TECHNICAL NOTE

Tze-Whay Phang,[1] *B.S.; Chen-Yang Shi,*[1] *Ph.D.; Jin-Ngee Chia,*[2] *Dip.; and Choon-Nam Ong,*[1] *Ph.D.*

Amplification of cDNA via RT-PCR Using RNA Extracted from Postmortem Tissues

REFERENCE: Phang, T.-W., Shi, C.-Y., Chia, J.-N., and Ong, C.-N., "**Amplification of cDNA via RT-PCR Using RNA Extracted from Postmortem Tissues,**" *Journal of Forensic Sciences,* JFSCA, Vol. 39, No. 5, September 1994, pp. 1275–1279.

ABSTRACT: Analysis of cDNA derived from messenger RNA is of advantage over using genomic DNA in genetic analysis of large genes, especially those with lengthy intron sequences. However, because of its instability and rapid degradation, RNA extraction from postmortem tissues has not been attempted. Here, we report the successful extraction of intact mRNA from various postmortem tissues from accidental and sudden death cases. Subsequently with reverse transcriptase-polymerase chain reaction (RT-PCR), we were able to amplify cDNA fragments of different lengths up to 0.9 kb. The described method therefore provides a useful tool in genetic analysis of postmortem tissues.

KEYWORDS: pathology and biology, postmortem tissues, RNA extraction, cDNA, RT-PCR

Genetic analysis of DNA has found wide applications in forensic medicine, not only in identification practices, but also in forensic pathological investigations [*1,2*]. DNA in forensic specimens can be readily extracted and purified [*3*]. Specific gene fragments can then be amplified using the polymerase chain reaction (PCR) for sequencing or fingerprinting. However, when a particularly large gene or a gene with numerous or long intron (noncoding) sequences is concerned, analysis of DNA could be laborious and time-consuming since the sequence would have to be divided into small fragments consisting of individual exons (coding sequences). A useful strategy would be to analyze cDNA fragments reverse-transcribed from mRNA, which contains only exon sequences. However, RNA is highly unstable and is rapidly degraded by the ubiquitous RNase. Painstaking efforts are always necessary to obtain fresh material for RNA extraction and to minimize contamination of exogenous RNase during sample processing. So far, little information is available on the stability of RNA in postmortem tissues and RNA extraction from such samples has not been attempted.

Received for publication 30 Aug. 1993; revised manuscript received 11 Feb. 1994; accepted for publication 7 March 1994.
[1] Research Assistant, Research Scientist, and Associate Professor, respectively, Department of Community, Occupational and Family Medicine, Singapore.
[2] Laboratory Technician, Institute of Molecular and Cell Biology; National University of Singapore.

Copyright © 1994 by ASTM International

The aim of this study was to test the stability of RNA in postmortem tissues and to develop a protocol for extraction of RNA that can be used for genetic analysis of cDNA sequences.

Materials and Methods

RNA Extraction

Tissue samples were obtained from persons who died accidental or sudden, unexplained deaths in Singapore. RNA extraction from these samples was carried out according to the method of Chomczynski and Sacchi [*4*] with modifications. Briefly, 0.2 g tissue was snap frozen in liquid nitrogen and homogenized with a mortar and pestle set. The powderized tissue was then placed in 2 mL of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). To the mixture, 0.2 mL of 2 M sodium acetate (pH 4.0), 2 mL water-saturated phenol and 0.4 mL chloroform/isoamyl alcohol were added sequentially with gentle mixing. After cooling on ice for 15 min, the tubes were centrifuged at 10,000 × g for 20 min at 4°C. The aqueous phase was then collected and the RNA precipitated by adding 2 mL ice-cold isopropanol and leaving at −20°C for at least 2 h. The RNA pellet was recovered by centrifugation, washed once with 75% ethanol and re-dissolved in 50 μL DEPC-treated dH_2O. Concentration and purity of extracted RNA was determined by UV spectrophotometry. RNA samples were stored at −20°C in DEPC-dH_2O instead of in SDS since it would inhibit reverse transcriptase and *Taq* polymerase [*5*] in subsequent reactions.

Agarose Gel Electrophoresis

Electrophoresis of the RNA samples were carried out as described previously [*6*]. Total RNA sample (2 to 4 μg) was heated in 5 μL formamide containing 0.005% bromophenol blue and 0.005% xylene cyanole at 60°C for 5 min. 1% agarose gels were prepared in PBS buffer containing 2.2 M formaldehyde and 0.5 μg/mL ethidium bromide. Electrophoresis was carried out at 100 V constant voltage for 45 min. Running buffer was 2.2 M formaldehyde in PBS.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For reverse transcriptase reaction, a mixture of 1 mM each dNTP, 1 U/μL of RNasin, 5 μM random hexamers, 10 U/μL of MoMuLV reverse transcriptase and 1 to 2 μg of total RNA extract (pre-heated at 90°C for 5 min and snap-cooled) was prepared in 20 μL 1X RT buffer containing 50 mM Tris-HCl, 75 mM KCl, 2.5 mM $MgCl_2$ and 10 mM DTT. The reaction mixture was incubated for 10 min at 23°C and then 60 min at 42°C. The mixture was then heated at 75°C for 10 min, snap-cooled, and used directly for PCR.

Fragments of the human cardiac β-myosin heavy chain gene were amplified by PCR in a thermal cycler (Thermolyne, U. S. A.). The primer sequences used for PCR are listed in Table 1. The PCR cocktail was made of 1X PCR buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mg/mL BSA) containing 10 pmol each of forward and reverse primers and 1.5 units of *Taq* polymerase in a volume of 30 μL. The cocktail was added directly to the RT mixture, making up to a final volume of 50 μL. The mixture was then overlaid with 2 drops of mineral oil and subjected to 30 cycles of PCR amplification. Initial denaturation was done at 95°C for 1 min. One cycle comprised of 1 min denaturation at 95°C, 30 s annealing at 55°C and 1 min extension at 72°C. After the last cycle, samples were incubated for an additional 10 min at 72°C. 2 μL of this reaction were used as template for another round of PCR reaction with identical conditions. 10 μL of the final PCR products were taken for agarose gel electrophoresis.

TABLE 1—*PCR primers for amplification of* β*-myosin cDNA.*

Exons	Product Size (bp)		Primer Pairs
3–5	404	Sense:	5′ TTGGCCCCTTTCCTCATCTGTAGACA 3′
		Antisense:	5′ CGAGTAGGTGTAGATCATCCAGGAGC 3′
4–9	471	Sense:	5′ GCTCCTGGATGATCTACACCTACTCG 3′
		Antisense:	5′ GATAGGTCTCTATGTCTGCAGATGCCA 3′
9–13	459	Sense:	5′ GCATCTGCAGACATAGAGACCTATCTTC 3′
		Antisense:	5′ GACGTACTCATTGCCCACTTTCACC 3′
13–15	358	Sense:	5′ AGTACGTCACCAAGGGGCAGAATGT 3′
		Antisense:	5′ ATGGGCTTCTCGATGAGGTCAATG 3′
4–13	904	Sense:	5′ GCTCCTGGATGATCTACACCTACTCG 3′
		Antisense:	5′ GACGTACTCATTGCCCACTTTCACC 3′
9–15	809	Sense:	5′ GCATCTGCAGACATAGAGACCTACTTC 3′
		Antisense:	5′ ATGGGCTTCTCGATGAGGTCAATG 3′

Results

Four kidney and five heart tissues samples were obtained during postmortem autopsies. Prior to reaching the morgue, the dead bodies had been exposed to different temperatures (20 to 33°C), humidity and light conditions for varying lengths of time (8 to 12 h). Exact conditions for individual bodies could not be determined. Tissue samples were stored at −20°C for three to eight months prior to RNA extraction.

RNA extracted from various tissue samples had satisfactory purity. The OD_{260}/OD_{280} ratios were in the range of 1.8 to 2.0, comparing to a ratio of around 2.0 for pure RNA [*7*]. The yield was about 300 to 400 ng RNA per mg tissue. Extracted RNA could be stored at −20°C for two months before RT-PCR.

When total RNA samples were electrophoresed on a 1% agarose-formaldehyde gel, typical RNA smears were observed, indicating certain extent of degradation (Fig. 1). A positive control was included by using RNA extracted from freshly harvested Mahlavu cell culture according to the same procedure. The positive control showed distinct 18S and 28S bands, which are characteristic of intact RNA. In comparison, RNA extracted from postmortem tissues showed faint but nonetheless recognizable 18S and 28S bands (Fig. 1). Subsequent experiments confirmed that the extracted RNA contained intact mRNA sequences that could be reverse transcribed.

Using the RT-PCR protocol, we had successfully amplified cDNA sequences of the human cardiac β-myosin heavy chain gene. cDNA sequences ranging from 358 bp to 904 bp were amplified with high specificity from both kidney and heart tissues (Fig. 2). Subsequent sequencing experiments confirmed the specificity of amplified cDNA sequences. In addition, genomic DNA was also used for PCR amplification and the quality of the product was comparable (data not shown).

Discussion

Results from this study demonstrated that although RNA was unstable and rapidly degraded in postmortem tissues, it was possible to extract intact mRNA for RT-PCR amplification of cDNA. This technique was successfully applied to amplify sequences of the human cardiac β-myosin heavy chain gene. The longest cDNA fragment amplified was 904 bp, which covers exons 4 to 13. Inclusive of introns, this corresponds to a 4002 bp segment in the genomic DNA sequence [*8*]. The amplification of cDNA has significantly facilitated our effort in sequence analysis of the β-myosin heavy chain gene. In contrast, if using genomic DNA and conventional PCR, each of the 10 exons would have to be amplified and sequenced separately. Therefore this method is labor- and time-saving as

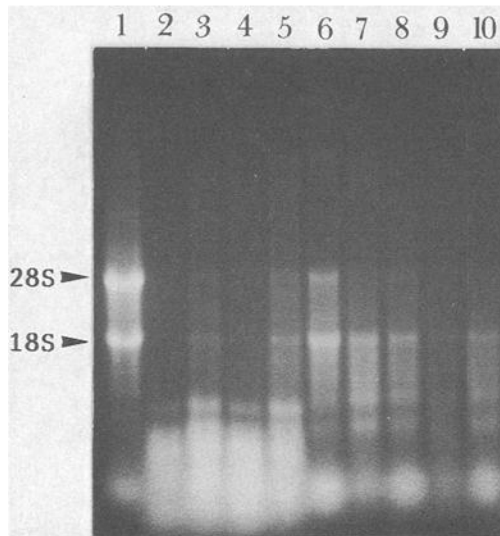

FIG. 1—*Analysis of total RNA electrophoresed on a 1% formaldehyde-agarose gel. Lane 1, RNA extracted from freshly harvested Mahlavu cell line using the same protocol. Lanes 2–5, total RNA extracted from different kidney tissues. Lanes 6–10, total RNA extracted from heart tissues. Tissue samples were stored for 8 months (Lane 2), 6 months (Lanes 3 and 4) and 3 months (Lanes 5–10) prior to RNA extraction.*

well as cost-efficient in genetic analysis of postmortem tissues. The length of cDNA fragments which can be amplified is suitable for a range of nucleic acid analysis such as hybridization, REA, and direct sequencing.

RNA of sufficient purity and yield could be extracted for RT-PCR. It was observed, however, that non-specific products were present after the first PCR amplification. This could be due to the fragmentation of RNA. In order to obtain high specificity and yield of the product, an additional round of PCR reaction was performed. It was also noted that degradation was more apparent in RNA extracted from kidney than that from heart. This could be due to the fact that the kidney tissues had been stored for longer period of time (5 to 8 months) than the heart tissues, which were three months old. Furthermore, cells at core body temperature with the last oxygenated blood would probably survive longer and preserve RNA better than cells elsewhere in the body.

The cardiac β-myosin gene is primarily expressed in the heart and not in the kidney. Despite the low level of expression, the fact (observation) that the β-myosin cDNA sequences could be amplified from kidney tissues indicated that this method was highly sensitive and could be used for detection of low level gene transcripts.

In summary, the current study showed that intact mRNA could be extracted from postmortem tissues and subsequently could be used for RT-PCR amplification of cDNA sequences. This method provides forensic pathologists a useful tool in genetic analysis of large genes. Potential applications include, but are not limited to, studies of sudden death cases in young athletes, migrant workers or infants (Sudden Infant Death Syndrome), which could be linked to certain genetic defects.

Acknowledgment

The authors wish to thank Dr. Danny Lo for providing assistance in sample collection.

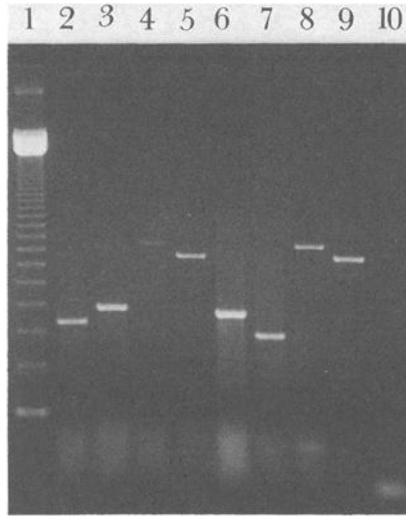

FIG. 2—*Amplification of human cardiac β-myosin heavy chain gene cDNA fragments using RT-PCR. PCR products were separated on a 1.2% agarose gel. Lane 1, DNA molecular weight markers. The bottom band represents 123 bp and each of the next bands is 123 bp longer in length. Lane 2, 404-bp fragment (exons 3–5). Lane 3, 471-bp fragment (exons 4–9). Lanes 4 and 8, 904-bp fragment (exons 4–13). Lanes 5 and 9, 809-bp fragment (exons 9–15). Lane 6, 459-bp fragment (exons 9–13). Lane 7, 358-bp fragment (exons 13–15). Lane 10, negative control (no DNA template added). RNA extracted from different kidney tissues were used for lanes 2–5. RNA extracted from different heart tissues were used for lanes 6–9.*

References

[1] Eastel, S., McLeod, N., and Reed, K., *DNA Profiling: Principles, Pitfalls and Potential*, Harwood Academic Publishers, Chur, Switzerland, 1991.

[2] Farley, M. A. and Harrington, J. J., *Forensic DNA Technology*, Lewis Publishers, Michigan, 1991.

[3] Akane, A., Shiono, H., Matsubara, K., Nakamura, H., Hasegawa, M., and Kagawa, M., "Purification of Forensic Specimens for the Polymerase Chain Reaction (PCR) Analysis," *Journal of Forensic Sciences*, Vol. 38, 1992, pp. 691–701.

[4] Chomczynski, P. and Sacchi, N., "Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction," *Analytical Biochemistry*, Vol. 162, 1987, pp. 156–159.

[5] Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., *PCR Protocols: A Guide to Methods and Applications*, Academic Press, 1990, p. 151.

[6] Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H., "RNA Molecular Weight Determinations by Gel Electrophoresis Under Denaturing Conditions, a Critical Re-examination," *Biochemistry*, Vol. 16, pp. 4743–4751.

[7] Sambrook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, 2nd. Ed., Cold Spring Harbor Laboratory Press, New York, 1989.

[8] Liew, C.-C., Sole, M. J., Yamauchi-Takihara, K., Kellam, B., Anderson, D. H., Lin, L., and Liew, J. C., "Complete Sequence and Organization of the Human Cardiac β-myosin Heavy Chain Gene," *Nucleic Acid Research*, Vol. 18, 1990, pp. 3647–3651.

Address requests for reprints or additional information to
Chen-Yang Shi, Ph.D.
Dept. of Community, Occupational and Family Medicine
National University of Singapore
Lower Kent Ridge Rd.
Singapore 0511
Phone: (65) 772-5239
Fax: (65) 779-1489