American Journal of Pathology, Vol. 153, No. 1, July 1998

Technical Advance

Analysis of mRNA from Microdissected Frozen Tissue Sections without RNA Isolation

Minh D. To,*† Susan J. Done,†‡§ Mark Redston,†‡§ and Irene L. Andrulis*†‡§

From the Departments of Molecular and Medical Genetics and Laboratory Medicine and Pathobiology,‡ University of Toronto, and the Samuel Lunenfeld Research Institute† and Department of Pathology and Laboratory Medicine,§ Mount Sinai Hospital, Toronto, Ontario, Canada*

Molecular study of gene expression in solid tumors is based largely on mRNA extracted from crushed frozen tumor samples. As most tumors are heterogeneous in composition, molecular alterations acquired by neoplastic cells may be masked by normal epithelial, stromal, and inflammatory cells, which may make up a significant volume of many tumors. We have developed a technique whereby reverse transcription polymerase chain reaction (RT-PCR) can be performed on lesions microdissected directly from frozen tumor sections. This allows for molecular analysis of mRNA from histologically homogeneous cell populations. Cryostat sections are placed onto a thin layer of 2% agarose on a glass slide and stained briefly. Microdissected tissue is immersed in a freezing solution to lyse the cells; aliquots are used directly in RT-PCR reactions without further purification. We successfully amplified cDNA fragments of the β_2-microglobulin, *p21*^{Waf1}, and *BRCA1* genes from small microdissected lesions. Also, we examined the effect of varying thickness of cryostat sections (20 *versus* 40 μm) and several tissue staining dyes. We estimate that a small microdissected region, containing no more than 200 cells, can provide enough mRNA to make cDNA for 80 to 100 PCR reactions. We believe that this technique will be a useful tool to study gene expression in histologically defined tissues. *(Am J Pathol 1998, 153:47–51)*

Many molecular studies of gene expression in cancer have relied on mRNA isolated from crushed tumor specimens for reverse transcription polymerase chain reaction (RT-PCR) analysis. A major concern when using this type of sample for quantification has been the inherent cellular heterogeneity that characterizes most tumors. In addition to neoplastic cells, a significant volume of a tumor mass may be composed of normal epithelial, endothelial, stromal, and inflammatory cells. Molecular alterations acquired by cancer cells that lead to deregulated gene expression can be potentially masked by mRNA contributed by normal cells. As well, variations in mRNA levels among different tumors detected by RT-PCR may not necessarily represent mutational events, but rather a reflection of differences in cellular composition, when gene expression is cell-type specific. Although *in situ* hybridization can overcome these concerns, the process can be time consuming when there is a large sample size and is less sensitive than RT-PCR in detecting small changes in mRNA levels and low-copy-number mRNA transcripts. On the other hand, tissue microdissection has proved to be a useful technique for analysis of DNA from small histologically identified lesions.[1,2] For these reasons, it has been desirable to characterize the optimal conditions for isolation of mRNA from microdissected regions of a tissue section to allow RT-PCR analysis of gene expression in a homogeneous population of cancer cells.[3–5]

Strategies for isolating pure RNA involve a number of steps, which can lead to degradation, and a lower recovery of an already limited amount of RNA. To overcome this we have modified a recent method,[6] describing RT-PCR analysis of cell line mRNA without RNA isolation, for the purpose of analyzing mRNA derived from microdissected regions of cryostat tumor sections. Briefly, microdissected cells are lysed by cycles of freeze-thaw to release RNA into a solution designed to minimize degradation. RT-PCR can be performed using the RNA solution without any additional processing. Using this method, we amplified different size fragments of the β_2-microglobulin (β_2m) gene mRNA transcript from microdissected regions of frozen breast carcinoma sections. As well, the methodology was used for amplification of *BRCA1* and

Supported in part by grants from the Canadian Breast Cancer Research Initiative and the National Cancer Institute of Canada (M. Redston and I.L. Andrulis). S.J. Done is a Research Fellow of the National Cancer Institute of Canada supported with funds provided by the Terry Fox Run.

Accepted for publication April 24, 1998.

Address reprint requests to Dr. Irene L. Andrulis, Room 870, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5 Canada. E-mail: andrulis@mshri.on.ca.

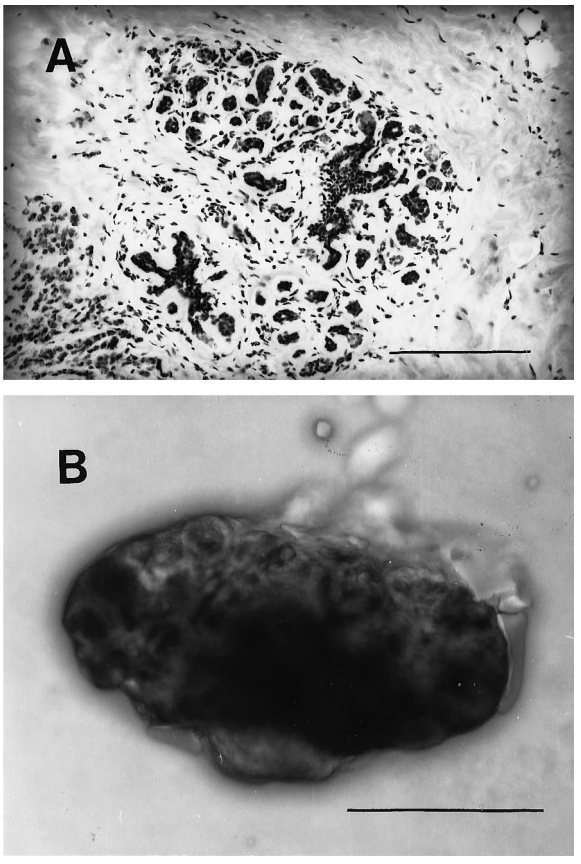

Figure 1. A normal breast terminal duct lobular unit before (A; H&E) and after (B; methylene blue) microdissection. Bar, 200 μm.

p21^{Waf1} cDNAs. The effects of different section thickness and various tissue staining dyes on the efficiency of RT-PCR were also assessed in the study.

Materials and Methods

Tissue Preparation and Microdissection

A block of fresh tissue (approximately 1 × 1 × 0.5 cm breast carcinoma or skin) was snap frozen in liquid nitrogen as close as possible to the time of surgical removal and stored at −70°C. The tissue block was embedded in OCT, and frozen sections were cut in a Reichert-Jung 2800 Figocut E cryostat. A single section of the frozen tissue was placed on a 2% agarose-coated glass slide and stained with 1% methylene blue or Harris's hematoxylin (unless specified otherwise) for 10 seconds and rinsed with water. Two 24-gauge needles were used to microdissect specific regions from the sections (Figure 1). The microdissected regions were immediately placed in a pre-chilled Eppendorf tube that was kept on ice at all times to minimize degradation.

Processing of Microdissected Regions

To the Eppendorf tube containing the microdissected tissue, 10 μl of freezing solution (0.15 mol/L NaCl, 10 mmol/L Tris, pH 8.0, 5 U of RNase inhibitor, 0.25 mmol/L dithiothreitol) was immediately added. In comparison with the published report,[6] we have used a smaller quantity of both RNase inhibitor and dithiothreitol, and to minimize any potential RNA degradation, we have included these reagents in the freezing solution rather than adding them after the cells have already been suspended. The tube was immediately frozen in an ethanol/dry ice bath and rapidly thawed in a 37°C water bath for at least two to three cycles of freeze-thaw to lyse the cells.

RT-PCR Analysis

The freeze-thawed cell suspension was briefly centrifuged to sediment cellular debris. The RNA-containing supernatant was used as template in a RT-PCR to amplify different fragments from the β_2m (267, 448, and 629 bp), BRCA1 (458 bp), and *p21*^{Waf1} (545 bp) mRNA transcripts. PCR primers were designed to target different exons of the gene to ensure the expected PCR products were derived from mRNA template and not from contaminating genomic DNA. As well, we used human genomic DNA as template in PCR amplification to confirm that the product was not due to pseudogene sequences. For cDNA synthesis, 2.9 μl of the supernant was incubated at 37°C for 1 hour in an 8-μl reaction containing 50 mmol/L Tris/HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 500 mmol/L of each dNTP, 40 ng of random hexamers, 4 U of RNase inhibitor, and 20 U of Moloney murine reverse transcriptase. We have supplied additional RNase inhibitor to the cDNA reaction as its activity can be inactivated by the freeze-thawing.[6] PCR amplification of β_2m was performed in a 12-μl reaction, containing 2 μl of the cDNA mix, 1X PCR buffer (10 mmol/L Tris/HCl, pH 8.3, 50 mmol/L KCl, 0.01% (w/v) gelatin), 112.5 μmol/L of each dNTP (Pharmacia Biotech, Piscataway, NJ), 1.0 mmol/L $MgCl_2$, 0.75 mmol/L of each primer, and 1 U of AmpliTaq polymerase (Perkin Elmer, Norwalk, CT). For different size fragments, different combinations of primers were used: β_2m1 (5′ ACC CCC ACT GAA AAA GAT GA 3′) and β_2m3 (5′ GGA GAC AGC ACT CAA AGT AG 3′) for the 267-bp fragment, β_2m4 (5′ CTC ACG TCA TCC AGC AGA GA 3′) and β_2m3 for the 448-bp fragment, and β_2m4 and β_2m5 (5′ CAA GCT TTG AGT GCA AGA GA 3′) for the 629-bp fragment. Amplification proceeded for either 35 or 40 cycles of 15 seconds at 94°C, 15 seconds at 56°C, and 20 seconds at 72°C in the Perkin Elmer 9600. PCR amplification of *BRCA1* and *p21*^{Waf1} were performed in a 20-μl volume containing 2 μl of the cDNA mix, 1X PCR buffer, $MgCl_2$ (0.8 mmol/L for *BRCA1* and 0.9 mmol/L for *p21*^{Waf1}), 100 μmol/L of each dNTP, 0.45 μmol/L of each primer, and 1 U of AmpliTaq polymerase. Primers used to amplify *BRCA1* cDNA were BRCA1–3F (5′ AGC AGA GGG ATA CCA TGC 3′) and BRCA1–6R (5′ CAA ATC GTG TGG CCC AGA CT 3′); primers used to amplify *p21*^{Waf1} cDNA were LinkA (5′ GCC GGA GCT GGG CGC GGA TT 3′) and Got-2 (5′ GGC TTC CTC TTG GAG AAG AT 3′). Amplification proceeded for either 35 or 40 cycles of 20 seconds at 94°C, 15 seconds at 56°C (for *p21*^{Waf1}) or 58°C (for

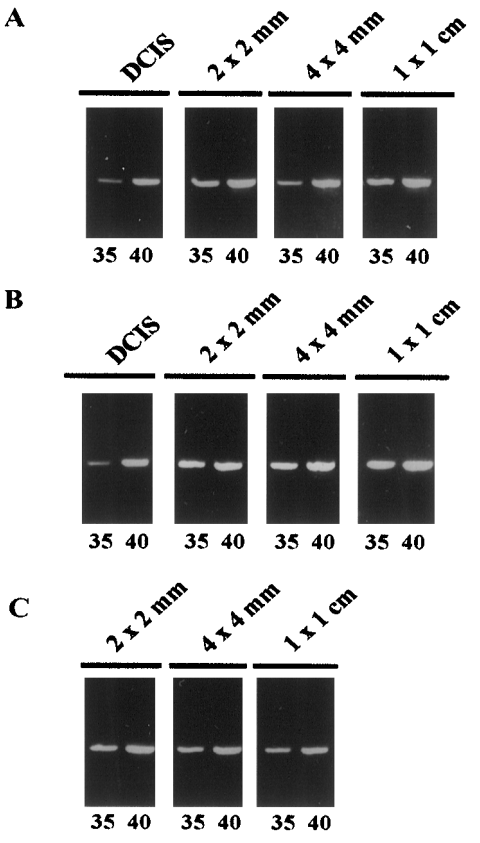

Figure 2. RT-PCR amplification (35 and 40 cycles) of a 267-bp fragment of the β_2m transcript using RNA from tissues of various sizes that were microdissected from frozen breast sections of 20 μm (**A**) and 40 μm (**B**) in thickness. In **C**, the 20-μm section was stained with Harris's hematoxylin.

BRCA1), and 25 seconds at 72°C in the Perkin Elmer 9600. PCR products were visualized on a 2% agarose gel stained with eithidium bromide.

Results

RT-PCR of β_2-Microglobulin Transcript

We successfully amplified a 267-bp region of the β_2m mRNA transcript using RNA from the supernatant of freeze-thawed microdissected cell suspensions as template. Figure 2 shows the ability to perform RT-PCR on microdissected regions with an area as small as 1 × 1 mm, the approximate size of a microdissected area of ductal carcinoma *in situ*. All sets of RT-PCRs contained negative controls for freezing buffer, cDNA synthesis, and PCR amplification to ensure that there was no contamination. In an attempt to increase the yield of RNA without having to expand the area of microdissection, we compared RT-PCR results of microdissected regions from sections of 20 μm (Figure 2A) and 40 μm (Figure 2B) in thickness. There was no significant difference in intensity of the β_2m RT-PCR product.

To identify the lower threshold from which RT-PCR could be reliably performed, we serially diluted the amount of microdissected RNA used in the RT-PCR. For a number of samples, including the ~1-mm^2 microdissected region, efficient RT-PCR amplification of the β_2m RT-PCR product was obtained when the volume of freeze-thawed cell suspension used in the cDNA synthesis was as low as 0.5 μl (Figure 3). These results indicate that a small microdissected region, containing no more than 200 cells, can provide adequate RNA for as many as 80 to 100 RT-PCRs. We were unsuccessful in quantifying the amount of RNA in the suspension because cellular debris and residual staining dye interfered with spectrophotometric analysis.

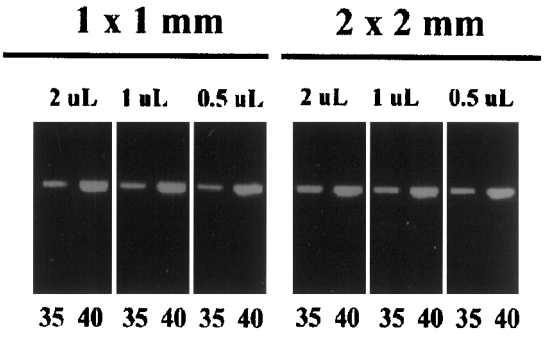

Figure 3. Effect of decreasing the volume of RNA-containing supernatant from flash-frozen microdissected tissues on RT-PCR. Water was added to make the final volume 2.9 μl for cDNA synthesis reaction (see Materials and Methods).

To assess the quality of the RNA in the freeze-thawed cell suspension, we designed PCR primers to amplify increasingly larger regions of the β_2m mRNA transcript. As shown in Figure 4, fragments as large as 629 bp were successfully amplified without any loss in efficiency. These results suggest that there is minimal RNA degradation during the preparation and processing of the tissue. It is likely that fragments significantly larger than 629 bp can be successfully amplified as well.

RT-PCR Amplification of p21^{Waf1} and BRCA1

To further validate the methodology we designed primers specific to the *p21*^{Waf1} and *BRCA1* cDNAs. Both *p21*^{Waf1} and *BRCA1* play important roles in breast cancer and are

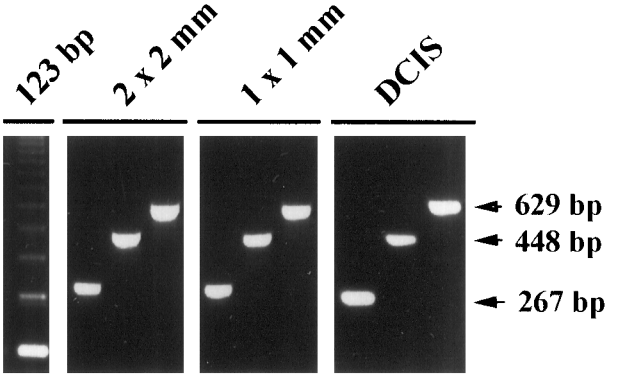

Figure 4. RT-PCR amplification the β_2m transcript using different primer combinations to give increasing product sizes.

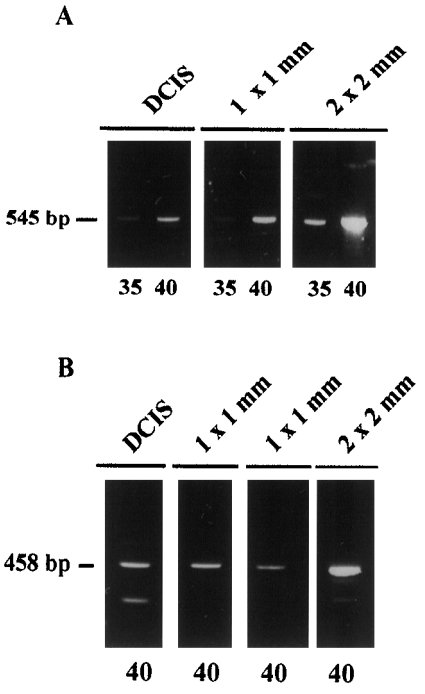

Figure 5. RT-PCR amplification (35 or 40 cycles) of a 545-bp fragment of $p21^{Waf1}$ (A) and a 458-bp fragment of BRCA1 (B) transcripts using RNA from tissues of various sizes that were microdissected from frozen breast carcinoma sections.

expressed at a lower level than β_2m. Nonetheless, a product as large as 545 bp of the *$p21^{Waf1}$* (Figure 5A) and 458 bp of the *BRCA1* (Figure 5B) mRNA transcripts was successfully amplified from microdissected specimens.

Effect of Various Tissue Dyes on RT-PCR Efficiency

To microdissect a region containing a specific cell type from a heterogeneous tissue, it is important to be able to recognize tissue architecture. A number of water-soluble dyes are available for the purpose of tissue staining, with the choice of dye depending on the specific tissue attribute of interest. To determine whether the choice of dye for tissue staining can interfere with RT-PCR, we added to a cell line RNA, an equal volume of serially diluted methylene blue (1%; Fisher Scientific, Fairlawn, NJ), Harris's alum hematoxylin (undiluted and filtered; Harleco, EM Diagnostic Systems, Gibbstown, NJ), light green (1%; BDH, Poole, UK), and neutral red (1%; Sigma, St. Louis, MO) before the RT-PCR assay (Figure 6). For Harris's hematoxylin, we found that there was no inhibition of RT-PCR until dye concentration exceeded 0.05% (1 in 2000 dilution from undiluted stock). In contrast, no RT-PCR product could be detected using methylene blue, light green, and neutral red at any concentration greater than 0.01%. We suspect that the amount of dye remaining on the tissue is lower than these inhibitory concentrations because the section is thoroughly rinsed with water

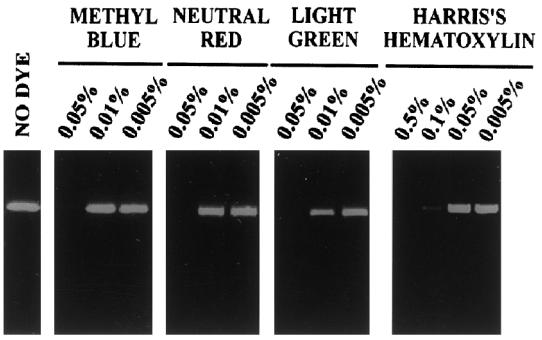

Figure 6. RT-PCR on RNA mixed with an equal volume of different tissue staining dyes that have been serially diluted from stock solutions. Percentages indicate final concentration of dye in RNA samples.

after staining. In fact, when using either methylene blue (Figure 2A) or Harris's hematoxylin (Figure 2C) for tissue staining, we did not observe any difference in efficiency of RT-PCR.

Discussion

We have modified and applied a recently described method of RT-PCR without RNA isolation to small microdissected regions from cryostat sections of frozen tissues for analysis of mRNA from a histologically defined sample of cells. We demonstrated that a microdissected region as small as 1 mm^2, containing a few hundred cells, can provide adequate RNA template for as many as 80 to 100 RT-PCRs. There is the potential to use RT-PCR to analyze many genes without the need to repeat the time consuming process of microdissection. In particular, this will allow for the study of small lesions, composed of few cells, that may not extend to deeper levels of a tissue block.

Although thicker sections (40 μm) contain more cells per unit area, they did not appear to provide more available RNA template as judged by the intensity of RT-PCR product. This may be due a lower lysing efficiency for cells lying in the center of the section. Sections of 20 μm may be the thickness of choice as they are technically easier to prepare and manipulate, as well as allowing for more sections to be cut from each block.

To microdissect tissue accurately it is necessary to stain the tissue to allow tissue architecture to be discerned. We found that at high concentration the four water-soluble dyes studied can inhibit the RT-PCR. However, in practice, this should not be a concern as tissues are stained briefly and washed in water to remove most of the dye.

In summary, we have described a method for analyzing mRNA transcripts from microdissected frozen tissue sections without the need for RNA isolation. The method can be used to study gene expression and mRNA structure and sequence in a histologically confirmed homogeneous cell population. This allows the opportunity to study RNA in small lesions that cannot be grossly identified. In addition to a variety of human cancers, the method can also be applied to other areas of research, such as developmental biology to allow for an analysis of

specific mRNA from different cell types within a developing embryo. In particular, we have found the methodology works equally well with microdissected epidermal cells from a section of skin (data not shown). With the methodology described here, there is the potential to correlate expression levels of mRNA, as well as structural variations, with particular histological phenotypes.

References

1. Zhuang Z, Bertheau P, Emmert-Buck MR, Liotta LA, Gnarra J, Linenhan WM, Lubensky IA: A microdissection technique for archival DNA analysis of specific cell populations in lesions <1 mm in size. Am J Pathol 1995, 146:620–625
2. Moskaluk CA, Kern SE: Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. Am J Pathol 1997, 150:1547–1552
3. Hiller T, Snell L, Watson PH: Microdissection RT-PCR analysis of gene expression in pathologically defined frozen tissue sections. Biotechniques 1996, 21:38–43
4. Houze TA, Gustavsson B: Sonification as a means of enhancing the detection of gene expression levels from formalin-fixed, paraffin-embedded biopsies. Biotechniques 1996, 21:1074–1082
5. Leygue ER, Watson PH, Murphy LC: Identification of differential expressed genes using minute amounts of RNA. Biotechniques 1996, 21:1008–1012
6. Klebe RJ, Grant GM, Grant AM, Garcia MA, Giambernardi TA, Taylor GP: RT-PCR without RNA isolation. Biotechniques 1996, 21:1094–1100