Rapid quantitation of mRNA species in ethidium bromide-stained gels of competitive RT-PCR products

Angelika Gebhardt,[1] Anja Peters, Daisy Gerding, and Axel Niendorf

Institute for Pathology, University of Hamburg, Martinistr. 52, 20246 Hamburg, Germany

Abstract A rapid method for quantifying the low-abundant mRNAs of the low density lipoprotein receptor and the 3-hydroxy-3-methylglutaryl coenzyme A reductase by competitive polymerase chain reaction is presented. This approach requires neither special labeling nor blotting procedures. For each analysis, a defined amount of total cellular RNA is co-reverse transcribed and co-amplified with a titration series of in vitro synthesized competitor RNA that carries an internal deletion. The equivalence point, which defines the amount of specific RNA in the sample, can be scored in ethidium bromide-stained agarose or polyacrylamide gels of the reaction products. As an example, responses to pravastatin, a competitive inhibitor of the HMG-CoA reductase, in a human tumor cell line were analyzed with this new technique. As a control, the expression of the unregulated gene, glyceraldehyde-3-phosphate dehydrogenase was measured in parallel using the same methodology. The results obtained were compared with those obtained by conventional Northern blotting.—**Gebhardt, A., A. Peters, D. Gerding, and A. Niendorf.** Rapid quantitation of mRNA species in ethidium bromide-stained gels of competitive RT-PCR products. *J. Lipid Res.* 1994. **35:** 976–981.

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Cholesterol, an essential component of membranes, can be acquired by cells of higher organisms through two different pathways (1). Cholesterol-rich low density lipoprotein (LDL) particles can be taken up from plasma through receptor-mediated endocytosis, or cholesterol can be synthesized intracellularly. The rate-limiting enzyme of the synthetic pathway is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which produces mevalonate, a precursor of both cholesterol and non-sterol isoprenoids that are involved in a variety of growth regulatory processes (2, 3). Thus, the LDL receptor and the HMG-CoA reductase are the two key proteins in cholesterol metabolism.

HMG-CoA reductase inhibitors are currently used to reduce plasma LDL levels of hypercholesterolemic patients (4). They block the endogenous synthesis of cholesterol, which in turn leads to an increased expression of LDL receptor molecules at the hepatocellular surface (5, 6). Additionally, their application as antineoplastic agents due to effects on the non-sterol branch of the mevalonate pathway has been proposed (2, 7, 8). A detailed analysis of possible feedback mechanisms upon reaction to these drugs, which interfere with a multitude of intracellular functions, is required. Such analysis is, however, hampered by the low abundance of the respective mRNAs.

The method of competitive (c) RT-PCR provides a powerful tool for quantifying very low-abundant species of mRNAs in small samples of total cellular RNA. Previously, a small artificial RNA was constructed to determine, using this approach, expression levels of the mRNAs of the LDL receptor and the HMG-CoA reductase (9, 10). The detection and quantitation of reaction products required the incorporation of labeled nucleotides and either counting of radioactively labeled bands or Southern blotting and use of a chemiluminescence detection system. We report here a variant of the method that uses as competitors for the RT-PCR reaction in vitro synthesized deletion mutants of the RNAs of interest. Detection of products requires neither blotting nor incorporation of labeled nucleotides, as the size differences between wild-type and competitor RNA allow easy discrimination on gels stained with ethidium bromide. Recently, a similar approach was used for quantifying human immunodeficiency virus type-1 DNA and RNA sequences in human blood specimens (11, 12).

As an example, we present the quantitative analysis of the regulation of the mRNA expression for the LDL

Abbreviations: cRT-PCR, competitive reverse transcriptase polymerase chain reaction; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; P, pravastatin.

[1]To whom correspondence should be addressed.

receptor, the HMG-CoA reductase and, as a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a human gastric tumor cell line that had been rendered resistant to the HMG-CoA reductase inhibitor, pravastatin, and we discuss further applications of this sensitive method.

MATERIALS AND METHODS

Cell culture and isolation of cellular RNA

The human gastric tumor cell line EPG 85-257 (13) was maintained in Leibovitz L15 medium (Boehringer Mannheim) supplemented with 10% fetal calf serum (FCS), 80 IU/l insulin, 2.5 mg/l transferrin, 6.25 mg/l fetuin, 1 mM glutamine, 1000 mg/l glucose, and 1.1 g/l $NaHCO_3$ (Boehringer Mannheim). A sub-population of cells was found to be resistant to, and able to grow in, 2000 μM pravastatin (obtained from Bristol-Myers Squibb) after a period of 6 weeks, in which the concentration of this drug in the medium had been increased in increments of 200 μM every 4 days.

Lipoproteins were removed from FCS by density gradient centrifugation using NaBr (14). Both lipoprotein-deficient serum (LPDS) as well as FCS used as control were dialyzed extensively against 154 mM NaCl, 15 mM EDTA, 10 mM Tris/HCl, pH 7.4. Total RNA was isolated from cells grown in FCS or after induction for 4 days with LPDS, or FCS/LPDS + 1000 μM pravastatin using the RNAzol™ method (Cinna/Biotecx) and quantified by measuring the absorbance at 260 nm.

Competitive RT-PCR

For quantitative RT-PCR, cellular RNA was titrated against in vitro synthesized RNAs that were homologous to the mRNAs of the HMG-CoA reductase, the LDL receptor, or GAPDH, but carry internal deletions. The in vitro transcription vector for the synthesis of competitor RNA for the HMG-CoA reductase mRNA was constructed by subcloning the 1.4-kb HindIII/BglII fragment of pHRed-102 (15, ATCC57043) into pTZ19R (Pharmacia), and subsequent deletion of the internal 98-bp PstI fragment. For competition of the LDL receptor mRNA, the 1.4-kb EcoRI/SphI fragment of pLDLR-3 (16, ATCC57005) was subcloned into pTZ19R, and the internal 264-bp PstI fragment was deleted. For GAPDH, a 983-bp cDNA fragment (17) was synthesized by PCR using a commercially available kit (Clontech). An 80-bp deletion was generated by PCR using a combination of the original 3'- and 5'-primers and internal primers. These internal primers were selected to generate XhoI sites that could be subsequently used to ligate the respective fragments (forward primer: 5'-GGCTCGAGAAATATGACA ACTCCCT-3', reverse primer: 5'-GGCTCGAGCCCCA GCCTTCTCCAT-3'). A 416-bp HindIII fragment was then ligated to pT7T3alpha19 (Gibco BRL). For generation of competitor RNAs, 500 ng of linear templates was incubated with 15 units of T7 RNA polymerase (Pharmacia) in the presence of 15 units of RNAguard™ (Pharmacia) in a total volume of 25 μl reaction buffer (40 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine/HCl, 5 mM NaCl, 10 mM DTT, 5 mM NTPs, and 100 μg/ml BSA). The template was subsequently digested for 30 min with 10 units of RNase-free DNaseI (Boehringer Mannheim); RNA was further purified by sequential extraction with phenol–chloroform and chloroform and precipitated with ethanol. Yield of competitor RNA was generally between 10 and 15 μg. One μg of total cellular RNA was used as template for the RT-PCR reaction for the determination of the LDL receptor and HMG-CoA reductase; 100 ng was used for the more abundant GAPDH mRNA. The RT reaction was set up in 20-μl mixes each containing the appropriate amount of total cellular RNA, competitor RNA as indicated, 1 × RT buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.3, 2 mM $MgCl_2$, 0.01% gelatin), 0.5 mM dNTPs, 8 units of RNAguard™ (Pharmacia), 20 pmol 3'-primer and 10 units of M-MLV Reverse Transcriptase (Gibco BRL), and incubated at 37°C for 30 min. For the amplification reaction, 30 μl of a mix containing 1 × Replinase buffer (Biozym), 20 pmol of the 5'-primer and 1 unit of Replitherm™ DNA Polymerase (Biozym) were added per reaction. Routinely, 35 cycles were performed with 1 min denaturation at 94°C, 2 min annealing at 60°C, and 3 min extension at 72°C. Reaction products were analyzed on 1.5% agarose (10 μl) or 5% polyacrylamide gels (3 μl) in Tris borate/EDTA buffer, visualized by staining with 1 μg/ml ethidium bromide and documented on Polaroid no. 665 film. For quantitation of relative band intensities, densitometric scanning of the negatives was performed using a CAMAG TLC Scanner II (CAMAG).

Northern blot analysis

Ten μg of total cellular RNA per sample was fractionated on denaturing agarose gels and subsequently transferred, using capillary blotting, to nylon membranes (Boehringer Mannheim) according to standard protocols (18). The digoxigenin chemiluminescent detection system of Boehringer Mannheim was used according to the manufacturer's specifications. Probes were made using a DIG random priming kit (Boehringer Mannheim). Template DNA was generated by PCR using the primers defined in Table 1 for the LDL receptor and GAPDH. The 2.5-kb BglII fragment of pHRed102 (15) was used as probe for the HMG-CoA reductase.

TABLE 1. Primer sequences and sizes of cRT-PCR products

mRNA Species	HMG-CoA Reductase	LDL Receptor	GAPDH
3'-Primer	5'-ATGCTCCTTGAACACCTAGCATCT-3'	5'-CTCTCACACCAGTTCACTCCTCTT-3'	5'-CATGCCAGTGAGCTTCCCGTT-3'
5'-Primer	5'-AGGTTCCAATGGCAACAACAGAAG-3'	5'-AAGATGCGAAGATATCGATGAGTG-3'	5'-GTGGAGTCTACTGGCGTCTTC-3'
Size of cRT-PCR products (bp)			
Wild-type	823	955	408
Deletion mutant	725	691	328

RESULTS AND DISCUSSION

To quantify the expression of HMG-CoA reductase, the LDL receptor, and GAPDH mRNAs by cRT-PCR, we used as competitors relatively large in vitro synthesized RNAs carrying internal deletions (**Table 1**). Wild-type and mutant RT-PCR products could therefore be separated easily and detected in 1.5% agarose or 5% polyacrylamide gels stained with ethidium bromide. This represents an improvement over a previously published approach by Wang, Doyle, and Mark (9), who used a small synthetic RNA as internal standard and based their detection system on radioactively labeled nucleotides that were incorporated during the amplification step. The same synthetic competitor RNA was later used by Powell and Kroon (10) who substituted radioactive compounds with a chemiluminescence detection system.

In order to be able to validate results obtained with our new technique by using the less sensitive method of Northern blotting, we used a cell line that was expected to possess a relatively high expression of both the LDL-receptor and HMG-CoA reductase. This line, EPG85-257PR, originating from a human gastric tumor cell line (13), had previously been cultured in the presence of increasing concentrations of the HMG-CoA reductase inhibitor, pravastatin. Cells were finally resistant to, and able to grow in, up to 2000 μM of this drug. We were interested in determining whether this resistance was due to a constitutive unregulated overexpression of the HMG-CoA reductase and/or the LDL receptor. Therefore, cells were cultivated either in LPDS to limit the external supply of cholesterol, or endogenous synthesis of cholesterol was blocked using the HMG-CoA reductase inhibitor, pravastatin. The expression of the mRNAs for the HMG-CoA reductase, the LDL receptor and, as control, GAPDH, was determined in parallel by cRT-PCR and Northern blot analysis.

Fig. 1a shows the analysis of cRT-PCR products on agarose (A, B, D) or polyacrylamide (C) gels stained with ethidium bromide. The method was used for quantitation of the mRNA for the LDL receptor (A), the HMG-CoA reductase (B, C) and, as control, GAPDH (D) in cells cultivated in FCS or LPDS with or without pravastatin as indicated. The equivalence point, which is characterized by equal intensities of bands for both wild-type and competitor sequences and defines the amount of specific RNA in the starting material, can be reliably detected by the eye. In addition, densitometric scanning of Polaroid film negatives was used to accurately quantify smaller differences in the expression of specific mRNAs (Fig. 1b and **Table 2**). **Fig. 2** shows Northern blot analysis performed on RNA samples in parallel.

The expression of both genes involved in cholesterol metabolism, LDL receptor and HMG-CoA reductase, is induced in response to a challenge with either LPDS or

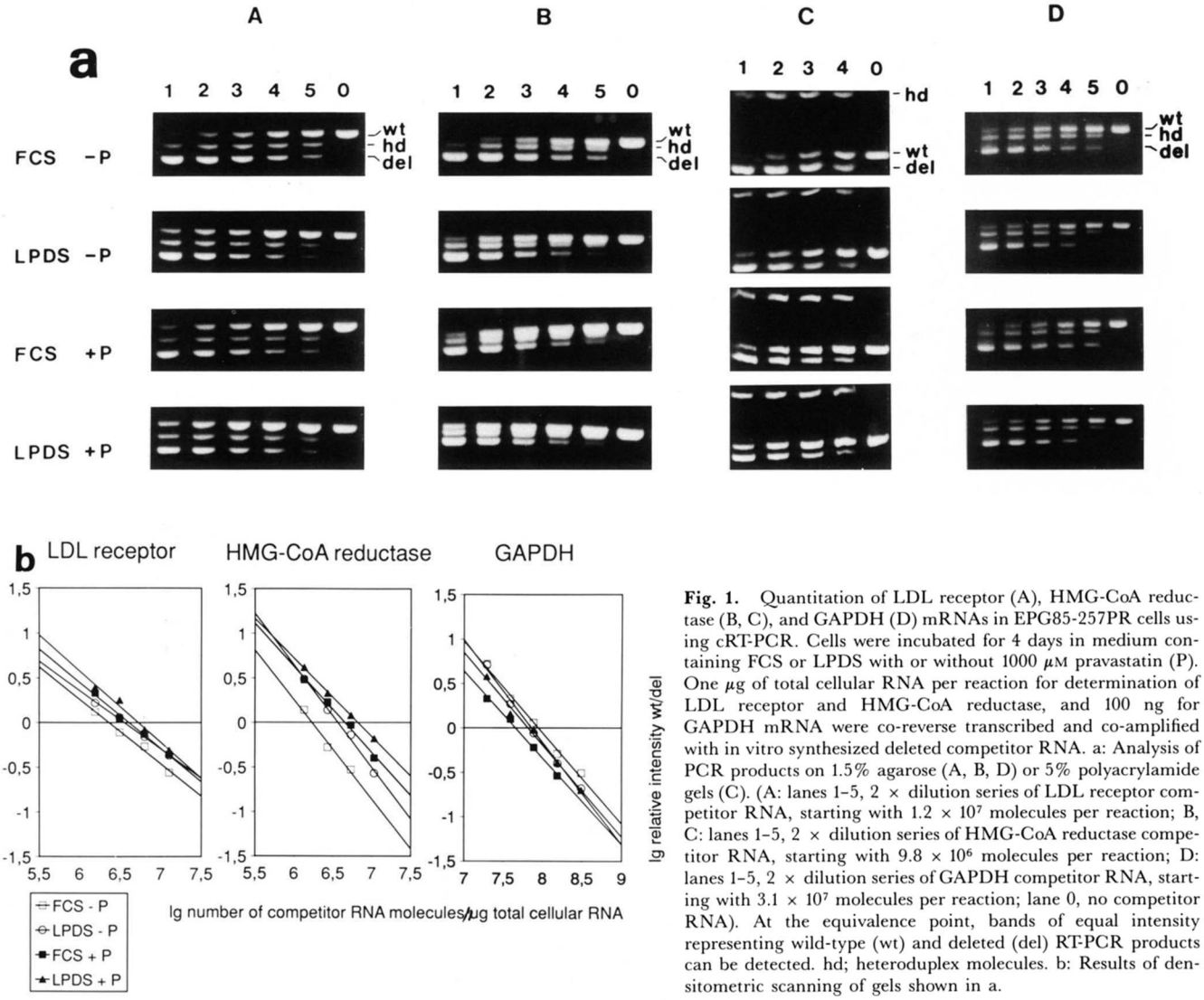

Fig. 1. Quantitation of LDL receptor (A), HMG-CoA reductase (B, C), and GAPDH (D) mRNAs in EPG85-257PR cells using cRT-PCR. Cells were incubated for 4 days in medium containing FCS or LPDS with or without 1000 μM pravastatin (P). One μg of total cellular RNA per reaction for determination of LDL receptor and HMG-CoA reductase, and 100 ng for GAPDH mRNA were co-reverse transcribed and co-amplified with in vitro synthesized deleted competitor RNA. a: Analysis of PCR products on 1.5% agarose (A, B, D) or 5% polyacrylamide gels (C). (A: lanes 1–5, 2 × dilution series of LDL receptor competitor RNA, starting with 1.2×10^7 molecules per reaction; B, C: lanes 1–5, 2 × dilution series of HMG-CoA reductase competitor RNA, starting with 9.8×10^6 molecules per reaction; D: lanes 1–5, 2 × dilution series of GAPDH competitor RNA, starting with 3.1×10^7 molecules per reaction; lane 0, no competitor RNA). At the equivalence point, bands of equal intensity representing wild-type (wt) and deleted (del) RT-PCR products can be detected. hd; heteroduplex molecules. b: Results of densitometric scanning of gels shown in a.

pravastatin. In contrast, there are no increases in the amount of GAPDH mRNA. This clearly demonstrates that the increases in LDL receptor and HMG-CoA reductase expression are not due to a nonspecific induction of gene expression. The effects of blocking external or internal supplies of cholesterol seem to be additive, as a combination of LPDS and pravastatin results in the highest induction. Both methods used for analysis of specific mRNA expression, cRT-PCR and Northern Blotting, gave comparable results; however, the cRT-PCR technique allows a more accurate quantification and requires less starting material. With this method, the maximal stimulation of the expression of the HMG-CoA reductase was found to be higher than that of the LDL receptor (maximal stimulation 3.9-fold versus 2.3-fold).

TABLE 2. Results from densitometric scanning and linear regression analyses[a]

Growth Conditions	LDL Receptor mRNA	HMG-CoA Reductase RNA (copy no./μg total RNA)	GAPDH
FCS – P	2.3×10^6	1.7×10^6	8.9×10^7
LPDS – P	3.8×10^6	3.6×10^6	7.2×10^7
FCS + P	4.2×10^6	4.5×10^6	4.6×10^7
LPDS + P	5.4×10^6	6.6×10^6	6.6×10^7

[a] All correlation coefficients were < -0.989.

The mechanism that enables EPG85-257PR cells to survive in high concentrations of pravastatin remains to be elucidated. Both the HMG-CoA reductase as well as the LDL receptor expression obviously still underlie normal feedback controls such as sterol-dependent repression of gene transcription (19) under conditions of sufficient supplies of exogenous cholesterol and in the absence of pravastatin. Furthermore, they do not overexpress these

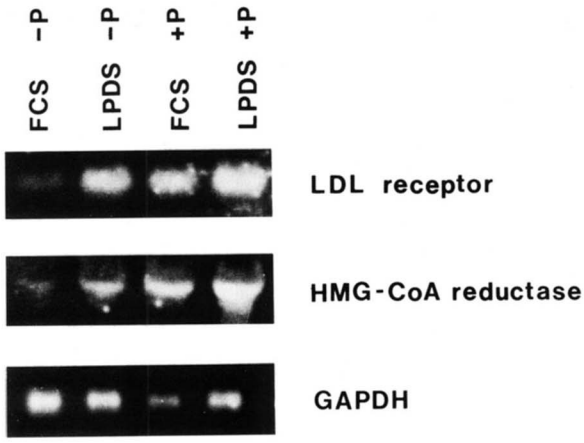

Fig. 2. Northern blot analysis of EPG85-257PR cells. Cells were incubated for 4 days in medium containing FCS or LPDS with or without 1000 μM pravastatin (P). Ten μg of total cellular RNA per lane was fractionated on 0.7% denaturing agarose gels, and blots were hybridized against Digoxigenin random prime labeled probes for LDL receptor, HMG-CoA reductase, and GAPDH as indicated.

two mRNA species when compared to sensitive cells under uninduced conditions (data not shown). Southern blot analysis did not reveal amplification of the HMG-CoA reductase gene (data not shown), a mechanism that has been observed in a Chinese hamster ovary cell line made resistant to a different HMG-CoA reductase inhibitor, compactin (20).

The cRT-PCR technique used in this study with deletion mutants as competitive templates provides several advantages when compared to other methods. First, although we routinely use 1 μg of total cellular RNA per reaction when measuring low-abundant mRNA species, this amount can be greatly reduced. The limitations of this method are determined more by the accuracy of reliably quantifying very low amounts of total cellular RNA than the amplification procedure itself. Much larger amounts of RNA are required for conventional techniques, which are also more time-consuming and do not allow the same precise quantification of mRNA species. Second, in contrast to non-competitive PCR methods, diverse parameters known to interfere with the efficiency of the reverse transcriptase and amplification reactions as well as plateau effects occurring at high cycle numbers can be neglected (21). Third, there is an important advantage in using as competitors deletion mutants rather than those with altered restriction sites. Frequently, heteroduplex molecules are formed between wild-type and mutant PCR products (22) during the course of competitive amplification reactions. When deletion mutants are used as competitors, heterodimers can be distinguished from homodimers on the basis of their altered gel mobilites. In cases where wild-type and mutant reaction products have relatively small differences in size, a good separation of bands can be achieved in polyacrylamide gels. We found that, due to their secondary structure, heteroduplex molecules have a much slower mobility than homodimeric DNA (compare Fig. 1a, blocks B and C) and therefore do not interfere with densitometric scanning procedures of the homodimeric products. Heteroduplices formed between PCR products derived from wild-type and deletion mutants therefore do not interfere with the determination of the equivalence point. Whereas, when point mutants with altered restriction sites are used, the analysis of cRT-PCR products requires restriction digests as an additional step after the amplification process in order to distinguish bands derived from wild-type or competitor RNA on gels. Such heteroduplices are, however, not susceptible to restriction digests, leading to an overestimation of the amount of uncleaved homodimeric DNA.

In summary, we present a new variation of cRT-PCR that allows the rapid determination and quantitation of the mRNAs for the LDL receptor, HMG-CoA reductase, and GAPDH without the need for radioactive compounds or time-consuming blotting and detection procedures. This sensitive method should enable us to analyze the expression of these two critical parameters of cholesterol metabolism not only in cell culture models, but also in vivo.

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