

The Human ABCG4 Gene Is Regulated by Oxysterols and Retinoids in Monocyte-Derived Macrophages

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Here we report the induction of gene expression of ABCG4, a member of the ABC transporter subfamily G, from human macrophages by oxysterols and retinoids, agonists of the nuclear receptors LXR and RXR. The cloned ABCG4 transcript has a size of 3.5 kb and contains an open reading frame which encodes a polypeptide of 646 amino acids. Structurally, the putative ABC transporter protein consists of a nucleotide binding fold followed by a cluster of six transmembrane-spanning domains and thus conforms to the group of half-size ABC transporters. Among the human ABC transporter subfamily G members the novel transporter shows highest protein sequence homology and identity to ABCG1 (84 and 72%, respectively). Analysis of the genomic organization demonstrates that the ABCG4 gene is composed of at least 14 exons which extend across a region of 12.6 kb in size on chromosome 11q23.3. Based on its structural features and an LXR/RXR-responsive regulation similar to the cellular lipid export protein ABCA1, we conclude that ABCG4 may be involved in macrophage lipid homeostasis.

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Key Words: cholesterol; differential gene expression; ABCG4; ABCG1; macrophage; foam cell; LXR; RXR; oxysterol; ABCA1.

Sequence data from this article have been deposited with the EMBL Nucleotide Sequence Database under Accession Nos. AJ308237–AJ308251.

Abbreviations used: 22R-HOC, 22R-hydroxycholesterol; 9cRA, 9-*cis* retinoic acid; ABC, ATP binding cassette transporter; acLDL, acetylated low-density lipoprotein; ATP, adenosine triphosphate; HDL, high-density lipoprotein; HMDM, human monocyte-derived macrophages; LDL, low-density lipoprotein; LXR, liver X receptor; RXR, retinoid X receptor.

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ATP binding cassette (ABC) transporters constitute a complex family of multispan transmembrane proteins which translocate a variety of substrates across cellular membranes including ions, peptides, sugars, vitamins and steroid hormones (1–4). The subgroup of full-size ABC transporters is structurally characterized by two nucleotide binding folds (NBF) with conserved Walker A and B motifs, signature sequences, and two clusters of typically six membrane-spanning domains (3). Half-size transporters consist of one NBF and one cluster of six membrane-spanning domains (3). This group of transporters may form either a homo- or heterodimeric complex in its active state (5, 6). The specificity for the transported substrates is thought to be determined by the membrane-spanning domains, whereas the energy required for the transmembrane transport is provided by hydrolysis of ATP at the two NBF (4). For more than a decade, ABC transporters have been studied extensively due to their ability to mediate multidrug resistance in tumor therapy. During the past few years, however, substantial information has accumulated to suggest that a large portion of the ABC transporter gene family exerts critical functions in the cellular transmembrane transport of endogenous lipid substrates (7, 8). This notion is supported by work from our laboratory and others providing evidence that ABCA1, the prototypic member of the ABC A subfamily, is a principal regulator of HDL levels in the circulation and functions as a facilitator of cellular phospholipid and cholesterol export (9–11). In addition to ABCA1, the transporter ABCG1 has been implicated in macrophage cholesterol and phospholipid export (12). Moreover, the recent finding that mutations in the half-size transporters ABCG5 and ABCG8 cause β -sitosterolemia, a rare autosomal recessive disorder which is characterized by hyperabsorption of sterols, hypercholesterolemia, decreased biliary excretion of dietary sterols and premature coronary atherosclerosis (13), has convincingly demonstrated a role for ABC transporters in intestinal sterol

uptake (14, 15). Database analysis showed that within the human genome five ABC transporter G-family members are present and thus the gene of a new family member was annotated as ABCG4 (4, 8).

In the present study, we report the regulation of gene expression of ABCG4 by oxysterols and retinoids in a similar manner as ABCA1. We cloned an ABCG4 cDNA from human primary macrophages which extends the known nucleotide and peptide sequence to a similar size as reported for other family members. Moreover, we present the structure of the human ABCG4 gene.

MATERIALS AND METHODS

Preparation of acetylated low-density lipoprotein (acLDL). LDL ($d = 1.019\text{--}1.063$ g/ml) was isolated from plasma of normolipidemic volunteers by sequential ultracentrifugation (16) and acetylated as described by Brown and colleagues (17).

Cells. Human monocytes were obtained from healthy volunteers by leukapheresis and counter current elutriation (18). Since the apoE genotype affects apoE secretion from macrophages (18), we took care that all donors had the apoE3/3 phenotype. Purity of the monocytes was controlled by FACS analysis and amounted to >95%. 10^7 monocytes per 75-cm² flask were differentiated into macrophages by 12 days cultivation in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 20% autologous human serum (Biochrom, Berlin, Germany), 1% nonessential amino acids and penicillin (100 units/ml)/streptomycin (0.1 mg/ml) (Sigma, Deisenhofen, Germany) as antibiotics. For some experiments human monocyte-derived macrophages (HMDM) were converted into foam cells by loading with RPMI 1640 containing 80 μ g/ml acetylated LDL (acLDL), 1% non-essential amino acids and penicillin (100 units/ml)/streptomycin (0.1 mg/ml) for 48 h as indicated.

Lipid analyses. Macrophages were washed twice with PBS, 2 ml of isotonic NaCl solution was added and cells were scraped off the plate. The cell suspension was homogenized by ultrasonication and protein content of each sample was measured by Pierce BCA kit using BSA as a standard. Aliquots of 1 mg protein were saponified with ethanolic potassium hydroxide and subsequently extracted with hexane. Samples were separated on a reversed phase column by high performance liquid chromatography (HPLC) as described previously (19).

Quantitative real-time PCR assay. Cells grown in a T75 tissue culture flasks were washed twice with PBS and lysed in 2 ml RLT-buffer supplemented with β -mercaptoethanol (Qiagen, Hilden, Germany). Total RNA was extracted from the lysates using QIAshredder columns, RNeasy mini kit and RNase-free DNase set (Qiagen) according to the manufacturers protocol. Five micrograms of total RNA was reverse transcribed using Superscript II RT and oligo-dT primers (Gibco BRL, Karlsruhe, Germany).

For quantification of gene expression the qPCR-core kit for SYBR-Green with ROX as a passive control and uracil-*N*-glycosylase (UNG) (Eurogentec, Herstal, Belgium) was used. Measurements were performed with a GeneAmp 5700 sequence detection system (Applied Biosystems, Weiterstadt, Germany). Twenty-five-microliter reactions were assayed according to the manufacturer's guidelines (Eurogentec) with a final concentration of 300 nM primer, 1.5 mM magnesium chloride, and 62.5 ng reverse transcribed RNA. Melting curves were recorded and size and specificity of the PCR-products was checked on a 4% agarose gel. Only reactions which produced a single band of expected size were used for analysis. Amplification of correct sequences was checked by direct DNA sequencing of purified PCR products. The dynamic range of detection was checked by serial

dilution of cDNA samples and amplification was found to be linear over a range of six orders of magnitude. The following primers were used for amplification: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TCA AGA AGG TGG TGA AGC AG-3', 5'-TCG CTG TTG AAG TCA GAG GA-3'; ABCA1, 5'-CCC TGT GGA ATG TAC CTA TGT G-3', 5'-GAG GTG TCC CAA AGA TGC AA-3'; ABCG1, 5'-CAG TCG CTC CTT AGC ACC A-3', 5'-TCC ATG CTC GGA CTC TGT G-3'; ABCG2, 5'-CAC TGT TGC ACA GCA GCA A-3', 5'-CCA TGG TTA CTG TCT GAG GAG AT-3'; ABCG4, 5'-CCT GGA GTT CAG GAA CCA AC-3', 5'-GTG AAG ATG CCA GCA TGG AG-3'; ABCG5, 5'-GCC TTG AAT GCA ATG GAA GT-3', 5'-GGT CCG CTC AGT CAC AAT TT-3'; and ABCG8, 5'-GGA GCA CTG TGC CTA CAT CA-3', 5'-ACA CCA GCA GGA AGT GCA G-3'.

5' RACE. total RNA was isolated as described above. 5' RACE was performed using the 5' RACE kit according to the manufacturer's guidelines (Gibco BRL). Five micrograms mRNA was reverse transcribed using the ABCG4 specific primer GSP1 (5'-CTG TAC CAG TAG TTG AGG T-3'). The cDNA was poly-G tailed and the PCR was then performed with the ABCG4 specific primer GSP2 (5'-CTT TAG ATA GGG GAT CAG GTT GGT-3'), and the linker-specific anchor primer AUAP (Gibco BRL) and HotStarTaq (Qiagen). *Taq* was activated by 10 min at 94°C and 35 consecutive cycles of PCR were carried out (94°C for 20 s, 53°C for 30 s, and 72°C for 2 min) in a final 50- μ l volume. A nested PCR was performed with 0.5 μ l of the above PCR as template, the linker specific UAP primer and an ABCG4-specific primer GSP3 (5'-ACT CAG CTT CAG GTT AGC AGA GAC-3') using the same conditions as in the above PCR. PCR products obtained were purified on columns (Qiagen) and sequenced directly using the ABI PRISM BigDye Terminators v3.0 cycle sequencing kit (Applied Biosystems). Sequencing reactions were separated on an ABI-Prism 3700 DNA analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

In the present study, we report the cloning of the cDNA of human ABCG4, a member of the ABC transporter G-subfamily, from macrophages, its differential gene expression and its genomic organization.

Differential Gene Expression Analysis

We screened for ABC transporters of the G subfamily which, like ABCG1 and ABCA1, are induced by the heterodimeric transcription factors LXR and RXR (20, 21). This identified the ABCG4 transcript as the second most strongly induced member of this subfamily upon treatment of human monocyte-derived macrophages (HMDM) with 9-*cis* retinoic acid (9cRA) and 22R-hydroxy cholesterol (22R-HOC), as shown in Fig. 1. The most strongly induced member was ABCG1. ABCG2 is the only non-inducible family member.

For analysis of differential gene expression we developed a real-time polymerase chain reaction (real-time PCR) assay. The read-out of this assay is linear within a range of six orders of magnitude and within the experimental conditions used (not shown). We used primer pairs specific for human ABCG1, ABCG2, ABCG4, ABCG5, ABCG8, ABCA1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

We checked differential gene expression in response to lipid loading of the macrophages with acLDL, treatment with 22R-HOC and 9cRA or cholesterol deloading

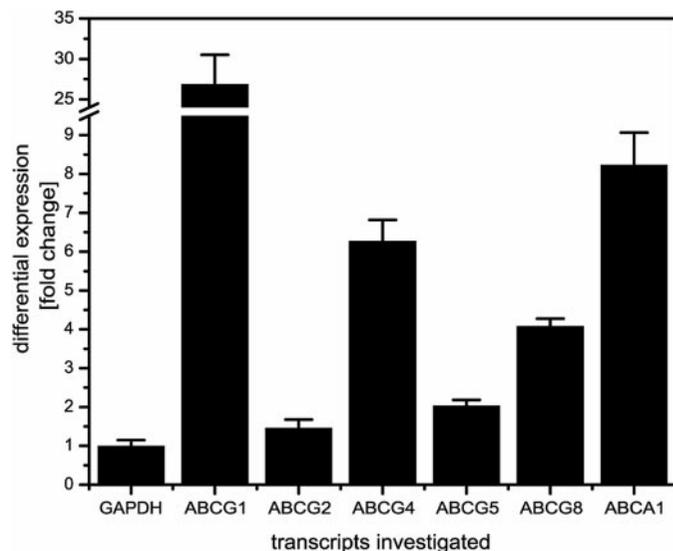


FIG. 1. Differential gene expression of human ABC transporter G-subfamily members. Expression levels of the indicated transcripts was measured in human monocyte-derived macrophages without or with treatment with a combination of 10 μ M 22R-hydroxycholesterol and 9-*cis* retinoic acid. Differential expression was measured by real-time PCR and calculated as fold changes, i.e., the ratio of expression levels of treated versus untreated samples normalized to the housekeeping gene GAPDH.

with cyclodextrin. As shown in Fig. 2, incubation of macrophages with acLDL increases the cellular free and esterified cholesterol content and incubation with cyclodextrin unloads cellular cholesterol and its esters, as expected. No treatment of the cells interfered with gene expression of GAPDH and thus the transcript of this enzyme can be regarded as a housekeeping gene and be used for normalization of the expression of the investigated genes. Expression of the ABCG4 gene is induced fivefold by treatment of the cells with LXR/RXR agonists like 22R-HOC and 9cRA. The change of expression levels is comparable to that observed for ABCA1 but approximately fourfold lower than that of ABCG1 (Fig. 2). Removal of cholesterol from macrophages mediated by cyclodextrin decreased ABCG4 message levels in a similar fashion to the decrease in message levels of ABCG1 and ABCA1. This behavior is common to oxysterol responsive genes. Lipid loading with acLDL increased ABCG4 transcript levels to a moderate extent.

ABCG4 cDNA and Predicted Polypeptide Structure

We amplified the 5'-end of the ABCG4-cDNA by RACE PCR using the sequence of AJ300465 for primer design. The cDNA of human ABCG4 (EMBL Nucleotide Sequence Database Accession No. AJ308237) has a length of 3.5 kb and the identified open reading frame codes for a polypeptide of 646 amino acids (AA) in length. Compared to GenBank entries AJ300465 and

NM_022169, the cDNA clone we report here is 12 base pairs longer and encodes a peptide that extends the reported ABCG4 sequence by 19 amino acids at the N-terminus.

The predicted ABCG4 polypeptide contains one nucleotide binding fold with the characteristic Walker A and B motifs and a signature sequence (Fig. 3) (4). The ABCG4 amino acid sequence predicts the existence of six transmembrane-spanning domains (TM) in the carboxy-terminal half of the peptide, a characteristic structural feature of ABC transporters of the G sub-

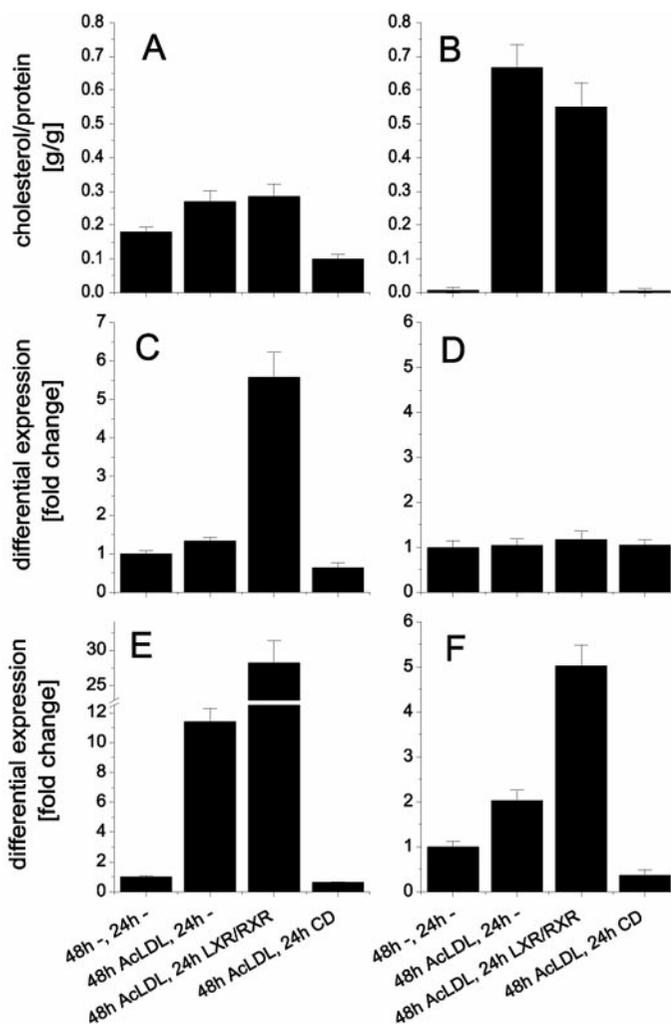


FIG. 2. Differential expression of the human ABCG4 gene. Human monocyte-derived macrophages were cultured in the absence (sample 1) or presence of 80 μ g acLDL (samples 2–4) for 48 h. Subsequently the cells were incubated for 24 h in the absence (samples 1 and 2) or presence of 10 μ M 22R-hydroxycholesterol and 9-*cis* retinoic acid, respectively (sample 3), or 4% cyclodextrin. Free cholesterol (A) and esterified cholesterol (B) were determined by HPLC. Differential expression of the ABCG4 (C), GAPDH (D), ABCG1 (E), and ABCA1 (F) transcript was measured by real-time PCR and calculated as fold changes, i.e., the ratio of expression levels of treated versus untreated samples normalized to the housekeeping gene GAPDH.

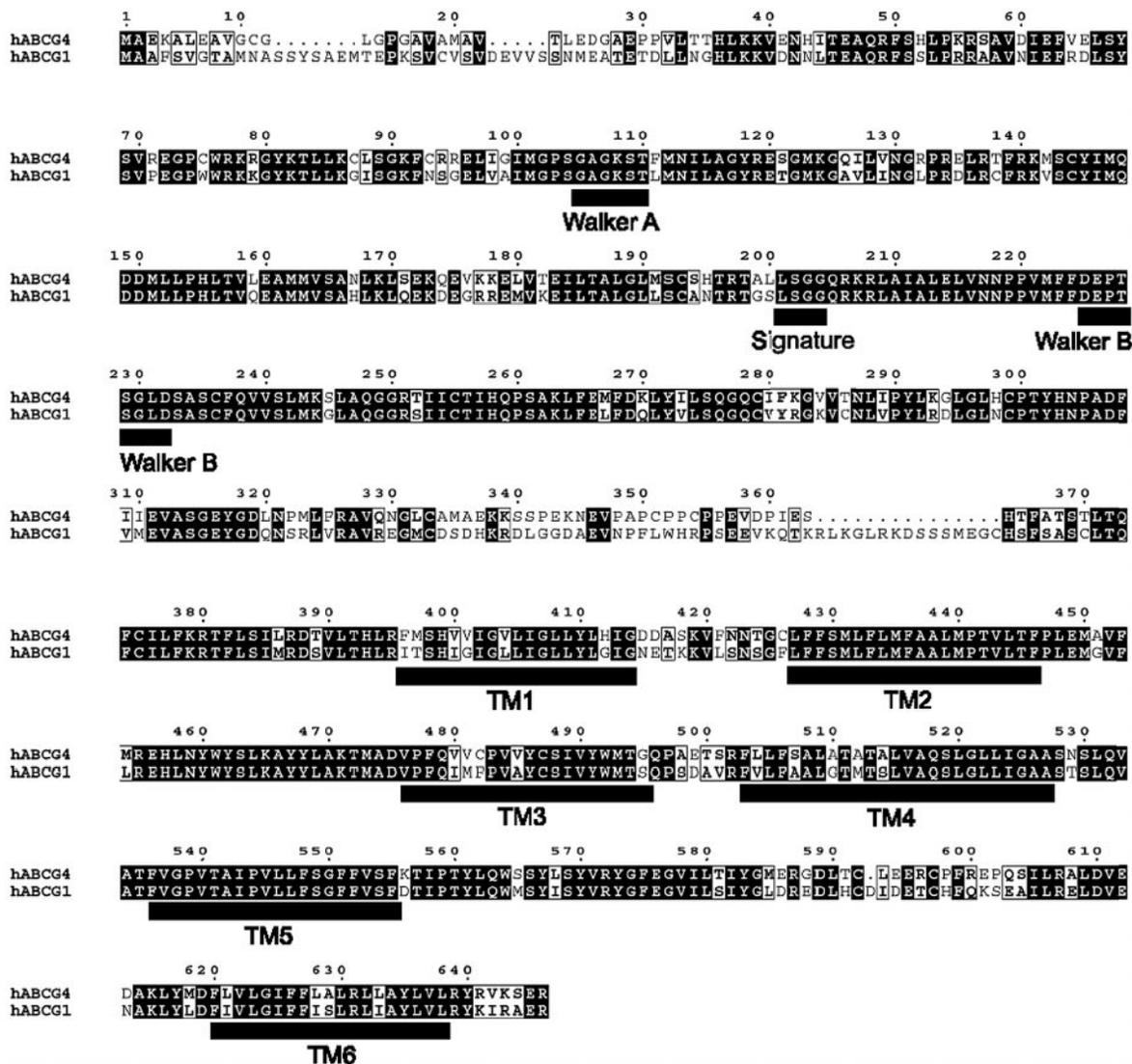


FIG. 3. ABCG4 homology plot. Human ABCG4 and ABCG1 protein sequence were aligned using the CLUSTAL tool. Alignments were formatted using ESPRIPT1.9 (http://www-pgm1.ipbs.fr:8080/cgi-bin/nph-ESPrIPT_exe.cgi). Amino acids are shown in single letter code, gaps due to optimal alignment are represented by dots. Identical amino acids are displayed in black boxes, homologous amino acids are boxed. Walker A, B, signature sequence and the six membrane-spanning regions are underlined.

family (4). The N-terminal extension of 19 aa reported here increases the length of the ABCG4 polypeptide to a size similar to that reported for other G-subfamily members (ABCG1, 666 aa; ABCG2, 655 aa; ABCG3, 650 aa; ABCG5, 651 aa; ABCG8, 673 aa) (14, 22–24).

Comparison of the predicted ABCG4 peptide sequence with known ABC half-size transporters of the G-subfamily revealed the highest degree of homology and identity to human ABCG1 (84 and 72%, respectively) whereas amino acid identities with the other ABC G subfamily members were far lower (human ABCG2, 28%; mouse ABCG3, 26%; human ABCG5, 24%; human ABCG8, 24%; Table 1). The strikingly high homology between ABCG4 and ABCG1 raises the possibility that both genes may have coevolved more recently than the other members of the ABC trans-

porter G subfamily, possibly through a multiplication event. TM 2 and 5 are identical in both transporters, whereas the other TM harbor several amino acid exchanges. TM2 and 5 are probably critical for the transporter's structure and function, whereas exchanges within the other TM may affect substrate specificity.

Compared to the ABCG1 peptide sequence amino acids are missing within the ABCG4 sequence at three positions (Fig. 3). These positions are located within regions of lower homology and may indicate domains which are critical for the transporters function. Strong sequence variability between the ABCG1 and ABCG4 peptide sequence is observed at the amino terminus (up to aa 35 of the ABCG4 peptide), between the NBF and the first transmembrane-spanning domain (aa 320–370) and between TM5 and TM6 (aa 580–608).

TABLE 1
Homology and Identity of ABC Transporter
G-Family Members^a

Homology	Identity					
	ABCG1	ABCG2	ABCG3	ABCG4	ABCG5	ABCG8
ABCG1	100	54	48	84	49	45
ABCG2	30	100	70	52	51	48
ABCG3	27	55	100	46	43	43
ABCG4	72	28	26	100	47	44
ABCG5	26	29	24	27	100	50
ABCG8	25	27	24	25	29	100

^a Homology and identity of ABC transporter G-family members at the protein level were calculated using the Blast 2 Sequences software with standard settings without the filter function (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>). The following GenBank database entries were used: human ABCG1, NM_004915; human ABCG2, NM_004827; mouse ABCG3, NM_030239; human ABCG4, AJ308237; human ABCG5, NM_022436; and human ABCG8, NM_022437.

Multiple transcripts of the human ABCG4 gene as were recently reported for the ABCG1 gene (25, 26) were not detected.

Mouse Homolog of Human ABCG4 cDNA

Mus musculus EST sequences homologous to ABCG4 were identified. AK018283 resembles with high homology the complete noncoding 3'-RNA sequence of the human ABCG4 transcript. Within the coding region of the ABCG4 cDNA EST-database entries BG298084, BB182384, and BE861999 are of relevance. BG298084 extends 110 nucleotides into the 5'-direction and contains the same open reading frame as is present in the reported human ABCG4 cDNA. Human and mouse ABCG4 protein are 98% identical as far as the sequence is known (not shown). We are now engaged in

studies in order to obtain the full sequence of the mouse ABCG4 cDNA.

Gene Structure and Exon/Intron Borders of Human ABCG4

Exon/intron boundaries were identified by alignment of our human ABCG4 cDNA (EMBL Nucleotide Sequence Database Accession Nos. AJ308238–AJ308251) with the human chromosomal contig in the HTGS Database (base pairs 26363–39051 of AC000384). Results are shown in Table 2. The ABCG4 gene consist of at least 14 exons. Corresponding entries AJ308238-51 have been deposited in the EMBL Nucleotide Sequence Database. The ABCG4 gene spans a region of 12.7 kb. All exons are relatively small, ranging between 51 and 250 nucleotides, except for the last exon which spans 1.8 kb. The initial and terminal dinucleotides of all introns showed the GT-AG configuration diagnostic of splice junctions (27). Most introns are shorter than 220 nucleotides; only 4 introns are larger than 1000 bp. Thus the genomic size is the smallest of all reported ABC transporter subfamily G members [ABCG1, 96 kb (25) or 40 kb (26); ABCG2, 66 kb; ABCG5 and ABCG8 together, 80 kb (14)].

Comparison of intron and exon size of the ABCG1 and ABCG4 gene reveals no conservation of intron size, whereas the size of 10 out of 14 exons (exons 2, 3, 4, 5, 6, 7, 10, 11, 12, and 13 of the ABCG4 gene, respectively) is conserved.

Analysis of the genomic sequence upstream of the 5'-end of the transcript reveals no transcription initiation elements such as a TATA-box or enhancer elements within 300 base pairs upstream of the start of transcription. A potential splice acceptor site was detected a four base pairs upstream of the transcripts 5'-end at a position where the nucleotide sequence of the mouse EST clone BG298084 is no longer homo-

TABLE 2
Exon–Intron Boundaries of the Human ABCG4 Gene

Exon	Exon size (bp)	5'-splice site	3'-splice site	Intron size (kb)
1	249	5'-UTR	CAAAAGGGgtagggaa	3.81
2	118	cactgcagGTTATAAG	GGATACAGgtttcctt	0.11
3	133	ccctgcagGGAGTCTG	CCATGATGgtgagggc	0.13
4	51	ggccccagGTCTCTGC	AGGAGCTGgtgagtgg	0.2
5	146	ggggccagGTGACAGA	CCCACCAGgtagtctt	1.41
6	124	ctggacagTGGTCTGG	TTGACAAGgtgagtgt	0.11
7	115	tgctgcagCTTACAT	TGACTTCAGtgagtgg	0.2
8	143	gtccccagTCATCGAG	GTCCTCCGgtgagttag	1.22
9	99	cttccccagGAAGTGGA	GGGACACGgtgagggc	0.22
10	169	ctcccaagTCCTGAC	GCTCACCTgtgagctg	0.1
11	101	gtcctcagTCCCCTTA	CCTTTCAGgtgggctt	1.27
12	159	ctccccagGTGGTGTG	CCCTACAGgtgggagg	0.15
13	119	ccctctagGTGCCAC	TATGTACAGgtcagtac	0.22
14	1773	ctgcctagGTATGGCT	3'-UTR	

gous to the human genomic sequence. Taken together these results indicate that either the ABCG4 gene contains a TATA-less promoter or, more likely, that the ABCG4 gene may contain at least one additional exon upstream of the exons reported in this study. We are performing further work to answer this question.

Chromosomal Assignment of the ABCG4 Gene

Using the Genome Browser software (<http://genome.ucsf.edu>) we allocated base pairs 26363–39051 of contig AC000384 to the chromosomal region 11q23.3 indicating that the ABCG4 gene is localized at this locus. 11q23.3 is of special interest, since it contains the locus for many other genes involved in cholesterol and lipid metabolism such as primary hypoalphalipoproteinemia, sterol-C5-desaturase-like protein, and the repressor of ABCA1 and ABCG1 expression, zinc finger protein 202 (28, 29).

In summary, we have identified a longer transcript of the human ABCG4 gene in macrophages which extends the reported ABCG4 peptide sequence to a length similar to that of other ABC transporter G family members. The detailed role of ABCG4 in transmembrane transport is presently unknown. Since we found that the ABCG4 gene is regulated by agonists of the nuclear receptors LXR and RXR in human macrophages (Fig. 1) it is possible that ABCG4 exerts functions in macrophage lipid export or metabolism, potentially in concert with other oxysterol-responsive ABC G subclass transporters such as ABCG1, ABCG5, or ABCG8 (12).

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