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A Dakhama, V Macek, J C Hogg and R G Hegele

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Technical Note

Amplification of Human β-Actin Gene by the Reverse Transcriptase–Polymerase Chain Reaction: Implications for Assessment of RNA from Formalin-fixed, Paraffin-embedded Material

AZZEDDINE DAKHAMA, VASILIJA MACEK, JAMES C. HOGG, and RICHARD G. HEGELE

University of British Columbia, Pulmonary Research Laboratory, St. Paul's Hospital, Vancouver, British Columbia, Canada.

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The polymerase chain reaction (PCR) is a powerful method that allows enzymatic amplification of rare target nucleic acid sequences. It has been applied to the amplification of viral genomes from paraffin-embedded pathology specimens. However, interpretation of negative results requires amplification of a housekeeping gene such as β-actin. In the present study we used specific oligonucleotide primers previously designed to amplify both the genomic DNA and the mRNA transcript from paraffin-embedded tissue. These products have predicted sizes of 250 bp and 154 bp, respectively, but our results showed that PCR amplification only (without reverse transcription) unexpectedly generated the 154-bp product. Further investigation of the nature of this product demonstrated that it originated from the amplification of DNA, not RNA. We conclude that the 154-bp product generated by these primers cannot be exclusively considered as β-actin RNA product and should not be used to assess successful extraction of RNA, to ascertain its integrity, or to normalize for the total amount of RNA assayed by RT-PCR from paraffin-embedded tissue. (J Histochem Cytochem 44:1205–1207, 1996)

KEY WORDS: Polymerase chain reaction; RNA; Paraffin-embedded material; Human β-actin.

Introduction

The polymerase chain reaction (PCR) is a sensitive method for detecting very small amounts of specific nucleic acid sequence (Saik et al., 1988) that is being increasingly used in the molecular diagnosis of infections, inflammation and genetic mutations (Adolf et al., 1991; Frye et al., 1989; Levi et al., 1989). PCR has been successfully used in retrospective analysis of DNA sequences in paraffin-embedded material (Shibata et al., 1988), and it was presumed that rare RNA can also be amplified from paraffin-embedded material by the reverse transcriptase–polymerase chain reaction (RT-PCR) (Aurer et al., 1993). However, the susceptibility of RNA to degradation, particularly with long-term storage, renders the interpretation of negative results inconclusive. The present study of formalin-fixed, paraffin-embedded human lung specimens was undertaken to determine whether RT-PCR amplification products of the human β-actin gene (Ben-Ezra et al., 1991) can be used to assess successful RNA extraction, to ascertain RNA integrity, and to normalize PCR results for the amount of RNA assayed in each patient sample.

Materials and Methods

Oligonucleotide Primers and Probe. The human cytoplasmic β-actin gene was amplified from formalin-fixed, paraffin-embedded material using the specific oligonucleotide primers A (5′-TCAJCAATGCGCAATGAG-3′) and B (5′-CACTTGCCCTACAGTG-3′), as previously described, to amplify a 154-bp (RNA) sequence and a 250-bp (DNA) sequence. The specificity of the amplification products was assessed by Southern blot analysis after hybridization with specific oligonucleotide probe (5′-GTCCACGCT-ACACTCATGATGGA-3′) (Ben-Ezra et al., 1991). Oligonucleotides were synthesized with an automated DNA synthesizer (University of Calgary; Calgary, Alberta, Canada).

Specimens and Amplification. Three human lung specimens were obtained from tissue resection for lung cancer. Five 5-μm sections (approximately 1 cm² total area/section) were cut from formalin-fixed, paraffin-embedded tissue and placed in Eppendorf tubes. After deparaffinization, the tissue sections were boiled for 10 min in 100 μl of diethyl pyrocarbonate (DEPC)-treated distilled deionized water to release nucleic acids. This crude extract was then centrifuged at 4°C for 5 min at 10,000 × g to sediment undissolved material. Five μl of cleared supernatant was used in a 50-μl PCR reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 200 μM each of dNTPs (Pharmacia; Montréal, Québec, Canada), 0.5 μM each primer, and 2 units of Taq DNA polymerase (Gibco BRL; Burlington, Ontario, Canada). For RT-PCR, 5 μl of sample was reverse-transcribed for 30 min at 37°C in a 20-μl reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 200 μM dNTPs, 20 units of...
performed at 37°C for 15 min with 50 units of reverse transcriptase (Gibco BRL). After reverse transcription, the entire DNA product was resolved by electrophoresis on ethidium bromide-stained 1.5% agarose gel and visualized by ultraviolet (UV) light.

Treatment with Nucleases. Pretreatment of samples with nuclease was performed at 37°C for 15 min with 50 μg of DNase-free RNase (Boehringer Mannheim; Laval, Québec, Canada) for RNA and 10 U of RNase-free DNase (Stratagene) for DNA in 50 μl reaction mixture consisting of 10 mM Tris-HCl, pH 8.3, containing 2.5 mM MgCl₂. After digestion, samples were incubated for 10 min at 99°C to inactivate nuclease before RT-PCR for RNA or PCR for DNA. No residual RNase or DNase activity was detected after heat inactivation, since addition of template after this step produced the same signal after amplification.

Southern Blot Analysis. After Southern transfer on Hybond-N nylon membranes (Amersham; Arlington Heights, IL) and crosslinking with UV light, PCR products were hybridized with the oligonucleotide probe which was end-labeled with [y-32P]-ATP using the T4 polynucleotide kinase reaction (Gibco BRL) following the manufacturer's instructions. Hybridization was carried out in a Hybaid hybridization oven (Stratagene) at 65°C for 18 hr in a solution consisting of 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 5 × Denhardt's solution [0.1% Ficoll 400, 0.1% bovine serum albumin (fraction V), 0.1% polyvinylpyrrolidone], 0.5% sodium dodecyl sulfate, and 100 μg/ml fragmented salmon sperm DNA. After hybridization membranes were washed in 6 × SSC three times for 5 min at room temperature and 30 min at 65°C before exposure to Kodak X-ray film.

Results

Amplification of β-actin by RT-PCR from formalin-fixed, paraffin-embedded human lung tissue consistently generated two products of 154 bp and 250 bp in length, in conformity with the predicted sizes for targeted RNA and DNA sequences, respectively (Figure 1A, Lane 2). However, amplification of equal amounts of template by PCR only (without reverse transcription) also generated both products, without any further enhancement in the intensity of the signal in comparison to RT-PCR products (Figure 1A, Lane 3). This clearly indicated that no additional copies of template could be made from the 154-bp fragment by the RT reaction, and suggested that both products might be generated during the PCR amplification step only. To examine this possibility, we investigated the nature of the templates (either RNA or DNA) by pretreating the samples with specific nuclease before amplification. As shown in Figure 1B (Lane 3), pretreatment with RNase had no effect on the template and both amplification products could be resolved on agarose gel. In contrast, when the samples were digested with DNase before amplification, no PCR product could be seen on agarose gel (Figure 1B, Lane 4), indicating that both products originated from the amplification of DNA templates but not from RNA. A Southern blot analysis further determined that both amplified fragments produced a positive signal after hybridization with an oligonucleotide probe specific for β-actin (Figure 1C).

Discussion

The results of this study demonstrate that both β-actin products (154 bp and 250 bp) generated by RT-PCR from formalin-fixed, paraffin-embedded human lung tissue specimens originated from the amplification of DNA, not RNA. The observation that direct amplification by PCR only (without reverse transcription) could generate these two different-sized fragments may be due to sufficient homology between the β-actin DNA sequence, which could shorten the length of the product, and/or to the existence of two different β-actin alleles. Although its product size is consistent with the predicted length of the RNA fragment, our results show that the 154-bp PCR product originally amplified from paraffin-embedded material (Ben-Ezra et al., 1991) cannot be unequivocally considered as originating from RNA. Moreover, the demonstration of this PCR product does not necessarily mean that intact RNA can be recovered from paraffin-embedded material. In a recent study, the investigators presumed that rare mRNA (i.e., IL2, IL4, and IL5) was successfully extracted and amplified from more than 10-year-old paraffin-embedded lung tissue (de Andrés et al., 1995). However, only β-actin amplification products (probably originating from DNA as in the present study) were shown. Because RNA is highly sensitive to degradation by endogenous ribonucleases present in tissue, some investigators have recognized the difficulty of using RNA from old paraffin-embedded tissues, although short fragments have occasionally been amplified (Hodges and Smith, 1995). This variability makes it difficult to analyze RNA retrospectively, either qualitatively or quantitatively, from paraffin-embedded specimens, in which the delay between the examina-
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Amplification and the processing of the tissue may be critical for the preservation of intact RNA. Because the interpretation of negative RT-PCR results is critically dependent on how well-preserved is RNA within a specimen, one must first assess in every individual sample that RNA is not degraded. Choosing the strategy of amplifying the β-actin housekeeping gene to assess successful extraction of "PCR-able" RNA from paraffin-embedded material may lead to misinterpretation of false-negatives, where the RNA might be totally degraded even though a β-actin product (in this case a DNA mimic of target RNA) could be demonstrated after amplification. Our results show that pretreatment of samples with RNase-free DNase to digest preexisting DNA before RT-PCR is necessary for the correct interpretation of negative results.

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Literature Cited


