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Short communication

A Simple Method for Elimination of False Positive Results in RT-PCR

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Discrimination between the amplification of mRNA and contaminating genomic DNA is a common problem when performing a reverse transcriptase-polymerase chain reaction (RT-PCR). Even after treatment of the samples with DNAse, it is possible that negative controls (samples in which no reverse transcriptase was added) will give positive results. This indicates that there was amplification of DNA, which was not generated during the reverse transcriptase step. The possibility exists that Taq DNA polymerase acts as a reverse transcriptase, generating cDNA from RNA during the PCR step. In order to test this hypothesis, we incubated samples with a DNAse-free RNAse after the cDNA synthesis. Comparison of the results that were obtained from these samples (incubated with or without DNAse-free RNAse) confirms that the reverse transcriptase activity of Taq DNA polymerase I is a possible source of false positive results when performing RT-PCR from intronless genes. Moreover, we describe here a simple and rapid method to overcome the false positive results that originate by this activity of Taq polymerase.

Keywords: RT-PCR, False positive elemination

Introduction

In the last few years, a number of molecular approaches that involve the detection and quantification of individual RNA molecules have been introduced. One of these techniques involves the amplification of target RNA molecules by sequentially combining reverse transcription (RT) and polymerase chain reaction (PCR). The reverse transcription-polymerase chain reaction (RT-PCR) was first described in 1987 (Veres *et al*., 1987), and its use for the analysis of steady-state messenger RNA levels has become increasingly widespread in recent years. This happened mainly because RT-PCR has significant advantages over more traditional RNA assays. First, RT-PCR is remarkably sensitive and can, in theory, detect single copies of a particular RNA in a highly complex sample. Second, reaction conditions in RT-PCR are easily optimized for maximum specificity, since tight control is maintained over the amplification kinetics. Finally, RT-PCR, perhaps more than any other technique currently available, is ideally suited for a rapid analysis of a large number of samples. Because of these advantages, biological questions that are difficult, or impossible, to answer using any other approach (eg. more traditional techniques such as Northern blotting and ribonuclease protection assays) can be addressed effectively with RT-PCR.

The major shortcoming of RT-PCR is derived from the possibility of contamination of the RNA preparation with genomic DNA. One way to distinguish an RNA template from a DNA template is to employ a pair of primers that are located in different exons. Thus, any contaminating DNA will be amplified at a size that is distinct from the cDNA (Chelly *et al*., 1988). However, identification of the gene expression from intronless genes, or when the genomic structure of the locus in question is unknown, is hindered by trace contaminants from genomic sequences. In this case, false positives can be caused by the contamination of RNA samples with minute quantities of DNA (Lo *et al*., 1988; Kwok and Higuchi, 1989; Sarkar and Sommer, 1990). To detect false positive results, multiple negative controls should be performed. First, the PCR should be performed directly, without the RT reaction (Menon *et al*., 1991). Second, an aliquot of the RNA sample should be treated with RNAse A prior to the RT reaction. Moreover, pretreatment of the RNA samples with RNAse-free DNAse has been used successfully to avoid false positive results (Grillo and Margolis, 1990; Dilworth and McCarrey, 1992).

We have been performing RT-PCR reactions in order to detect the gene expression of intronless genes. We found that even after treatment of the RNA samples with RNAse-free DNAse, there is in some cases a band that is present in the negative controls (samples in which no RT reaction is

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performed). This indicates that there is amplification of DNA that is not generated during the RT step.

Loeb *et al*. (1973) reported that *E. coli* DNA polymerase I has reverse transcriptase activity *in vitro*. Recently, it was shown that *Thermus aquaticus* DNA polymerase also has reverse transcriptase activity at 68°C *in vitro* (Jones and Foulkes, 1989). So, it is possible that Taq DNA polymerase, acting as a reverse transcriptase, is generating cDNA from mRNA during PCR.

To test this hypothesis, the following experimental procedure was performed. After treatment of the total RNA samples with RNAse-free DNAse (in order to remove the contaminating DNA from the RNA preparation) the mRNA was reverse transcribed and specific cDNA was amplified by PCR, with or without incubation with an DNAse-free RNAse after the RT reaction. In both experimental conditions, the negative controls were performed (no RT).

The primers that were used do not amplify a spliced sequence from the mRNA, so they do not discriminate between mRNA and/or the contaminating genomic DNA. Primers for the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (RG (5'-TCCACCACC CTGTTGCTGTA-3') and FG (5'-ACTGGCGTCTTCACCA CCAT-3)) should produce a band of 683 bp following PCR. Primers for a transport protein from the rat kidney (UST1; 11) ((RU (5'-ATTGGCTGGAATGGTGAT-3') and FU (5'-GACC AACAAACTCCAGAA-3)) should produce a band of 316 bp following PCR.

Materials and Methods

Rat kidney or adrenal total RNA (±20 µg) were treated with RNAse-free DNAse (1 U/10 µg total RNA in 100 µl of 50 mM triethanolamine/HCl pH 7.5 (37°C) and 5 mM $MgCl_2$; incubation for 30 min at 37°C). Aliquots of DNAse-treated RNA (about 5 µg) were treated as follows: (a) with 100 units of reverse transcriptase (RT; Superscript II™) and 200 pmol random hexamers in 20 µl of a RT buffer (75 µM KCl, 50 µM triethanolamine/HCl (pH 8.7 at 26°C), 100 µM dithiothreitol, 1.5 mM dNTPs, 7 mM $MgCl_2$, 15 U RNAse inhibitor (RNAseOUT™) for 60 min at 45°C, followed by 10 min at 95°C (to inactivate RT)); (b) the same as for (a), but with the following incubation with DNAse-free RNAse A 0.01 or 0.1 mg/ml (1 µl of DNAse-free RNAse A in 10 mM Tris.HCl (pH 7.5) and 50% glycerol was added to 4 µl of the RT product; incubation for 30 min at 37°C). The samples that were subjected to treatments (a) and (b), but without RT (negative controls) were performed in parallel. After the reverse transcription step (or reverse transcription step followed by incubation with DNAse-free RNAse A, 3.8 units of Taq DNA polymerase I and 0.5 µM of primers were added to 4 µl of the product in a final volume of 50 µl of the PCR buffer (50 mM KCl, 10 mM Tris.HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.3 mM $MgCl_2$, 0.2 mM dNTPs). Amplification consisted of denaturation (94°C, 30 s), annealing (55 or 58°C, 1 min), and elongation (72°C, 1 min) for 28, 34, or 38 cycles, using a Thermal Cycler (Techna). A final elongation was performed at 72°C for 10 min. Then 10 µl of the PCR product was analyzed in a 1.6% agarose gel that was stained with ethidium bromide.

Taq DNA polymerase I, RNAseOUT™, and Superscript II™ were obtained from Gibco BRL (Life Technologies, Gaithersburg, USA). RNAse-free DNAse and DNAse-free RNAse A were obtained from Sigma (Sigma, St. Louis, USA). All of the other reagents were obtained from Sigma.

Results and Discussion

Analysis of Fig. 1A shows that when the sample is not subjected to the DNAse-free RNAse treatment, there is a band that is present in the negative control (lane 2). Because the sample was previously treated with RNAse-free DNAse, the band observed in the negative control (lane 2) cannot be derived from the contaminating genomic DNA. In agreement with this assumption, when aliquots of the sample were treated with 0.01 mg/ml DNAse-free RNAse A, a decrease in the band of the negative control (lane 4) was observed. After

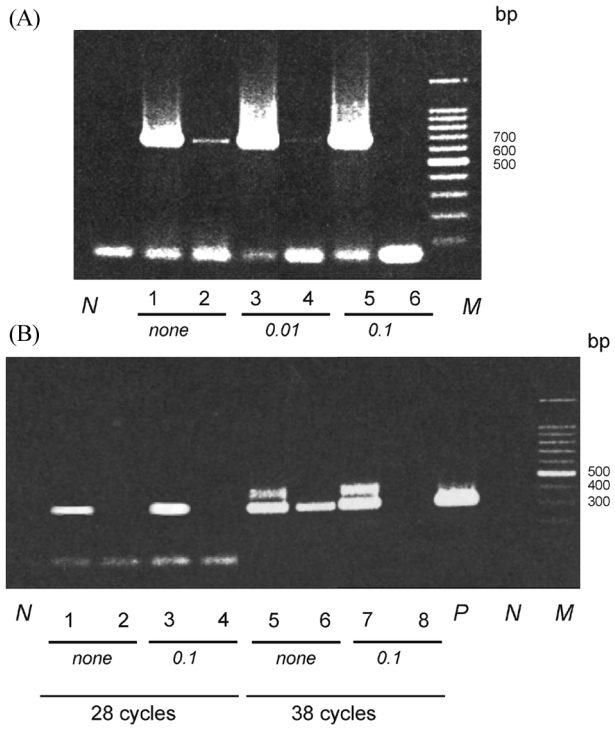

Fig. 1. Effect of DNAse-free RNAse A treatment on the detection of specific mRNA sequences. Samples were subjected to treatment (a) no incubation with RNAse A; *none*, or (b) treatment with 0.01 or 0.1 mg/ml RNAse A (0.01 or 0.1), respectively. Lanes 1, 3, 5, and 7 correspond to the samples in which RT was added; lanes 2, 4, 6, and 8 correspond to the samples in which no RT was added. Lane *M*, DNA 100 bp ladders, lane *N*, negative control, lane *P*, positive control. (A) PCR products for GAPDH (683 bp) that were obtained from rat adrenal total RNA. Amplification consisted of 34 cycles with an annealing temperature of 55°C. (B) PCR products for UST1 (316 bp) that were obtained from rat kidney total RNA. Amplification consisted of 28 or 38 cycles with an annealing temperature of 58°C.

treatment with 0.1 mg/ml DNAse-free RNAse A, the band in the negative control (lane 6) completely disappeared. These results show that the DNA band in the negative control is derived from RNA that is present in the sample when PCR is performed. The same effect of the samples' treatment with DNAse-free RNAse A was observed with UST1 (Fig. 1B). When PCR was performed for 38 cycles, there was a band in the negative control, when the sample was not subjected to treatment with DNAse-free RNAse A (lane 6). However, incubation of the sample with 0.1 mg/ml of DNAse-free RNAse completely abolished the band in the negative control (lane 8). These results confirm the ones that were obtained with GAPDH (Fig. 1A). An extra band of about 350 bp is present in kidney samples, but not in the positive control. This might indicate the presence of a transporter that is homologous to UST1 in the sample, or it could be the result of alternative RNA splicing. When PCR was performed for 28 cycles (Fig. 1B), there was no band in the negative control (lane 2). This indicates that the number of PCR cycles is critical for the appearance of false positive results. In other words, the reverse transcription of RNA by Taq polymerase I during the PCR reaction does not appear to be a very efficient process. Also, Figs. 1A and 1B show that treatment of the samples with DNAse-free RNAse A originates an improvement of the amplification band in the PCR. This can be explained by the fact that the reverse transcriptase and DNA polymerase activities of Taq DNA polymerase I compete during the PCR. So, the absence of reverse transcription leads to a higher DNA polymerase activity of the enzyme

In conclusion, this study confirms the reverse transcriptase activity of Taq polymerase I as a possible source of false positive results when performing RT-PCR from intronless genes. Moreover, we describe here a simple and rapid method to overcome the false positive results that can be originated by this activity of Taq polymerase. Incubation of the samples after a reverse transcriptase reaction with 0.1 mg/ml DNAse-free RNAse A (37ºC, 30 min) efficiently eliminated the generation of DNA from RNA during PCR (by *Taq* polymerase I) and the subsequent amplification of DNA by this enzyme. It also simultaneously improved the amplification of the specific DNA during PCR.

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