IMPROVED TISSUE PREPARATION FOR *IN SITU* LOCALIZATION OF SPECIFIC mRNA USING NON-RADIOACTIVE DNA PROBES: EFFECTS OF PROTEASE DIGESTION AND PROBE SIZE ON SIGNAL DETECTION IN FROZEN AND PARAFFIN SECTIONS OF RAT PITUITARY GLANDS

TAKEHIKO KOJI, TETSUYA MORIUCHI AND PAUL K. NAKANE

Department of Cell Biology, Tokai University School of Medicine,
Bohseidai, Isehara 259-11

To better describe the physiological state of cells, detection of specific mRNA by *in situ* hybridization at the cellular level is often demanded. In order to accomplish this reliably, various factors such as morphological preservation, retention of mRNA, accessibility of the labeled probe to the target mRNA and efficiency of the hybridization should be considered during the tissue processing. In this paper, using non-radioactive probes, we investigated the effects of protease treatment and the size of probe on the efficiency of *in situ* hybridization with mRNAs in frozen and paraffin-embedded tissue sections of the rat pituitary glands. As non-radioactive haptenic DNA probes, we used dinitrophenyl (DNP)-labeled pro-opiomelanocortin (POMC) DNA and thymine-thymine (T-T) dimerized prolactin cDNA. The signals were visualized by the indirect enzyme-immunohistochemistry. The best results were obtained when frozen sections of tissues fixed by 4% paraformaldehyde in phosphate buffered saline were mildly digested with protease and hybridized with the haptenized DNAs of about 200–400 base pairs.

During the past decade, much effort has been made on the analysis of expression of specific mRNA in cells and tissues by the Northern blot analyses. Through these analyses, it soon became apparent that the study of individual cells rather than the study of a mass of cells or tissues was required, since the expression of mRNA varies considerably from cell to cell. To accomplish this task, the localization of mRNA by means of *in situ* hybridization was found to be the most effective method. The latter studies have yielded information on the state of intracellular synthesis of peptide, regardless of the states of peptide secretion, peptide degradation, peptide transport and post-translational modification of the peptide.

For the *in situ* localization of mRNAs, cDNAs labeled with radio-isotopes have been used as probes and the sites of mRNAs are visualized with radioautographic techniques (3, 7, 9, 12). More recently, in order to obtain better resolution and to minimize the cumbersome procedures associated with the handling of radioactive compounds, non-radioactive probes have been introduced. These non-radioactive probes are usually labeled with haptens such as sulfon (23), biotin (15), dinitrophenyl (DNP) (28), acetylaminofluorene (AAF) (31), mercury-sulfhydryl-hapten (11) or are made im-

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munoreactive by forming T-T dimers (22). When these probes are used, the sites of mRNA are recognized immunohistochemically by localizing the haptens.

There are several basic differences in methods of tissue preparation depending upon the type of labels used. For example, fixatives which generate background silver grains should be avoided when the radioactive probes are used, whereas one should be concerned with non-specific adsorption of antibodies when the non-radioactive probes are used. As non-radioactive probes are expected to have superior resolution to radioactive probes, the criteria for the degree of morphological preservation of cells and tissues become more stringent. Most of information on the methods for tissue preparation were obtained from the experiments using the radioactive probes and require some modification to be applicable for use with the non-radioactive probes. From our previous experience with non-radioactive probes, we recognized that the new set of condition is needed for the maximal retention of target mRNA in cells and tissues, the optimal morphological preservation of the cells and tissues, the maximal accessibility of probe DNA to target mRNA and the efficient hybridization between the probe and mRNA. To this end, we investigated the effects of protease treatment and the size of the non-radioactive probe on the efficiency of in situ hybridization with mRNAs in frozen and paraffin-embedded tissue sections of the rat pituitary glands. As non-radioactive haptenic DNA probes, DNP-labeled proopiomelanocortin (POMC) DNA and thymine-thymine (T-T) dimerized prolactin cDNA were used. The signals were visualized by indirect enzyme-immunohistochemistry. The best results were obtained when frozen sections of tissues fixed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) were mildly digested with protease and hybridized with haptenized DNAs of about 200–400 base pairs (bp).

MATERIALS AND METHODS

Materials: Following reagents were purchased; formamide from Nakarai Chemical Co., Japan, 3,3'-Diaminobenzidine/4 HCl from Dojin Chemical Co., Japan, bovine serum albumin (BSA) (98–99% pure), yeast tRNA, DNase I (bovine pancreas), heparin (porcine intestinal mucosa, grade II), proteinase K (18–20 units/mg protein), horseradish peroxidase (type VI) (HRP) and polyvinylpyrrolidone (MW 360,000) from Sigma Chemical Co., USA, Ficoll-400 from Pharmacia Co., Sweden, salmon sperm DNA from Wako Pure Chemical, Co., Japan, diethylpyrocarbonate from Aldrich Chem. Co., USA, and human placental ribonuclease inhibitor (RNin) from Takara Shuzo Co., Japan. All chemicals and biochemicals used were of analytical grade.

DNA probes used: Human POMC DNA (genomic clone) (1.16 kbp) (30), rat prolactin cDNA (823 bp) (5), Hind II fragment of pBR 322 DNA (1107 bp) and lambda DNA were used. The plasmids containing POMC DNA or prolactin cDNA were purified according to the method described in the textbook of molecular cloning (17). Finally, the inserts of POMC DNA and prolactin cDNA were excised from the plasmids by appropriate restriction endonucleases, purified and used. The DNAs were dissolved in 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM EDTA (TE) and kept at -20°C.

Antibody used: Rabbit anti-DNP was prepared by immunizing rabbits with DNP labeled BSA as detailed previously (27). Rabbit anti-T-T dimer was prepared
by immunizing rabbits with a mixture of T-T dimerized salmon sperm DNA and methyl-bovine serum albumin (a gift from Dr. H. Yoshida, Kyoto University, School of Medicine) (16). IgG from the rabbit serum was isolated and used as the first antibody. Fab fragment of goat IgG against rabbit IgG conjugated with HRP was prepared according to the method of Wilson and Nakane (34).

Tissue preparation: Adult male Wistar rats weighing 250-350 g were used in this experiment. For paraffin sections, rats were killed by cervical dislocation, and pituitary glands were quickly excised and immersed in 4% PFA in PBS, adjusted to final pH of 7.4, or immersed in ethanol/acetic acid (3:1) mixture for about 15-17 hr at room temperature (RT; 25°C-28°C). Unless otherwise specified, the following experiments were carried out at RT. The fixed tissues were dehydrated by serial ethanol and xylene, and embedded in a mixture of paraffin and Paraplast. For frozen sections, rats were anesthetized with ether and perfused intracardially with about 100 ml of 4% PFA in PBS. The pituitary glands were isolated and fixed further in the 4% PFA in PBS for 2 hr at 4°C. Then the glands were immersed in 30% (w/v) sucrose solution containing 0.02% (v/v) diethylpyrocarbonate at 4°C until the glands sunk to the bottom of the fixing jar (about 2 hr), and embedded in OCT compound. After freezing in ethanol/dry ice, the glands were kept under −80 to −60°C until sectioned.

Labeling of probe DNA: DNP labeling of probe DNAs was carried out as detailed previously (27). T-T dimer was introduced into probe DNAs by UV-irradiation as described in detail previously (22). The optimal UV dose for prolactin cDNA as well as for lambda DNA was 5,000 J/m² when it was determined by the dot blot hybridization method.

DNase digestion of DNAs: To shorten the length of DNA, the probe DNAs were digested with DNase I at a concentration of 0.1-0.3 µg/ml for 10 min at 37°C. The reaction was initiated by mixing 9 vol. of DNA solution with 1 vol. of 2× SSC (SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7.0) containing 50 mM MgCl₂ and 1 to 3 µg/ml of DNase I, and the reaction was terminated by heating in a boiling water bath for 10 min.

Determination of DNA size: The probe DNAs were heated in a boiling water bath for 10 min, mixed with bromophenol blue-xylene cyanol, and applied to 1% agarose gel. Electrophoresis was carried out at 40 volts in an ice bath. As a size marker, a mixture of Hind III digest of lambda DNA and Hae III digest of φX174 DNA, and Alu I digest of pBR 322 were used. After electrophoresis, the gel was stained with ethidium bromide and analyzed under UV illumination.

In situ hybridization:
Coating of glass slide; three different methods of coating glass slide were used. 1) Denhardt’s solution coating; Glass slides were coated by a mixture of 0.02% (w/v) BSA, 0.02% (w/v) polyvinylpyrrolidone and 0.02% (w/v) Ficoll-400 according to the method of Brahic and Haase (3). 2) Gelatin coating; pre-cleaned glass slides were coated with 1% gelatin/0.1% CrK(SO₄)₂ for 15 min at 40-50°C and fixed with 1% formaldehyde for 10 min at RT. After baking the slides for 15 hr at 45°C, the slides were stored at RT until used. 3) Albumin coating; pre-cleaned glass slides were coated with egg albumin (21). A drop of 25% glutaraldehyde solution was placed on the surface of slide for 10 min, then the slide was immersed totally in a solution of 2.5% glutaraldehyde for 10 min. After rinsing with distilled water (DW), the slides
were used immediately.

Pretreatment; the frozen sections (5–6 μm) were mounted onto the gelatin coated glass slides, dried and baked at 40°C for 4–15 hr, or onto the other coating slides, dried and kept at -60°C until used. At the time of in situ hybridization, they were rehydrated with PBS. Some of the hydrated sections were treated with 0.3% Triton X-100 in PBS for 10 min. The specimens were digested with various concentrations of proteinase K dissolved in PBS for 10–30 min at 37°C. After washing 3 times with PBS (5 min each), the specimens were fixed in 4% PFA in PBS for 5 min, washed twice with PBS (5 min each) and the remaining aldehyde was quenched twice by immersion in 2 mg glycine/ml in PBS for total of 30 min. Then the slides were washed twice with PBS (5 min each), immersed in 40% (v/v) deionized formamide in 2×SSC and kept in the formamide until hybridized. The formamide was deionized with mixed bed resin (Bio-Rad). With the paraffin embedded tissues, 5–6 μm sections were cut, placed on the coated glass slides and dried. After deparaffinization with xylene and toluene, the slides with sections were hydrated until 70% ethanol, immersed in ethanol/acetic acid (3:1) mixture for 20 min and were washed with DW. Some of them were further treated with 0.2 N HCl for 20 min in order to remove basic proteins such as histone and RNase. The specimens were digested with various concentrations of proteinase K dissolved in PBS for 10 min at 37°C. After washing with three changes of PBS (5 min each), the specimens were fixed in 4% PFA in PBS for 5 min, washed twice with PBS (5 min each) and the excess aldehyde was quenched twice by immersion in 2 mg/ml glycine in PBS for 15 min each. Some of them were kept in 2×SSC until hybridized, and the others were dehydrated with serial ethanol, air dried and kept at RT until hybridized.

Hybridization; DNP-labeled or T-T dimerized probe DNAs were mixed with deionized formamide and the other constituents, boiled for 10 min, and quickly chilled in an ice water bath. The final hybridization mixture was as follows; 10 mM Tris/HCl (pH 7.3), 1 mM EDTA, 0.6 M NaCl, 1×Denhardt’s solution, 40% (v/v) deionized formamide, 250 μg/ml yeast tRNA, 125 μg/ml sonicated salmon sperm DNA and 2–4 μg/ml DNP-labeled or T-T dimerized probe DNA. Yeast tRNA and salmon sperm DNA had been re-purified in our laboratory. In some cases, RNin (1,000 units/ml) or heparin (5,000 units/ml) was included in the hybridization mixture. Twenty μl of the mixture was applied to each section, mixed and incubated in a moist chamber for 15 hr at 42°C. Then the specimens were washed with 50% (v/v) formamide in 2×SSC with four changes for 1 hr interval at 37°C, and twice with 2×SSC at RT for 15 min each.

Enzyme-immunohistochemistry; for immunohistochemical detection of DNP-DNA or T-T dimerized DNA, the specimens were treated with PBS (pH 7.2) containing 5% (w/v) BSA, 500 μg/ml normal goat IgG, 100 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA and 0.05% NaN₃ for 1 hr in order to block the non-specific binding of antibodies to the specimens. Then the specimens were reacted with rabbit anti-DNP IgG or rabbit anti-T-T dimer IgG dissolved in PBS containing 5% (w/v) BSA, 100 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA and 0.05% NaN₃ for 15 hrs. After 1 hr wash with PBS, the specimens were reacted for 1 hr with HRP-conjugated Fab of goat IgG against rabbit IgG dissolved in PBS containing 5% (w/v) BSA, 100 μg/ml yeast tRNA and 100 μg/ml sonicated salmon sperm DNA and washed with PBS for 1 hr. In some cases, RNin or heparin was added to
the antibody solutions. The sites of HRP were visualized histochemically using 5 mg/ml 3,3'-diaminobenzidine/4HCl and hydrogen peroxide with or without nickel and cobalt ions (1). In some experiments, silver enhancement was carried out (25). Without counterstaining, the slides were dehydrated, cleared and mounted as usual.

RESULTS

Glass slide coating:

Glass slides coated with a variety of materials were examined for their ability to retain tissue sections during the procedure. Those slides coated with Denhardt’s solution which has been adapted effectively for the adhesion of alive cells or fresh frozen tissue sections on the slide, failed to retain paraffin sections. The best retention was obtained by the slide coated with egg albumin. The sections remained firmly attached to the slide even after proteolysis (100 μg/ml, 37°C, 10 min) (Fig. 1). The gelatin coated slide retained frozen sections of 4% PFA in PBS fixed tissues (Fig. 2), but not retain paraffin sections.

The effects of protease treatment and probe size:

The paraffin sections of rat pituitary glands fixed with 4% PFA in PBS were first treated with various concentrations of proteinase K. The sections were then hybridized in situ with DNP-POMC DNA or DNP-pBR 322 DNA. POMC mRNA was localized in most cells of intermediate pituitary and some cells (very weak, but significant signals) in anterior pituitary when the sections were treated with 100 μg/ml of proteinase K (Fig. 1g). However, using the lower concentrations (0–10 μg/ml) of proteinase K (Figs. 1a, 1c, 1e), little or no signals were detected both in the intermediate and anterior pituitaries. On the other hand, the staining in the intermediate pituitary was strongest with the DNP-pBR 322 DNA in the undigested section and decreased as the concentration of proteinase K was increased (Figs. 1b, 1d, 1h). Little or no staining was observed in the section digested with 100 μg/ml of proteinase K and reacted with the DNP-pBR 322 DNA. The cells in the intermediate pituitary were stained evenly with DNP-pBR 322 DNA, whereas with DNP-POMC DNA the staining was focal and the intensity varied from cell to cell. When the sections of pituitary which were fixed with ethanol/acetic acid mixture and embedded in paraffin, high nonspecific staining was observed both in the anterior and intermediate pituitaries (data not shown). The sizes of DNP-labeled DNA probe were not determined in this study, since DNP-labeled DNA in agarose gel slab could not be visualized with routine ethidium bromide staining.

In order to examine the intensity of hybridization signal as a function of probe size, the frozen sections of pituitary glands fixed with 4% PFA in PBS were hybridized with various sizes of T-T dimerized prolactin cDNA. The T-T dimerized prolactin cDNA (823 bp) was first digested by DNase I and the sizes of the digested DNA were determined by the electrophoretic pattern on agarose gel. The size of DNA varied from <150 to 410 bp depending upon the concentration of DNase I and the duration of digestion. With sections of pituitary untreated with proteinase K, the short (180–280 bp) (Fig. 2a) and medium (220–410 bp) (Fig. 2c) size T-T dimerized prolactin cDNA hybridized with the cytoplasmic area of cells which could be identified as typical prolactin cells from their location and morphology (20). However, no signals were detected with the undigested whole prolactin cDNA (823 bp) (Fig. 2e). When
Fig. 1. Effect of increasing concentrations of proteinase K on in situ detection of POMC mRNA in paraffin sections of rat pituitary gland. The paraffin embedded tissue sections were treated with proteinase K at the concentration of 0 µg/ml (a, b), 1 µg/ml (c, d), 10 µg/ml (e, f) or 100 µg/ml (g, h), and were hybridized in situ with DNP-labeled POMC DNA (a, c, e and g) and DNP-labeled pBR 322 DNA (b, d, f and h). A: anterior pituitary, I: intermediate pituitary, P: posterior pituitary.

the specimen was treated with proteinase K (1 µg/ml, 37°C, 15 min), the intensity of the specific staining increased slightly with the short probe (Fig. 2b), considerably with the medium size probe (Fig. 2d), and a slight but significant staining of prolactin mRNA could be detected with the whole cDNA (Fig. 2f). On the other hand, with the probe less than 100-150 bp, no constant specific staining was attained. When the sections were hybridized with various sizes of lambda DNA, no specific staining was observed (data not shown).
FIG. 2. Effects of protease digestion and probe size on in situ detection of prolactin mRNA in frozen sections of rat pituitary gland. The frozen sections were hybridized in situ with various sizes of T-T dimerized prolactin cDNA; 180–280 bp (a and b), 220–410 bp (c and d) and 823 bp (e and f). Some of them (b, d and f) were treated with proteinase K before hybridization. The cytoplasmic area of typical prolactin cells was darkly stained. ×400
Other special remarks on in situ hybridization:
(a) In order to increase the diffusibility of probe DNAs, several investigators recommended the use of detergent such as sodium dodecyl sulfate (SDS) (12) and Triton X-100 (4). When these detergents were used in conjunction with the above experiments, no beneficial effect on the intensity of staining was observed. On the contrary, the non-specific staining of nuclei was sometimes observed (data not shown).
(b) There is an inconsistency with the published protocols as for the beneficial effect of drying tissue sections prior to hybridization (3, 4, 10, 12, 14). We often found some heterogeneity when the sections were dried, hence it appears that drying the sections offers no positive effect, at least in our system.
(c) To inhibit RNase activity, heparin (29) or RNin (17) was added to the hybridization mixture and to the antibody solutions in some experiments. Again, we found no significant benefit when the inhibitors were used.
(d) The sites of HRP were visualized using an incubation solution containing 3,3'-diaminobenzidine, nickel ions, cobalt ions and hydrogen peroxide (1). The addition of both ions to the classical Graham and Karnovsky solution (8) resulted in about a 10 fold increase in the sensitivity. The silver enhancement (25) introduced the non-specific staining of nuclei in the pituitary system and the signal/noise ratio was not increased.

DISCUSSION

In situ hybridization method using non-radioactive probes is considered to be the most appropriate technique to analyze the expression of specific mRNA sequences at the cell level. However, in spite of its potential application, systematic studies to establish a tissue processing procedure best suited for in situ hybridization are rare. While utilizing non-radioactive probes, we have been making efforts to establish such conditions for some time. During the course of our studies, we recognized the importance of the collision frequency of the probe DNA with the target mRNA for the successful implementation of in situ hybridization techniques. Any time the frequency was lowered, such as when the numbers of probe DNA or target mRNA were reduced, the diffusion of probe DNA was retarded, or some interfering material was present between the probe DNA and the target mRNA, the hybridization signal was reduced. In well preserved tissues and cells such as with paraffin sections and frozen sections of fixed tissues, materials which prevented the diffusion of probe DNA and interfered with the hybridization of the probe DNA with the target mRNA were present. Thus, the signal was reduced. With large-sized DNA probes (more than about 1 kbp) which may not have diffused through the matrix of well preserved cells and tissues, little or no hybridization signal was attained. To increase the collision frequency between the probe DNA and the target mRNA or the accessibility of the probe DNA to the target mRNA, there are two logical procedures. Aside from increasing the number of probe DNA, one is to use smaller probe DNA, and the other is to make mRNA more accessible to the probe DNA by removing materials which retard the diffusion of the probe DNA or mask the target mRNA. Both of these approaches were tried in this study.

As shown in Figs. 2a, 2c and 2e, the intensity of the hybridization signal depended on the size of the probe cDNA used. As the size of probe was reduced, we found more signal and the signal was maximum when the size was about 200–400 bp, but
when the size was less than 150 bp the signal was again less. Others also found similar optimal sizes, i.e. about 50–300 bp for DNA probes (2, 3, 13, 19, 26) and 400 bases for single stranded RNA probe (10).

The biphasic nature of the signal as the size of probe was changed may have resulted from various factors. As the size of probe was reduced, the probe diffused through the cellular matrix more easily and gained access to the target mRNA more frequently, thus more signal was generated. There are two possible reasons why when the size of probe DNA was reduced to less than 150 bp, the signal was less. One is that, since only 2–3 haptens are introduced per 100 bases of probe DNA (22), if only a portion of the larger probe DNA hybridizes with the target mRNA, there will be more hapten per target mRNA than with the smaller probe DNA. Consequently, down to a certain size of probe DNA, the hybridization signal is increased, but beyond a certain size, the hybridization signal is decreased. Another possibility is that, since we used heat denatured double stranded DNA as the probe, one expects that when the hybridization solution was applied to the sections, the probe DNA to hybridize with the target mRNA as well as to reanneal with another probe DNAs and form complicated DNA matrixes. Also a portion of the probe DNA hybridizes to the target mRNA and another portion reanneals with another probe DNA. The formation of matrix in fact has been attributed to the reason why when double stranded DNA is used, more signal is generated than when single stranded DNA or cRNA is used (14). However, when the size of the probe DNA is reduced to beyond a certain size, one can speculate that the entire length of the probe DNA is involved with the hybridization with the target mRNA and will not anneal with another probe DNA, thus no DNA matrix is formed adjacent to the target mRNA, less hapten at the site and less signal is generated. Either one or both of the above possibilities may have contributed to the result that when the size of probe DNA was less than 150, there was less signal in our experiments. Because of the biphasic nature of the signal as the probe size was changed, regardless of the reason, our experiments point to a necessity of obtaining an optimal size of probe DNA for a given set of experiments. This is particularly important when non-radioactive probes which were haptenized either chemically (DNP, AAF, Hg-sulphhydryl hapten, etc.) or physically (photobiotin (6), T-T dimer) are used, since no nuclease is used during the haptenization and the length of DNA strands may stay unchanged.

The mild proteolysis of sections also resulted in an increase of the hybridization signals. The beneficial effect was more apparent when the larger probe DNA was used (Fig. 2). With the 1.16 kb probe DNA, more drastic digestion by protease was required to generate specific hybridization signals (Fig. 1). These results demonstrated the presence of proteinacious materials which reduce the collision frequency between the probe DNA and target mRNA and should be removed in order to increase the hybridization signals. It appears that the amount of the interfering material varies considerably depending on how the tissues were fixed and processed (14, 18, 32). Generally, tissues fixed with cross-linking fixatives such as those containing formaldehyde or glutaraldehyde require the protease treatment (Figs. 1, 2). On the other hand, those fixed by protein precipitating fixatives such as ethanol/acetic acid (3:1) do not always require this treatment (Koji et al., Histochem. J., in press), in agreement with Lawrence & Singer (14) and Pringle et al. (24).

In addition to the above proteolytic effect, the protease treatment removed the
specific, but hybridization independent binding of DNP-labeled pBR 322 DNA to the sections of rat pituitary glands (Figs. 1b, 1d, 1f, 1h). Since pBR 322 DNA contains several glucocorticoid-receptor binding sequences (33), DNP-pBR 322 DNA may have reacted with the glucocorticoid-receptors in the untreated sections. And the proteolysis of the sections removed the receptors from the section and rendered the DNP-pBR 322 unreactive with the sections. Treatment of cells and tissue sections with 0.2 N HCl is an alternative method to remove proteins, especially basic proteins such as histone and RNase. We found this treatment to have a little value with the paraffin sections of formaldehyde fixed tissues, although there was a remarkable effect with ethanol/acetic acid (3:1) fixed fresh frozen sections and cells (Koji et al., Histochem. J., in press). In a preliminary experiment with the frozen sections of formaldehyde fixed tissues, the treatment with 0.2 N HCl resulted in an increase in a signal/noise ratio (data not shown).

**In situ** hybridization with non-radioactive probes has proved to be a powerful technique in investigating gene expression at the cell level when appropriate conditions are established.

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