Differential regulation of macrophage scavenger receptor isoforms: mRNA quantification using the polymerase chain reaction

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Abstract There are two isoforms of the macrophage scavenger receptor (MSR I and II). Both are expressed on macrophages and mediate internalization of oxidized lipoproteins and several other ligands. MSR expression is regulated by cytokines but the individual regulation of each isoform is not well documented. We have therefore developed a PCR method to quantify mRNA levels of MSR isoforms. The analysis is based on relating the amount of reverse transcribed and amplified human macrophage MSR transcripts to a synthetic internal standard, using a ^{32}P-labeled 5′- primer to allow quantitation of the products. Each MSR isoform and its corresponding standard amplified with equal efficiency and the amount of MSR mRNA could be determined from 1 to 100 ng of total RNA. Using this method, we estimated that each monocyte-derived macrophage contains 10–130 molecules of MSR I and 30–640 copies of MSR II mRNA. Both isoforms were down-regulated by bacterial endotoxin (LPS), but the effect was more pronounced for MSR II transcripts. However, cycloheximide induced a selective degradation of MSR I transcripts, leaving MSR II levels unaltered. This suggests that both transcriptional and posttranscriptional control mechanisms are important in the regulation of MSR expression.—**Dufva, M., A. Svenningsson, and G. K. Hansson.** Differential regulation of macrophage scavenger receptor isoforms: mRNA quantification using the polymerase chain reaction. *J. Lipid Res.* 1995. **36:** 2282–2290.

Supplementary key words reverse transcription • lipopolysaccharide • lipoprotein receptors

Scavenger receptors are found on the surface of macrophages (1). By uptake of oxidized low density lipoproteins (oxLDL), the macrophage scavenger receptor (MSR) is believed to be the most important receptor mediating cholesterol accumulation in atherosclerosis (2). There are two isoforms of MSR (MSR I and II) with no, as yet discovered, differences in biological activity. They internalize particles that expose clustered negative charges on their surfaces, such as oxidized LDL and endotoxins (3, 4). The two MSR isoforms are produced by alternative splicing of a common transcript (ref. 5; **Fig. 1**). The first eight exons are shared by both isoforms and encode an intracellular domain, a transmembrane domain, an α-helical coiled coil domain, and a collagen coil-like domain. In addition, MSR II contains a short domain (6 amino acids) encoded by exon 9 in its C-terminal end. MSR I, in contrast, has a 110 amino acid-long cysteine-rich C-terminal domain that is encoded by exons 10 and 11 (6, 7). The regulation of each isoform is unknown in detail. There is, however, evidence that the ratio between isoforms changes during differentiation from monocyte to macrophage (8). The two isoforms are produced by alternative splicing, implying that the regulation of the respective isoform is posttranscriptional. A technique to quantify expression is, however, required to characterize the regulation of the MSR isoforms.

We have chosen to use the reverse transcription–polymerase chain reaction method (RT-PCR) to quantify MSR isoforms. One major advantage of quantitative RT-PCR is its sensitivity. As little as 10^4 target molecules can be measured with quantitative RT-PCR, implying a 1000-fold increase in sensitivity compared to a dot blot assay (9, 10). In contrast to other methods for quantifying mRNA expression, such as Northern blot and the nuclease protection assay, RT-PCR can be made independent of endogenous standards like "house-keeping genes" to normalize for differences between samples.

Abbreviations: MSR, macrophage scavenger receptor; PCR, polymerase chain reaction; LPS, bacterial endotoxin (lipopolysaccharide); oxLDL, oxidized low density lipoprotein; RT, reverse transcription.

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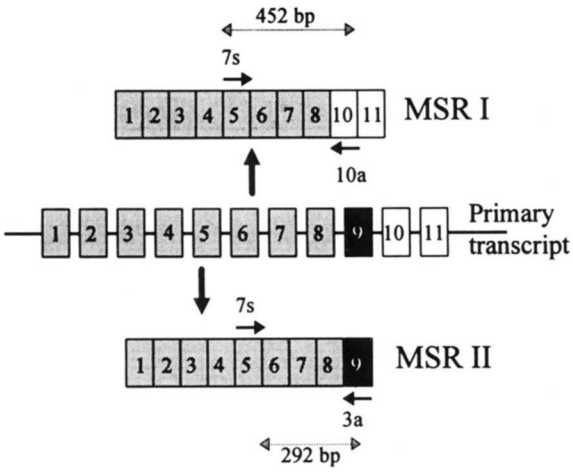

Fig. 1. Splicing pattern of MSR transcripts. The two isoforms are produced by alternative splicing. Gray boxes denote exons common to both isoforms while open boxes show MSR I-specific exons and black boxes show the MSR II-specific exon. Each isoform can be detected by reverse transcription–polymerase chain reaction using 7s together with 10a or 3a as primers (see Material and Methods). The obtained fragments differ in length (gray double-headed arrows), which makes it possible to detect both isoforms in the same RT-PCR.

PCR amplifies the product exponentially until lack of one of the reagents is limiting the reaction. It is assumed that during the exponential phase, *i)* the efficiency of amplification is equal between cycles, and *ii)* two targets with the same primer complementary sequences but different intervening sequences amplify equally well. A co-amplified standard using the same primer pair would therefore permit quantitation in absolute numbers.

We have designed an RT-PCR method for quantitative analysis of MSR mRNA and show *i)* that our standards amplify with the same efficiency as their respective MSR isoforms, *ii)* that quantitation of MSR I and II transcripts is possible at least within the range of 1 to 100 ng of total macrophage RNA, and *iii)* that twofold differences in MSR transcripts can be distinguished. In human monocyte-derived macrophages, both MSR isoforms showed a dose-dependent down-regulation by lipopolysaccharide (LPS). However, they differed significantly in sensitivity to LPS and to an inhibitor of protein synthesis. These data suggest that MSR isoforms are regulated both transcriptionally and at the posttranscriptional level.

MATERIAL AND METHODS

Synthesis and purification of oligonucleotides

All oligonucleotides were prepared on an Applied Biosystems DNA synthesizer (PCR-Mate, Applied Biosystems Inc., Foster City, CA) and purified on an oligonucleotide purification cartridge (OPC-column, Applied Biosystems) according to instructions from the manufacturer. The primer sequences for MSR I and II were as follows: 5′ common type I and type II sense primer (7s): TGG GAA CAT TCT CAG ACC TTG AG, 3′ type I-specific antisense primer (10a): TTG TCC AAA GTG AGC TGC CTT GT, and 3′ type II-specific antisense primer (3a): TGC CCT AAT ATG ATC AGT GAG TTG (ref. 11; Fig. 1). For use in quantitative RT-PCR, the 7s primer was end-labeled with [^{32}P]γ-ATP using T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany).

Construction of the internal standard pAM I

A plasmid was desired that contains multiple primer sites for the amplification products of interest under a prokaryotic promoter. This would allow for simultaneous transcription of both RNA standards and thus ensure equal concentrations of the two internal standards in the subsequent RT-PCR reaction. The plasmid pAW 108, which contains multiple cytokine primer sites under the T7 promoter (9), was obtained from American Type Culture Collection, Rockville, MD. New primer sites were introduced into pAW 108 by repetitive PCR reactions using oligonucleotides specific for existing sequences in the plasmid and tailed with the desired primer sequence to be introduced (**Fig. 2**). After the first two primer sites had been introduced, an aliquot was transferred to another tube with fresh PCR reagents and new oligonucleotides were added that were tailed with the next primer sequences to be introduced. Five new primer sites and new restriction enzyme sites were introduced in this way without the need for purification between the steps. The entire inserted fragment was purified through electrophoretic separation on an agarose gel followed by isotachophoretic purification of the excised band. Finally, the purified fragment was ligated into the pT7Blue T-vector (Novagen, Madison, WI) using the introduced restriction enzyme sites. The new construct was designated pAM I. The introduced primer sites were checked by PCR reactions using the specific MSR primers followed by detection on ethidium bromide-stained agarose gels, which yielded bands of the expected sizes (data not shown). Furthermore, the sequence of the manipulated fragment was confirmed by DNA sequencing on an Applied Biosystems model 373 DNA sequencer.

Macrophage cultures

Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats of human donor blood (Blood Center, Sahlgrenska Hospital) by Ficoll-paque (Pharmacia Biotechnology, Uppsala, Sweden) separation. The concentration and viability of PBMC were

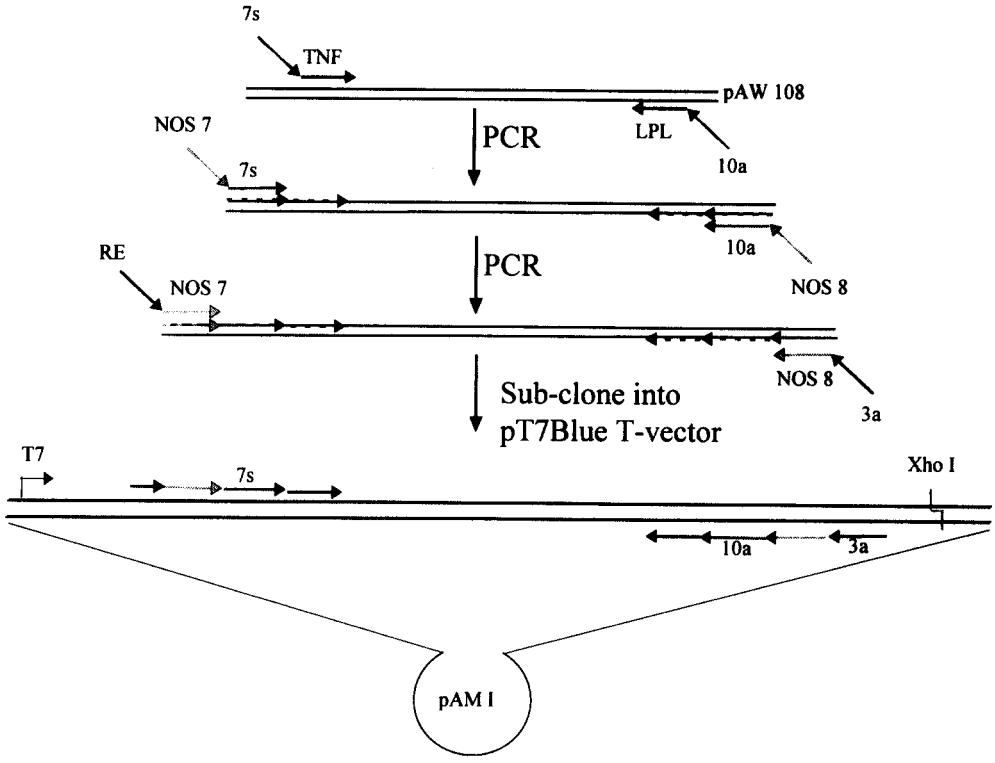

Fig. 2. Strategy for the production of the standard. In the first PCR, a part of the plasmid pAW 108 was amplified using primers that where tailed with the 7s and 10a sequences. Using an aliquot (10 μL) of the first PCR as a template, a second PCR was performed using 7s primers tailed with NOS 7 (sense primer to NO synthase: ACC AGC CTG ATC CAT GGT GG) and 10a primers tailed with NOS 8 (antisense primer to NO synthase: CAA CAT GCG GTG GAC ACC CTC). The third PCR introduced 3a in the 3′ end and a stretch of restriction sites (RE) in the 5′ end. To be able to clone the fragment into the pT7Blue T-vector, additional restriction enzyme sites were inserted in the 3′ end (not shown). Dotted arrows indicate reverse complementary sequences. When amplifying pAM I with primers to MSR I and MSR II, the obtained fragments were 300 bp and 352 bp, respectively, which is different from the fragments generated from each native MSR isoform (Fig. 1). Thus, the standard and MSR can be coamplified in the same tube and the resulting fragments are easily separated on a gel.

determined in a Bürker chamber after trypan blue staining. PBMC were plated into 80 cm^2 tissue culture flasks (NUNC, Roskilde, Denmark) at a density of 2×10^5 cells/cm^2 in RPMI-1640 culture medium (Gibco, Paisley, Scotland). The monocytes were allowed to adhere for 3 h at 37°C in 5% CO_2. Nonadherent cells were discarded and the monocytes were washed twice with PBS (140 mM NaCl, 8.6 mM Na_2HPO_4, 3.6 mM KCl, 2 mM KH_2PO_4, pH 7.2 at 20°C). The monocytes were allowed to differentiate into macrophages in culture medium (RPMI-1640 containing 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Gibco) and 5% heat-inactivated human serum (pooled serum from 50 donors, Blood Center, Sahlgrenska Hospital)) for 7 days at 37°C in 5% CO_2. The medium was changed after 3 days of culture. It always contained less than 68 pg/mL LPS, after as well as before culture.

In experiments where the macrophages were subjected to LPS or cycloheximide treatment, *E. coli* (serotype 0111:B4)-derived LPS (Sigma Chemical Company, St. Louis, MO) or cycloheximide (Sigma) was added 24 h before harvest. The final concentration of LPS used was 500 pg or 100 ng per mL and the final concentration of cyclohexamide was 10 μg/mL.

More than 95% of the cells were viable after 7 days of culture. LPS did not reduce viability of the macrophages but when using cycloheximide in the media, the viability was reduced to 80%.

Cell harvest and RNA preparation

The culture medium was poured off and the macrophages were washed once with PBS. They were harvested by addition of 1 mL guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.1 M 2-mercaptoethanol, and 0.5% *N*-laurolylsarcosine) to the tissue culture flasks followed by scraping the bottom with a rubber policeman. The RNA was extracted and precipitated as described (12). The obtained RNA pellet was washed twice with 75% ethanol and dried in a vacuum exsiccator. The RNA concentra-

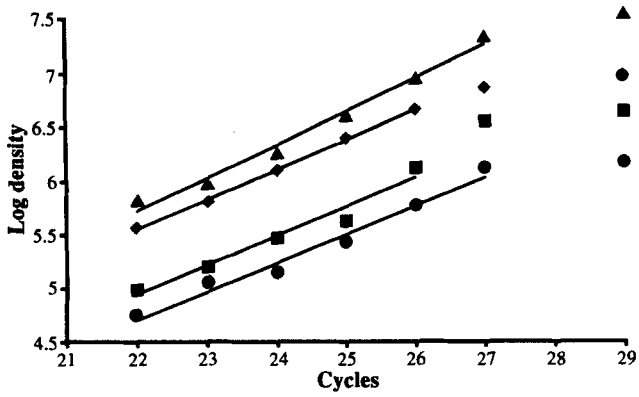

Fig. 3 Relationships between PCR cycles and product accumulation. Total macrophage RNA (67.5 ng) was spiked with 50,000 molecules of pAM I transcripts. The target and standard were reverse-transcribed and amplified together for 22-29 cycles. Linear regression was performed under conditions of exponential PCR amplification. MSR I (◆) and its standard (■) showed a linear amplification up to the 26th cycle whereas MSR II (▲) and its standard (●) were linear to the 27th cycle. After that, the accumulated products of MSR isoforms and their standards exhibited different kinetics of amplification, suggesting that the PCR had left the exponential phase.

tion was determined spectrophotometrically at A_{260}. The background absorbance, measured at A_{310}, was subtracted from A_{260} before calculation of the RNA concentration.

Production of pAM I cRNA

The pAM I plasmid was linearized by using the *Xho*I cleavage site 3′ of the 3a primer site (Fig. 2) to allow a run-off in vitro transcription. A 100-µL transcription reaction containing 5 µg pAM I linearized plasmid, 50 U of T7 RNA polymerase (Promega, Madison, WI), 100 U RNase inhibitor (Boehringer Mannheim), and 1X reaction buffer (500 µM NTP, 40 mM Tris-HCl, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, and 2 mM spermidine) was incubated at 37°C for 120 min. After transcription, the pAM I plasmid was removed by DNase treatment (5 U RQ I DNase (Promega)) for 15 min at 37°C. The RNA was extracted with one volume acidic phenol and 0.2 volume chloroform–isoamylalcohol 49:1 and precipitated with 2-propanol. The pellet was washed once with 75% ethanol, dried, and dissolved in RNase-free glass-distilled water. The amount of RNA recovered was determined spectrophotometrically.

Characterization of the pAM I transcript

One µg RNA was mixed with sample buffer (1.6% Ficoll, 6% formaldehyde, 47% formamide in MOPS buffer) and incubated for 3 min at 70°C, followed by quickly chilling on ice. The samples were run for 2 h at 80 V in a denaturing gel (1% (w/v) agarose MP (Boehringer Mannheim), 0.7% (v/v), formaldehyde, 145 ng/mL ethidium bromide in MOPS buffer (200 mM MOPS, 50 mM sodium acetate, and 10 mM EDTA, pH 7.0)).

Reverse transcription–polymerase chain reaction

This standard protocol was followed unless otherwise specified. Target molecules were mixed with a reverse transcription mixture (1 mM dNTP, 2 U/µL RNase inhibitor (Boehringer Mannheim), 2.5 µM random hexamers (Pharmacia), 2 U/µL M-MuLV reverse transcriptase (Boehringer Mannheim), 25 mM Tris-HCl, 20 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, and 10 µg/mL bovine serum albumin, pH 8.3, at 37°C) to a final volume of 10 µL. The target molecules were reverse-transcribed for 60 min at 42°C and the reaction was stopped by denaturation of the reverse transcriptase for 5 min at 95°C.

A master mix (PCR mix) was made with PCR reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3 (20°C)), 0.037 U/µL *Taq* DNA polymerase (Boehringer Mannheim), and 1.0 pmol/µL of each sense and antisense primer. Forty µL of the PCR mix was added to the reverse transcription mix and 60 µL of mineral oil was overlaid the reaction mixture. The PCR tubes (Perkin Elmer, Norwalk, CT) were immediately put into a prewarmed (95°C) Perkin-Elmer Cetus thermocycler. The following cycle profile of the PCR was used: denaturation at 95°C for 30 sec, annealing of primers at 60°C for 30 sec, and elongation at 72°C for 30 sec. In the first cycle, incubation times of 2 min instead of 30 sec were used at the described temperatures. The last elongation step was prolonged to 10 min. In cases where the reverse transcription step was omitted, the PCR mix was complemented with dNTP to a final concentration of 200 µM.

Quantitative RT-PCR

Target molecules were subjected to RT-PCR as described but the PCR mix was spiked with 10^6 cpm 5′-end ^{32}P-labeled 7s primer (25,000 cpm/pmol). The PCR fragments were separated on a sieving gel with 2% (w/v) NuSieve agarose (FMC, Rockland, ME) and 0.7% (w/v) agarose MP (Boehringer Mannheim). The gel was put on two sheets of 3MM Whatman filter paper, dried in a Model 583 gel dryer (Bio-Rad, Hercules, CA) at 55°C for 3–4 h, and exposed to a phosphor storage screen (Molecular Dynamics, Sunnyvale, CA) for 24–48 h. The screen was scanned in a Phosphor Imager (Molecular Dynamics) set at 700 V laser scanning voltage and 174 µm resolution. Images were analyzed with Image Quant software (IQ, Molecular Dynamics) and quantitation of the bands was performed with the "integrate volume" method.

Statistics

The differences between paired data were tested using either non-parametric or parametric tests. MSR mRNA expression varies greatly between individuals (Table 1). Wilcoxon-Mann-Whitney non-parametric significance test for unmatched data was used when analyzing data that could not be normalized for interindividual variations (Fig. 5). Student's *t*-test was used when testing differences between paired data which could be normalized to a control (Figs. 6A and 6B). Finally, linear regression was used in Figs. 3, 4B and 4C.

RESULTS

We have chosen to use a standard that contains primer sites homologous with elements of the MSR mRNA but that differs completely in other respects. The quantitation of a target with RT-PCR by using an internal standard requires that several parameters of the system are characterized. These are described below.

Production of cRNA copies of standard molecules

The pAM I plasmid was constructed to have primer sites identical to those found in the target (Figs. 1, 2). These primer sites were chosen to result in fragment sizes that differ between target and standard. In this way the standard and the target could be coamplified and distinguished by size.

The RT step can vary in efficiency and the standard should therefore be of cRNA type instead of cDNA. The standard fragment was transcribed in vitro using a T7 promoter located 5′ of the fragment. The obtained

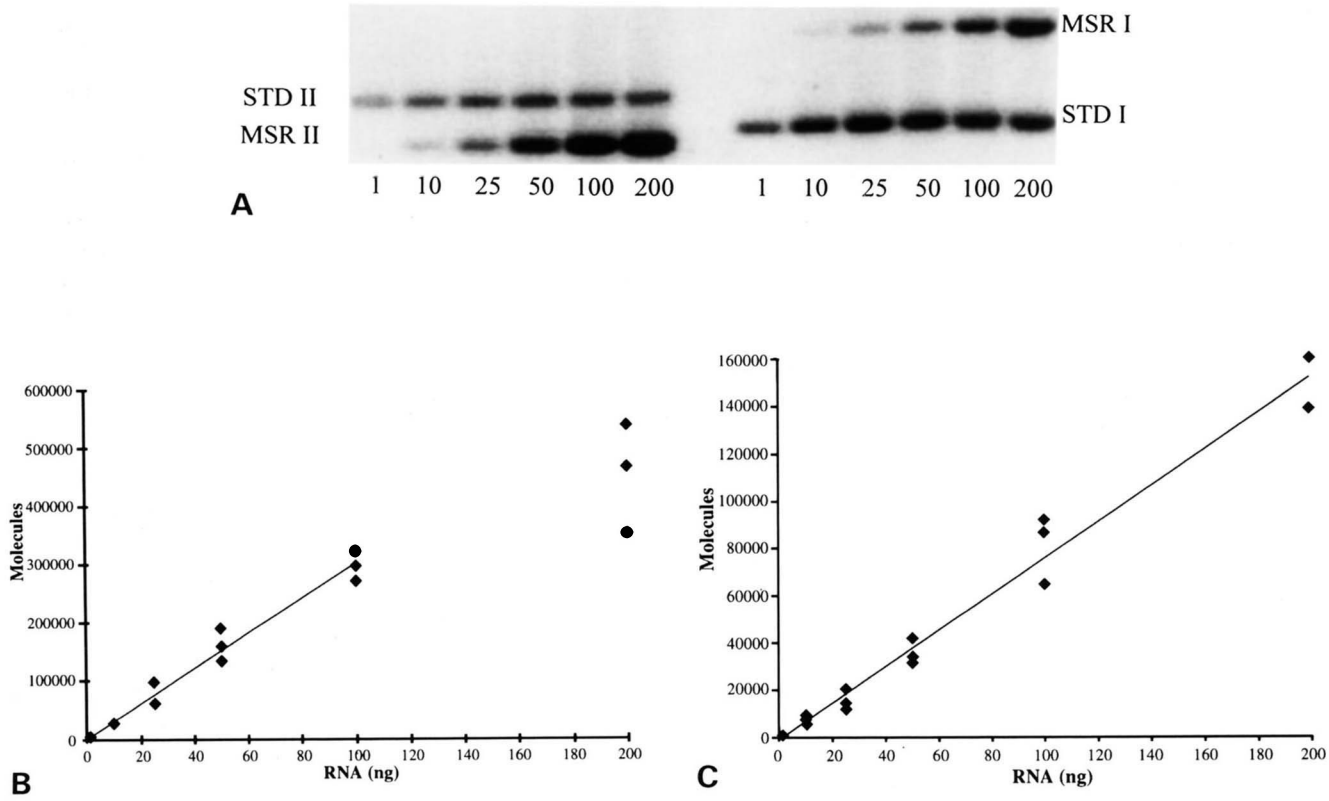

Fig. 4. Relationships between total RNA, PCR products, and the estimated number of specific mRNA copies. A: Autoradiogram of electrophoretic separation of PCR products of MSR I and II and their respective standards (STD I and STD II), Phosphor Imager visualization. A dilution series of macrophage RNA (1–200 ng) was spiked with 100,000 molecules of standard molecules and subjected to RT followed by 25 cycles of amplification. Graphs B and C show the correlation between input RNA and calculated number of molecules. The linearity of the relationship between input RNA and number of molecules was determined using linear regression. MSR I transcripts shows linearity from 1 ng to 200 ng of total RNA (r=0.98) (C) and the more abundant MSR II shows linearity from 1 ng to 100 ng of total RNA (r=0.99) (B).

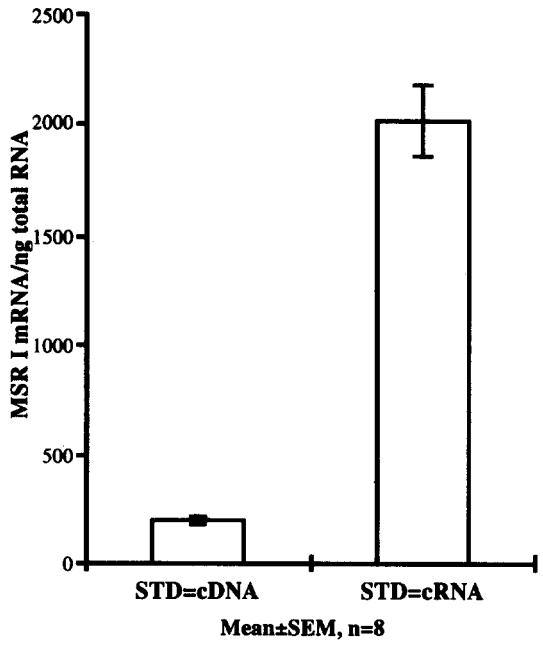

Fig. 5. Evaluation of the RT step. Total macrophage RNA was spiked either with cRNA as a standard prior to the RT step or with cDNA as a standard (linearized plasmid) after the RT step. The number of molecules was calculated as described (equation 4). The number of molecules of MSR I determined by using the cDNA standard was significantly lower than the number of molecules determined by using cRNA as a standard ($P<0.001$).

cRNA was quantified spectrophotometrically and its quality was evaluated by determining whether transcripts were full-length and whether any contaminating plasmid could be detected. On a denaturing gel, one sharp band was obtained at approximately 470 bp, corresponding to a full-length transcript of the cDNA insert (data not shown). No contaminating plasmids could be detected in a PCR using 7s, 10a and 3a primers (data not shown).

Evaluation of the efficiency of amplification

The product in a PCR accumulates exponentially according to equation 1:

$$P = M_{start} \cdot (1 + E)^n \qquad Eq.1)$$

Where P is the accumulated product, M_{start} is the number of starting molecules, E is the efficiency of the PCR, and n is the number of cycles. The PCR is in the exponential phase as long as the efficiency remains constant between cycles. Note that the PCR can be in the exponential phase although the efficiency is less that 100%. During the exponential phase, log P exhibits a linear relationship with n (eq. 2):

$$\log_{10} P = \log_{10} M_{start} + n \cdot \log_{10} (1 + E) \qquad Eq.2)$$

As long as E is constant, equation 2 will describe a line determined by $\log_{10} M_{start}$ as the intercept with the Y-axis and with $\log_{10} (1 + E)$ as the coefficient. Hence, equation 3 could be used to calculate the efficiency of the PCR as long as it is in the exponential phase.

$$E = 10^z - 1 \qquad Eq.\ 3)$$

Where z is the slope of the line. With an efficiency of 100% the product will be exactly doubled in each cycle. The slope of the line (z) will therefore have a maximum of log 2.

We determined these characteristics in our PCR system in the following way. An RT master mix including total RNA and cRNA transcripts was made to ensure that equal amounts of molecules were put in each tube. The cDNA obtained after the RT step was subjected to 22–29 cycles of amplification and the logarithms of the densities of the bands were plotted against the number of cycles as shown in **Fig. 3.** The slope of the linear regression line reflects the efficiency of amplification (eqs. 2 and 3). Thus, parallel slopes indicate equal efficiencies of amplification of the two targets. Four experiments were performed with the amount of standard ranging between 10,000 and 740,000 copies. Under these conditions, linear amplification could be identified within the investigated range of cycles. The efficiency of amplification varied between 75% and 100%. However, the efficiency ratio between the target and the standard was 1.00 ± 0.02 for MSR I and 1.06 ± 0.07 for MSR II (means ± SEM).

Determination of the detection range

Three dilution series of total RNA were spiked with pAM I and subjected to quantitative RT-PCR. One of the resulting gel images is shown in **Fig. 4A.** Although the bands derived from 1 ng were very weak for the eye, the Posphor Imager analysis clearly showed that the activity was above background.

The product of the RT-PCR is proportional to the band densities and the number of starting molecules can be calculated using equation 4. Besides being used for this purpose, equation 4 compensates for amplification efficiencies between tubes.

$$MSR_{Molecules} = STD_{Molecules} \cdot \frac{Density_{MSR}}{Density_{STD}} \qquad Eq.\ 4)$$

The calculated number of molecules shows a linear relationship with the total RNA input (ng) between 1 and 200 ng for MSR I and 1 and 100 ng for MSR II (Figs.

4B, C). Clearly, twofold differences of input molecules could be distinguished.

Evaluation of the RT step: comparison between cDNA and cRNA as a standard

The efficiency of the RT step cannot be assumed to be 100%. If the standard is omitted in the RT step and first provided in the PCR mixture, an efficiency less than 100% of the RT step would underestimate the number of target molecules. Notice that the density is affected not only by the efficiency of the PCR but also by the efficiency of the RT step. The latter was therefore investigated by using a cDNA or cRNA standard (**Fig. 5**). The estimated number of target molecules when using a cDNA standard was only a small fraction of that obtained when using a cRNA standard. Thus, the efficiency of the RT step was less than 100%.

MSR expression in macrophages: mRNA levels and LPS regulation

Monocyte-derived macrophages cultured for 7 days showed substantial variation in MSR expression. As indicated in **Table 1,** MSR I mRNA varied between 1043 and 12722 molecules per ng total RNA and MSR II mRNA between 2900 and 64131 molecules per ng total RNA. If one assumes that the average RNA content in a cultured macrophage is 10 pg/cell (13), then MSR I mRNA levels varied from 10 to 130 molecules per cell and MSR II levels from 30 to 640 molecules per cell.

TABLE 1. MSR mRNA levels in monocyte-derived macrophages from eight donors

	Buffy Coat	
Donor	MSR I	MSR II
	molecules/ng	
A	3172	5846
B	7051	64131
C	3456	12215
D	4896	12662
E	6471	24456
F	3409	10224
G	12722	23654
H	1043	2900

The number of molecules was calculated using equation 4.

The addition of *E. coli* LPS to the culture medium reduced mRNA expression significally (**Fig. 6A**). The LPS effect was dose-dependent and already detectable at a concentration of 500 pg LPS per mL medium. Both isoforms were reduced, although the relative effect on MSR II transcripts was larger than that on MSR I (Fig. 6A). For the latter, addition of cycloheximide abolished the effect of LPS, suggesting that LPS acts via a protein synthesis-dependent mechanism (Fig. 6B). Cycloheximide alone did not affect MSR II levels but reduced MSR I levels compared to control (Fig. 6B).

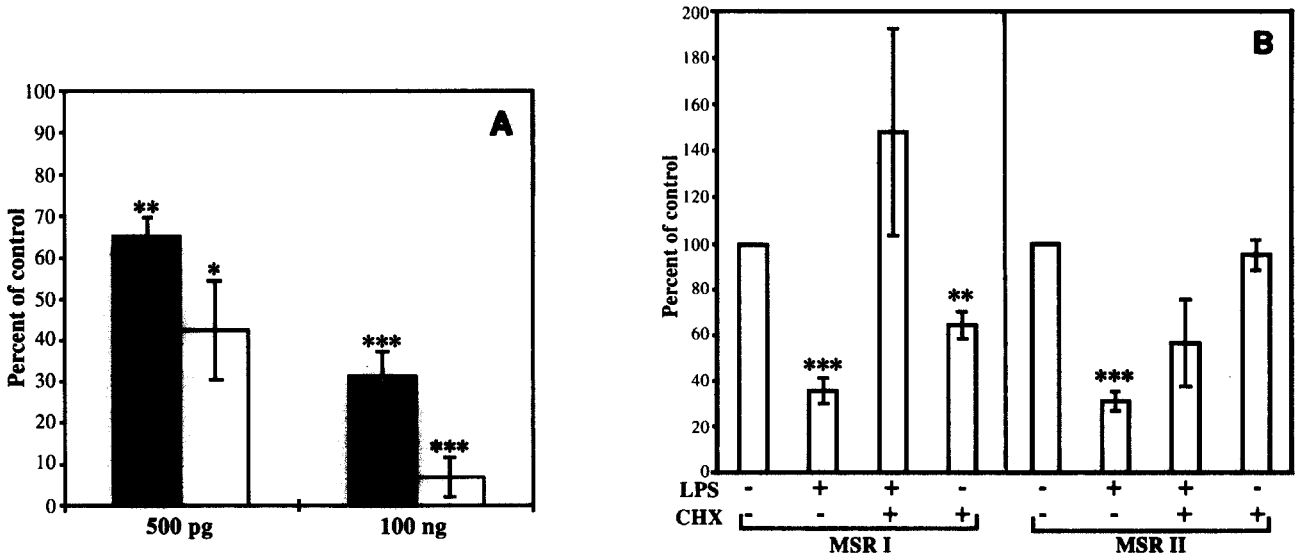

Fig. 6. Effects of LPS and cycloheximide on MSR levels. The bars represent the change in MSR levels compared to control (mean±SEM, n=4 (*, $P<0.05$; **, $P<0.01$; ***, $P<0.005$)). A: Dose dependency of LPS effects on MSR mRNA. At 48 h before harvest, LPS was added to the cultures at 500 pg or 100 ng per mL medium. At both concentrations of LPS, both isoforms showed a significant decrease in compared to control (100%). The effect of LPS was dose-dependent as both MSR I (■) and MSR II (□) transcripts showed a significantly reduced expression when treated with 100 ng LPS per mL medium compared to 500 pg LPS per mL medium ($P<0.001$ for MSR I and $P<0.01$ for MSR II). B: Effects of LPS (100 ng per mL medium for 24 h) and cycloheximide (10 μg per mL medium for 24 h) on MSR expression. MSR I mRNA levels were selectively reduced by cycloheximide treatment of macrophages. The difference of response to cycloheximide between the isoforms was significant ($P \leq 0.005$).

DISCUSSION

Several investigators have shown that PCR can be used to quantify DNA and mRNA-derived cDNA (9, 14, 15). Many settle with the fact that they can quantify changes in the relative amounts of RNA/DNA. Such semiquantitative analysis is usually backed up by the use of a "housekeeping gene", i.e., an mRNA that is assumed to remain in constant concentration under the different experimental conditions used in the study. This assumption is, however, often incorrect. Besides, using a housekeeping gene usually requires that two pairs of primers must be used. Such multiplex PCR can make the quantitation more complex. To avoid these limitations, an artificial standard with homologous primer sites can be used. This increases the likelihood that the standard and target amplify equally well (9, 16). If the standard is quantified in absolute numbers, the amount of target molecules can be quantified.

We have used cRNA copies of the standard to quantify MSR mRNA as a cRNA standard could compensate for efficiency differences in the RT step. Our data suggest that a cDNA standard underestimates the number of target molecules by a factor of 10. The low efficiency of the RT step could be compensated for if a cRNA is used. As in vitro transcription can result in a truncated cRNA, analysis of the cRNA on a denaturing gel was performed to verify that full-length transcripts were obtained. The assumption that the two targets amplify equally well in our system was confirmed by experimental data showing that efficiency changes affected the target and the standard equally much. This was further supported by the fact that the ratio between the efficiency of the standard and the target was 1.

The major strength of PCR is its sensitivity. The dilution experiments show that quantitation is possible from as little as 1 ng of total RNA. That corresponds to 100 macrophages if one assumes that a macrophage has 10 pg total RNA per cell. The sensitivity can be pushed even further by increasing the number of cycles of amplification but the PCR should always remain within the exponential phase. In our system, amplification was exponential up to 26–27 cycles and we have chosen to quantify at 25 cycles of amplification. The range of input molecules that could be quantified was derived from 1 ng to 100 ng of total macrophage RNA for MSR II and from 1 to 200 ng total RNA for the MSR I isoform. In conclusion, our data show that by using an artificial internal standard it is possible to quantify MSR mRNA in absolute numbers.

MSR mRNA expression was fairly low in monocyte-derived macrophages, with 10–130 transcripts per cell of MSR I and 30–640 MSR II copies per cell. The large variation among donors confirms previous observations using Northern blot (17) and suggests that individual-specific factors may be important in macrophage cholesterol metabolism.

MSR II transcripts were always more abundant than MSR I. As both isoforms are derived from the same gene, this suggests that posttranscriptional regulation is important for the regulation of isoform expression. This is also supported by our observation that LPS reduced MSR II transcripts more than the less abundant MSR I isoform. While the fact that LPS inhibited the expression of both species suggests a transcriptional LPS regulation of the MSR gene, the more prominent effect on MSR II points to an additional, posttranscriptional effect of LPS. It is thus possible that LPS affects the splicing pattern of the primary MSR transcript; alternatively, MSR II may be more susceptible to LPS-induced mRNA degradation than MSR I. Signals for mRNA degradation are found in the 3′ untranslated end of mRNA and it is noteworthy that the two MSR isoforms differ in this part of the mRNA molecule.

The effects of LPS on MSR I levels could be reversed by cycloheximide, suggesting that the down-regulation of MSR I transcripts is dependent on protein synthesis (Fig. 6B); this could not be confirmed for MSR II. Interestingly, the basal levels of MSR I, but not MSR II, seemed to be dependent on protein synthesis (Fig. 6B). This suggests that MSR I transcripts are stabilized by a protein that is normally expressed in cultured macrophages. LPS had an opposite effect on the MSR isoform ratio compared to cycloheximide, suggesting that there could be two pathways of MSR regulation: one utilized by LPS that preferentially degrades MSR II transcripts and an LPS-independent pathway that primarily degrades MSR I transcripts and is inhibited by a labile protein.

In conclusion, the effect of LPS on MSR expression appears to be complex and may involve both transcriptional and posttranscriptional regulation of MSR mRNA. The finding that MSR isoforms are differentially expressed, both during cellular differentiation (8) and in response to LPS and cycloheximide (this report) also emphasizes the complexity of regulation of this gene. As MSR is of paramount importance in foam cell transformation of macrophages during atherosclerosis, it will be necessary to analyze MSR expression under such pathological conditions. The RT-PCR method described here permits analysis of very small samples and should be useful when studying MSR expression in atherosclerotic plaques.

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