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Measurement of mRNA by quantitative PCR with a nonradioactive label

Elizabeth E. Powell and Paulus A. Kroon

Department of Biochemistry, The University of Queensland and Clinical Research Centre, Royal Brisbane Hospital Foundation, Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia

Summary This report describes the development of a method to measure mRNA in small samples of human tissue by the polymerase chain reaction with a nonradioactive label. In this method RNA is reverse-transcribed in the presence of a control RNA, and subsequently amplified by the polymerase chain reaction during which a nonradioactive label (digoxigenin-11-dUTP) is incorporated. Gel blotting and immunological detection of digoxigenin followed by a chemiluminescent reaction provide an intense signal on film. This allows the detection and quantitation of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase mRNA in 12 ng of RNA. We demonstrate that this is a sensitive and reproducible method, and that quantitation is linear with respect to the amount of mRNA present. The application of this method to the measurement of low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA levels in circulating peripheral blood mononuclear cells and human liver biopsy samples is discussed. The use of chemiluminescent reagents instead of radioactive labels allows this procedure to be performed safely in laboratories not equipped for radioactivity.—**Powell, E. E., and P. A. Kroon.** Measurement of mRNA by

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The key regulatory steps in the cellular pathway for cholesterol metabolism involve the rate-limiting enzyme for cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), and the receptor-mediated endocytosis of low density lipoproteins (LDL) by the LDL receptor (LDL-R) (1). Knowledge of the way in which these genes are regulated is of vital importance, not only for an understanding of lipoprotein and cholesterol metabolism, but also for delineating new ways to influence plasma LDL-cholesterol levels and thus interfere with the process of atherogenesis. A major difficulty in studying the regulation of HMG-CoA reductase and LDL-R gene expression in humans in vivo has been the limited amount of tissue available for RNA analysis. Conventional methods of mRNA analysis such as Northern and dot-blot hybridization and nuclease protection analysis are often not sensitive enough to detect rare mRNA species in samples limited by size (2, 3). In a study of lymphocyte LDL-R gene regulation using the nuclease protection method, small amounts of LDL-R mRNA were detected, but this required 10 μg of total RNA (4). The polymerase chain reaction (PCR) has become an invaluable tool for the study of low-copy number mRNA transcripts, but in most cases has provided only qualitative results. Recently Wang, Doyle, and Mark (5) have described a method for the quantitation of specific mRNA species by PCR using a synthetic RNA as an internal standard. This approach involves coamplification of a target mRNA with a synthetic RNA that utilizes the same primer sequences but yields a PCR product of a different size. However, the use of high specific activity radioisotopes as a label in these PCR reactions remains problematical. In addition to concerns about safety, ^{32}P-labeled primers and PCR products have a short half-life. We have adapted the method of Wang et al. (5) by the incorporation of a nonradioactive label during amplification followed by detection and quantitation using chemiluminescence (6, 7). In this report, we describe the application of this method to the measurement of LDL-R and HMG-CoA reductase mRNA levels in circulating peripheral blood mononuclear (PBMN) cells and human liver biopsy samples. We suggest that this technique may be useful in clinical laboratories for the quantitative analysis of gene expression in the small tissue samples available from in vivo sources.

MATERIALS AND METHODS

RNA preparation

Total cellular RNA was prepared from lymphocytes in 10 ml venous blood samples and from 5- to 10-mm needle liver biopsies obtained from patients who had undergone cholecystectomy for gallstones. The basic procedure of Chirgwin et al. (8) and Glisin, Crkvenjakov, and Byus (9) was used. After homogenization in 1.2 ml guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM dithiothreitol, 0.5% N-lauryl sarcosine) the lysates were layered over a 1-ml 5.7 M cesium chloride cushion and spun in a TLS-55 Rotor at 33,000 rpm in a TL100 centrifuge (Beckman) for 15–18 h. The PBMN cell RNA pellets were resuspended in 90 μl TES buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% sodium dodecyl sulfate), precipitated twice with 0.1 vol 3 M sodium acetate/2.5 vol ethanol, and finally resuspended in 10 μl of sterile deionized water. The liver RNA pellets were resuspended in 200 μl TES buffer, extracted with phenol–chloroform–isoamyl alcohol 1:0.95:0.05 (v/v), then with chloroform–isoamyl alcohol 0.95:0.05 (v/v) and finally resuspended in 10 μl of sterile deionized water. The RNA concentration was measured spectrophotometrically at 260 nm.

PCR amplification with a nonradioactive label

Aliquots of total cellular RNA were reverse-transcribed into cDNA along with 10^4 molecules of AW109 cRNA. This cRNA is available commercially from Perkin-Elmer Cetus. AW109 contains primer sites for a number of cytokines, apolipoprotein E and lipoprotein lipase, in addition to the LDL-R and HMG-CoA reductase. A 20-μl reverse transcription reaction mixture containing up to 120 ng of total cellular RNA, 10^4 molecules of AW109 cRNA, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 20 units of RNasin (Cetus), 2.5 μM random hexanucleotide

Abbreviations: AMPPD, disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL-R, low density lipoprotein receptor; PCR, polymerase chain reaction; PBMN cells, peripheral blood mononuclear cells; DIG, digoxigenin-11-dUTP.

primers (Cetus), 1 mM each dGTP, dATP, and dCTP, 0.975 mM dTTP (Boehringer Mannheim), and 50 units of Moloney Murine Leukemia Virus reverse transcriptase (Cetus) was incubated at room temperature for 10 min, 42°C for 15 min, 95°C for 5 min, and then quick-chilled on ice. Digoxigenin-11-dUTP (DIG) (Boehringer Mannheim) was incorporated as a nonradioactive label during PCR amplification of the reverse-transcribed RNA. A DIG concentration in the PCR mix of 50 μM was recommended by the manufacturer. To reduce the cost of this reagent, we have successfully reduced the concentration of DIG down to 14 μM. In the current experiments, PCR was performed at a final concentration of 1 × PCR buffer, 1.25 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer/Cetus), 14–25 μM DIG, with the addition of 10 μl of reverse transcription reaction mixture in a total of 50 μl. A total of 15 pmol each of 5′ and 3′ LDL-R or HMG primers was added to the PCR mixture. The primer sequences are shown in **Table 1.** The mixture was overlaid with 20 μl of Nujol mineral oil (Cetus) and then amplified with a Bioquest thermal cycler. The amplification profile involved denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 1 min.

PCR product detection by chemiluminescence

Ten μl of each PCR reaction mixture was electrophoresed in a minigel apparatus at 90V for 90 min in 3% agarose (Pharmacia) gels (10 cm × 7 cm) in Tris-borate/EDTA buffer, to allow adequate separation of the target and synthetic amplification products. The DNA was transferred to a nylon membrane (Boehringer Mannheim) by blotting for 3 h in 10 × SSC (20 × SSC: 3 M sodium chloride, 0.3 M sodium citrate, pH 7). The blot was baked under vacuum at 120°C for 20 min and rinsed in 2 × SSC. Detection of the PCR products was performed directly on the nylon membrane. The blot was washed briefly in buffer A (maleic acid, 0.1 M; sodium chloride 0.15 M, pH 7.5, plus Tween 20, 0.3%) and incubated for 30 min at room temperature in blocking buffer (1% blocking reagent (Boehringer Mannheim) in buffer A). This was followed by a 30-min incubation with an antidigoxigenin-IgG conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1 in 10^4 in blocking buffer. The blot was then washed twice for 15 min in buffer A and incubated in buffer B (Tris-HCl, 0.1 M; sodium chloride, 0.1 M; magnesium chloride, 50 mM, pH 9.5) for 5 min. Chemiluminescent detection was performed by incubating the membrane for 5 min in a solution of AMPPD (100 μg/ml in buffer B). The diluted substrate solution can be stored at 4°C and reused at least six times. The membrane was blotted lightly and sealed in a clear plastic bag. Exposure to X-ray film (Agfa Curix MR4) was performed in an X-ray cassette at room temperature. Sharp distinct signals corresponding to the target mRNA and the synthetic internal control were obtained without background signals within 2–10 min. Typically, several exposures were made for the same blot to ensure that band intensities were within an appropriate range for densitometric analysis (see below).

Quantitation

Each pair of signals corresponding to the target mRNA and the internal control was scanned by a laser densitometer (Helena Laboratories, Rapid Electrophoresis Analyzer). Quantitation of the target mRNA was performed by comparison with the cRNA internal standard, and expressed per μg of total cellular RNA. Analysis was generally limited to exposures that gave absorbance readings of < 1.0 for individual bands. Integration of peak areas was performed using programs provided with the densitometer. The concentration of LDL-R or HMG-CoA reductase mRNA was calculated from the relative sample and control peak areas(R) and the known number of molecules of cRNA added to the PCR reaction:

$$\text{mRNA molecules/μg RNA} = \frac{R \times (\text{copies of cRNA added})}{(\text{μg of human RNA added}).}$$

RESULTS AND DISCUSSION

Reverse transcription and coamplification of synthetic cRNA and human RNA corrects for variables

TABLE 1. Primer sequences for amplification of human and synthetic RNA

mRNA Species	5′ Primers	3′ Primers	Size of PCR Product	
			mRNA	cRNA
			base pairs	
LDL-R	5′-CAATGTCTCACCAAGCTCTG-3′	5′-TCTGTCTCGAGGGGTAGCTG-3′	258	301
HMG	5′-TACCATGTCAGGGGTACGTC-3′	5′-CAAGCCTAGAGACATAATCATC-3′	246	303

due to differences in sample preparation, conditions of reverse transcription, and PCR amplification. Since the same primers are used in the PCR amplification of both templates, there are no differences in primer efficiency for cDNA derived from cRNA or mRNA. **Fig. 1A** illustrates the chemiluminescent image obtained on X-ray film from PCR-amplified cRNA and mRNA separated on an agarose gel and transferred to a nylon filter. These data demonstrate the sensitivity of chemiluminescence as a detection method for PCR products. The direct incorporation of DIG-labeled dUTP makes it possible to compare the relative intensities of the cRNA and mRNA bands to calculate absolute copy numbers of specific mRNAs. Densitometric scans of cRNA and mRNA bands shown in Fig. 1B demonstrate that the band separation is adequate to allow quantitation of relative areas. The result of an analysis by nonlinear curve fitting (Peakfit, Jandel Scientific) is shown and demonstrates that the scans can be fitted to two individual peaks.

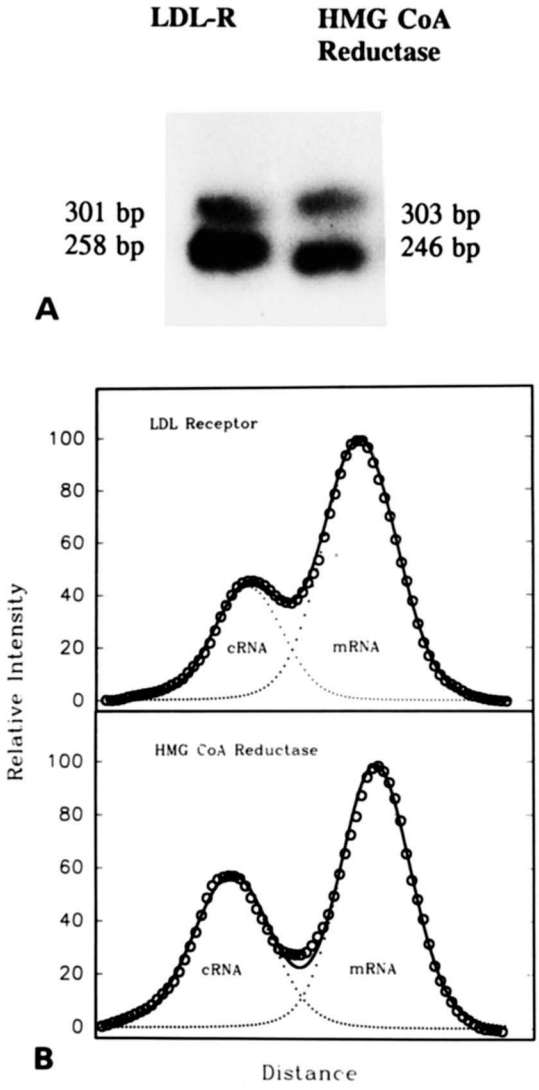

Fig. 1. Chemiluminescent images and densitometric scans of PCR amplified cRNA and mRNA. A: Chemiluminescent image. RNA isolated from PBMN cells was reverse-transcribed in the presence of 10^4 copies of AW109 cRNA and subjected to 25 cycles of PCR amplification with LDL-R or HMG-CoA reductase primers. PCR products were separated by agarose gel electrophoresis, blotted onto a nylon membrane and detected by chemiluminescence as described in Materials and Methods. Lane 1: PCR with LDL-R primers; lane 2: PCR with HMG-CoA reductase primers. The upper band in each case corresponds to cRNA and the lower band to the specific mRNA. B: Densitometric scans of the chemiluminescent images in A. Digitized densitometric scans (○) were analyzed using a nonlinear curve-fitting algorithm (Peakfit, Jandel Scientific), Individual peaks (.....) and the summation of these peaks (——) are shown.

To assess the utility of DIG-PCR as a quantitative method, we addressed the following questions: *i)* is quantitation affected by the number of PCR cycles; *ii)* is quantitation linear with respect to the amount of mRNA present; and *iii)* is the method reproducible. The first question is important because the efficiency of PCR amplification decreases at high cycle numbers (10). As a result, the number of cycles in quantitative PCR reactions is usually limited to keep amplification exponential. To determine whether the cycle number affected the ratio of PCR products formed, 120 ng of total PBMN cell RNA and 10^4 copies of AW109 cRNA were subjected to 24, 26, 28, or 30 cycles of amplification using the LDL-R specific primers. The intensity of the resulting chemiluminescent bands increased markedly for both templates between 24 and 28 cycles and then leveled off. The amount of target mRNA at each cycle number was calculated from appropriate X-ray film exposures and is summarized in **Table 2.** These data show that the ratios between the mRNA and cRNA band intensities and the calculated LDL-R mRNA copy number vary little between 24 and 28 cycles. Even at 30 cycles, when the amplification efficiency has leveled off dramatically, the calculated copy number remains similar. We surmise that the use of identical primers for both PCR products is responsible for the insensitivity to the number of cycles. For subsequent reactions reported here we have used 25 cycles. Clearly, if substantially smaller amounts of RNA

TABLE 2. Effect of increasing number of PCR cycles on the relative intensity of the cRNA and mRNA bands and the calculated mRNA copy number

Number of Cycles	Relative Intensity of PCR Bands		mRNA Copy Number × 10^{-4} per μg RNA
	cRNA	mRNA	
24	17.1	79.4	23.2
26	16.4	83.6	25.5
28	16.1	83.9	26.1
30	18.9	81.1	21.5

are used, the number of cycles could be increased without a significant loss in accuracy.

To demonstrate that the DIG-PCR method is linear with respect to the amount of target mRNA, we performed a series of experiments in which 10^4 molecules of AW109 cRNA were combined with either 6 ng, 9 ng, 12 ng, 15 ng, 21 ng, or 24 ng of human total liver RNA. After reverse transcription, PCR amplification was performed with HMG-CoA reductase-specific primers for 25 cycles and the products were analyzed by chemiluminescence and densitometry. The results shown in **Fig. 2** illustrate that the intensity of the mRNA band is directly proportional to the amount of RNA used for reverse transcription and PCR amplification. This fulfills an essential requirement for the detection of variable amounts of mRNA in RNA preparations.

To assess the reproducibility of the DIG-PCR method, two separate lymphocyte and RNA samples were prepared from 20 ml of fasting venous blood and analyzed separately for HMG-CoA reductase and LDL-R mRNA content. The results, summarized in **Table 3,** show that the two measured LDL-R and HMG-CoA reductase mRNA levels varied by 13% and 2%, respectively. In a separate study of ten patients, the variation between duplicate measurements of HMG-CoA reductase and LDL-R mRNA levels was found to be 30%. For more precise measurements, a range of RNA concentrations was used and the results were calculated by a least squares analysis of the data as shown in Fig. 2. Copy numbers in this study were found to fall within

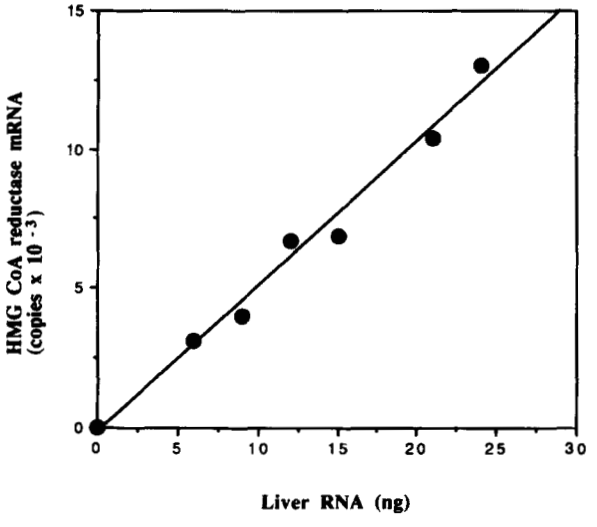

Fig. 2. Relationship of intensity of mRNA band to amount of RNA used for reverse transcription and PCR amplification. One $\times 10^4$ molecules of AW109 cRNA were combined with either 6 ng, 9 ng, 12 ng, 15 ng, 21 ng, or 24 ng of human total liver RNA. After reverse transcription, PCR amplification was performed with HMG-CoA reductase specific primers for 25 cycles and the products were analyzed by chemiluminescence and densitometry.

TABLE 3. Specific mRNA levels in two separate lymphocyte and RNA preparations from one blood sample

Sample	mRNA Copies $\times 10^{-4}$/μg RNA	
	LDL-R	HMG
RNA-1	12.5	81.9
RNA-2	14.2	83.7

quite a narrow range, varying between 7 and 35×10^4 copies for LDL-R mRNA and 5 and 22×10^5 copies for HMG-CoA reductase mRNA. Using similar PCR technology, Wang et al. (5) have calculated that there are 1.3×10^4 copies of LDL-R mRNA in a normal human coronary artery.

The above experiments have shown that mRNA can be quantitated by DIG-PCR with the use of an internal cRNA standard. The incorporation of DIG as a label during amplification allowed direct comparison of the final PCR products. The use of this nonradioactive label has a number of distinct advantages. The detection process is rapid and safe, eliminating the need for radioactivity or generation of probes. The newer chemiluminescent substrates such as AMPPD markedly enhance the sensitivity of detection. No background staining was observed during the brief X-ray film exposures (<10 min) required to produce strong signals. The high intensity of the emitted blue light also ensures that the response of film, which has not been preflashed, is linear to incoming light (11). The labeled PCR products can be stored indefinitely for use as controls or comparison in subsequent experiments, in contrast to ^{32}P-labeled primers which have a short half-life.

This method of quantitative PCR with a nonradioactive label has important implications for the study of human pathology in clinical laboratories. The measurement of a number of specific mRNA molecules can be readily performed in small samples of human tissue such as that obtained by liver biopsy. Replacement of radioactive labels by chemiluminescent reagents allows this procedure to be performed safely in laboratories not equipped for radioactivity. The ease and rapidity of the detection method makes it possible to process samples quickly and it may thus be used to follow changes in the expression of specific RNA molecules, for example during treatment of metabolic disorders.

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