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 μ l 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 μ l of 2 M NaAc (pH 4.5), 500 μ l of water-saturated phenol, and 100 μ l 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 μ g 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 μ l of diethylpyrocarbonate-sterilized water. Standard isolations were performed on 4–10 $\times 10^6$ cells.

cDNA Synthesis

Prior to adding reverse transcriptase, 1 μ l of an antisense 18S rRNA primer (50 pmoles/ μ l) was added to 5 μ l of a nucleic acid extract (see above) in a total volume of 11 μ l. This mixture was incubated at 80°C for 2 min and quenched on ice. Subsequently, 14 μ l of a mixture containing 90 mM Tris-HCl (pH 8.3), 200 mM KCl, 5 mM MgCl₂, 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 μ l containing 8 mM Tris-HCl (pH 9.0), 40 mM KCl, 2 mM MgCl₂, 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-

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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