

Improvement of *In Situ* PCR by Optimization of PCR Cycle Number and Proteinase K Concentration: Localization of X Chromosome-Linked Phosphoglycerate Kinase-1 Gene in Mouse Reproductive Organs

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Received March 13, 2009; accepted March 26, 2009; published online April 23, 2009

In situ polymerase chain reaction (*in situ* PCR), which can detect a few copies of genes within a cell by amplifying the target gene, was developed to better understand the biological functions of tissues. In this study, we optimized the protocol conditions for the detection of X chromosome-linked phosphoglycerate kinase-1 (pgk-1) gene in paraffin-embedded sections of mouse reproductive organs. The effects of various concentrations of proteinase K (PK) and PCR cycle numbers were examined. To label the amplified DNA, we used digoxigenin-dUTP (Dig), Cy-3-dUTP (Cy-3), or FluorX-dCTP (FluorX). The optimal concentration of PK was 50 µg/ml for the ovary and 10 µg/ml for the testis. Ten PCR cycles were optimal for Dig and 25 cycles were optimal for FluorX and Cy-3 in the ovary and testis. The signal-to-noise ratio of FluorX and Cy-3 for ovarian tissue was better than that of Dig. Using the above conditions, we detected 1–4 and 1–2 spots of pgk-1 in the nuclei of granulosa and germ cells, respectively. Our results indicate that *in situ* PCR is useful for detecting a specific gene in paraffin-embedded sections under optimized conditions of both PCR cycle number and PK concentration.

Key words: *in situ* PCR, pgk-1, ovary, testis, proteinase K

I. Introduction

The polymerase chain reaction (PCR) method is suitable for detection of a few copies of a particular gene based on its extremely high sensitivity. In fact, the PCR method allows amplification of a single gene copy in a solution up to a large quantity sufficient for detection by conventional gel electrophoresis and Southern blot hybridization [16]. However, the PCR method itself cannot detect the site of a target gene at the cellular level. On the other hand, *in situ* hybridization (ISH) is considered a powerful technique for

localization of specific nucleic acid sequences at the level of individual cells with excellent detection specificity [7–10]. However, the detection sensitivity of ISH is sometimes limited. The combination of these two methods, called *in situ* PCR, first described by Haase *et al.* in 1990 [6], is an extremely highly sensitive technique to localize a single gene copy at the individual cell level [1–4, 11–13, 20, 22]. However, *in situ* PCR has not gained wide acceptance by the scientific community, partly because of the significant variations in the optimal conditions of the protocol, which depend on the tissues used for analysis [11, 12].

In the present study, we optimized the *in situ* PCR protocol for paraffin-embedded sections of the mouse reproductive organs using X chromosome-linked phosphoglycerate kinase-1 (pgk-1) gene. Pgk-1 gene within the X chromosome is ubiquitously expressed in the euchromatic X

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chromosome and is also used as a marker for detection of mouse sex chimeras [17, 24]. In this study, *pgk-1* gene expression was analyzed in several applications as follows: (1) the effects of various concentrations of proteinase K (PK), (2) the amplification of PCR cycles, and (3) visualization dyes using digoxigenin-11-dUTP (Dig), Cy-3-dUTP (Cy-3), and FluorX-10-dCTP (FluorX). After optimization of these applications, *in situ* PCR was found to be excellent for detection of a specific gene in paraffin-embedded tissue sections.

II. Materials and Methods

Chemicals and biochemicals

Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany). PK was from Wako Pure Chemicals (Osaka, Japan). Dig was from Roche Diagnostics (Mannheim, Germany). Cy-3 and FluorX were from Amersham Biosciences Corp. (Piscataway, NJ). TaKaRa Ex Taq (Taq DNA polymerase, dNTP mixture, 10×PCR buffer, and MgCl₂) was from Takara Bio Inc. (Shiga, Japan). 3,3'-diaminobenzidine-4HCl (DAB) was from Dojin Chemical Co. (Kumamoto, Japan). All other reagents used in this study were from Wako Pure Chemicals or Sigma Chemical Co. (St. Louis, MO) and were of analytical grade.

Animals and tissue preparation

The study was conducted in 6- to 8-week-old ICR mice. All experiments were conducted according to the principles and procedures outlined in the Guidelines for Animal Experimentation of Nagasaki University with the approval of the Institutional Animal Care and Use Committee. For tissue sampling, the ovaries and testes were harvested under ether anesthesia. They were cut into small pieces and divided into two groups. Tissue sections of the first group were frozen immediately in liquid nitrogen and later used for standard PCR. Those of the second group were fixed in 4% PFA in phosphate buffered saline (PBS) at room temperature overnight and embedded in paraffin. Sections (5-µm-thick) were prepared and then mounted on 3-aminopropyltriethoxysilane-coated glass slides and used for *in situ* PCR.

PCR method

Genomic DNA was prepared from the testis or ovary of ICR mouse with AquaPure Genomic DNA Kits (Bio-Rad, Richmond, CA) according to the instruction manual provided by the manufacturer. When the PCR conditions were optimized, the band intensity corresponding to the increased amount of DNA correlated linearly with the PCR cycle number. The bands were quantitated by densitometry (NIH Image J software, version 1.08i, <http://rsb.info.nih.gov/ij/>) and each value was expressed relative to that of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers (BEX Co., Japan) corresponding to mouse *pgk1* were 5'-CACGCTTCAAAGCGCACGTCT-3' and 5'-CTTGAGGGCAGCAGTACGGAAT-3', while those for

mouse GAPDH were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The Gene Amp PCR System 9400 (PE Applied Biosystems, Foster City, CA) was used for gene amplification. PCR was carried out in a 20-µl final volume of reaction mixture, containing 0.5 units of TaKaRa ExTaq polymerase, 0.2 mM of each dNTP, ExTaq buffer (1×), 1 µM of each specific primer described above and 100 ng of testis or ovary DNA as a template. Each cycle of PCR included 20 sec of denaturation at 94°C, 20 sec of annealing at 55°C, and 30 sec of extension at 72°C. After 28 reaction cycles, the PCR products were separated on a 1.5% TAE-agarose gel and stained with ethidium bromide. The amplified PCR products with *pgk-1* primers were loaded onto a 3% agarose gel, and the DNA bands of the predicted size were cut, purified with glass powder and integrated into pGEM-easy (Promega, Madison, WI). BigDye terminator cycle sequencing was performed against the *pgk-1* plasmid and the sequence was determined with ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) using the instructions provided by the supplier.

In situ PCR method

The oligo-DNAs that can amplify part (170 bp) of the mouse X chromosome-linked *pgk-1* gene were used as primers as described above. The tissue sections were deparaffinized and rehydrated using standard procedures. These sections were digested with 0–100 µg/ml of PK (37°C, 15 min). After post-fixation with 4% PFA in PBS (5 min), the sections were washed in PBS and immersed in 50% formamide in 2×SSC (1×SSC=0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) overnight at 4°C. After washing with double-distilled water (DDW, 5 min, 3 times), the sections were completely desiccated. For each slide, the amplification mixture was prepared in a final volume of 100 µl, containing 1×PCR buffer, 1.0 µg/ml forward primer, 1.0 µg/ml reverse primer, 0.2 mM dNTP, 2.5 mM MgCl₂, and 2.5 U/100 µl Taq DNA polymerase. To label the amplified DNA, 5.0 µM Dig, 2.0 µM Cy-3, and 0.2 µM FluorX were used. The slides were sealed with EasiSeal (Hybaid, UK), and then placed on the heated plate of the OmniSlide System (Hybaid); the DNA in the tissues then was denatured at 94°C for 3 min. As for PCR cycle studies, 5, 10, 15, 20, 25, or 30 cycles of amplification (denaturation for 15 sec at 94°C, annealing for 15 sec at 56°C, extension for 60 sec at 72°C) were run. The slides were then heated at 72°C for 5 min. After amplification, the cover slips were removed and the slides were washed with 2×SSC (37°C, 15 min, 4 times). As a negative control, *in situ* PCR was performed with the omission of Taq DNA polymerase and the primers.

Visualization and image analysis

The Dig-labeled signals were detected by enzyme immunohistochemistry, and the images of the Dig-labeled sections were analyzed using an Olympus BH-2 microscope connected to a Canon CCD camera, and an Olympus SP-500 Image analyzer with Image Command program 5098 as described in detail previously [26]. Cy-3 and FluorX signals

were detected using a Zeiss fluorescence microscope connected to a CCD camera (AxioCam, Carl Zeiss, Jena, Germany).

Statistical analysis

All data were expressed as mean \pm SD. Differences between groups were examined for statistical significance using the unpaired Student's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference. All analyses were performed with a statistical software package (StatView, version 5.0; Abacus Concepts, Berkeley, CA).

III. Results

PCR products

PCR products comprising 170 bp were obtained (Fig. 1) and were confirmed by DNA sequencing to be part of the *pgk-1* gene. Semiquantitative analysis of these products using the NIH Image analysis system showed that the quantity of the *pgk-1* gene product from the ovary was about 2-fold more than that from the testis.

PK concentration

The effect of digestion by PK at concentrations from 0 to 100 μ g/ml was evaluated using FluorX. The optimal concentration of PK, as evaluated by the signal-to-noise ratio, was 50 μ g/ml for the ovary (Fig. 2). One to 4 *pgk-1* spots were detected in the nuclei of granulosa cells

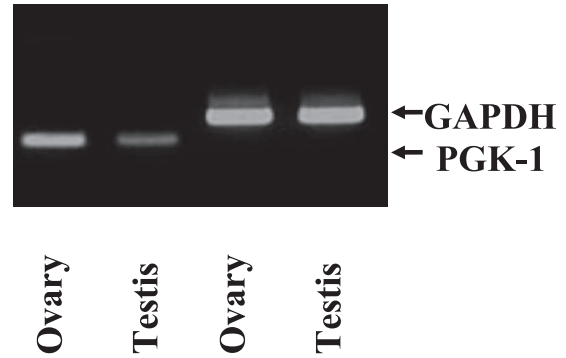


Fig. 1. PCR of *pgk-1* gene in mouse ovary and testis. PCR products of *pgk-1* (170 bp) were found in mouse ovary and testis.

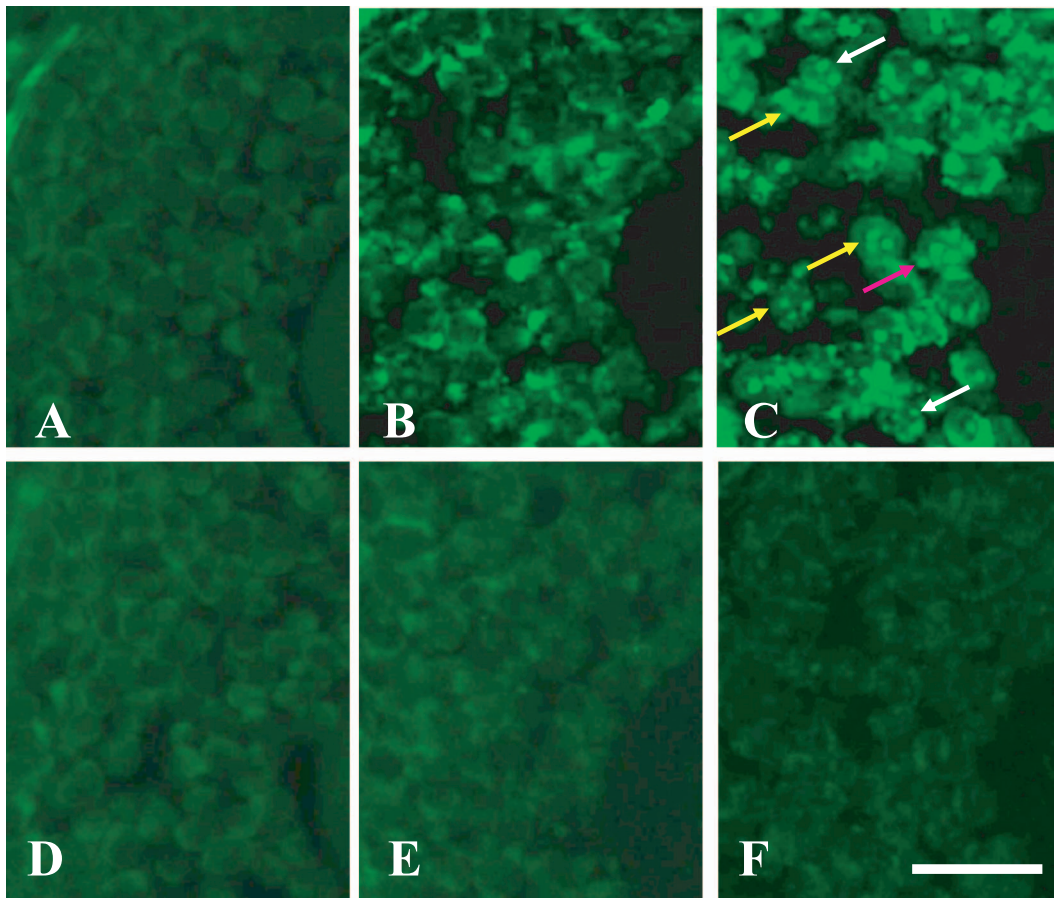


Fig. 2. Effects of various concentrations of PK in mouse ovary using FluorX. Upper panels (A, B, C) were carried out Taq polymerase and lower panels (D, E, F) were performed omitting Taq polymerase. PK was evaluated at varying concentrations from 5 μ g/ml (A, D), 20 μ g/ml (B, E), and 50 μ g/ml (C, F). One to 4 green spots were detected in the nuclei of granulosa cells (C). Red arrow; 4 spots. Yellow arrows; 3 spots. White arrows; 2 spots. Bar=20 μ m.

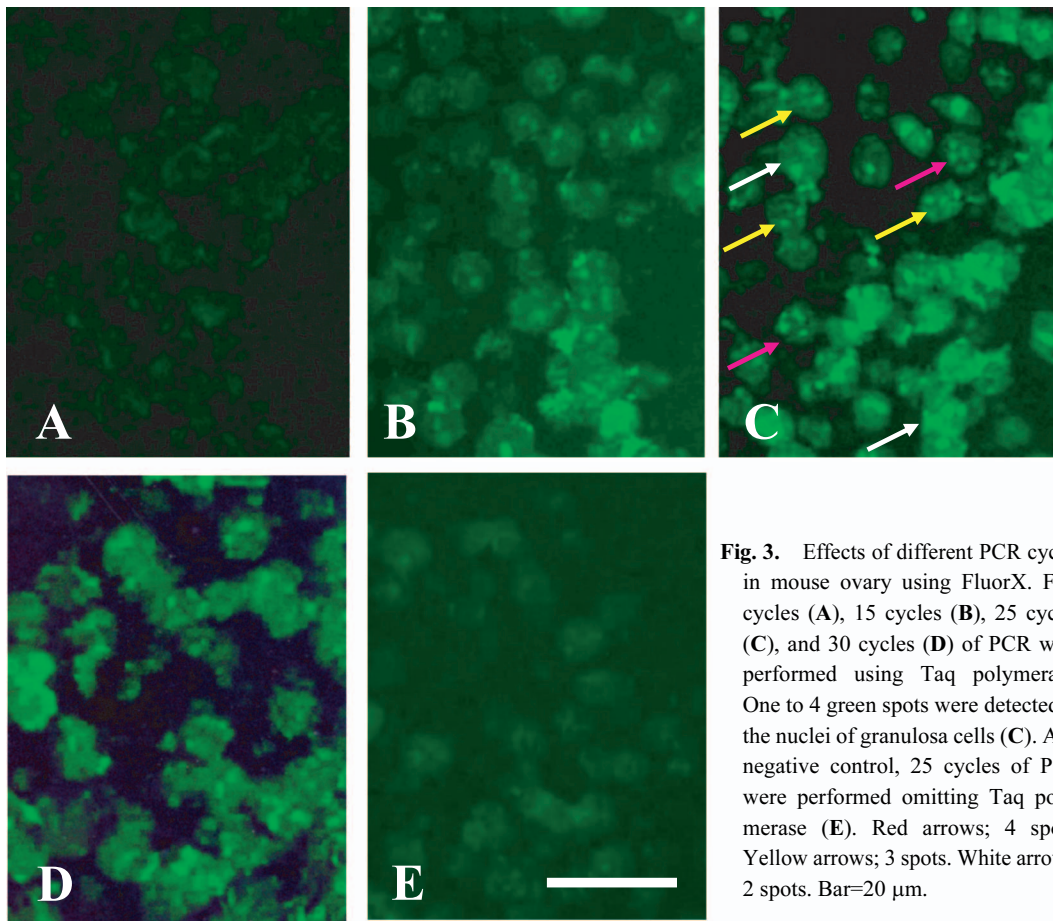


Fig. 3. Effects of different PCR cycles in mouse ovary using FluorX. Five cycles (A), 15 cycles (B), 25 cycles (C), and 30 cycles (D) of PCR were performed using Taq polymerase. One to 4 green spots were detected in the nuclei of granulosa cells (C). As a negative control, 25 cycles of PCR were performed omitting Taq polymerase (E). Red arrows; 4 spots. Yellow arrows; 3 spots. White arrows; 2 spots. Bar=20 μ m.

(Fig. 2C). Treatment of the sections with 100 μ g/ml PK resulted in diffuse spreading of the signal (data not shown). There was no difference between the use of FluorX and Cy-3 dye for *in situ* PCR.

PCR cycle

The effect of different PCR cycles (from 5 to 30 cycles) was evaluated using FluorX, Cy-3 and Dig dyes. For FluorX, 25 cycles were optimal for the detection of the *pgk-1* gene in the nuclei of granulosa cells (Fig. 3C). Ten cycles was the optimum number of PCR cycles necessary to detect *pgk-1* gene in the nuclei of ovarian granulosa cells using Dig (Fig. 4A). The signal-to-noise ratio of FluorX and Cy-3 for granulosa cells was better than that of Dig (Fig. 3C and Fig. 4A). Amplification over 10 cycles for Dig or over 25 cycles for Cy-3 and FluorX significantly increased the background signals due to the diffusion of PCR products into the surrounding tissues (Fig. 3D). For the testis, the concentration of PK was 10 μ g/ml and 25 cycles of PCR was optimal, and 1–2 spots were detected in the nuclei of some germ cells using Cy-3 dye (Fig. 5A). Testicular sections treated with 20 μ g/ml PK showed no signal for *pgk-1* gene (data not shown).

Number of *pgk-1*-positive spots in mouse granulosa cells

Finally, we determined the percentage of granulosa cells containing one or more *pgk-1*-positive spots after employing 15 cycles and 25 cycles. Five tissue samples were used in these experiments. The number of *pgk-1*-positive spots in granulosa cells was 1.6 ± 0.1 spots/cell in the ovary using 15 cycles for *in situ* PCR and 2.5 ± 0.1 spots/cell using 25 cycles ($P < 0.0001$) (Fig. 6A). The percentage of *pgk-1*-positive granulosa cells after using 25 cycles for *in situ* PCR was as follows: $15.8 \pm 5.8\%$ of the cells showed 1 spot in the nuclei, $37.2 \pm 7.1\%$ of the granulosa cells showed 2 spots, $18.7 \pm 8.3\%$ of the cells had 3 spots, and $27.7 \pm 4.5\%$ had 4 spots (Fig. 6B).

IV. Discussion

In the present study, we assessed the utility of *in situ* PCR in detecting a specific gene using paraffin-embedded sections of mouse reproductive organs. Using *in situ* PCR, we found 1 to 4 *pgk-1* spots in the nuclei of granulosa cells and 1 to 2 *pgk-1* spots in the nuclei of the germ cells under optimal number of PCR cycles and PK concentration.

In practice, *in situ* PCR is technically difficult, and there is no standard protocol for optimal detection, especially

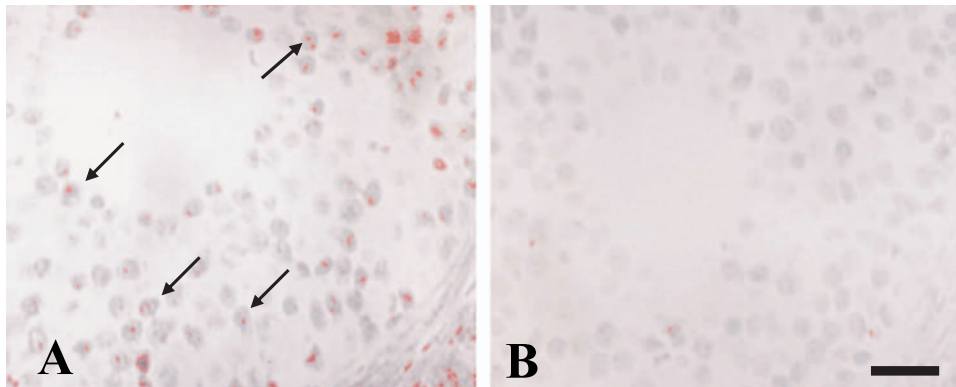


Fig. 4. Detection of pgk-1 in mouse ovary using Dig. One to 2 red spots were detected in the nuclei of granulosa cells using image analysis system (A). PCR cycles were 10 times, and PK concentration was 50 $\mu\text{g/ml}$. Taq polymerase was omitted as a negative control (B). Bar=20 μm .

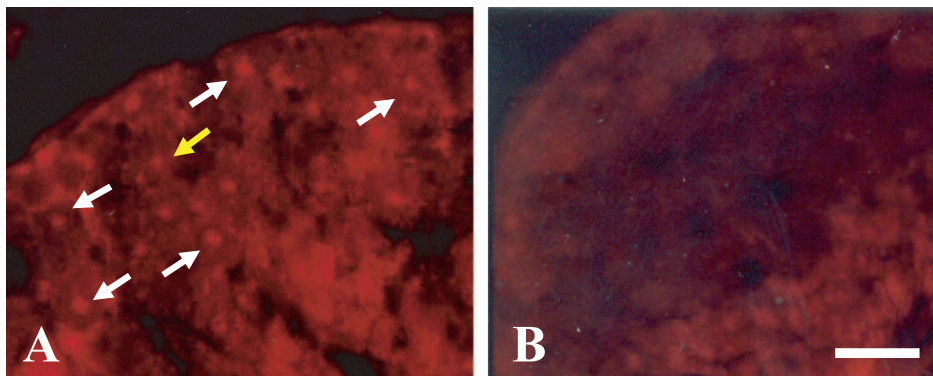


Fig. 5. Detection of pgk-1 in mouse testis using Cy-3. One to 2 red spots of P_{gk}-1 gene were detected in the nuclei of germ cells with 10 $\mu\text{g/ml}$ PK concentration and 25 cycles (A). Yellow arrow; 2 spots. White arrows; 1 spot. Taq polymerase was omitted as a negative control (B). Bar=20 μm .

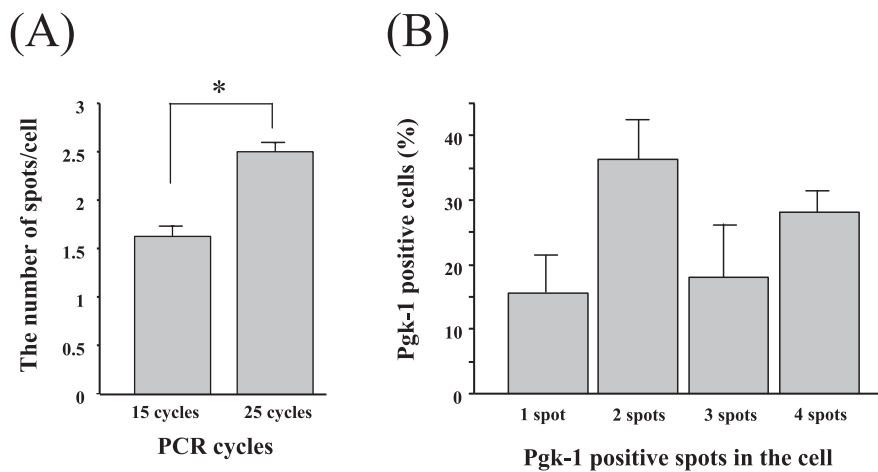


Fig. 6. P_{gk}-1-positive spots in mouse granulosa cells. The number of pgk-1-positive spots after employing 15 cycles and 25 cycles (A). The percentage of pgk-1-positive granulosa cells after using 25 cycles for *in situ* PCR (B). Data represent mean \pm SD. * P <0.0001.

when using paraffin-embedded tissue sections. We were able to detect 1 to 4 spots of pgk-1 in granulosa cells using our optimal conditions. However, when the PCR cycles were increased to more than 25 cycles for Cy-3 and FluorX,

the signals were diffusely spread in the sections. In fact, the diffusion of the amplified products from the target cells is a major problem in this method [11, 25]. The factors that influence diffusion include excessive protease digestion

[5, 18] and the use of an excessive number of PCR amplification cycles [5, 11, 21, 27]. In relation to the diffusion of the PCR products into the surrounding tissue, non-specific incorporation of labeled nucleotides into fragmented DNA, and the inhibitory effects of cross-linking of histones to DNA or PCR amplification are problematic. Long *et al.* [13] reported some false positive results, especially when using tissue sections, which were related to non-specific incorporation of labeled nucleotides into cellular DNA through internal priming and DNA repair. This was due, at least in part, to the exonuclease activity of Taq DNA polymerase. In our experiments, however, the diffusion of PCR products could be minimized by reducing the number of PCR cycles and the use of appropriate concentrations of PK.

Recently, *in situ* reverse transcription-PCR (*in situ* RT-PCR) has been used to detect low levels of mRNA within cells and tissue sections [2, 14, 15, 20, 23]. However, this method has certain limitations in detecting specific mRNAs. This is because detection of very few copies of mRNA in tissues or cells by this method requires the PCR reaction to be performed after carrying out reverse transcription of the specific mRNA to cDNA using reverse transcriptase. Then, it is necessary to determine whether this reaction truly occurs within the individual cell. Moreover, it is also important to establish that the RT-PCR reaction is of genomic DNA origin, and that the products truly originated from the target mRNA. For these reasons, we considered the *in situ* RT-PCR method to be unsuitable for evaluating the experimental results obtained by the *in situ* PCR method.

For *in situ* PCR, two main protocols, direct and indirect *in situ* PCR, have been described [11, 13, 25]. In the direct detection approach, the labeled nucleotides are directly incorporated into the PCR products, while the indirect method consists of an initial PCR reaction without labeled nucleotides, followed by *in situ* hybridization using labeled probes to the PCR products. The direct *in situ* PCR has been reported to increase the turn-around time and ease of *in situ* PCR studies due to hot-start modifications of *in situ* PCR without jeopardizing specificity [19].

In conclusion, *in situ* PCR is a potentially useful method for detecting a specific gene at cellular and sub-cellular levels, and may prove suitable for evaluation of the functional organization of various genes in paraffin tissue sections when used with critical PK concentration and PCR cycle number.

V. Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 18390060 to T.K.).

VI. References

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