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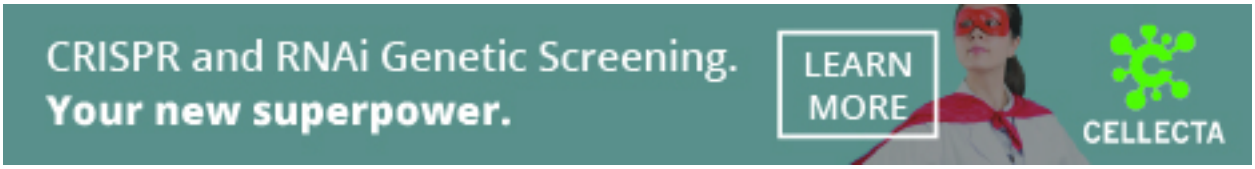

Differential Detection of Colinear Genes and RNA Transcripts by Modified Reverse Transcription and PCR

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The combination of reverse transcription of RNA into DNA and subsequent amplification of the cDNA by PCR is a very powerful procedure for the analysis of gene expression.[1] However, studies on gene expression of infectious agents in the context of their clinical manifestations require adequate control measures. To assess the quality of the RNA preparations, it is necessary to study the integrity of an abundant RNA species originating from the infected individual or the pathogen itself. Obviously, mRNA molecules deriving from genes that are expressed in most tissue and cell types are preferred. Examples of these target molecules are keratin mRNA,[2] actin mRNA,[3] or other mRNA species.[4] Detection of such an "internal control RNA molecule" is essential to prove that failure to detect the mRNA of interest is not due to poor quality or low quantity of the RNA preparation. The control assays are constructed in such a way that the length of the PCR fragment to be synthesized indicates whether the template was genomic DNA or cDNA deriving from a spliced mRNA. In general, this involves the presence of an intron sequence in the gene, positioned in such a way that the cDNA-deriving PCR fragment is significantly shorter than its genomic counterpart. It is difficult to discriminate PCR products deriving either from genomic DNA or from a cDNA molecule in the case where genes and transcripts are colinear. In this paper, an alternative procedure is described for the selective detection of cDNA molecules originating from mRNA molecules transcribed from colinear genes. The latter is frequently the case when expression studies are carried out in bacterial or viral systems lacking strategically situated intron sequences.

MATERIALS AND METHODS

Nucleic Acid Isolation

Nucleic acid extracts were prepared using an adapted version of a protocol described before.[5] Briefly, 0.25 ml of a suspension of human cervical carcinoma cells (CaSki cells) were centrifuged for 10 min at 3000 rpm in an Eppendorf centrifuge. The supernatant was discarded and 500 µl of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol) was added. Also added were 50 µl of 2 M NaAc (pH 4.5), 500 µl of water-saturated phenol, and 100 µl chloroform/isoamylalcohol (49:1). The mixture was kept on ice for 10 min and subsequently centrifuged for 20 min at 12,000 rpm at 4°C. The water phase was transferred to a new tube and 50 µg of tRNA was added. Nucleic acids were precipitated by addition of 1 ml of ethanol (96%), incubation at −20°C for 1 hr, and centrifugation. After vacuum drying, the pellet was dissolved in 50 µl of diethylpyrocarbonate-sterilized water. Standard isolations were performed on 4–10 × 10^6 cells.

cDNA Synthesis

Prior to adding reverse transcriptase, 1 µl of an antisense 18S rRNA primer (50 pmoles/µl) was added to 5 µl of a nucleic acid extract (see above) in a total volume of 11 µl. This mixture was incubated at 80°C for 2 min and quenched on ice. Subsequently, 14 µl of a mixture containing 90 mM Tris·HCl (pH 8.3), 200 mM KCl, 5 mM $MgCl_2$, 16 mM dithiothreitol, and 0.8 mM of the various desoxynucleotide triphosphates (dNTPs) was added. Finally, 30 units of RNasin (Promega) and 200 units of MMLV reverse transcriptase (BRL) were added and cDNA synthesis proceeded for 1 hr at 42°C. The enzyme was inactivated by an incubation at 95°C for 5 min, and the resulting mixture was stored on ice. Incubations containing all ingredients except the reverse transcriptase were included as negative controls. Primers A and C were used for cDNA synthesis. Primer A is fully complementary to the 18S rRNA, whereas primer C has mismatches with the RNA molecule in 9 out of 25 positions.

PCR and Southern Blot Analysis

To the cDNA synthesis mix 75 µl containing 8 mM Tris·HCl (pH 9.0), 40 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.1 mM dNTPs, 0.25 units of *Taq* DNA polymerase (Sphaero Q, The Netherlands), and 50 pmoles of the sense primer were added. PCR was performed in a Biomed thermocycler (model 60). The PCR program consisted of 40 cycles of repeated denaturation (1 min at 94°C), primer annealing (2 min at 52°C), and extension (3 min at 74°C. When cDNA synthesis was directed by primer A, PCR was performed in combi-

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nation with primer B. cDNA synthesis primer C was combined with primer D during PCR. The expected length of the fragment to be amplified is 197 bp (see Fig. 1). DNA fragments synthesized during the PCR were analyzed by electrophoresis in 3% agarose gels in 40 mM Tris·acetate (pH 7.8), 2 mM EDTA, followed by ethidium bromide staining. DNA fragments were identified by standard Southern blotting procedures.[6] The human 18S rRNA probe (see Fig. 1) was labeled by 5′ phosphorylation using T_4 polynucleotide kinase and γ-labeled ATP (Amersham, 3000 Ci/mmole).

RESULTS AND DISCUSSION

This new approach takes advantage of the differences in annealing temperature during reverse transcription and the *Taq* DNA polymerase-mediated DNA synthesis. As a result, hybridization mismatches in the oligonucleotides that are within the target hybrids at which DNA synthesis initiates enable a "cDNA versus genomic DNA" differentiation. Figure 1 gives the sequence of four oligonucleotides (A to D) that have been used for the detection of 18S rRNA sequences. The oligonucleotides A and C can both be used as efficient cDNA synthesis primers, initiating cDNA synthesis from the same site in the RNA molecule. If oligonucleotide A, which is 100% complementary with the rRNA gene, is used as cDNA synthesis primer and is combined with oligonucleotide B during PCR, both cDNA and genomic DNA serve as PCR templates. In Figure 1B, lanes 1 and 2, it can be seen that no difference is observed when the PCR products, which are synthesized with or without prior cDNA synthesis, are compared. The PCR products are identified by hybridization with a radioactively labeled ribosomal DNA specific probe (see Fig. 1). If, on the other hand, primer C is used for cDNA synthesis and is combined with primer D during PCR, apparently the PCR with genomic DNA is abolished: no hybridization signal is found when no previous reverse transcription reaction is carried out (see Fig. 1, lane 4). The identity of the PCR product found in lane 4 as being of ribosomal origin has been confirmed with direct sequencing. The nucleotide order determined was in complete agreement with the previously published sequence of the human gene.[7]

In conclusion, it is possible to discriminate colinear cDNA molecules from their genomic counterparts. The rationale is that, first, cDNA molecules are tagged with unique sequences that are not present in the gene itself. Second, at the PCR annealing temperature, additional discrimination takes place. Neither of the PCR primers is fully complementary to the gene sequence, whereas in the case of a cDNA molecule one of the primers matches perfectly. This implies that PCR on a cDNA molecule will be preferred. This procedure, described here for ribosomal RNA, can be extrapolated to the detection of other RNA molecules, being high or low in abundance, which are colinear with their encoding genes. Differences in homology between cDNA synthesis primers and RNA templates should be clustered at the 5′ terminus of the primer and differences of at least 10°C in melting temperature

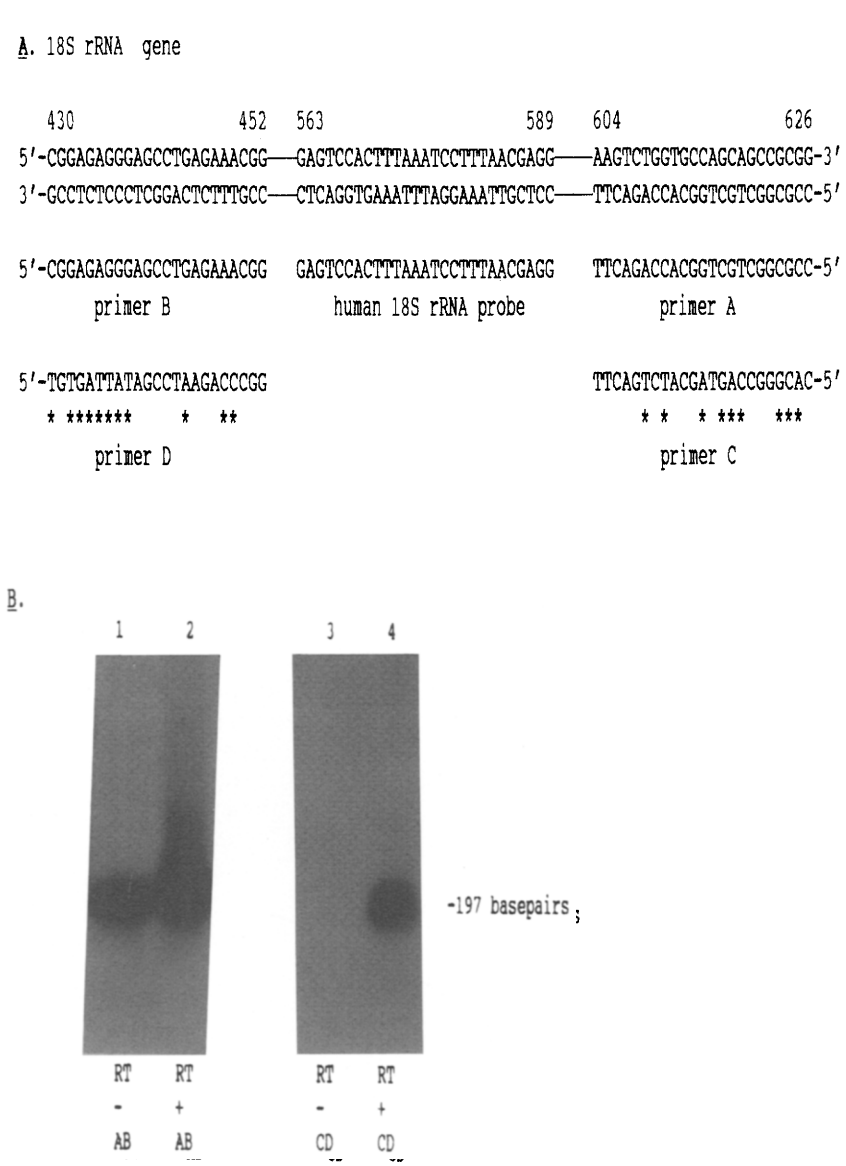

FIGURE 1 Differential detection of cDNA and genomic DNA encoding the 18S rRNA. (*A*) Part of the sequence of the human 18S rRNA gene is given (nucleotide numbering is indicated). The sequences of the primers discussed in the text are shown. Mismatches between primers and the gene are highlighted by an asterisk. Primers A and C are the antisense primers, and primers B and D have a sense orientation. (*B*) Detection of the 197-nucleotide-long 18S rRNA PCR product. If the different cDNA synthesis primers (A and C) are used for cDNA synthesis (RT+) and if they are combined with primers B and D, respectively, cDNA molecules are specifically amplified when the combination of oligonucleotides C and D is used during PCR.

should be predictable. Present studies on human papillomavirus type 16 (HPV-16) gene expression have already revealed that the procedure described in this paper, adapted for detection of HPV-16 mRNA, is at least as sensitive as an ordinary splice site-mediated PCR (unpublished results).

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