

Detection of Target mRNAs by Non-RI Hybridization Technique : With Special Reference to Factors Affecting Hybridization Signals

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Abstract

Factors affecting the Northern blot hybridization signals were examined using synthetic oligonucleotides as probes and the digoxigenin labeling and detection system. The factors were incubation time for labeling probes, substrates for alkaline phosphatase, irradiation intensity of ultraviolet (UV) rays for cross-linking RNA to Nylon membrane, and concentrations of target mRNAs. (1) Labeling efficiency of probes was increased by extending the incubation time with terminal deoxynucleotidyl transferase (TdTase); 15, 30, and 60 minutes. The 60-minute incubation produced the labeled probes with longer tails and high detection efficiency. (2) Probes labeled by 60-minute incubation were detected at as low as 10^{-16} mol/ μ L/dot on Nylon membrane in combination with NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate), Vector Blue, or Vector Red for enzyme substrates. Digital images of the signals processed by NIH Image showed the graded sensitivity of the enzyme-mediated precipitations depending on the substrates (NBT/BCIP \geq Vector Blue $>$ Vector Red). (3) The intense signals of the Northern blot hybridization were obtained from membranes irradiated by UV rays at 30×10^3 to 120×10^3 μ J/cm². UV rays at either low or high energy beyond the optimal range reduced the signals. (4) In the Northern blot analysis, poly(A)⁺ RNA samples showed high sensitivity for detecting target mRNAs in comparison with total RNA analysis. (5) The protocol established in the present study detected tissue-specific expression of β - γ -actin mRNA, glycerol-3-phosphate dehydrogenase-1 (G3PDH-1) mRNA, preproenkephalin (PPE) mRNA, preprodynorphin (PPD) mRNA in four tissues; brain, liver, kidney, and testis.

Key Words : hybridization, oligonucleotide, digoxigenin, alkaline phosphatase,
tissue-specific mRNA expression

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Introduction

The distinctive functions and phenotypes of cells and tissues depend on the gene expressions of constituting cells, the activities of which are maintained in steady-state levels for performing the normal functions. In addition, those cells continue to alter the metabolisms in response to stimuli arising in the inside as well as the outside of the body, and then keep the homeostasis from cell to body. Such dynamics of the gene expressions have been studied in the pre- and post-natal development,^{14, 15)} a pathological condition,²⁵⁾ a stress condition,²⁾ virus infection cases, and various experimental conditions.^{7, 16~18)}

Detecting the expression of target mRNAs implies that the cells and tissues have potentials to produce physiologically active substances through the intracellular metabolic pathways. The intracellular localization of target mRNAs indicates the phenotype of the cells indirectly, and the temporal fluctuation of mRNA expression reflects the activities of transcription factors in nuclei indirectly. Detecting the dynamics of mRNA expression is, thus, important to know the physiological and pathological conditions of cells and tissues.

At first, techniques detecting target DNA or RNA by hybridization started using the cloned cDNAs and radioisotopes as labeling and detection materials. Thereafter, the technology evolved various new ideas and advanced the improvement and diversification in materials and techniques for use. Nowadays, available are cDNA, cRNA and synthetic oligonucleotide as probes and various radioisotopes (RIs) as well as non-RI materials (enzymes, haptens, fluorescent materials, and so on) for labeling and detection. It is important to note that the materials and techniques employed should be arranged in an optimal way for purposes of investigations. The present study examined several factors affecting the intensity of hybridization signals using synthetic oligonucleotides and non-RI labeling and detection materials.

Materials and Methods

1. Probe

Antisense oligonucleotides (45 mers) were synthesized against six RNA sequences of the rat: 18s- and 28s-ribosomal RNA, β - γ -actin, glycerol-3-phosphate dehydrogenase-1 (G3PDH-1, EC.1.1.1.8), preproenkephalin (PPE), and preprodynorphin (PPD). The specificity of the probes were analyzed on the computer-assisted program (Blast Search = [BLASTN]) available at the internet site of the National Center for Biotechnology Information (NCBI). However, the sequence of β - γ -actin probe are homologous to both cytoskeletal β - and γ -actin mRNAs (100%), vascular α -actin mRNA (88.9%), cardiac α -actin mRNA (84.4%) skeletal α -actin mRNA (82.2%) because of sequence similarity between the isoforms. The antisense probes were chosen from the following sequences (accession number and site): 18s-rRNA: Acc. #. V01270[1006-1050], 28s-rRNA: Acc. #. V01270[6048-6092], β - γ -actin: Acc. #. NM 031144[536-580], G3PDH-1: Acc. #. NM 022215[701-745], PPE: Acc. #. K02807[787-831], PPD: Acc. #. M10088[929-973].

2. Probe Labeling

The antisense probes were labeled by incubating with terminal deoxynucleotidyl transferase (TdTase) and a mixture of digoxigenin-linked dUTP and dATP for 15 minutes at 37°C (3'-end tailing kit, Roche Diagnostics). In addition to a recommended protocol of the kit, the incubation time was extended to either 30 or 60 minutes. After labeling, probes were purified routinely and diluted to concentrations of 10^{-13} ~ 10^{-18} mol/ μ L for assessing labeling efficiency. One microliter from each vial was blotted on pieces of non-charged Nylon membrane (Immobilon-Ny, Millipore Inc.). The Nylon membranes were baked in an oven at 80°C for 20 minutes, and then exposed to UV rays (254 nm, 60×10^3 μ J/cm²). The labeled probes on the membranes were detected according to a detection protocol of the kit; the membranes were incubated with alkaline phosphatase-conjugated

anti-digoxigenin antibody (dilution 1 : 1,000~1 : 4,000) for 1 hour at room temperature and then the enzyme reaction was carried out with NBT/BCIP (1 : 50). In addition, two other substrates for the enzyme were used as chromogens (Vector Blue and Vector Red, Vector Inc.).

The elongations of labeled probes (3 pmol) were analyzed by polyacryl amide 15~25% gradient gel electrophoresis, and visualized by silver staining for nucleic acids.

3. Detection By Labeled Probe

The detection ability of labeled probes was examined by hybridizing rat liver total RNA with 18s-rRNA probes (15-or 60-minute labeling) and 28s-rRNA probes (30-or 60-minute labeling). Total RNA (5 μ g per lane) was electrophoresed in the formalin-denatured agarose gel, and then transferred and cross-linked to Nylon membrane as described above. The membranes were incubated with each labeled probe (1 pmol/mL) in hybridization solution (4 \times SSC [600 mM NaCl, 60 mM sodium citrate], 50% formamide, 0.12 M phosphate buffer [pH 7.2], 1% sarcosyl, 1 \times Denhardt's solution [0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone K-90], 30 μ g/mL carrier DNA, 200 μ g/mL poly(A) RNA) for 18~21 hours at (T_m -25) $^{\circ}$ C. Posthybridization wash was carried out in 1 \times SSC at (T_m -25) $^{\circ}$ C for 1 hour. Then the membranes were processed for enzyme detection described above.

The melting temperature (T_m) values of probes were calculated according to the following formulas :

- (a) For Na^+ concentrations above 0.4 M ;
 $T_m = 81.5 + 0.41(\% \text{ GC}) - 0.72(\% \text{ formamide})$
 (b) For Na^+ concentrations of 0.01~0.2 M ;
 $T_m = 81.5 + 16.6(\log[\text{Na}^+]) + 0.41(\% \text{ GC}) - \frac{675}{n}$
 (n = probe length)

4. Cross-linking under UV irradiation

For cross-linking of RNA to Nylon membrane, 254 nm UV rays were used. The membranes were prepared for the Northern blot analysis

using rat liver total RNA (3 μ g per lane). The membranes were exposed to UV rays at different levels of energy ; 0, 15×10^3 , 30×10^3 , 60×10^3 , 120×10^3 , 240×10^3 , 480×10^3 , and $960 \times 10^3 \mu\text{J}/\text{cm}^2$. The effects of UV ray irradiation were examined by hybridizing the membranes with 28s-rRNA probe (60-minute labeling).

5. RNA Extraction and Analysis

Animals used were Sprague-Dawley male rats at the age of postnatal 10 weeks (SPF/VAF Crj : CD[SD], 350 ± 20 g, Charles River Inc.). The rats were kept for at least 1 week in an artificial condition of 12-hour day/night cycle and 25 $^{\circ}$ C. The animals were anesthetized with pentobarbital sodium (Nembutal, 1 mL/kg body weight, i.p., Dinabott Inc.) before sacrificing. Brain, liver, kidney and testis were removed, frozen in powdered dry ice, and then kept at -80 $^{\circ}$ C until use. The handling and treatment of the animals conformed to the guidelines of the Animal Study Committee of the Kagawa Prefectural College of Health Sciences.

RNA extraction from tissues was carried out according to a protocol of Chomczynski and Sacchi¹⁾ with an additional step of deproteinization. Portions of total RNA from the 4 tissues were purified to obtain poly(A)⁺ RNA by passing through the oligo(dT)-cellulose column.

Both total RNA and poly(A)⁺ RNA were used for the Northern blot analysis. Total RNA was prepared at 5, 10 or 20 μ g per lane and poly(A)⁺ RNA was prepared at 2.5 μ g per lane. The Northern-transferred membranes were baked at 80 $^{\circ}$ C and then exposed to 254 nm UV rays at $60 \times 10^3 \mu\text{J}/\text{cm}^2$. The protocol of hybridization and posthybridization wash were carried out as described above.

6. Image Processing

Signals of enzyme-mediated precipitations in various experiments were processed in the Plot Profile program of the NIH Image (ver. 1.56). The processed signal intensity was visualized as a scheme.

RESULTS

1. Probe Labeling

The 3'-end tailing reaction with TdTase elongated the probe length by adding digoxigenin-conjugated dUTP at 3'-end. The elongations of probes depended on the duration of enzyme incubation (15, 30, or 60 minutes) and

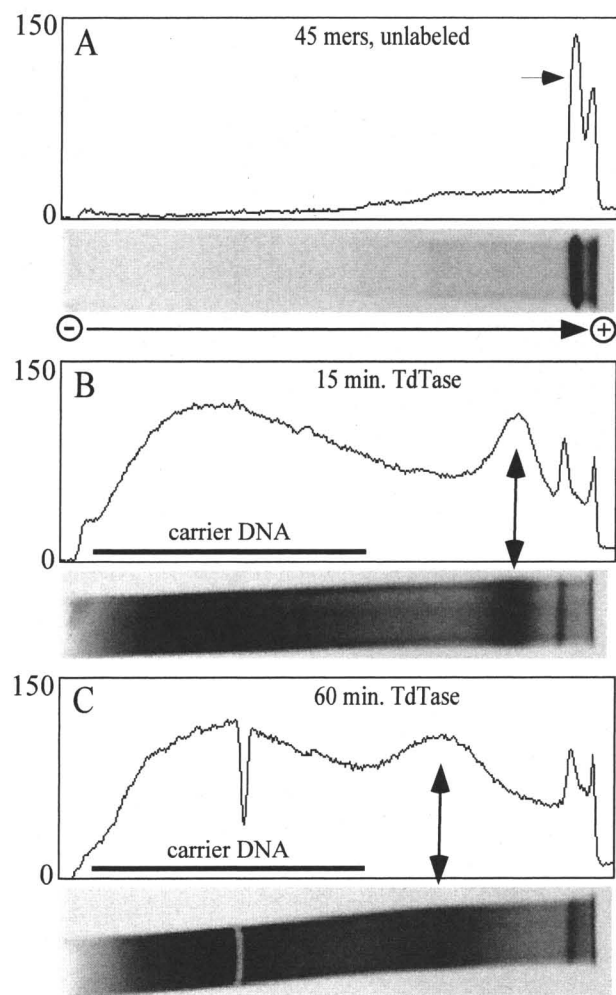


Fig. 1 Elongation of probe length by 3'-end tailing. A : 45-mer oligonucleotide unlabeled (arrow) . B & C : Oligonucleotides incubated with TdTase for 15 and 60 minutes, respectively. Double-headed arrows indicate elongated oligonucleotides by adding nucleotides at 3'-end. The upper case of each panel is the density profile of electrophoresed probes processed by NIH Image. The relative density of accumulated pixels is represented on the Y-axis. Carrier DNA used for ethanol precipitation is indicated by solid bars in panel B and C.

were confirmed by PAGE gel electrophoresis (Fig. 1). The extended, or 60-minute, incubation generated longer and sensitive probes and vice versa.

For examining the labeling efficiency, the probes of 60-minute labeling were diluted to concentrations of $10^{-13} \sim 10^{-18}$ mol/ μ L, and 1 μ L sample of each dilution vial was dot-blotted to Nylon membrane. The activity of alkaline phosphatase was visualized by incubating with NBT/BCIP, Vector Blue or Vector Red, which yielded insoluble precipitations with different colors (brown-purple, blue or red, respectively) on the membrane (Fig. 2). The precipitations

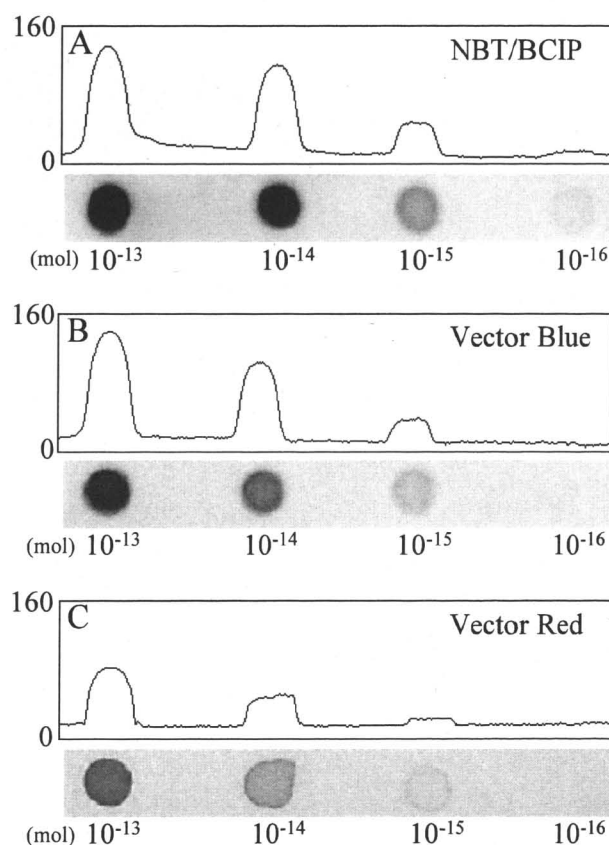


Fig. 2 Signals of digoxigenin-labeled probes detected with different substrates for alkaline phosphatase ; (A) NBT/BCIP, (B) Vector Blue and (C) Vector Red. NBT/BCIP and Vector Blue showed higher sensitivity than Vector Red. The upper case of each panel indicates the density profile of the enzyme-mediated precipitations processed by NIH Image. The relative density of accumulated pixels is represented on the Y-axis.

were recognized at as low as 10^{-16} mol/ μ L/dot under a careful inspection. The digital image processing by the NIH Image showed the differential intensity of those signals; the signals of NBT/BCIP was highly intense whereas those of Vector Red showed a slightly decreased intensity (signal intensity : NBT/BCIP \geq Vector Blue $>$ Vector Red) (Fig. 2).

2. Detection Efficiency

The detection of target RNA was examined by hybridizing probes of different incubation time with rat liver total RNA (5 μ g/lane). The hybridization signals by 15-minute labeling of

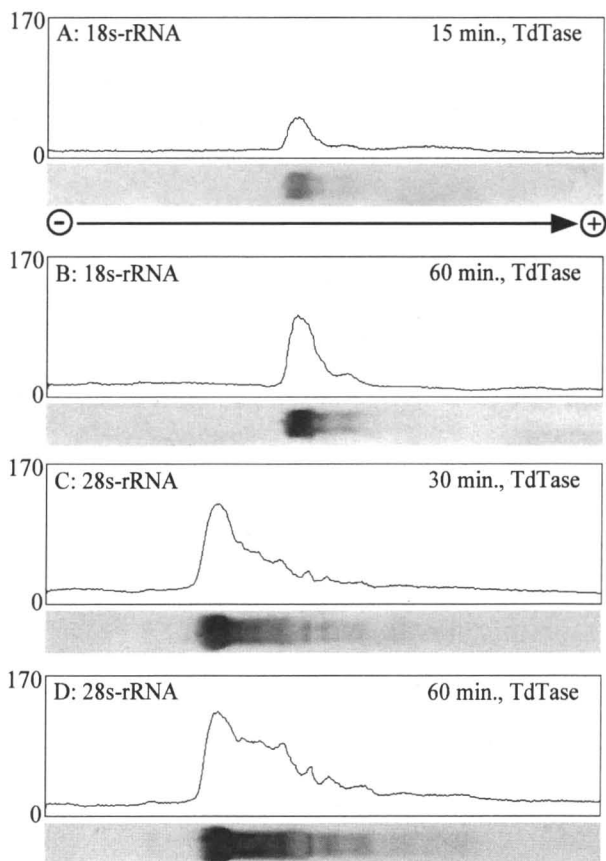


Fig. 3 Detection of 18s- and 28s-rRNA with their probes labeled for different incubation time. Both probes (B : 18s-rRNA and D : 28s-rRNA) labeled for 60 minutes exhibited intensified hybridization signals in comparison with those of 18s-rRNA (A : 15 minutes) and 28s-rRNA (C : 30 minutes). The upper case of each panel indicates the density profile of hybridization signals processed by NIH Image. The relative density of accumulated pixels is represented on the Y-axis.

18s-rRNA probe and 30-minute labeling of 28s-rRNA probe were compared with those of 60-minute labeling, respectively (Fig. 3A vs. B for 18s-rRNA probes; Fig. 3C vs. D for 28s-rRNA probes). In both ribosomal RNA species, the probes of 60-minute labeling exhibited stronger signals than their counterparts.

3. Effects of Ultraviolet Ray Irradiation

For cross-linking Northern-transferred RNA to Nylon membrane, 254 nm wave length UV rays were effective in the cases that the membranes of rat liver total RNA (3 μ g/lane) were exposed to UV rays at $30 \times 10^3 \mu$ J/cm², $60 \times 10^3 \mu$ J/cm² or $120 \times 10^3 \mu$ J/cm² (Fig. 4C, D and E). The membranes exposed to UV rays at low energy ($15 \times 10^3 \mu$ J/cm² and no exposure) showed less intense signals with the same positive areas to the optimal exposure cases (Fig. 4A and B). The high energy irradiation of UV rays beyond the level of $120 \times 10^3 \mu$ J/cm² decreased hybridization signals in both intensity and positive areas (Fig. 4F, G and H).

4. Detection of Target mRNA

In the Northern blot analysis, total RNA from the 4 tissues was prepared at 5 μ g, 10 μ g, or 20 μ g per lane for electrophoresis. Following capillary transfer and cross-linking of RNA to Nylon membrane, the membranes were processed for hybridization using 1 pmol/mL β - γ -actin probe, G3PDH probe, PPE probe or PPD probe. In this experiment were chosen the antisense probes labeled by 60-minute incubation and NBT/BCIP for the substrate of alkaline phosphatase. The β - γ -actin probe alone exhibited hybridization signals in total RNA analysis. The signal intensity varied from one tissue to another (Fig. 5A-D). Testis showed the relatively high expression of β - γ -actin mRNA (ca. 2,100 bases) and feeble signals of small-sized mRNA (ca. 1,800 bases) detectable with the β - γ -actin probe (Fig. 5D : asterisk).

To improve the intensity of hybridization signals and the signal versus noise ratio (S/N ratio), poly(A)⁺ RNA of the 4 tissues was prepared at 2.5 μ g per lane for electrophoresis and then processed for Northern blot analysis with

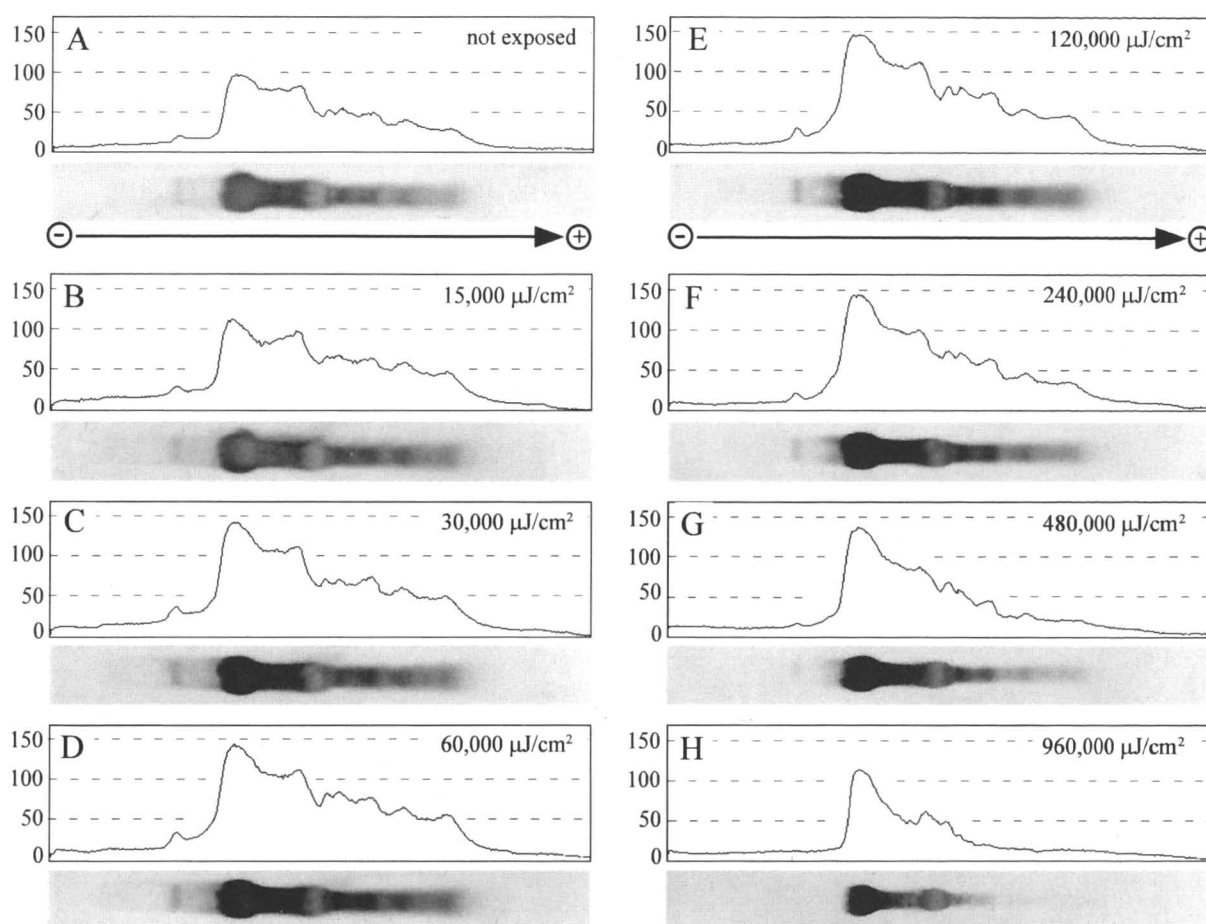


Fig. 4 Effects of ultraviolet (UV) rays affecting hybridization signals of 28S-rRNA. The cross-linking between RNA and Nylon membrane by UV rays is quite effective at optimal energy levels (C, D, and E). No or low energy exposure resulted in insufficient cross-linking of RNA to Nylon membrane (A, B). Overexposure to UV rays disintegrated RNA and decreased the hybridization signals of 28S-rRNA (F, G, H). The upper case of each panel indicates the density profiles of hybridization signals processed by NIH Image. The relative density of accumulated pixels is represented on the Y-axis. Note that degraded fragments of 28S-rRNA were electrophoresed along the lanes but this rather served the purpose to analyse the effects of UV rays.

the 4 antisense probes. In contrast to the total RNA analysis, the 4 probes exhibited hybridization signals with tissue specific expression of their target mRNAs (Fig. 6). The increased intensity of $\beta\cdot\gamma$ -actin mRNA signals was obtained in all lanes of the 4 tissues, and the signal patterns in the lanes were the same to the cases of the total RNA analysis (Fig. 5E-H; Fig. 6A). The expression of small-sized mRNA detectable with the $\beta\cdot\gamma$ -actin probe was also exaggerated in testis (Fig. 5H, asterisk; Fig. 6A-a).

In poly(A)⁺ RNA analysis, the expression of

G3PDH-1 mRNA was detected in the 4 tissues with stronger signals in the liver and kidney (Fig. 6B-b, c). The estimated size of G3PDH-1 mRNAs were about 3,700~4,200 bases; ca. 3,700 bases in brain and liver, ca. 4,000 bases in kidney and ca. 4,200 bases in testis. The PPE mRNAs were detected in the testis and brain, where the hybridization signals indicated different sizes of mRNA species (Fig. 6C-a, d). The PPE mRNA in the brain was about 1,300~1,400-base long as reported previously and the transcripts in testis were slightly bigger (ca. 1,600~1,800 bases). The quite feeble expres-

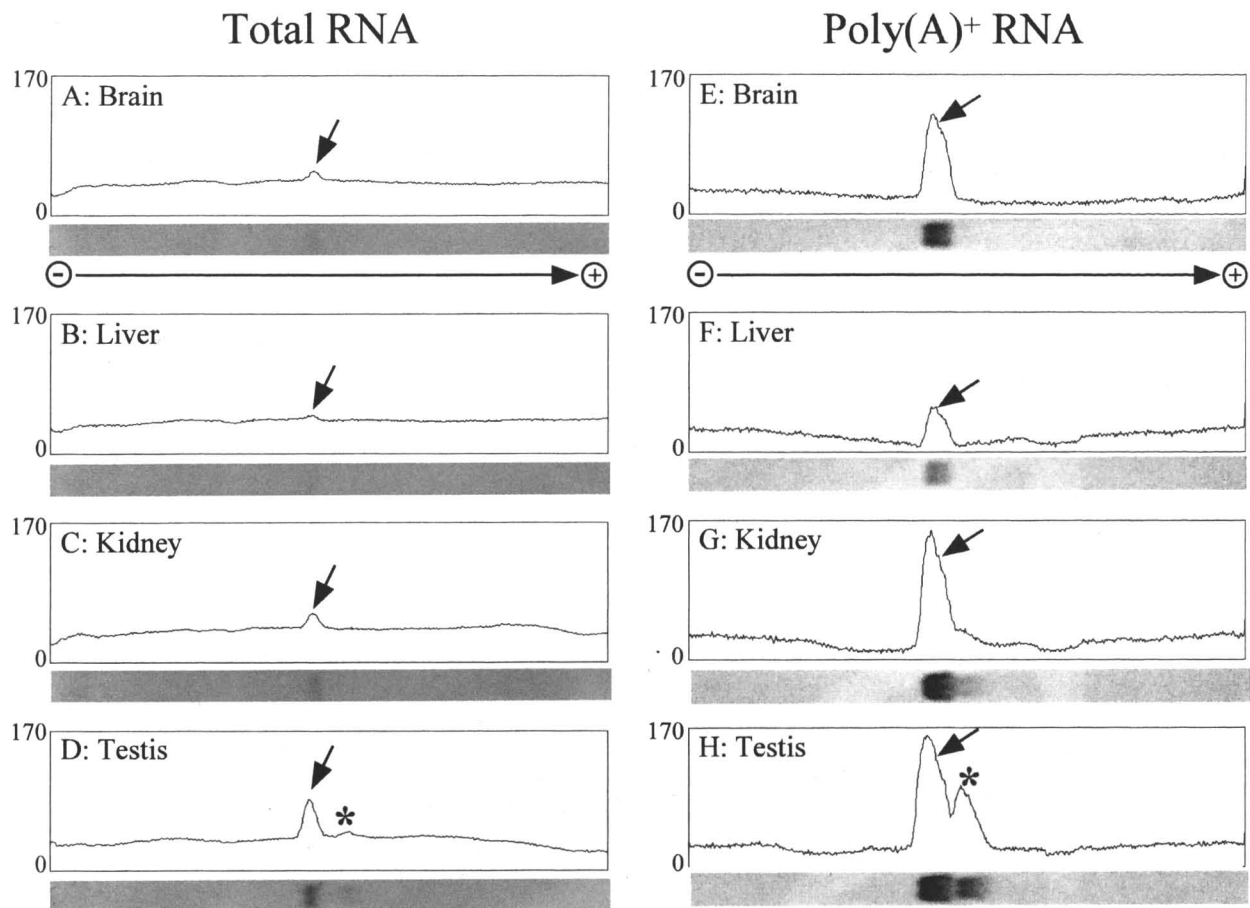


Fig. 5 Northern blot analysis of $\beta \cdot \gamma$ -actin mRNA (arrow) in total RNA (A~D: 10 μ g/lane) and poly (A)⁺ RNA (E~H: 2.5 μ g/lane) from brain, liver, kidney and testis. Hybridization signals were apparently increased in poly(A)⁺ RNA analysis. In testis, a small-sized species of $\beta \cdot \gamma$ -actin mRNA (asterisks in D and H) was detected in both total and poly(A)⁺ RNA analyses. The upper case of each panel indicates the density profile of hybridization signals in the lane (by NIH Image). The relative density of accumulated pixels is represented on the Y-axis.

sion of PPD mRNA was recognized in testis and brain under a careful inspection, although the level of the mRNA expression appeared close to the detection limit. The estimated size of PPD mRNA was about 2,600 bases. The resolution of digital image (1.3 million pixels) and the image processing by the NIH Image failed to show any specific signals (Fig. 6D).

Discussion

In hybridization experiments, the technologies continue to evolve with new materials, and new ideas and techniques one after another. Appropriate materials and technologies should be selected for the purposes of investigations and

according to the availabilities. In particular, it is inevitably necessary that the hybridization with non-RI materials should be optimized by selecting materials and techniques.

Hybridization technologies with non-RI labeling and detection systems are timesaving and economic and also give the advantages of safety and no half-life of materials in comparison with techniques with RI. However, the intensity of hybridization signals varies depending on materials and protocols employed. In the present study, synthetic oligonucleotides (45 mers), digoxigenin-linked dUTP, and alkaline phosphatase-conjugated antibody were used as pivotal elements. The other materials and techniques were, thus, examined as parameters affecting the intensity of

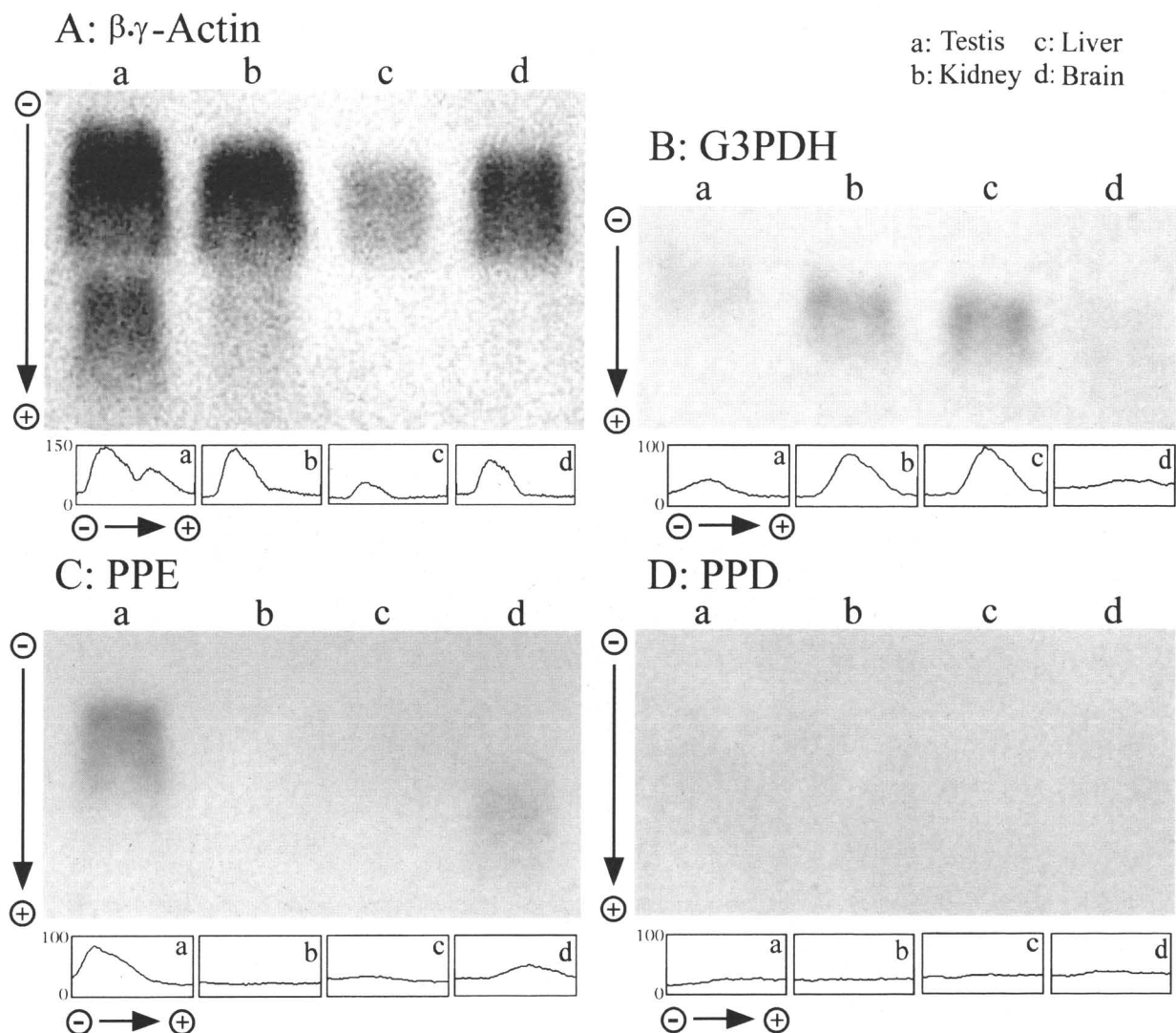


Fig. 6 Northern blot analysis of poly(A)⁺ RNA ; $\beta \cdot \gamma$ -actin, G3PDH-1, PPE and PPD mRNAs from testis(a), kidney(b), liver(c) and brain(d). A : Hybridization signals of $\beta \cdot \gamma$ -actin mRNA showed that the expressions were differentially regulated in the four tissues. B : G3PDH-1 mRNA was also expressed in four tissues with slightly higher signals in the liver and kidney. C : Signals of PPE mRNA were detected in brain and testis. The PPE mRNA species in testis is longer in size than the brain one. D : Feeble signals of PPD mRNA were recognized in the lanes of brain and testis under a careful inspection, but the feebleness was beyond the ability of the digital pick-up device(1.3 million pixels). The lower cases of each panel indicate the density profiles of hybridization signals processed by NIH Image. The relative density of accumulated pixels is represented on the Y-axis.

hybridization signals; incubation time for labeling, temperature for hybrid formation, and substrates for enzymatic detection.

Probe

The probes are essential elements in the hybridization experiments identifying target RNAs. To date, a variety of probes, such as

cDNA, cRNA, oligonucleotide and PCR fragment, are available for specific purposes of investigations and in different lines of protocols. Some probes have the advantages of originality, priority and availability, and the others have the advantages of easiness, promptness and economy for use. In addition to some advantages,

synthetic oligonucleotides are particularly useful to generate specific probes to target molecules. The probes can be designed to identify the distinctive parts of the target molecules among the associated, homologous sequences, for instance in a large superfamily of the gene^{3, 19)}. Therefore, it is indispensable to maximize the advantage that oligonucleotide probes distinguish the target molecules specifically and produce the optimal or intense hybridization signals by improving labeling and detection protocols.

Probe Labeling

Various labeling methods of oligonucleotides are available in consonance with the purposes of investigations. The 3'-end tailing would be suitable to increase the sensitivity of probes by adding labeled dNTPs at 3'-end. The length of tailing can be controlled, but care should be paid for potential non-specific binding properties of the probes deriving from additional tails, for instance hydrophobicity. The other enzymatic labeling with ddNTPs/TdTase or ATP/T4 polynucleotide kinase is able to add only one labeled molecule at either 3'-or 5'-end of the probes, respectively. These probes might be less sensitive but suitable for certain purposes, such as quantification, using sensitive detection devices. Chemical modification of probes is also available by directly introducing photo-biotin, and antigenic thymine dimmer and sulfonic group. The modified probes are supposed being improved in mobility, penetration and hybridization ability.⁹⁾ Furthermore, marker molecules such as haptens, enzymes or fluorescence materials can be incorporated directly into oligonucleotides on synthesis.

Effect of UV Rays

For *in vitro* hybridizations, different types of membranes have been used; nitrocellulose filter, charged and uncharged Nylon membranes and others. These membranes have different physico-chemical properties, which require different handling and treatment for use and also affect the intensity and S/N ratio of hybridization signals.

Uncharged Nylon membrane is known to be useful for cross-linking RNA when it is exposed to UV rays at optimal energy levels. In the present study, 254 nm wave length UV rays were used at different levels of energy from 0 to $960 \times 10^3 \mu\text{J}/\text{cm}^2$. Optimal signal intensity was obtained in cases of UV ray irradiation at $30 \times 10^3 \mu\text{J}/\text{cm}^2 \sim 120 \times 10^3 \mu\text{J}/\text{cm}^2$. UV ray irradiation beyond the optimal range of energy resulted in decreased intensity of hybridization signals. No or low energy of UV rays appeared insufficient for cross-linking RNA to membrane, because the overall intensity of signals was declined without any significant reduction of positive areas. On the other hand, the high energy exposure appeared to cause RNA molecules severe damage, because reduction of signal areas was conspicuous. This can be ascribed to disintegration of RNA by hazardous UV rays.

T_m value for hybridization

The efficiency of hybrid formation between probes and target molecules depend on temperature of the hybridization solution, as well as the amount of cation and the types of probes. In the present study, several factors of the solution were fixed to a standard formula,^{14, 15)} that is, 600 mM Na⁺, 50% formamide, and pH 7.2. Since the maximum ratio of hybridization of DNA is at *T_m*-25°C,⁴⁾ the *T_m* value of each probe was estimated on the basis of the length and GC content of the probe, and applied to the temperatures of hybridization and posthybridization wash.

Posthybridization washes were also performed at different *T_m* values, or *T_m*-15°C and *T_m*-5°C, showing no significant differences of signals and background noise between the 3 cases. This also supports that *T_m*-25°C is suitable for hybridization and posthybridization wash in consonance with detection of a single species of target mRNA.

Substrates of Alkaline Phosphatase

In the detection process of hybridization protocol, three different substrates were examined. Comparison of the intensity of enzyme-mediated precipitations showed the graded sensitivity

depending on the substrates; NBT/BCIP (purple-brown) \geq Vector Blue (blue) $>$ Vector Red (red). However, the precipitations with different colors may be suitable for experiments of multiple labeling and detection. Furthermore, the detection system using alkaline phosphatase, like peroxidase, can be transferred to a chemiluminescence detection protocol, which may be comparable to or superior to RI-generated signals.

Detection of mRNAs

Using our protocol established on the basis of factor analysis, the 4 oligonucleotide probes (β - γ -actin, G3PDH-1, PPE and PPD) were used to detect their target mRNAs in both total and poly(A)⁺ RNA samples from brain, liver, kidney and testis of rat. In total RNA analysis, β - γ -actin probe alone exhibited hybridization signals of about 2,100-base long mRNA in the 4 tissues investigated. The estimate of molecular size is based on the size-markers of 18s- and 28s-ribosomal RNAs by gel electrophoresis. The size of β - γ -actin mRNA agrees with the size of ca. 2,200 bases reported previously⁸⁾ and indicates cytoskeletal β - and/or γ -actin mRNAs⁸⁾. Although the cytoskeletal isoforms of actins are assumed to be expressed constantly in non-muscle tissues, the relative amount of the mRNA in total RNA varied from one tissue to another. In addition, testis exhibited a small-sized mRNA (ca. 1,500 bases) detectable with β - γ -actin probe. Owing to the size⁸⁾ and the probe property (described in materials and methods), the signals may indicate the muscle-specific α -actin mRNA deriving from peritubular myoid cells surrounding seminiferous tubules and/or vascular smooth muscle cells.

In poly(A)⁺ RNA analysis the hybridization signals of β - γ -actin mRNA were exaggerated with the same pattern to those of total RNA analysis in the 4 tissue samples.

The other 3 probes required poly(A)⁺ RNA selection for concentrating their target mRNAs. Hybridization signals of G3PDH-1 mRNA were observed in liver and kidney, while the signals in brain and testis were fairly feeble. The size

of G3PDH-1 mRNA ranged from ca. 3,700 bases to ca. 4,200 bases in the present study; ca. 3,700 bases in brain and liver, ca. 4,000 bases in kidney and ca. 4,200 bases in testis. The variety of molecular sizes of G3PDH-1 mRNA have been reported (ca 3,500 bases^{6, 10)} and 4,700 bases¹²⁾, even though RNA investigated was purified from the same tissues (liver and brain) of the same animals (mouse and rat). The discrepancy of the mRNA size shouldn't be simply concluded as tissue-specific variations, since there might be either possible technical error such as estimates of molecular size by gel electrophoresis or variable contribution of the poly(A) tail.

The present study, however, revealed not only 3 different G3PDH-1 mRNA species but also different magnitudes of the mRNA expressions in the 4 different tissues. Isozymes of G3PDH-1 have been investigated in several animals that the gene expression is differentially regulated at each developmental stages and in various tissues.¹¹⁾ The steady-state level of the enzyme activity of one isozyme (Gdc-1) varies over 2 orders of magnitude among different tissues of the adult mouse.²³⁾ Furthermore, the expressions of multiple isozymes have been reported to change over 2 to 3 orders during development and differentiation.^{11, 23)} Since these modifications are seen under experimental conditions which result in changes in cell phenotypes, it seems that the regulation of the G3PDH-1 gene is coupled to the state of differentiation of a cell.^{23, 29)} Thus, it is intriguing to know what controls the developmental and tissue-specific activations of G3PDH-1 genes and how the G3PDH-1 genes achieve their levels of expression in different tissues.

The PPE and PPD genes encode 2 of 3 endogenous opioid precursor proteins. PPE codes Leu- and Met-enkephalins and PPD codes dynorphin, neo-enkephalin, and Leu-enkephalins. The third one is the preproopiomelanocortin (PPOMC) gene coding β -endorphin, melanocyte-stimulating hormones and adrenocorticotrophic hormone. Various endogenous

opioid peptides are active substances functioning as neurotransmitters and/or neuromodulators in the brain and spinal cord, based on their distinctive distribution^{14, 15, 20-22)} and association with the specific sensory modality (pain).²⁴⁾ The size of PPE mRNA in brain is about 1,300~1,400 bases and coincides with that reported previously. On the other hand, PPE mRNA in testis is about 1,600~1,800 bases in length. This may be related to the disparity between the amounts of PPE mRNA and Met-enkephalins reported in testis and heart.⁵⁾ Extracts from these tissues contains large amounts of PPE mRNAs and only small amount of PPE products.⁵⁾ Signals of PPD mRNA were detected in brain and testis but quite feeble in intensity, in spite of the distinctive distribution of PPD mRNA expressing neurons in rat brain.¹⁵⁾ The estimated size of PPD mRNA in both tissues was about 2,600 bases in length as reported previously.

To understand the central opioidergic systems, the metabolic processing of the opioid precursor proteins must be considered. Weber et al.,^{26, 27)} Seizinger et al.,²⁴⁾ and Zamir et al.³⁰⁾ have reported that several brain regions contain prodynorphin-derived peptides in different molar ratios, suggesting that in certain PPDergic systems prodynorphin may be processed completely, while in the other systems the processing may be terminated with the formation of large intermediates. Similarly, White et al.²⁸⁾ have shown that the relative ratio of proenkephalin-derived peptides in 3 different opioidergic systems of rat brain is distinctive for each system. Furthermore, Liston et al.¹³⁾ have demonstrated clearly that proenkephalin synthesized in the bovine supraoptic nucleus neurons is processed during the axonal transport through hypothalamo-hypophyseal system. They also showed that the metabolic intermediates in the supraoptic nucleus differed in molecular size and ratio from those in the adrenal medulla.¹³⁾ Taken together, these studies indicate the presence of different post-translational processing pathways of proenkephalin and pro-

dynorphin in different types of cells in the brain as well as non-neuronal tissues.

In conclusion, the present study provides a hybridization protocol using synthetic oligonucleotide, digoxigenin-linked dUTP, and alkaline phosphatase-conjugated antibody as pivotal elements in technology. The various parameters were established by comparing factors in each step of the protocol. The protocol allowed the 4 antisense oligonucleotides to demonstrate tissue-specific expression of the target mRNAs in 4 different tissues; brain, liver, kidney and testis.

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