Rapid quantification of murine ABC mRNAs by real time reverse transcriptase-polymerase chain reaction

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Abstract   Several ATP-binding cassette (ABC) transporters are critically involved in cholesterol and phospholipid efflux, reverse cholesterol transport, and play an important role in the development of atherosclerosis. Quantification of ABC mRNA is important in studying the regulation of cellular cholesterol homeostasis and mechanisms related to the pathogenesis of atherosclerosis. We have developed a one-step real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method for measuring mRNA levels of ABCA1, ABCG1, and ABCA2 in murine tissues using the TaqMan™ technology. It has significant methodological benefits when compared to classic Northern blotting or semi-quantitative RT-PCR analysis. Using this method, we found high expression levels of ABCA1 in liver and macrophages, and of ABCG1 in the brain and macrophages. The expression of ABCA1 and ABCG1 were further induced in macrophages loaded with acLDL. In contrast, ABCA2 was expressed exclusively in the brain with low expression levels in the macrophages.██ This method provides a rapid, highly sensitive, specific, and reproducible quantification of ABC mRNA, and can be performed with nanograms of total RNA sample, thus making it a superior method for studying the regulation of ABC transporters in cholesterol efflux and its role in the pathogenesis of atherosclerosis in murine models.—Su, Y. R., M. F. Linton, and S. Fazio. **Rapid quantification of murine ABC mRNAs by real time reverse transcriptase-polymerase chain reaction.** *J. Lipid Res.* **2002.** 43: 2180–2187.

Supplementary key words   quantification of mRNA • TaqMan™ • cholesterol transport • macrophage foam cells

The ATP binding cassette (ABC) super family comprises a large number of membrane proteins, which transport a variety of substrates across the cell membranes (1). Several members of this gene family are involved in transporting cholesterol and phospholipid into or out of the cell. ABCA1 is thought to be critical in apolipoprotein A-I-mediated cholesterol and phospholipid efflux, and to play an important role in HDL maturation and reverse cholesterol transport (2–8). Mutations in ABCA1 lead to familial HDL deficiency and Tangier disease, a rare genetic disorder characterized by severe reductions in plasma HDL levels, accumulation of lipids in peripheral tissues and macrophages, and premature atherosclerosis (9–13). ABCG1 is the founding member of the ABCG family that consists of half-size ABC proteins with a high sequence similarity and expression pattern to ABCA1 (14). Upregulation of ABCG1 was observed exclusively during monocyte differentiation into macrophages and foam cell formation after acetylated LDL (acLDL) loading (15, 16). Over expression of ABCG1 was found in macrophages from patients with Tangier disease (17). Another member of the ABC gene family, ABCA2, was recently identified as a cholesterol-responsive gene in human macrophages. It is localized within a genomic region only 21 kb from ABCA1 and is also upregulated in human macrophages loaded with enzymatically modified LDL, suggesting a potential role for ABCA2 in macrophage lipid metabolism (18).

It has been shown that excess accumulation of cholesterol in macrophages can lead to foam cell formation and contribute to the development of atherosclerotic lesions. To elucidate the pathological pathways involved in cholesterol and lipid efflux in macrophages, it is important to quantify the expression of ABC transporters involved in this process. Several methods can be used for quantitative measurement of the mRNA level of a specific gene, including Northern blotting analysis, in situ hybridization, cDNA arrays, and reverse transcriptase-polymerase chain reaction (RT-PCR). However, quantitative RT-PCR offers a number of advantages over the other approaches, especially when dealing with small samples. The recent development of fluorescence-based 5′ nuclease assay (TaqMan™ technology) represents a significant advance in

Abbreviations: ABC, ATP-binding cassette; C_T, threshold cycle; RT-PCR, reverse transcriptase-polymerase chain reaction.

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mRNA quantification (17). It provides a highly sensitive, specific, reproducible, and accurate quantification of mRNA levels. Here we report the development of a real time RT-PCR method for the measurement of mRNA quantity of ABCA1, ABCG1, and ABCA2 in murine tissues and peritoneal macrophages. It is simple, accurate, and will have broad applications in future studies of the regulation of cholesterol efflux and the role of ABC transporters in the development of atherosclerosis.

MATERIALS AND METHODS

Total RNA preparation from murine tissues and peritoneal macrophages

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were maintained in microisolator cages on a rodent chow diet (4.5% fat; Purina Mills. Inc.) and autoclaved acidified water ad libitum. All the experimental protocols were performed according to the guidelines of Vanderbilt University's Institutional Animal Care Committee. Peritoneal macrophages were collected 3–4 days after injection with 3% thioglycollate intraperitoneally. Cells were plated in 6-well plates in DMEM media with 10% FBS and incubated at 37°C with 5% CO_2. After 4 h, the media was removed and replaced with DMEM-4% FBS (controls) or DMEM-4% FBS-50 μg/ml acLDL. Cells were cultured for 24 h before collection. Tissues were collected in RNAlater solution (Qiagen) followed by homogenization and cell lyses. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The integrity of the total RNA samples isolated from all tissues used in the real time RT-PCR assay was verified by 1.2% formaldehyde agarose gel. After staining with ethidium bromide, no degradation of the 28S and 18S RNA was observed.

Design and synthesis of TaqMan™ probes and oligonucleotide primers

The primers and probes (**Table 1**) were designed using Primer Express 1.5 Software (ABI) based on the sequence entries in the GenBank (GenBank accession numbers: X75926 for Mus musculus ABCA1, AF323659 for Mus musculus ABCG1, X75927 for Mus musculus ABCA2). The primers were designed to be located in two adjacent exons to avoid the amplification of potentially contaminating genomic DNA in the total RNA sample (**Fig. 1**). The information for intron/exon boundaries of mouse ABCA1, ABCG1, and ABCA2 was obtained based on alignment of mouse cDNA sequence against the human genomic se-
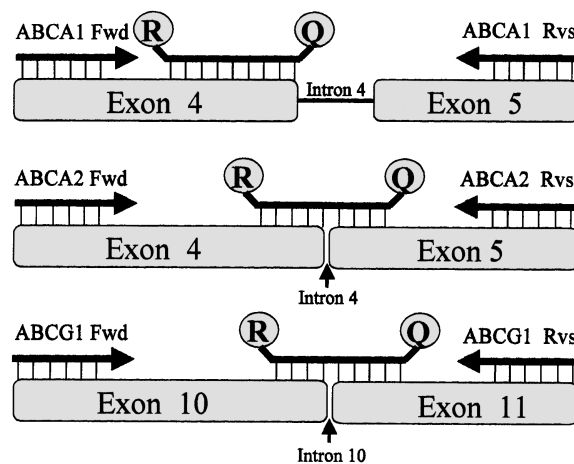
Fig. 1. Location of the primers and probes of the ATP-binding cassette (ABC) transports. The figure illustrates that the primer set for each ABC transporters are located in two adjacent exons with the TanMan™ probe situated in between the primers. R, reporter fluorescent dye; Q, quencher fluorescent dye; Fwd, forward primer; Rvs, reverse primer.

quence, and the genomic structure of the human sequence (17–19). The primers and TaqMan™ probe for 18S RNA was obtained from ABI. The probes for ABCA1, ABCA2, and ABCG1 were labeled with a reporter dye (FAM) on the 5′ end and quencher dye (TAMRA) on the 3′ end. The probe for 18S RNA was labeled with a reporter dye (VIC™) on the 5′ end and TAMRA on the 3′ end. The primers were synthesized by Invitrogen and probes were made by ABI.

One step RT-PCR amplification and data analysis

The one step RT-PCR reaction was achieved by using ABI Prism 7700 Sequence Detection System (ABI). The TaqMan™ one-step RT-PCR master mix reagent kit (ABI, P/N 4309169) was used for RT-PCR. The amplification of ABC transporters and 18S RNA were run in separate tubes using the same amount of total RNA retrieved from the same sample. The experimental conditions were extensively optimized for each ABC transporter. Reactions were prepared in 96-well optical grade PCR plate in a total of 50 μl containing the following components: 20 ng of total RNA, 25 μl of 2× TaqMan™ Universal Master Mix, 1.25 μl of 40× Multiscribe™ and RNAse inhibitors, 250 nM of probe, 600 nM of each primer for ABCA1 and ABCG1, 900 nM of each primer for ABCA2, 40 nM of each primer, and 50 nM of probe

TABLE 1. TaqMan™ probe and primer sequence

Target Gene	Primer/Probe Sequence	Length of Amplicon	Starting[a] Position in mRNA
ABCA1	Forward: 5′-GGACATGCACAAGGTCCTGA-3′	76 bp	452
	Reverse: 5′-CAGAAAATCCTGGAGCTTCAAA-3′		506
	Probe: 5′-6FAM-AATGTTACGGCAGATCAAGCATCCCAAC-TARMA-3′		473
ABCA2	Forward: 5′-CTGGCACCTGGGAGAGTCA-3′	73 bp	401
	Reverse: 5′-TGGCCACCGAGTCCAGAG-3′		449
	Probe: 5′-6FAM-TCAGCCAGACCTGCAGTTTCATCCTTC-TARMA-3′		421
ABCG1	Forward: 5′-CCTTCCTCAGCATCATGCG-3′	67 bp	1316
	Reverse: 5′-CCGATCCCAATGTGCGA-3′		1366
	Probe: 5′-6FAM-CTCGGTCCTGACACATCTGCGAATCAC-TARMA-3′		1338

[a] GenBank accession numbers: X75926 (Mus musculus ABCA1), AF323659 (Mus musculus ABCG1), X75927 (Mus musculus ABCA2).

for 18S RNA. Thermal cycling conditions consisted of an initial reverse transcription step at 48°C for 30 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C for both ABCA1 and ABCG1. The cycling conditions for ABCA2 were 40 cycles of 15 s at 95°C and 1 min at 58°C. The adjustment of baseline and threshold was performed according to the manufacturer's instructions.

Relative quantification of ABCA1, ABCA2, and ABCG1

The relative quantities of ABCA1, ABCG1, and ABCA2 message from all tissue samples used in the real time RT-PCR were normalized with 18S ribosomal RNA to compensate for variations in input RNA amounts. The data were analyzed using both relative standard curve method and comparative threshold cycle (C_T) method. (See details on ABI User Bulletin #2). In the relative standard curve method, serial dilutions of known amounts of total RNA sample isolated from mouse peritoneal macrophages were used to construct the standard curve for each of the ABC transporters as well as for 18S RNA. A linear amplification was obtained over a wide range of input total RNA (3 logs). For each unknown sample, the relative amount is calculated using linear regression analysis from their respective standard curves (**Fig. 2**). A relative ABC expression value was then obtained by dividing the value for the gene of interest (ABC) by the value for the 18S RNA. In the comparative C_T method, standard curves were eliminated. The relative quantification of ABC gene expression is calculated from the threshold cycle (C_T) values for each sample. The threshold cycle is the cycle at which a statistically significant increase in ΔR_n is first detected. Therefore, samples with higher initial template concentrations reach the threshold value at lower cycle numbers during PCR than samples containing lower initial template concentrations. The ΔR_n is the intensity of reporter dye emission of each reaction containing all components including the template (R_n^+), minus the fluorescent intensity of an unreacted sample (R_n^-). The value for R_n^- may be obtained from the early cycles of a real time run or from a reaction not containing template. The relative expression value of each ABC gene was obtained by evaluating the C_T values for the unknown samples using the equation $2^{-\Delta\Delta C_T}$. Briefly, ΔC_T for the calibrator and samples in each ABC gene expression assay were obtained by subtracting the VIC C_T value of 18S RNA from the FAM C_T value of the ABC gene. $\Delta\Delta C_T$ was calculated by subtracting the average $\Delta C_{T(calibrator)}$ values from the $\Delta C_{T (sample)}$. The relative quantification was then calculated by $2^{-Average \Delta\Delta C_T}$. The mRNA quantity for the calibrator is expressed as 1× sample and all other quantities are expressed as a number of fold differences relative to the calibrator.

RESULTS

The primer and probe sequences for ABCA1, ABCA2, and ABCG1 as well as their positions in the mRNA sequence are listed in Table 1. The location of the primers and probes for each of the ABC transporters are illustrated in Fig. 1. Representative amplification plots for each of the ABC transporters and 18S RNA are shown in **Fig. 3**. Each line represents a variable starting total RNA concentration (60 ng, 30 ng, 12 ng, 6 ng, 3 ng, 0.6 ng). The plot is shifted to the left with an increasing initial template concentration used for the reaction.
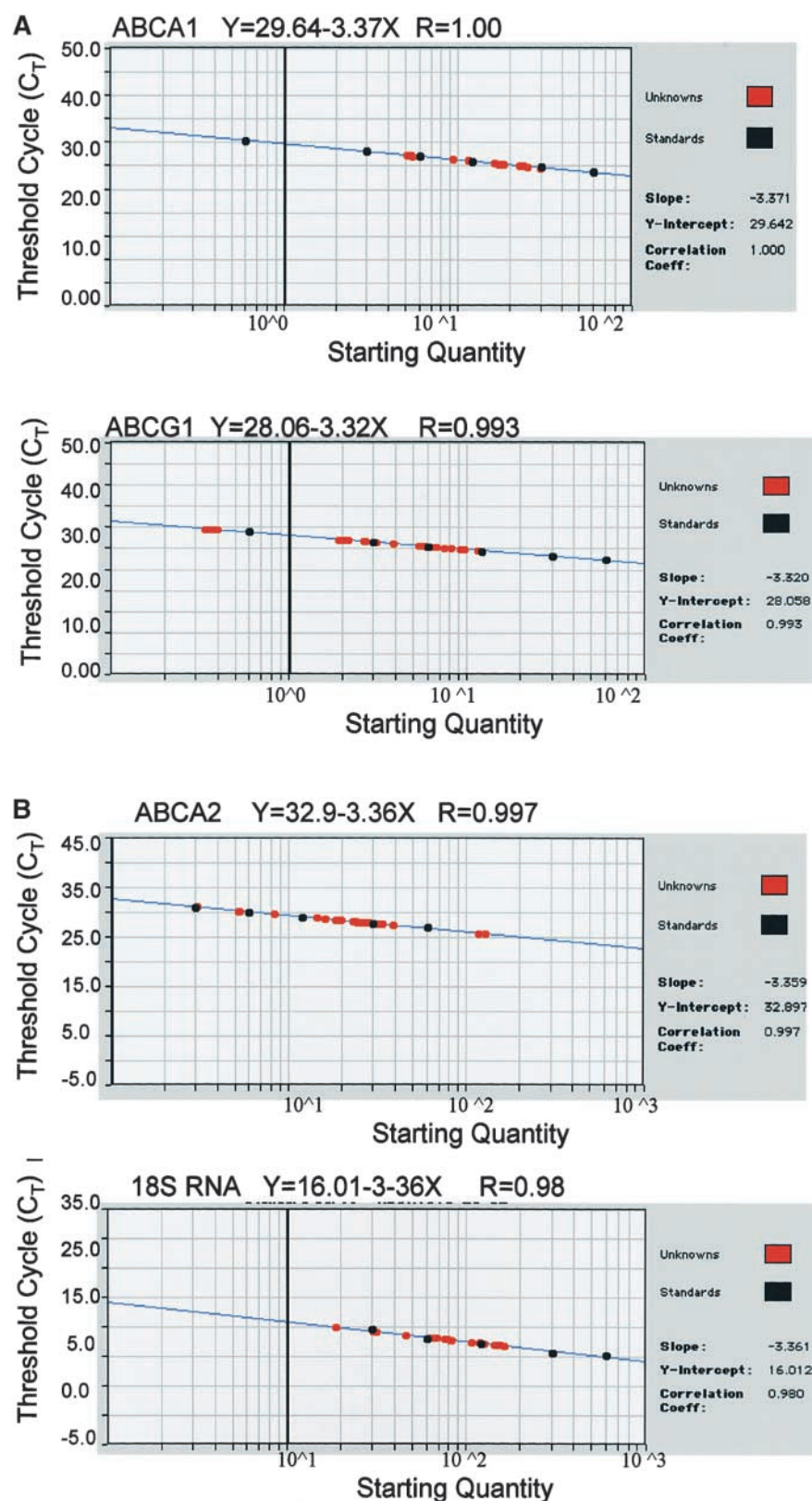
Relative standard curve

The standard curve for each of the ABC transporters is illustrated in Fig. 2. Serial dilutions of the total RNA sample isolated from mouse peritoneal macrophages were used to
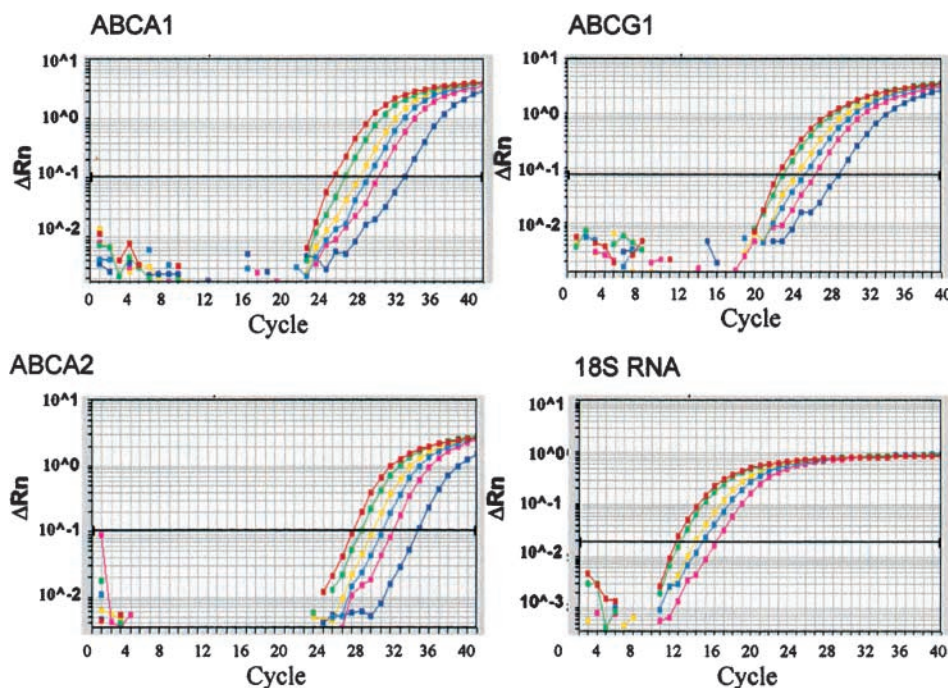
Fig. 2. Representative amplification plots of real time quantification of ABCs and 18S RNA. Serial dilution (60 ng, 30 ng, 12 ng, 6 ng, 3 ng, 0.6 ng) of total RNA isolated from macrophages was used for real time RT-PCR amplification. Each plot represents a variable starting total RNA concentration. The plot is shifted to the left with an increasing initial template concentration used for the reaction. ΔRn: Normalized reporter signal. ΔRn is the intensity of reporter dye emission of each sample containing all components minus the fluorescence intensity of reactions containing no template.

Fig. 3. Standard curves for ABCA1, ABCG1, ABCA2, and 18*S* RNA. The standard curves were generated by real time reverse transcriptase-polymerase chain reaction (RT-PCR) amplifying with 60 ng, 30 ng, 12 ng, 6 ng, 3 ng, and 0.6 ng of total RNA sample from macrophages. The threshold cycle (C_T) values are plotted against the logarithm of the input amount of total RNA. The slope for 18*S* RNA and ABC genes were about −3.3, indicating approximately equal amplification efficiency for both target gene and endogenous control. The C_T value in the regression equation inversely correlates with the sensitivity of the RT-PCR reaction.

construct the standard curve. Each standard curve was constructed as the log of the quantity of total RNA (*x* axis) versus the threshold cycle (*y* axis). All four standard curves give slopes close to -3.3, indicating similar amplification efficiency for both ABCs and the 18*S* rRNA. The relative quantity of ABCs is expressed as normalized to 18*S* rRNA.

Validation of the comparative C_T method

For the comparative C_T method to be valid, the efficiency of the target amplification and the efficiency of the reference (endogenous control) amplification must be approximately equal. One sensitive method for assessing this is to look at the relationship of ΔC_T value with respect to template dilution. If two amplicons have approximately equal amplification efficiency, the plot of log input amount versus ΔC_T has a slope of approximately zero. Validation experiments for ABCA1, ABCG1, and ABCA2 were performed to evaluate the amplification efficiency for target and reference. A relative efficiency plot of the log of total RNA (ng) verses ΔC_T of ABCA1, ABCA2, and ABCG1 was plotted and the absolute value of the slope was 0.038 for ABCA1, 0.087 for ABCG1, and 0.056 for ABCA2. As described in ABI user's bulletin #2, an absolute slope value <0.1 is considered ideal.

Intra- and interassay precision

The intra-assay (within-run) precision test was determined by calculating mean, SD, and coefficient of variation (CV) of the C_T value. Six replicates of total RNA sample from macrophages (12 ng) were measured for ABCA1, ABCA2, ABCG1, and 18*S* RNA. The mean of $\Delta C_{T(ABCA1)}$ was 15.96 ± 0.059 (SD) with CV of 0.37%; the mean of $\Delta C_{T(ABCG1)}$ was 12.37 ± 0.103 (SD) with CV of 0.83%; and the mean of $\Delta C_{T(ABCA2)}$ was 17.40 ± 0.095 (SD) with CV of 0.55%. The inter-assay (between run) precision test was performed on each of the ABC genes on four separate days, using four dilutions of total RNA (60 ng, 30 ng, 12 ng, 6 ng). The CV for ABCA1 (n = 16) was $<4\%$, for ABCG1 (n = 16) was $<6\%$, and for ABCA2 (n = 16) was $<5\%$.

Tissue expression levels of ABC transporter

High expression levels of ABCA1 and ABCG1 were observed in peritoneal macrophages (**Fig. 4A**, **B**) and the mRNA levels were further induced in macrophages loaded with acLDL. ABCA2 is primarily expressed in the brain, with low expression level in the peritoneal macrophages. AcLDL loading had no significant effect on ABCA2 expression in macrophages (Fig. 4C).

DISCUSSION

Quantification of mRNA levels is often required in studying mammalian gene expression. Several methods can be used for quantitative measurement of mRNA of a target gene, such as Northern blotting analysis, RNAse protection assay, in situ hybridization, and quantitative RT-PCR. In the past few years, a rapid development in methodology has made quantitative RT-PCR a sensitive and powerful tool for analyzing RNA. A number of applications have been described to quantify the PCR product. Semi-quantitative RT-PCR measures the product during the exponential phase of the reaction by interrupting the PCR after fixed number of cycles, and only provides a relatively small linear range. Alternatively, the competitive quantitative RT-PCR has been widely used for mRNA analysis; it requires co-amplification of a known amount of RNA sample or a "competitor" with the target gene in the same tube. The amount of mRNA in the target gene is measured by comparing to the known internal competitor or the known RNA sample added to the reaction. Although competitive quantitative RT-PCR provides better sensitivity and specificity compared to semi-quantification, it requires extensive optimization of the experimental conditions and is limited in its reproducibility and accuracy (20–22). The recent development of the fluorescence-based 5′ nuclease assay (TaqMan™ technology) represents a significant advance in mRNA quantitation (23). This approach is based on the 5′-3′ exonuclease activity of the Taq polymerase to cleave a dual-labeled probe annealed to a target sequence located between the two primers during the amplification. The release of the fluorogenic tag from the 5′ end of the probe is proportional to the target sequence concentration and can be measured "real time" in every PCR cycle. To date, TaqMan technology is the most rapid, reproducible, and accurate method for mRNA quantification.

Real time quantification of mRNA can be either absolute or relative. Absolute quantification is used to determine the copy numbers of a specific RNA per cell or unit mass of tissue. It usually requires additional steps to construct a standard curve with in vitro transcribed cRNA or single-stranded sense-strand oligodeoxynucleotides (21, 24). Relative quantification determines the difference in expression level of a target gene among different samples, and is adequate for the majority of gene expression studies. The method we described here is based on relative quantification, in which the quantity of each ABC transporter mRNA is expressed relative to a calibrator sample. Therefore, the calibrator becomes the $\times 1$ sample, and all other experimental sample quantities are expressed as n-fold difference relative to the calibrator. The data was analyzed by both Standard Curve method and Comparative C_T method (See ABI user bulletin #2 for detailed instruction). Similar results were obtained using both methods.

To validate the currently developed real time RT-PCR method, we have performed independent semi-quantitative RT-PCR for each of the ABC transporters using the same forward primer and different reverse primer further downstream of the cDNA sequence. The PCR product for ABCA1 was 512 bp (exons 4–8), for ABCG1 was 681 bp (exons 10–14), and for ABCA2 was 588 bp (exons 4–8). For endogenous control, a 488 bp 18*S* RNA was amplified using the same amount of total RNA as used for ABC
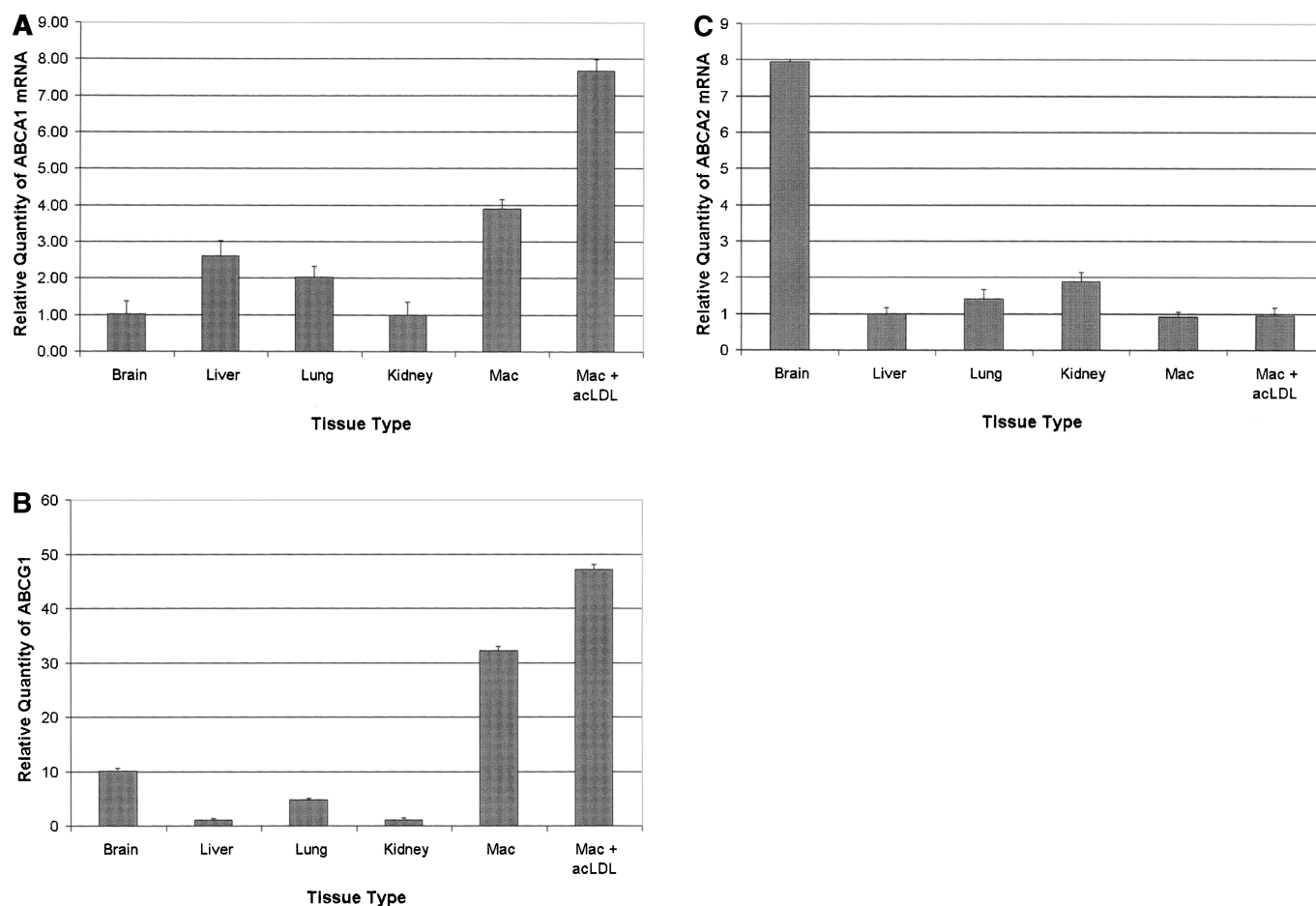
Fig. 4. Tissue expression patterns of murine ABC transporters. Tissues and peritoneal macrophages used to isolated total RNA samples were from C57BL/6 mice. Bars represent the mean and standard error of five independent assays. The ABCA1 mRNA expression level in each tissue was expressed as relative expression levels compared to the expression in the kidney. The ABCG1 and ABCA2 mRNA levels were relative expression levels compared to the expression in the liver. Results were analyzed using comparative C_T method. Similar results were obtained by the relative standard curve method.

transporters. The relative mRNA expression level was quantitated by densitometry. We used the same total RNA samples that were used to generate the real time RT-PCR data and found similar expression patterns using the two methods (data not show).

A reliable quantitative RT-PCR method requires correction for experimental variations, such as the amount of starting material used in individual reactions. To normalize the RT-PCR reaction for the amount of RNA input or the efficiency of reverse transcription, a housekeeping gene is usually used as internal control. An ideal housekeeping gene should be expressed at a constant level among different tissues of an organism at all stages of development, and its expression should not be affected by experimental treatment. The selection of controls for quantitative RT-PCR has been reviewed in detail elsewhere (21, 25). We have compared the use of GAPDH versus 18S RNA as an internal control for ABC mRNA quantification, and found that GAPDH is more variable, especially in samples treated with acLDL (data not shown). 18S RNA, on the other hand, is more consistent.

The occurrence of alternatively spliced transcripts of ABCA1 has been reported in the literature (26, 27). One alternatively spliced transcript, which was found in human skin fibroblasts, endothelial cells, smooth muscle cells, and HepG2 cell lines, completely skips exon 4 leading to a premature stop and a predicted translation product of 74 amino acids presumably lacking function. More splicing variations were observed by 5′ RACE PCR in HepG2 cells, with truncated version of exon 2 and lacking of exon 3 (1). The forward primer for the real time RT-PCR amplification of ABCA1 was sitting on exon 4, which will eliminate the amplification of the 74 amino acid truncated non-functional protein. The human ABCG1 gene structure has been characterized extensively (28). The gene consists of 23 exons spanning 98 kilobase pairs (kb). Alternatively, spliced transcripts were reported in regions upstream of exon 10. Human ABCA2 gene consists of 48 exons spanning 7.3 kb; no alternative spliced transcript has been reported. Because ABC transporters are larger membrane proteins, it is possible that more alternatively spliced transcripts may exist. It is important to keep in

mind the location of the primer set used in this method when quantifying ABC mRNAs in tissues or cell lines other than the tissues reported in this paper.

The tissue expression patterns of human ABCA1 and ABCG1 and ABCA2 have been reported using Northern blot analysis (1, 15, 29). ABCA1 is ubiquitously expressed. ABCG1, also a cholesterol-responsive gene, is highly expressed in human macrophages (14, 15). The highest expression of ABCA2 mRNA was found in human brain and was induced in human macrophages loaded with enzymatically modified LDL (18, 29). We observed similar expression patterns of all three ABC transporters in murine tissues. Interestingly, the ABCA2 expression in murine macrophages was relatively low, and no significant induction was found after acLDL loading. Human ABCA1 mRNA tissue distribution patterns have recently been reported using real time quantitative RT-PCR (30). Our results show that the tissue expression pattern of ABCA1 mRNA is the same in mice as has been previously reported in humans. Furthermore, because discordance between mRNA and protein expression levels has been observed in some tissues (31), studies of the regulation of ABC transporters should include measurements of protein expression levels as well.

The ABC transporters are critically involved in cellular cholesterol homeostasis and multiple drug resistance. This report describes a simple, reliable, and accurate mRNA quantification method that will provide a powerful tool for further investigation of the role of ABC transporters in regulating cholesterol efflux and their involvement in the development of atherosclerosis.⬛

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