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Improved specificity of RT-PCR amplifications using nested cDNA primers

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Received November 10, 1992; Revised and Accepted February 1, 1993

Quantitative and sensitive methods are needed for the analysis of gene transcripts that are differentially expressed in various cells and tissues or under altered conditions of differentiation and activation. The reverse transcriptase-polymerase chain reaction (RT-PCR) technique, a highly sensitive method for detecting and characterizing RNA transcripts in small samples of cells or tissues, employs reverse transcription of the target mRNA followed by PCR amplification of its cDNA. The nonspecific nature of oligo (dT) or random primers that are widely used in the first step of cDNA synthesis often results, however, in coamplification of nonspecific templates. This is evident in Figure 1A, where the use of such cDNA synthesis primers for the amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gave, in addition to the expected ~300-base pair fragment, additional bands (lanes 1 and 2). Alternatively, the use of the same gene-specific 3′-primer for both cDNA synthesis and PCR amplification likewise resulted (Figure 1, lane 3) in an even more pronounced coamplification of nonspecific products due to the low annealing stringency at 37–42°C (the temperature at which reverse transcription is performed) as well as in a reduced yield of the desired amplification product.

Here we report a modification that increases the specificity of RT-PCR amplification by using, first, a gene-specific primer (termed nested cDNA primer) for cDNA-synthesis. This primer lies adjacent to, and downstream of, a pair of PCR primers which are used in the second step for PCR amplification. The increased specificity afforded by this methodology is clearly evident from the elimination of the nonspecific bands amplified by the other methods and the presence of only one detectable PCR product having the expected size (Figure 1A, lane 4). This increased specificity is achieved without an increase in time and effort. It is of particular advantage during RT-PCR-enhanced gene cloning since it offers a specificity level comparable to a two-round nested PCR amplification while, at the same time, employing only one round of PCR amplification, thereby reducing significantly the reaction time and the probability of point mutations introduced into the PCR product by the error-prone *Taq* polymerase. Likewise, this novel modification can be used very efficiently in semi-quantitative mRNA measurements. An example is given in Figure 1B, where the expression of a recently isolated novel human protein kinase C (PKC) isoform, PKCθ (1), is quantitated in various human cell lines. The linearity of PKCθ mRNA quantitation by the RT-PCR were determined by mixing RNA from a leukemic T cell line (Jurkat), that expresses abundant levels of PKCθ (Figure 1B), with HeLa (PKCθ-negative)-derived RNA at different ratios. PKCθ expression correlated directly with the amount of Jurkat-derived RNA added (Figure 1C). As an interal control that allows sample-to-sample comparison and normalization of expression levels, we used in parallel a 'housekeeping' gene, GAPDH. The expression level of this gene, in contrast to the often used β-actin, remains relatively constant and is refractory to many common activating agents, including tumor-promoting phorbol esters and various cytokines (2, 3). The GAPDH primers described here were used successfully in RT–PCR experiments employing not only human, but also murine, rat, hamster and monkey mRNA from tissues and cell lines (data not shown).

Total RNA was isolated as described (4). Briefly, 10^6 cells were lysed in 100 μl RNazol solution (Cinna/Biotecx, Friendswood, TX), followed by phenol/chloroform extraction and isopropanol precipitation according to the manufacturer's protocol. Oligonucleotide primers specific for human PKCθ (1) correspond to nucleotide positions 1695–1711 (PKCθ-1, 5′ACC-ACCAGTCCACAGAG-3′; nested cDNA primer), 139–156 (PKCθ-2, 5′GAGAACGGGCAGATGTAT-3′; sense PCR primer) and 1067–1085 (PKCθ-3, 5′ TTATCCACCTCATCC-AACG-3′; antisense PCR primer). GAPDH-specific (5) oligonucleotides correspond to nucleotide positions 340–359 (GAPDH-1, 5′-GAGATGATGACCCTTTTGGC-3′; nested cDNA primer), 4–22 (GAPDH-2, 5′-GTGAAGGTCGGAGT-CAACG-3′; sense PCR primer) and 286–303 (GAPDH-3, 5′-GGTGAAGACGCCAGTGGACTC-3′; antisense PCR primer). Reverse transcription of 2 μg total RNA was performed at 42°C for 60 min. followed by 56°C for 30 min., using a mixture of 10 ng each primers PKCθ-1 plus GAPDH-1 and 500 units Mu-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD) in a single tube. Primers PKCθ-2 and GAPDH-2 were kinased prior to their use with [γ^{32}P]ATP and T4 polynucleotide kinase in order to radiolabel the PCR products synthesized in each round of amplification.

Amplifications of divided cDNA samples representing approximately 0.4 μg total RNA were carried out separately for 20 (GAPDH) or 25 (PKCθ) cycles, respectivelly, using a TwinBlock™ thermal cycler (Ericomp, San Diego, CA). Prior titration experiments indicated that under these conditions, amplification was quantitative. The PCR mixture contained 100 ng unlabeled plus 30 ng ^{32}P-labeled PCR primers and all four dNTPs (each at 200 μM) in 50 μl *Taq* polymerase buffer (1.5 mM $MgCl_2$ and 2.5 units of *Taq* polymerase; Promega, Madison, WI). Temperature cycles were set as follows: 94°C

for 1 min., 50°C for 2 min. and 72°C for 1 min. One fifth of the PCR products were resolved by 2.4% agarose gel electrophoresis, dried, visualized directly by autoradiography and quantified by laser scanning densitometry.

REFERENCES

1. Baier,G. *et al.* (1993) *J. Biol. Chem.*, in press.
2. Zentella,A. *et al.* (1991) *Mol. Cell. Biol.* **11**, 4952–4958.
3. Bosma,P.J. and Kooistra,T. (1991) *J. Biol. Chem.* **266**, 17845–17849.
4. Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.* **162**, 156–159.
5. Tso,J.Y. *et al.* (1988) *Nucl. Acids Res.* **13**, 2485–2505.

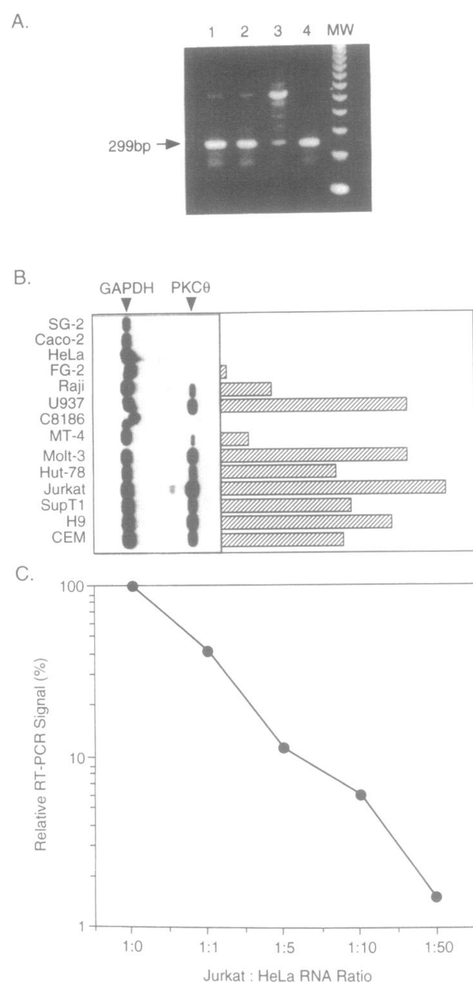

Figure 1A. Comparison of four distinct RT-PCR methods used for the amplification of a GAPDH fragment. Total RNA from murine spleen cells was employed for cDNA synthesis using 200 ng random primers (lane 1), 200 ng oligo (dT) (lane 2), 10 ng GAPDH-3 (lane 3) or 10 ng GAPDH-1 (lane 4). In each case, cDNA synthesis was followed by a GAPDH-specific PCR using 100 ng each of primers GAPDH-2 (sense) and GAPDH-3 (antisense). MW, molecular weight markers (1/2/3 ladder, Gibco-BRL). The amplified GAPDH fragment is indicated by an arrowhead. **B.**Quantitation of the PKCθ PCR products by laser densitometry. The bars indicate the relative expression level of PKCθ obtained by dividing the PKCθ signal by that of the corresponding GAPDH control. Autoradiograms are shown on the left. The human cell lines used include carcinomas (SG-2, Caco-2, HeLa and FG-2), Burkitt lymphoma (Raji), histiocytic lymphoma (U-937) and T cell leukemias/lymphomas (all others). **C.** Linearity of PKCθ quantitation by the RT-PCR. A total of 400 ng RNA per sample representing a mixture of Jurkat- and HeLa-derived RNA at the indicated ratios was used. The PKC signal in each sample is depicted as percentage of the maximum signal (100% Jurkat RNA).