextran sulfate, and 200 units/ml heparin. After appropriate washings with 50% formamide in 2 X SSC at 37°C and 2X SSC alone, the sections were incubated for 1

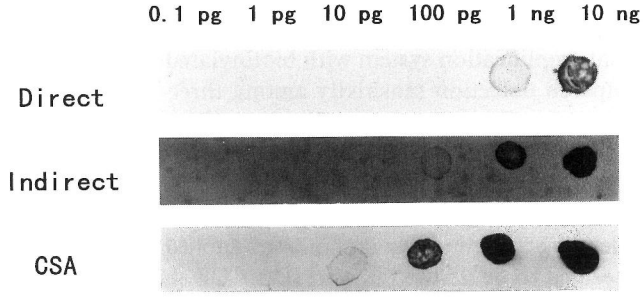

Fig. 2. Immunodetection of T-T dimerized DNA on nitrocellulose filters using various detection methods. An aliquot (2 μl) of T-T dimerized salmon testis DNA solution was dotted onto nitrocellulose filters at 0.1 pg-10 ng/dot. The sites of T-T dimer were detected with the direct, the indirect and the CSA methods, as described in Materials and Methods.

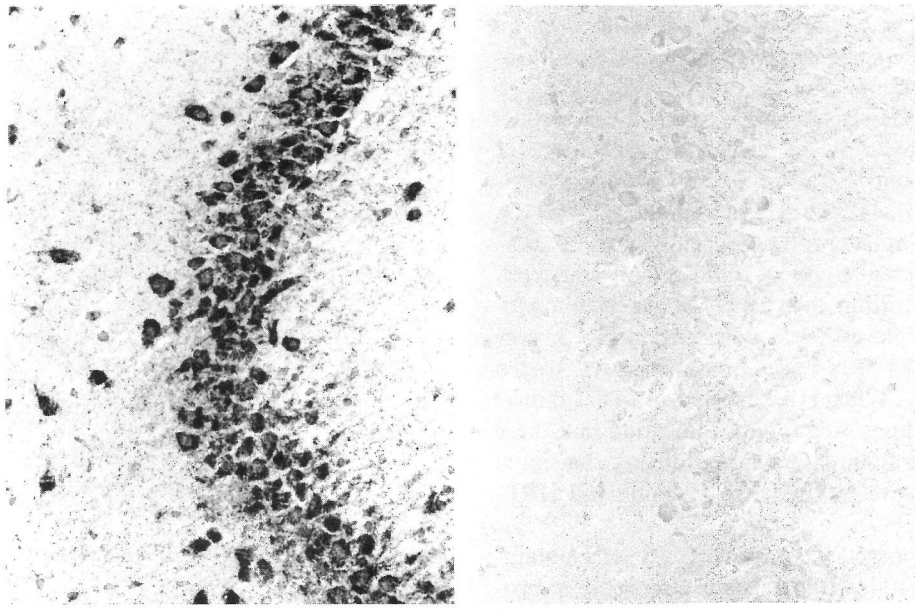

Fig. 3. 28S rRNA staining by *in situ* hybridization with catalyzed signal amplification in the frozen sections of rat brain. The frozen sections of rat brain fixed with 4% paraformaldehyde in PBS were hybridized with the T-T dimerized oligonucleotide probe complementary to rat 28S rRNA and the signal was detected (the left panel) with biotinylated tyramine, as detailed in Materials and Methods. In the right panel, only the reaction with HRP-anti-(T-T dimer) was omitted, as negative control. Magnification; ×180.

hr with PBS containing 5% BSA, 100 μg/ml salmon testis DNA and 100 μg/ml yeast tRNA and 500 μg/ml of normal mouse IgG (for the direct and CSA methods) or normal rabbit IgG (for the ABC method). For the direct method, the sections were reacted overnight with HRP-anti-(T-T dimer) and the signal was visualized as detailed above. For the CSA method, the sections were further reacted with biotinylated tyramine and HRP-streptavidin, successively, as described above. The principle of our signal amplification system with biotinylated tyramine was shown in Fig. 1. The sections reacted with normal rabbit IgG were subjected to the ABC method according to the manufacture' s instruction and the sites of HRP were detected, as described above.

III. Results and Discussion

First of all, to evaluate the usefulness of catalyzed signal amplification system with biotinylated tyramine, we compared detection sensitivity among three protocols including the direct, the indirect or the CSA method in the immunohistochemical detection of T-T dimerized DNA, which was dotted onto nitrocellulose filters. As shown in Fig. 2, with the direct method 100 pg spot was barely detectable. When the indirect method was applied, the sensitivity seemed to be a little bit increased. On the other hand, remarkable amplification of signal was found with the CSA method, showing a faint but significant staining of 1 pg spot. Therefore, we confirmed about 100-fold increase in the sensitivity, compared to that of the usual direct method.

Next, we tried to merge the CSA method to our nonradioactive *in situ* hybridization protocol, which had been already established [10, 12]. The principle of the combined protocol was summarized in Fig. 1. As an evaluation system of the usefulness of the combined protocol, 28S rRNA staining by *in situ* hybridization was employed, because the rRNA staining turned out to be a convincing tool not only to assess the retention of hybridizable RNA, but also to optimize various pretreatment conditions [23]. Although we conduct 28S rRNA staining routinely at a probe concentration of 0.5–1.0 μg/ml, in the present study we did it at only 10 ng/ml, a much less concentration than usual to generate a suboptimal condition. Nevertheless, as shown in Fig. 3, very intense signal for 28S rRNA was obtained in the frozen sections of rat brain. When HRP-labeled anti-(T-T dimer) was omitted, no staining was found, indicating that there was no background staining generated during the signal amplification steps with biotinylated tyramine and HRP-labeled streptavidin.

Finally, we compared the degree of 28S rRNA staining in the frozen sections of rat brain among three protocols, i.e., the usual direct method, the ABC method and the CSA method, which were used as an immunohistochemical step after hybridization with the low concentration of 28S rRNA probe. As shown in Fig. 4, essentially no staining was observed with the direct method. When we applied the ABC method, a weak staining for 28S rRNA came out. Dramatically, however, very intense staining was found with the CSA method and the signal was localized to the cytoplasmic area as well as nucleoli of nerve cells. Thus, we should say that the signal amplification system with biotinylated tyramine works very well on tissue sections as well as on nitrocellulose filters. We can reasonably assume that now the sensitivity of nonradioactive *in situ* hybridization is high enough to analyze a single copy of specific RNA at the level of individual cells.

The localization of the signals was strictly restricted without any visual diffusion. The reason might be explained by the postulated mechanism, in which biotinylated

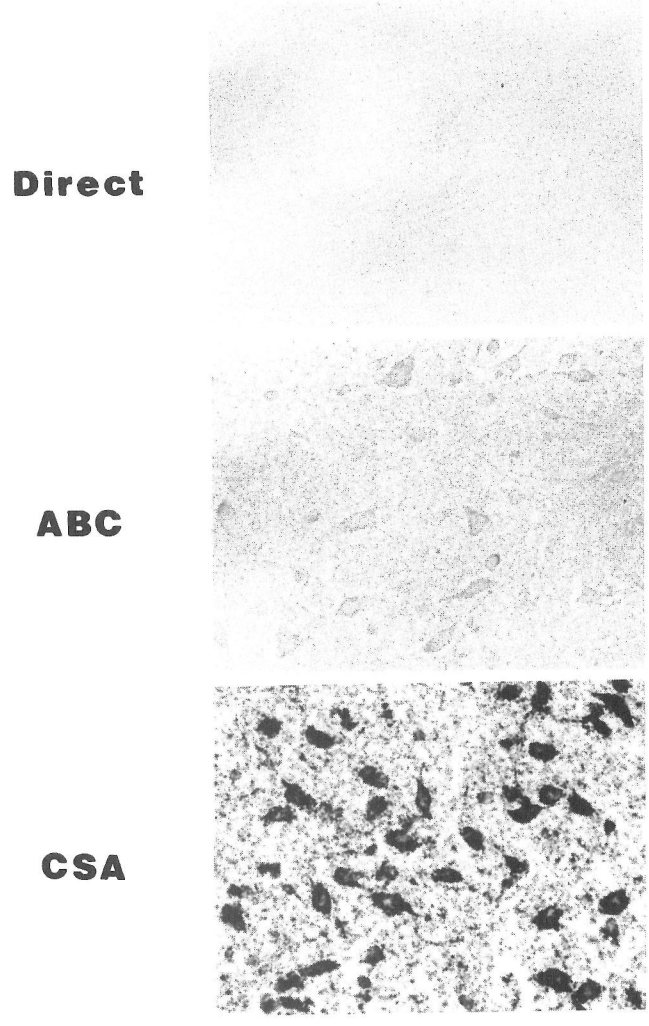

Fig. 4. Comparison of 28S rRNA staining by *in situ* hybridization with different detection protocols. The frozen sections of rat brain fixed with 4% paraformaldehyde in PBS were hybridized with the T-T dimerized oligonucleotide probe complementary to rat 28S rRNA and the signal was detected by the direct, the ABC and the CSA methods, as detailed in Materials and Methods. Magnification; ×350.

tyramine is deposited via free radical formation and reaction with electron rich moieties such as tyrosines, tryptophans, etc., of protein molecules surrounding the sites of HRP. As the free radical is short lived, the reaction with the solid-phase should only occur in the location where it is generated [3].

The catalyzed signal amplification system with biotinylated tyramine was originally reported by Bobrow *et al.* [3, 4] as a novel method using catalyzed reporter deposition to increase detection limits in microplate immunoassays [4] and membrane immunoassays [3]. Later, it was applied to immunohistochemistry [2] and chromosomal *in situ* hybridization [9]. Very recently, De Haas *et al.* [5] have succeeded to apply the method to fluorescence *in situ* hybridization for specific RNA in cultured cells. In this study, we further extended the application of the signal amplification system to colorimetric *in situ* hybridization for RNA molecules in tissue sections, much more complicated structurally than cultured cells, resulting in a remarkable increase in signal sensitivity. Additionally, successful use of the amplification system in nonradioactive *in situ* hybridization should also bring about to decrease a concentration of probe in the hybridization solution and to save time by shortening the hybridization reaction, while we must optimize various conditions to manage the development of background staining for a wide application.

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V. References

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