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Quantitation of mRNA by the polymerase chain reaction

(transcriptional phenotype/cytokine/macrophage/atherosclerosis)

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ABSTRACT **A method for the quantitation of specific mRNA species by the polymerase chain reaction (PCR) has been developed by using a synthetic RNA as an internal standard. The specific target mRNA and the internal standard are coamplified in one reaction in which the same primers are used. The amount of mRNA is then quantitated by extrapolating against the standard curve generated with the internal standard. The synthetic internal standard RNA consists of a linear array of the sequences of upstream primers of multiple target genes followed by the complementary sequences to their downstream primers in the same order. This quantitative PCR method provides a rapid and reliable way to quantify the amount of a specific mRNA in a sample of <0.1 ng of total RNA. In addition, the same internal standard RNA is used, with appropriate primer pairs, to quantitate multiple different mRNA species.**

Polymerase chain reaction (PCR) is a powerful tool to amplify small amounts of DNA or mRNA for various molecular analyses. It has been used for RNA blot analysis (1), nuclease protection analysis (2), and mRNA phenotyping (3) for the study of short-lived, low-copy number, mRNA transcripts. It has widespread applications in genetic disease diagnosis (4, 5), disease susceptibility (6, 7), and cancer diagnosis (1, 8). However, in most instances, the PCR technique has only provided qualitative results. The availability of quantitative PCR should provide valuable additional information for these and other applications.

Quantitative PCR analyses have been used to study the mRNA levels for dystrophin in different tissues (9) and for thymidylate synthase in tumors (10). However, these studies provide only relative comparisons of the amounts of mRNA. It has been difficult to quantitate the absolute amount of specific mRNA without an internal standard of known concentration. Because PCR amplification is an exponential process, the extent of amplification (N) is given by the equation $N = N_0(1 + \text{eff})^n$, where N_0 is the initial amount of material, eff is the efficiency, and n is the cycle number. Small differences in efficiency could lead to large differences in the yield of PCR product. Previous attempts to quantitate PCR amplification of mRNA sequences have involved the use of a relatively invariant mRNA such as β-actin or an unrelated template as an internal standard (9, 11). However, this approach provides only comparative data, in part because of differences in efficiency between the primer pairs for the standard and the target mRNAs. Ideally, target mRNA could be quantified most accurately by using an internal standard with the same sequence as the target itself. However, to control for "tube effects," the standard and the target RNAs must be amplified in the same reaction tube. Unfortunately, in this case, their PCR products cannot be distinguished. A second approach is to generate an allelic variant (e.g., a small deletion or insertion in the gene of interest) such that there is a small difference in the size of the PCR product of this internal standard and the PCR product of the native mRNA. Creation of a restriction enzyme site in the target gene is another method that would permit distinction between the PCR products of the standard and target RNAs. These approaches require that a new standard be constructed for each target gene. We report here a technique in which a synthetic RNA is used as an internal standard for quantitating the amount of specific mRNA by PCR. The technique involves coamplification of a target mRNA with the internal standard. This standard utilizes the same primer sequences as the target mRNA but yields a PCR product of a different size. The two PCR products can then be easily separated by gel electrophoresis after amplification. In the exponential phase of the amplification, the amount of target mRNA can be quantitated by extrapolating against the standard curve. In addition, the internal standard developed here contains the primer sequences for multiple genes so that the same standard can be used to quantitate a number of different mRNAs of interest.

To demonstrate its utility, this technique has been applied to the quantitation of the mRNA levels for several lymphokines in human monocyte-derived macrophages in response to stimulation with lipopolysaccharide (LPS) and to the expression of mRNAs in human atherosclerotic plaque tissue.

MATERIALS AND METHODS

RNA Preparation. A synthetic gene was constructed by a technique of oligonucleotide overlap extension and amplification by PCR. Our procedure was similar to the one recently published by Ho *et al.* (12) for use in site-directed mutagenesis. After construction, the synthetic gene was subcloned into an Okayama–Berg vector containing the T7 polymerase promoter and a polyadenylylated sequence. This plasmid, pAW108, was used as a template for transcription by the T7 polymerase according to the transcription protocol of Promega Biotec. The resulting AW108 complementary RNA (cRNA) product was purified by oligo(dT) chromatography and quantitated by absorbance at 260 nm. Total cellular RNA was isolated from macrophages and tissues by the method of acid guanidium thiocyanate/phenol/chloroform extraction (13).

Oligonucleotides Used for Amplification. Oligonucleotides were synthesized on a Biosearch DNA synthesizer. Most of the primers are RNA-specific primers. The 5′ primers spanned the junction of the first two exons and the 3′ primers spanned the junction of the next two exons. Alternatively, the 5′ primers spanned the junction of the first and second

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Abbreviations: PCR, polymerase chain reaction; LPS, lipopolysaccharide; cRNA, complementary RNA; IL-1α, interleukin 1α; PDGF, platelet-derived growth factor.

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exons, and the 3′ primers spanned the junction of the second and third exons. These sequences are shown in Table 1 (14–24).

Amplification Method. RNA was reverse transcribed into cDNA as described (25). A 10-μl reverse transcription reaction mixture containing 1 μg of total cellular RNA, 1.77 × 10^2–10^6 molecules of AW108 cRNA, 1× PCR buffer (20 mM Tris·HCl, pH 8.3/50 mM KCl/2.5 mM $MgCl_2$/100 μg of bovine serum albumin per ml), 1 mM dithiothreitol, 0.5 mM dNTP, 10 units of RNasin (Promega Biotec), 0.1 μg of oligo$(dT)_{12-18}$, and 100 units of BRL Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) was incubated at 37°C for 60 min, heated to 95°C for 5–10 min, and then quick-chilled on ice. PCR was performed at a final concentration of 1× PCR buffer/50 μM dNTPs/0.1 μM each 5′ and 3′ primers/1 × 10^6 cpm of ^{32}P-end-labeled primer/1 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin–Elmer/Cetus) in a total vol of 50 μl. The mixture was overlaid with mineral oil and then amplified with the Perkin–Elmer/Cetus 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. Oligonucleotides were labeled with [γ-^{32}P]ATP by using polynucleotide kinase, and unincorporated nucleotides were removed on a Bio-Gel P-4 column.

Quantitative Analysis. Ten microliters of each PCR reaction mixture was electrophoresed in 8% polyacrylamide gels in Tris borate/EDTA buffer. Gels were stained with ethidium bromide and photographed. Appropriate bands were cut out from the gel and radioactivity was determined by Cerenkov counting. The amount of radioactivity recovered from the excised gel bands was plotted against the template concentrations or the number of PCR cycles.

Northern Blot Analysis. RNA was electrophoresed in a 1.5% agarose gel containing formaldehyde and was transferred to a nitrocellulose filter in 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate). The blot was hybridized with 2 × 10^6 cpm of ^{32}P-end-labeled oligonucleotides per ml. Hybridization was for 4 hr at 55°C in 0.75 M NaCl/0.075 M sodium citrate, pH 7.0/20 mM sodium phosphate, pH 7.0/5 mM EDTA/200 μg of yeast RNA per ml/1% Sarkosyl (Sigma). The blot was washed in 1× SSC at 55°C for 30 min and was autoradiographed with intensifying screens at −70°C.

Macrophage Cultures. Human peripheral blood monocytes were isolated from buffy coat preparations by Ficoll/Hypaque gradient centrifugation followed by adherence to plastic for 1 hr. Adherent cells were then removed and replated at 10^6 cells per well onto six-well plates in RPMI 1640 medium supplemented with 2% fetal calf serum and 2000 units of recombinant macrophage colony-stimulating factor per ml (Cetus). After 10 days, half of the cultures were treated with 5 μg of LPS per ml (Sigma). All the cultures were harvested for nucleic acid isolation 5 hr later.

Human Tissue Samples. The carotid endarterectomy sample was obtained through the courtesy of Dale Strawn (Veterans Administration Hospital, Martinez, CA) during the course of a surgical operation and with the informed consent of the patient. The RNA preparation of a histologically normal coronary artery from a heart transplant recipient was a generous gift from the laboratory of Russell Ross (University of Washington).

RESULTS

Internal Standard AW108 cRNA. The AW108 cRNA was synthesized as a sense strand from pAW108 by T7 polymerase. The structure of the plasmid pAW108 is shown in Fig. 1. There are two functions of this AW108 cRNA involved in the quantitation method. First, it serves as an internal mRNA control for the reverse transcription reaction. Second, it is used to generate a standard curve for quantitating the specific target mRNAs from experimental samples. The plasmid encoding this synthetic cRNA has the 5′ primers of 12 target mRNAs connected in sequence followed by the complementary sequences of their 3′ primers in the same order (see Table 1). The PCR product from each primer set is 300–308 base pairs (bp) depending on the primer used and is designed not to overlap in size with the PCR products from these target mRNAs. The size difference between the PCR products permits easy separation of the cRNA product from the target mRNA product by gel electrophoresis. Linkers containing unique restriction enzyme recognition sites were placed after the set of 5′ primers and after the set of 3′ primers to allow insertion of additional pairs as needed. The unique *Bam*HI site is used to linearize the plasmid to produce run-off transcripts. The AW108 cRNA contains a polyadenylylated sequence at the 3′ end to facilitate purification of the *in vitro* synthesized cRNA and to permit reverse transcription together in the same reaction with the target mRNA. Since the same primers are used in the PCR amplification of both templates, there are no primer efficiency differences between the standard and the target RNAs. When dilution series of mixtures of the target mRNA and AW108 cRNA are coamplified in the same tube and the reaction is terminated in the exponential phase of amplification, the amount of target

Table 1. Oligonucleotides of 5′ primers and 3′ primers of 12 target genes

mRNA species	5′ primers	3′ primers	Size of PCR product, bp	
			mRNA	cRNA
TNF	5′-CAGAGGGAAGAGTTCCCCAG-3′	5′-CCTTGGTCTGGTAGGAGACG-3′	325	301
M-CSF	5′-GAACAGTTGAAAGATCCAGTG-3′	5′-TCGGACGCAGGCCTTGTCATG-3′	171	302
PDGF-A	5′-CCTGCCCATTCGGAGGAAGAG-3′	5′-TTGGCCACCTTGACGCTGCG-3′	225	301
PDGF-B	5′-GAAGGAGCCTGGGTTCCCTG-3′	5′-TTTCTCACCTGGACAGGTCG-3′	217	300
apoE	5′-TTCCTGGCAGGATGCCAGGC-3′	5′-GGTCAGTTGTTCCTCCAGTTC-3′	270	301
LDL-R	5′-CAATGTCTCACCAAGCTCTG-3′	5′-TCTGTCTCGAGGGGTAGCTG-3′	258	301
HMG	5′-TACCATGTCAGGGGTACGTC-3′	5′-CAAGCCTAGAGACATAATCATC-3′	246	303
IL-1α	5′-GTCTCTGAATCAGAAATCCTTCTATC-3′	5′-CATGTCAAATTTCACTGCTTCATCC-3′	420	308
IL-1β	5′-AAACAGATGAAGTGCTCCTTCCAGG-3′	5′-TGGAGAACACCACTTGTTGCTCCA-3′	388	306
IL-2	5′-GAATGGAATTAATAATTACAAGAATCCC-3′	5′-TGTTTCAGATCCCTTTAGTTCCAG-3′	222	305
PDGF-R	5′-TGACCACCCAGCCATCCTTC-3′	5′-GAGGAGGTGTTGACTTCATTC-3′	228	300
LPL	5′-GAGATTTCTCTGTATGGCACC-3′	5′-CTGCAAATGAGACACTTTCTC-3′	277	300

TNF, tumor necrosis factor (14); M-CSF, macrophage colony-stimulating factor (15); PDGF-A, platelet-derived growth factor A (16); PDGF-B (17); apoE, apolipoprotein E (18); LDL-R, low density lipoprotein receptor (19); HMG, 3-hydroxy-3-methylglutaryl coenzyme A reductase (20); IL-1α, interleukin 1α (21); IL-1β (21); IL-2 (22); type β PDGF receptor (23); LPL, lipoprotein lipase (24).

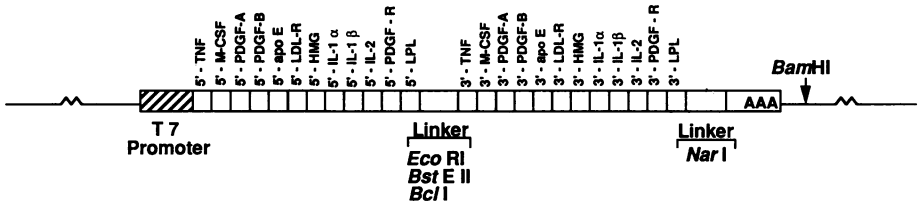

FIG. 1. Structure of pAW108. The plasmid contains 5′ primers of 12 target genes connected in sequence followed by the complementary sequences of the 3′ primers in the same order. Restriction enzyme linkers are placed after the set of 5′ primers and after the set of 3′ primers. The multiple primer region is flanked upstream by the T7 polymerase promoter and downstream by polyadenylylated sequences.

mRNA can be determined by extrapolating against the AW108 cRNA standard curve.

Quantitative Analysis. As an example of this approach to mRNA quantitation, we have used the AW108 internal standard to determine the amount of interleukin 1α (IL-1α) mRNA isolated from LPS-induced cultures of human macrophages. Two different protocols were used to conduct this analysis. (*i*) Protocol A: 50 ng of total macrophage RNA and 1.77×10^6 molecules of AW108 cRNA were combined and then reverse transcribed into cDNA. Serial 1:3 dilutions of one-tenth of the cDNA mixture were amplified by using the IL-1α specific primers listed in Table 1. ^{32}P-end-labeled 5′ primer (1×10^6 cpm) was included in the amplification. Reaction products were resolved by gel electrophoresis and visualized by ethidium bromide staining (Fig. 2*A*). The amounts of radioactivity recovered from the excised gel bands were plotted against the template concentrations (Fig. 2*B*). In this experiment, target mRNA and AW108 cRNA were amplified after serial 1:3 dilutions, and the results demonstrate that the method can resolve <3-fold differences in RNA concentrations. The fact that the reaction rates of AW108 cRNA and IL-1α mRNA amplification are identical within this exponential phase of the PCR reaction allows construction of a standard curve for AW108 cRNA and extrapolation to a copy number for the IL-1α mRNA present in the macrophages. As shown in Fig. 2*B*, 1 ng of LPS-induced macrophage total RNA and 1×10^4 molecules of AW108 cRNA gave the same amount of IL-1α PCR product. In other words, 1 ng of LPS-induced macrophage RNA contained 1×10^4 molecules of IL-1α mRNA. (*ii*) Protocol B: 500 ng of total macrophage RNA was reverse transcribed with 1.77×10^6 molecules of AW108 cRNA. Aliquots containing one-tenth of the cDNA mixture each were subjected to 14, 16, 18, 20, 22, 24, 26, or 28 cycles of amplification under the same conditions as in protocol A. The amounts of radioactivity recovered from the excised bands were plotted as a function of the number of cycles (Fig. 2*C*). The rates of amplification were exponential between 14 and 22 cycles for both templates. At 24, 26, and 28 cycles, the rates decreased drastically and approached a plateau (data not shown). The efficiencies of amplification were calculated from the slopes of these curves and found to be 88% for both AW108 cRNA and IL-1α mRNA. Since the amplification efficiency was the same for both coamplified targets within the exponential phase, the amount of IL-1α mRNA can be calculated by comparison with the AW108 cRNA internal standard. The amount of IL-1α mRNA in 1 ng of LPS-induced macrophage total RNA calculated by this method was 1.1×10^4 molecules. Thus, the results using either of these two alternative protocols for quantitation are the same.

Northern Blot Analysis. The amount of IL-1α mRNA in LPS-induced macrophages determined by the quantitative PCR method was verified by Northern blot analysis. The PCR analysis (see above) demonstrated that 1 ng of macrophage RNA and 1×10^4 molecules of AW108 cRNA produced the same amount of IL-1α PCR product. Thus, 5 μg of macrophage RNA and 5×10^7 molecules of AW108 cRNA should give similar signal intensities by Northern blot analysis. Serial dilutions (1:2) of macrophage RNA and AW108

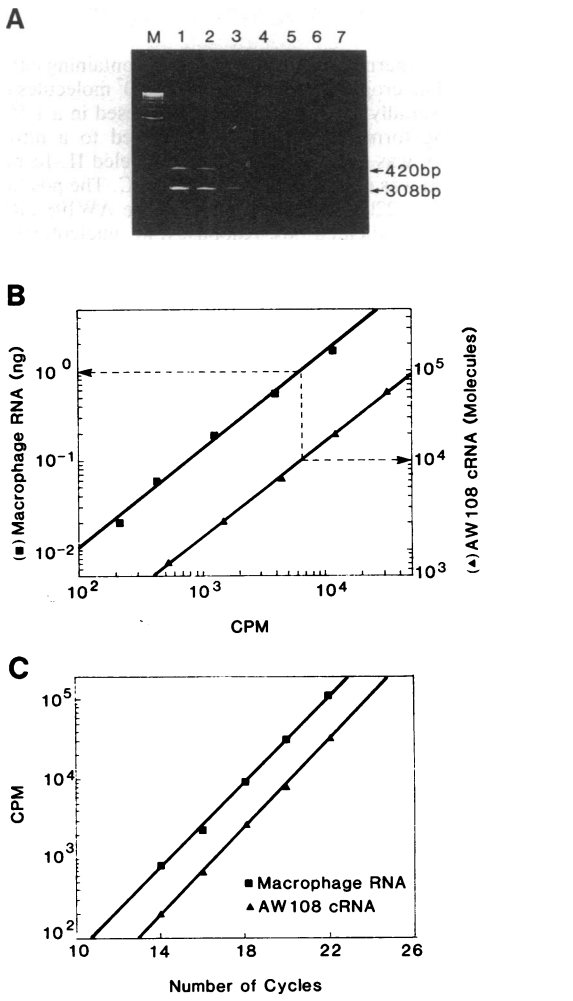

FIG. 2. Quantitative analysis of IL-1α mRNA levels in LPS-induced macrophages. (*A*) Ethidium bromide staining of PCR products separated in 8% polyacrylamide. Lanes: M, molecular weight markers; 1–6, IL-1α PCR products from serial 1:3 dilutions of a mixture containing 5 ng of LPS-induced macrophage total RNA plus 1.77×10^5 molecules of AW108 cRNA after amplification for 25 cycles; 7, control reaction without template and amplified for 25 cycles. The 420-bp products from macrophage RNA and the 308-bp products from AW108 cRNA are indicated. (*B*) Bands shown in *A* were cut out from the gel and radioactivity was determined by Cerenkov counting. The variable template concentrations of the internal standard AW108 cRNA and macrophage total RNA were plotted against the radioactivity of their PCR products. (Data from gel lane 1 were not plotted.) (*C*) The IL-1α PCR products of a mixture containing 50 ng of LPS-induced macrophage total RNA plus 1.77×10^5 molecules of AW108 cRNA, quantitated by counting the radioactivity of the excised gel bands, were plotted against the number of amplification cycles.

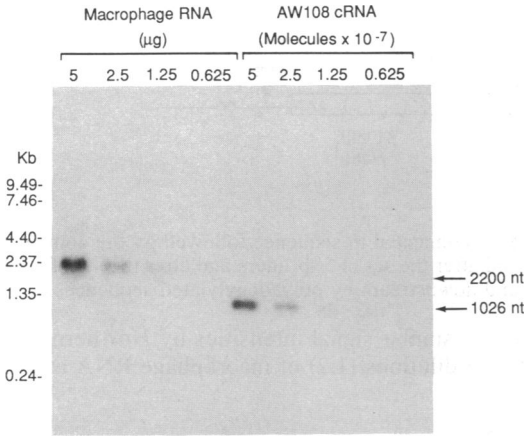

FIG. 3. Northern blot analysis. Samples containing either 5 μg of LPS-induced macrophage total RNA or 5×10^7 molecules of AW108 cRNA were serially diluted 1:2, electrophoresed in a 1.5% agarose gel containing formaldehyde, and transferred to a nitrocellulose filter. The blot was hybridized with a ^{32}P-labeled IL-1α probe. The autoradiogram was exposed for 72 hr at −70°C. The positions of the IL-1α mRNA (≈2200 nucleotides) and of the AW108 cRNA (1026 nucleotides) are indicated. kb, Kilobases; nt, nucleotides.

cRNA were subjected to Northern blot analysis by probing with the IL-1α 3′ primer. The sizes of the target RNA molecules were estimated to be ≈2200 nucleotides for IL-1α mRNA in macrophages and 1026 nucleotides for AW108 cRNA. Positive hybridization signals of equal intensities were detected at all the dilutions of macrophage RNA and AW108 cRNA as shown in Fig. 3. This result demonstrates that the amount of mRNA estimated by the quantitative PCR method correlates with the results of Northern blot analysis.

Quantitation of Specific mRNAs in Untreated and LPS-Induced Macrophages. A major advantage of this PCR quantitative technique is the ability to analyze several target mRNA species in parallel. Table 2 shows the results from quantitation of the expression levels of six cytokine mRNAs in human macrophages in response to LPS treatment. Quantitative analysis in the exponential reaction phase was performed by using serial dilutions of the cDNA reaction mixtures and PCR amplification for 25 cycles. The levels of IL-1β and IL-1α mRNAs, after LPS induction, increased ≈50-fold. The levels of mRNAs for platelet-derived growth factor type A (PDGF-A), macrophage colony-stimulating factor, and tumor necrosis factor increased 5- to 10-fold. However, the PDGF-B mRNA level remained constant for control and LPS-treated cells. This approach produces a detailed, yet multifaceted, picture of the transcriptional phenotype in both the resting and the induced states and uses only fractions of micrograms of total RNA.

Quantitative Analysis of Normal and Atherosclerotic Human Blood Vessels. Because accurate quantitative results can be obtained by this PCR technology even with small amounts of material, it provides an important tool for the analysis of samples that are in limited quantity. As an example, Table 3 compares the results of quantitation of six different mRNA species from a human atherosclerotic carotid artery with those of a normal coronary artery. The data obtained from the quantitative analysis show a 3- to 5-fold enhancement in the level of PDGF-A and -B mRNAs, no change in the type β PDGF receptor, and a 3-fold decrease in the low density lipoprotein receptor in the atherosclerotic vessel. There were increases in the levels of IL-1α and IL-1β mRNAs in the diseased tissue. Histologically, the atherosclerotic tissue showed a fibrous cap, some neovascularization, and areas of calcification and cholesterol clefts.

Table 2. Specific mRNA levels (molecules per cell) in LPS-induced and uninduced human macrophages

mRNA species	Uninduced	Induced	Induced/uninduced
IL-1α	1.4	69	49
IL-1β	51	2950	58
PDGF-A	0.05	0.48	10
PDGF-B	0.47	0.47	1
M-CSF	0.06	0.47	8
TNF	1.8	8.4	4.7

Molecules per cell = molecules per μg of RNA (calculated as described in Fig. 2) × μg of RNA isolated per cell. Monocyte-derived macrophages were cultured for 10 days; 5 hr prior to harvest, half of the cultures were exposed to 5 μg of LPS per ml. M-CSF, macrophage colony-stimulating factor; TNF, tumor necrosis factor.

Table 3. Specific mRNA levels (molecules per μg of total RNA) in a normal and in an atherosclerotic blood vessel

mRNA species	Atherosclerotic	Normal
PDGF-A	1.8×10^5	3.3×10^4
PDGF-B	7.6×10^4	2.2×10^4
PDGF-R	1.1×10^4	1.4×10^4
LDL-R	4.0×10^3	1.3×10^4
IL-1α	1.0×10^2	ND
IL-1β	6.4×10^4	1.0×10^2

mRNA levels were calculated as described in Fig. 2. ND, not detectable; LDL-R, low density lipoprotein receptor.

DISCUSSION

The quantitative PCR method presented here can accurately determine the amounts of low abundance specific mRNAs in very limited amounts (<0.1 ng) of sample. By reverse transcription and amplification of the target mRNA and AW108 cRNA internal standard in the same tube, variable effects due to differences in sample preparation, conditions of the reverse transcription, or the PCR amplification are internally controlled and will affect the yield of PCR product equally for the target mRNA and the standard cRNA. The synthetic internal standard contains multiple primer sets, which allows this single cRNA standard to be used to quantitate a number of different mRNAs in parallel.

Accurate quantitation of mRNA levels by the techniques described here is affected by the efficiencies of both cDNA synthesis and PCR amplification. Differences in nucleotide sequences, length of poly(A) tails, and the distance between the PCR primers and the poly(A) tail are among the factors that may affect the efficiency of cDNA synthesis of the target mRNA and the AW108 cRNA. The agreement between the results of the Northern blot (Fig. 3) and the PCR quantitation (Fig. 2 *B* and *C*) indicates that there were no apparent differences in the cDNA synthesis and the PCR amplification efficiencies for IL-1α mRNA and the AW108 cRNA. A similar correlation of results was found with the quantitation of IL-2 mRNA in T cells (unpublished data). Although there were no problems for either of these two mRNA species, possible differences in the efficiency of cDNA synthesis should be tested for each target mRNA.

There are many variables that could influence the efficiency of the PCR amplification. Some of the parameters that can be easily controlled are the concentrations of template, dNTPs, $MgCl_2$, primers, polymerase, and the PCR cycle profile. However, differences in primer efficiency are difficult parameters to regulate for quantitative analyses. The concentration of AW108 cRNA required to yield an exponential reaction phase by using different primer sets and the same PCR amplification conditions varied over a range of several orders of magnitude. For instance, the IL-1β primers were 10^5-fold more efficient than the apoE primers (unpublished data). Thus, it is critical to use the same primers for

amplification of the target mRNA and the internal standard in any attempts to quantitate mRNA expression by PCR.

It has been shown that the amount of an amplified DNA fragment in a given sample has a prevailing influence on the amplification efficiency (26). When a high template concentration is used or occurs as a result of the PCR amplifications, phenomena such as the substrate saturation of enzyme, product inhibition of enzyme, incomplete strand separation, and product strand reannealing can be limiting factors for efficient amplification. Accordingly, to obtain reliable quantitative evaluation of specific mRNA by using this internal standard, the range of concentrations for both of the templates as well as the number of amplification cycles should be such that the reactions remain within the exponential phase.

Smith *et al.* (27) have used an RNA dot blot assay to quantitatively assess the expression level of the two IL-1 mRNAs in human macrophages. Comparison of their results with the data in this paper shows that the levels of IL-1β in LPS-treated macrophages agree within a factor of 2. However, the level of IL-1α mRNA in uninduced macrophages was below the limits of detection of their RNA dot blot assay, while it was readily measured by the quantitative PCR method. As shown here in Fig. 2 *B* and *C*, the PCR quantitation technique can readily measure 10^4 molecules, which is 1000 times more sensitive than the dot blot assay. Moreover, the sensitivity of this method could easily be increased by using higher specific activity ^{32}P-labeled primers or more cycles of amplification.

Our results on the quantitative PCR analysis of human atherosclerotic lesions and normal blood vessels, while obviously preliminary and limited in sample number, are instructive in emphasizing the sensitivity and power of this approach to investigating the biology of cells and tissues *in vivo*. The observation of increased PDGF-A and -B chain expression in the diseased vessel is supported by and extends the work of J. Masuda and R. Ross (personal communication) and of Barrett and Benditt (28). Barrett and Benditt demonstrated a 5-fold increase in PDGF-B gene expression in lesion samples. Our work showed a 3.5-fold increase. Their results suggested that PDGF-A gene expression might be enhanced (29). Our data demonstrate that PDGF-A mRNA levels were 6-fold greater in this diseased tissue than in the normal one. The increased levels of IL-1α and -β mRNAs in the atherosclerotic tissue are intriguing and, if confirmed, could provide additional evidence for an inflammatory or immunological component of the pathogenesis of this disease. Information about the relative cellularity and the types of cells present in the samples is critical for comparative analysis of different lesion tissues. One significant problem encountered in the quantitative analysis of such small samples is the determination of the amount of total RNA. Preliminary observations indicate that quantitative PCR analysis to measure the amount of a specific genomic DNA target (e.g., HLA-DQα) can be performed directly on dilutions of the guanidium thiocyanate-treated sample. Therefore, by quantitating the copies of HLA-DQα DNA in the sample, the results of quantitative mRNA analyses can be expressed on a per genome basis.

Due to its sensitivity, speed, and accuracy, the quantitative PCR method can be used to study gene expression in a more extensive way than has been possible to date, allowing quantitative measurements of gene expression in a very small number of cells and from small amounts of tissue samples available from *in vivo* sources. This technique can also provide information on changes in expression levels of specific RNA molecules, which may be valuable in the diagnosis and analysis of, for example, cancer, metabolic disorders, and autoimmune diseases.

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