Coverslip Mounted-Immersion Cycled In Situ RT-PCR for the Localization of mRNA in Tissue Sections

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ABSTRACT

An improved method was developed for in situ reverse transcription-polymerase chain reaction (RT-PCR) to detect and localize mRNA in tissue sections. The coverslip mounted-immersion cycled (COSMIC) in situ RT-PCR technique combines the advantages of solution-phase PCR with the tissue immobilization necessary for in situ analysis. The tissue specimen is mounted on an AES-silane-coated coverslip, excess glass is removed and the sample is immersed in reaction mixture in a PCR tube and subjected to thermal cycling. Processing the section on the coverslip is efficient, the thin glass withstands the high temperature cycling and the tissue adheres securely through the process. The specimen is fully exposed to the reagents, and is heated uniformly and accurately according to temperatures programmed into the thermal cycler. An application is described for the detection and localization of the mRNA for surfactant protein A (SP-A) in fetal rat lung tissue.

INTRODUCTION

In situ reverse transcription-polymerase chain reaction (RT-PCR) is an emerging methodology that combines the high sensitivity of PCR amplification with the cell-localizing ability of in situ hybridization (8). The method has potential uses as a research and diagnostic tool in the detection of rare copies of RNA in organogenesis and the pathogenesis of viral, neoplastic and other diseases (6,7). In situ RT-PCR is performed on tissue specimens by the “slide-PCR” method: the section is mounted on a glass microscope slide, overlaid with a thin film of reaction mixture and covered with a glass slip, which is secured to the slide to create a reaction chamber; the slide is heat-cycled on a standard tube-PCR or a dedicated slide-PCR thermal block (1,8). The accumulated DNA product can be visualized either directly by adding labeled nucleotides in the PCR step or indirectly by in situ hybridization (ISH) with a labeled probe that is specific for the amplified sequence (6). Despite the conceptual simplicity of the process, in situ RT-PCR has proven difficult, to a large degree due to physical deficiencies of the glass microscope slide as a high-temperature reaction chamber (1, 6,11,12). Spann et al. described a method with isolated cells mounted on slide fragments and reacted in solution (10) that eliminated some of the mechanical problems of slide-PCR. However, applying this technique to tissue sections proved difficult and unreliable, and thus was used on a limited basis (9).

In this report, we describe an improved in situ RT-PCR method for tissue sections. The coverslip mounted-immersion cycled (COSMIC) technique has the following advantages: (i) efficient and dependable processing of the sections on a thin glass matrix; (ii) uniform exposure of the histological sample to the reagents; (iii) accurate thermal conduction; (iv) high throughput of up to 48 samples is possible using a standard thermal cycler. We applied the technique and describe a protocol for the detection and localization of surfactant protein A (SP-A) mRNA in fetal rat lung, which, to the best of our knowledge, is the first application of in situ RT-PCR to section from fetal tissue.

MATERIALS AND METHODS

Specimen Preparation

Pregnant (21-day gestation) Sprague-Dawley rats were anesthetized, the fetuses removed and the fetal lungs were rapidly excised and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.5) for 4 h at 4°C. The specimens were washed in PBS and dehydrated in an ethyl alcohol series. Samples were cleared in xylene, embedded in 56°C paraffin wax and cooled. Under RNase-free conditions, 5-μm sections were cut and mounted on No. 1.5 coverslips (18 × 18-mm, 0.16–0.19-mm thickness, premium

Table 1. Thermocouple Readings at the Level of the COSMIC Chip

<table>
<thead>
<tr>
<th>Block Set</th>
<th>52°C</th>
<th>72°C</th>
<th>94°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Begin</td>
<td>End</td>
<td>Begin</td>
</tr>
<tr>
<td>Control</td>
<td>52.9 ± 0.2</td>
<td>52.0 ± 0.1</td>
<td>69.8 ± 0.6</td>
</tr>
<tr>
<td>COSMIC chip</td>
<td>52.4 ± 0.1</td>
<td>51.8 ± 0.1</td>
<td>69.8 ± 0.7</td>
</tr>
</tbody>
</table>

Thermal block was programmed for 1-min plateaus and cycled as described in Materials and Methods.

Temperature measurements in 100 µL of solution in thin-wall PCR tubes. (*n* = 3, ± standard deviation)

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grade; Fisher Scientific, Pittsburgh, PA, USA) that had been previously treated with 2% 3-aminopropyltriethoxysilane (AES; Sigma Chemical, St. Louis, MO, USA) (1) and stored at 4°C until use. The specimens were deparaffinized by heating at 60°C for 1 h, xylene-treated and rehydrated in a graded alcohol series. The samples were dried at 80°C in a vacuum oven for 15 min and used immediately or stored under vacuum at 4°C. Using a diamond-tip slide marker (Thomas Scientific, Swedesboro, NJ, USA) and an aluminum block (both chemically or heat-treated to be RNase-free) (Figure 1a), chips approximately 3 × 7 mm containing the lung section were cut out from the coverslip (Figure 1b). The glass was lightly scored using a template that matches the inside of a 0.5-mL, thin-wall PCR tube, and the excess glass was broken off by using the edge of the aluminum block for leverage. The top edge of the chip has a strip of glass that is a “handle”, by which it can be manipulated with forceps and it is asymmetric (e.g., one corner is notched) for orientation purposes, indicating the side containing the tissue section (Figure 1c). All operations such as vacuum aspiration are done on the glass side to avoid scratching the tissue. Once the chips are prepared, all subsequent reactions are done in microtubes. The sections were treated with a solution of predigested (30 min at 37°C) proteinase K (Sigma Chemical) at a concentration of 5 µg/mL in PBS for 1 h at room temperature in a volume of 100 µL. The sections were washed in PBS and then heated at 95°C for 2 min in PBS to inactivate the enzyme, followed by a final wash in PBS and then in H2O. The specimens were incubated overnight in a DNase solution containing 1 U/µL RNase-free DNase (10 U/µL stock; Boehringer Mannheim, Indianapolis, IN, USA) and 3 U/µL RNase inhibitor (40 U/µL stock; Promega, Madison, WI, USA) in a total volume of 100 µL of DNase buffer (10 mM Tris-HCl, pH 7.5, 5 mM CaCl2, 2 mM MnCl2, 1 mM dithiothreitol [DTT]) at 37°C. The sections were rinsed in three changes of DNase buffer (5 min each) and finally in water.

Oligonucleotide Sequences

A 22-base sense primer (5’-TTTC-CAGCTTACCTGGATGAGG-3’; positions 350–369 of the full-length rat SP-A cDNA) and a 22-base anti-sense primer (5’-GGAGTCTGTCTCTCATTGC-3’; positions 620–639) were designed to specifically amplify a 290-bp segment. A 20-base sense oligonucleotide, 5’ biotin-labeled (5’-AGGAT-TCCATGCTGTCAGTGG-3’), specific for the amplification product was used as a probe in the hybridization step.

In Situ RT-PCR

Reverse transcription was performed for the mRNA of rat SP-A, a protein expressed in the Type II cells of the lung (14). A volume of 90 µL of RT mixture was used per section and consisted of the following reagents: 20 µL of MgCl2 (25 mM stock), 9 µL of 10× PCR Buffer II (Perkin-Elmer, Norwalk, CT, USA), 42 µL of water, 1 µL each of dATP, dCTP, dGTP and dTTP (dNTP set, 100 mM stock; Boehringer Mannheim), 5 µL of RNase inhibitor (40 U/µL stock; Promega), 5 µL of avian myeloblastosis virus (AMV) reverse transcriptase (25 U/µL stock; Boehringer Mannheim) and 5 µL of antisense primer (15 pmol/µL stock). The sections were incubated at 42°C for 1 h, and then the RT solution was removed.

The PCR mixture contained the following in a final volume of 100 µL: 10 µL of 10× PCR Buffer II, 18 µL of MgCl2 (25 mM stock), 65 µL of tetramethylammonium chloride (TMAC) (3 mM in water stock), 1 µL of each dNTP, 1 µL antisense primer (as above) and 1 µL of sense primer (15 pmol/µL stock). A 9-µL aliquot of solution was removed, placed on ice, and 1 µL of Taq DNA Polymerase (Perkin-Elmer) was added and saved for manual “hot start” procedure (7). Ninety microliters
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of PCR mixture were added to the tube containing the section and incubated in a thermal cycler (DNA Thermal Cycler; Perkin-Elmer) with an initial time delay file of 95°C for 5 min. When the block temperature reached 80°C, 10 µL of PCR mixture containing Taq DNA polymerase were added as per “hot start” procedure, and the solution was overlaid with wax to prevent evaporation. The following program was used: an initial denaturing step of 94°C for 4 min, followed by 25 cycles of: 55°C for 1 min, annealing segment; 72°C for 1 min, extension segment; and 94°C for 1 min, denaturing segment.

In Situ Hybridization

The specimens were washed with PBS twice and transferred to screw-cap or lock-top tubes to prevent boil-overs in the next step. The prehybridization buffer contained in a final volume of 100 µL: 50 µL of formamide, 10 µL of 20× standard saline citrate (SSC), 20 µL of 50× Denhardt’s, 10 µL of heated-denatured salmon sperm DNA (stock 10 mg/mL; Boehringer Mannheim), 1 µL of 10% sodium dodecyl sulfate (SDS) and 8 µL of water. A 9-µL aliquot was removed and combined with 1 µL of 5’-biotin-labeled SP-A probe (15 pmol/µL stock) and then stored to be used for the hybridization step. Ninety microliters of the prehybridization solution were added to the section and incubated at room temperature for 1 h, and then 10 µL of hybridization buffer containing the probe were added. The tubes were heated to 95°C for 5 min to denature the DNA strands and hybridized overnight at 37°C.

The sections were washed 2 times for 5 min each in 2× SSC/30% formamide at 37°C and twice in PBS for 5 min each at room temperature. Modified avidin-fluorescein conjugate (Neu-tralite™; Molecular Probes, Eugene, OR, USA), diluted 1:250 in PBS, was added for 30 min at room temperature, followed by 2 washes in PBS for 5 min each, and a water wash. The chips were mounted on a microscope slide as follows: glass strips measuring 2×18 mm were cut from No. 1.5 coverslips, arranged in a rectangular perimeter on the slide and adhered with nail polish to create a well for the chips, which were arrayed side by side (Figure 1d). DAB-CO mounting solution (Sigma Chemical) was added, and the top of the well was sealed with a coverslip. Slides were examined with a Leitz Phase/Fluorescent Microscope (Leitz, Wetzlar, Germany) and a double optical filter (450–490-nm wavelength illumination).

Immunohistochemistry

Immunohistochemistry for Type II cells was performed as described previously (5). Briefly, frozen sections (3 µm) of 21-day rat fetal lung were stained with rT2 rat monoclonal antibodies, which are specific for apical markers of pulmonary Type II cells. The anti-rat secondary antibody was conjugated with fluorescein isothiocyanate (FITC).

RESULTS AND DISCUSSION

Although encouraging results have been reported with in situ RT-PCR with isolated cells in suspension (2), designing a reliable protocol for tissue sec-

Figure 2. Electrophoretic characterization of amplification product for SP-A. (A) Solution phase RT-PCR for SP-A from isolated total RNA in the presence (+) of COSMIC chips (lanes 3 and 4, duplicates) or absence (-) (lanes 5 and 6, duplicates); efficiency of amplification is similar. Lane 1 is 100-bp MW marker, lane 2 is blank. (B) In situ RT-PCR for SP-A from tissue sections mounted on chips results in single 290-bp band (lanes 3 and 4, duplicates), identical to solution phase RT-PCR product. Lane 1 is MW marker V, and lane 2 is blank. Electrophoresis in 2% agarose for both Panels A and B.
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Sections with high affinity. Tissue mounted on AES-coated coverslips adhered securely during the enzymatic digestions, high temperature cycling and ISH steps, provided the sections were mounted and used within 2–3 weeks of subbing. The manipulation of specimens on the coverslips, including mounting, deparaffinizing and subse-

Table 1. We tested amplification efficiency in the presence or absence of COSMIC chips by solution-phase RT-PCR for SP-A mRNA and found no difference in the amount of final product (Figure 2A).

AES forms a positively charged aminopropyl derivative of glass (1), which binds negatively charged tissue sections with high affinity. Tissue mounted on AES-coated coverslips adhered securely during the enzymatic digestions, high temperature cycling and ISH steps, provided the sections were mounted and used within 2–3 weeks of subbing. The manipulation of specimens on the coverslips, including mounting, deparaffinizing and subse-

Figure 3. In situ RT-PCR amplification of SP-A mRNA in 21-day fetal rat lung. (A) The tissue section was digested with proteinase K for 1 h, with DNase overnight at 37°C, followed by RT and PCR amplification (25 cycles) and in situ hybridization as described in Materials and Methods: green fluorescent (FITC) staining is seen in cells that dot the alveolar lining (arrows) (the yellow stain is autofluorescence in red blood cells). (B) Processed in the same way as the tissue in Panel A with the exception that no reverse transcriptase was included in the step before amplification (RT negative control). (C) No DNase treatment. (D) No avidin-FITC added (autofluorescence control). (E) Higher magnification of positive cells showing cytoplasmic FITC signal and corresponding phase contrast (F). (G) Immunohistochemical staining with Type II cell-specific antibody and corresponding phase contrast (H); note the Type II cells in the alveolar lining and the similarity in distribution of FITC fluorescence between Panels E and G. Magnification Panels A–D: 700×; Panels E–F: 1400×.
quently cutting out the section from the thin glass can be accomplished efficiently with minimal practice. We tested COSMIC glass chips for up to 35 heating cycles, using parameters as described in Materials and Methods, without significant cracking. A prior technique used for detecting viral DNA in isolated cells (10) involved cutting microscope slides and then mounting the specimens on the glass fragments (4). Technically, this was a difficult procedure in all phases and proved cumbersome and unreliable for tissue sections, as confirmed by Patel et al. (9).

We tested the COSMIC in situ RT-PCR method by detecting the mRNA for SP-A in 21-day fetal rat lung. SP-A is a pulmonary protein that is induced late in gestation and is known to be expressed in the Type II cells that dot the alveolar lining (14). To confirm the specificity of amplification from mRNA in tissue, 3-µm frozen sections were extensively permeabilized with proteinase K to make them “leaky”, and in situ RT-PCR was performed using primers and conditions as described in Materials and Methods. After 25 cycles of amplification, the supernatant was removed and cycled an additional 25 times. The resulting product was a single band at 290 bp (Figure 2B), which was identical to the band amplified by solution-phase RT-PCR from isolated total RNA (Figure 2A). To localize the mRNA, indirect in situ RT-PCR was performed on 5-µm paraffin sections followed by ISH with a specific, biotin-labeled probe as described in Materials and Methods. An intense, green fluorescent signal indicating amplified cDNA product for SP-A was observed in cells that line the alveoli (Figure 3A). The signal, as seen on higher magnification, was localized to the cytoplasm (Figure 3E), consistent with amplification from target mRNA, and not from genomic DNA or cDNA product, which had contaminated nontarget cells (this is further supported by the RT negative controls as discussed below). The tissue distribution of positive cells followed the pattern of distribution of Type II cells in the alveolar lining as determined by immunostaining of antigens specific for Type II cells (Figure 3G) and by electron microscopy (3).

We determined that digestion with DNase was essential for getting an acceptable signal with in situ RT-PCR; when this step was omitted, no specific signal was detected (Figure 3C). This empirical result, also observed by others (1), may be due to a number of possible factors, including: the chromosomal DNA acts as a “sink”, which depletes the factors or co-factors necessary for the amplification steps; the DNA presents a viscous barrier that limits permeability to the site of amplification; or the DNA nonspecifically binds the amplification product and reduces the final yield. We considered the possibility that DNase treatment of the chromosomes produces short-length DNA sequences, which are then amplified. However, this can be ruled out be-
cause sections processed without the RT step before amplification (negative control) showed only background fluorescence (Figure 3C), indicating that the source of the PCR product is mRNA and not genomic DNA.

For the rat fetal lung tissue sections that we examined, a 1-h treatment with proteinase K resulted in an appreciable signal without significant deterioration of morphology (Figure 3, E and F). Longer reactions with protease (or increasing the concentration of enzyme) produced a stronger signal but compromised morphology (data not shown). Proteinase K treatment is critical and needs to be optimized for different tissues (1). Background tissue fluorescence was enhanced by the in situ RT-PCR procedure. An indication that this is auto-fluorescence is demonstrated by the fact that it is present in tissue that has not been treated with fluorescent stains (Figure 3D).

Once in situ RT-PCR conditions were optimized for our tissue specimens, there was uniform signal detection among sections on different chips, comparable to successful immunohistochemistry or in situ hybridization. This was in contrast to our experience with slide in situ RT-PCR where, in any given experiment, a significant portion of sections (20%–30%) did not work at all due to the mechanical difficulties discussed above, and the ones that did work showed a lack of uniform signal detection across a section, most likely due to reagent diffusion problems (12). One limitation of the COSMIC method is the size of the specimen that can be analyzed. Although the 3 x 7-mm area is sufficient for the fetal rat experiments that are being performed in our laboratory, the size limitation is a disadvantage in studies where larger areas of tissue must be analyzed.

In summary, we have shown the feasibility of performing in situ RT-PCR on histological sections supported on coverslips and cycled immersed in solution. The COSMIC method combines the advantages of solution-phase PCR with the requirements of tissue immobilization for in situ analysis and provides conditions for improved efficiency for in situ RT-PCR protocols. In this article, we present the method as applied to RNA targets in tissue specimens, but it can also be used with isolated cells (R. Ertsey and L.M. Scavo, unpublished data) and would be applicable to in situ PCR for the amplification of genomic or viral DNA sequences with simple modifications of the procedure.

REFERENCES


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