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Sterol-dependent Transactivation of the *ABC1* Promoter by the Liver X Receptor/Retinoid X Receptor*

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Tangier disease, a condition characterized by low levels of high density lipoprotein and cholesterol accumulation in macrophages, is caused by mutations in the ATP-binding cassette transporter ABC1. In cultured macrophages, ABC1 mRNA was induced in an additive fashion by 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid (9CRA), suggesting induction by nuclear hormone receptors of the liver X receptor (LXR) and retinoid X receptor (RXR) family. We cloned the 5′-end of the human ABC1 transcript from cholesterol-loaded THP1 macrophages. When transfected into RAW macrophages, the upstream promoter was induced 7-fold by 22(*R*)-hydroxycholesterol, 8-fold by 9CRA, and 37-fold by 9CRA and 22(*R*)-hydroxycholesterol. Furthermore, promoter activity was increased in a sterol-responsive fashion when cotransfected with LXRα/RXR or LXRβ/RXR. Further experiments identified a direct repeat spaced by four nucleotides (from −70 to −55 base pairs) as a binding site for LXRα/RXR or LXRβ/RXR. Mutations in this element abolished the sterol-mediated activation of the promoter. The results show sterol-dependent transactivation of the *ABC1* promoter by LXR/RXR and suggest that small molecule agonists of LXR could be useful drugs to reverse foam cell formation and atherogenesis.

Plasma HDL[1]-cholesterol levels are inversely related to the incidence of coronary artery disease (1). Two genetic diseases illustrate this phenomenon, the rare Tangier disease and the more common familial HDL deficiency. Tangier disease is characterized by an extremely low concentration of circulating HDL and the accumulation of cholesteryl esters in tonsils, liver, spleen, and intestinal mucosa, mostly in macrophage foam cells (2). Patients with familial HDL deficiency exhibit a low concentration of HDL particles and an increased risk of coronary artery disease (3). A common explanation for the cardioprotective effect of HDL is the major role it plays in reverse cholesterol transport (4). It is commonly accepted that the efflux of cholesterol from cells is caused by two different pathways: the first is passive and promotes efflux from the cell membrane to HDL and the second is energy-dependent and apolipoprotein-mediated (5). The latter was characterized in fibroblasts and macrophages and involves lipid-poor or -free apolipoproteins such as apoA-I, apoA-II, and apo-E (5–7). This active pathway has been reported to be defective in both Tangier disease and familial HDL deficiency (8–10). It was recently demonstrated that *ABC1* is a key gene in this process (11) and that mutations of *ABC1* are the major cause of both Tangier disease and familial HDL deficiency (3, 12–17).

ABC1 (ABCA1) belongs to the large ATP-binding cassette transporter family. These transmembrane proteins transport many diverse substrates across membranes because of their channel-like topology (18, 19). The human *ABC1* gene was assigned to chromosome 9q31, spanning a minimum of 70 kilobases and containing at least 49 exons (14, 16, 20). Whereas its expression is ubiquitous, the highest levels of human or murine mRNAs were found in placenta, fetal tissues, liver, lung, and adrenal glands (21, 22). The predicted human protein contains 2201 amino acids (220-kDa protein) (21).

The expression of hABC1 is induced by cholesterol loading of human macrophages. Both the protein and the mRNA are up-regulated in the presence of acetylated LDL, and down-regulated by cholesterol unloading via HDL3 (21). Whereas the cholesterol-mediated regulation of genes involved in cholesterol uptake or biosynthesis via sterol regulatory element binding protein (SREBP) pathways is well understood (23), much less is known about direct mechanisms of sterol-mediated up-regulation of gene expression. Two families of nuclear receptors are known to be activated by oxysterols and to mediate a positive response by binding to specific DNA elements, the liver X receptor (LXR) and steroidogenic factor 1 (SF1) (24–27). SF1 acts as a monomer and has been implicated in the regulation of steroidogenic acute regulatory protein gene expression (StAR) activity (26). Recently, two different genes involved in the reverse cholesterol transport pathways, cholesterol 7α-hydoxylase (24) and cholesterol ester transfer protein (*CETP*) (25), have been shown to be up-regulated by the heterodimer LXR·RXR. This suggests the hypothesis that LXRs might coordinate different steps of reverse cholesterol transport (25). LXRα (NR1H3) and LXRβ (NR1H2) heterodimerize with their partner RXR. The resulting complex up-regulates genes through binding sites typically composed of direct repeats (DR) of the motif AGGTCA, spaced by 4 nucleotides (LXRα and LXRβ) or 1 nucleotide (LXRβ) (28–30). The dimer can be activated by both the ligands of RXR (retinoids) and LXR (oxysterols) separately or together (29, 31, 32).

Here we report the sequence of the *hABC1* promoter and show that this promoter is active in macrophages and that its sterol-mediated activation depends on the binding of LXR/RXRα to a DR4 element.

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[1] The abbreviations used are: HDL, high density lipoprotein; 9CRA, 9-*cis*-retinoic acid; 22(*R*)-Hch, 22(*R*)-hydroxycholesterol; 25-Hch, 25-hydroxycholesterol; 7-Kch, 7-ketocholesterol; LXR, liver X receptor; RXR, retinoid X receptor; LXR/RXR, cotransfected mixture of two receptors; LXR·RXR, putative heterodimer complex; SF1, steroidogenic factor 1; CETP, cholesterol ester transfer protein; DR, direct repeat; RACE, rapid amplification of cDNA ends; bp, base pair(s); PCR, polymerase chain reaction; LDL, low density lipoprotein.

MATERIALS AND METHODS

5′-RACE PCR—5′-RACE PCR was performed with the SMART RACE cDNA kit (CLONTECH, Palo Alto, CA) using 1 μg of poly(A)$^+$ mRNA from HepG2 and THP-1 cells that were differentiated into macrophage with phorbol 12-myristate 13-acetate and exposed to acetylated LDL (25 μg/ml) for 48 h. After reverse transcription (M-MLV reverse transcriptase, Life Technologies Inc.), a first PCR (hot start, 94 °C 30 s, 65 °C 30 s, 72 °C 3 min, 25 cycles, and then 72 °C 10 min) was performed using the reverse primer 5′-CCCCCTCCCTCGGGATGCCCGCAGACAA-3′. A second PCR (hot start, 94 °C 30 s, 55 °C 30 s, 72 °C 3 min, 25 cycles, and then 72 °C 10 min) was done on 2.5 μl of the 50×-diluted first PCR sample with the nested primer, 5′-GCCTCCGAGCATCTGAGAACAGGC-3′. The forward primers were provided by CLONTECH.

Cloning of the hABC1 Promoter and Introns 1 and 2—The screening of the human RPC.11 BAC clone library was performed (Research Genetics, Inc., Huntsville, AL) with a 68-mer oligonucleotide probe corresponding to nucleotides 11–79 of the published *hABC1* sequence. Two BAC clones were recovered that were positive by PCR for exon 1 (BAC553F19) and exon 3 (BAC 522C12). After digestion by *Pst*I, a Southern blot was performed using the ^{32}P-radiolabeled probes generated by PCR with the previously cited exons. Positive bands were cloned in pBluescript KS(+) (Stratagene, La Jolla, California). A colony hybridization (probes used for Southern blot) (33) allowed us to isolate positive clones for the *hABC1* promoter (5 kilobases) and intron 2. Sequencing performed on both strands showed that we also cloned intron 2 from BAC 522C12. The sequences of these introns are contained in the sequence of human genomic clone RP11–1M10, which also contains exons 1, 2, and 3 (see Fig. 2*a*).

Cell Cultures and Transfection Experiments—The cell lines were purchased from ATCC (Manassas, VA). The murine RAW 264.7, African green monkey CV-1, and human 293 or HepG2 cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. THP-1 cells were maintained in RPMI 1640 containing L-glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin supplemented with 0.5 μM β-mercaptoethanol. Confluent cells were differentiated with 0.2 μM phorbol 12-myristate 13-acetate (Sigma) in ethanol over 72 h. Thioglycolate-elicited peritoneal macrophages were isolated from C57 Bl/6 mice as described previously (34).

Transfections were performed in 24-well plates with LipofectAMINE reagent (transactivation experiments in CV-1 and 293 cells, see Figs. 4 and 5) or LipofectAMINE-Plus reagent (basal activation experiments in RAW 264.7, see Figs. 4, 6, and 8) according to the manufacturer's instructions (Life Technologies Inc.). For basal activation experiments, a total of 0.15 μg of reporter DNA and 0.05 μg of PRL-CMV (*Renilla*, Promega) per well were used. For transactivation studies, we used 0.025 μg/well PRL-CMV, 0.2 μg of reporter DNA, and 0.1 μg of each receptor (CMX-hRXRα, CMX-hLXRα, CMV-mLXRβ). pcDNA3.1 plasmid was included to obtain a final quantity of 0.45 μg of total DNA per well. The transfected cells were cultured in lipoprotein-deficient serum medium in the presence of 4 μg/ml (see Figs. 4, 6, and 8) or 2 μg/ml (transactivation experiments, see Fig. 5) of 22(*R*)-hydroxycholesterol (22(*R*)-Hch), 25-hydroxycholesterol (25-Hch), or 7-ketocholesterol (7-Kch), 10 μM 9-*cis*-retinoic acid (9CRA, Sigma) or ethanol alone for 24 h. The luciferase activities were measured using the Promega dual luciferase assay system. A reporter plasmid used to analyze the activity of the *hABC1* promoter was constructed by subcloning a 1029-bp PCR fragment of the *hABC1* promoter (from −928 to +101 bp) into the pGL3-Luc basic vector (Promega). A shorter promoter (from −469 to +101 bp) was generated by digestion of this plasmid with *Sac*I. Deletions (see Fig. 7) were performed by enzymatic digestion (*Apa*I, from −156 to +101 bp) or PCR. The sequence of the PCR fragments were verified. Where shown, *error bars* represent S.D. Non-parametric Mann Whitney tests were performed to obtain *p* values.

Northern Blot Analysis—Total RNA was isolated with RNAzol B reagent (TEL-TEST, Inc., Friendwood, TX). Northern blots were performed as described previously (33). A human *ABC1* probe corresponding to exons 2–8 of the published sequence was synthesized by reverse transcriptase-PCR using the forward primer, 5′-AGGTGGCCTGGCCTCTATTTATCTTC-3′ and the reverse primer, 5′-GCCTCCGAGCATCTGAGAACAGGC-3′. LXR probes were synthesized from human *LXRα* and mouse *LXRβ* sequences (28, 35). A mouse glycerol-3-phosphate dehydrogenase probe was used as an internal standard (reverse transcriptase-PCR synthesized fragment: forward primer, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′). Signals were quantitated with phosphor imaging.

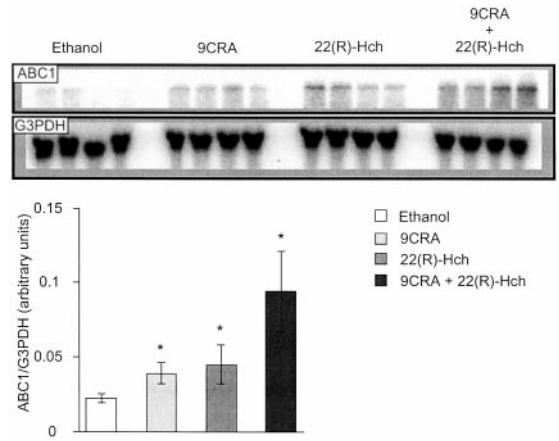

FIG. 1. **Expression of hABC1 in THP1 macrophages.** THP-1 cells were exposed for 72 h to phorbol 12-myristate 13-acetate to induce differentiation in macrophages. On day 4, cells were treated for 24 h with vehicle (ethanol), 22(*R*)-Hch (10 μM), and/or 9CRA (10 μM) ($n = 4$ per treatment). *Top*, Northern blot with 40 μg of total RNA from each sample. The membrane was hybridized with hABC1 probe and mouse glycerol-3-phosphate dehydrogenase (*G3PDH*) as an internal standard. *Bottom*, quantitation of blot, *error bars* represent S.D. Significance of treatment *versus* ethanol is indicated by *, $p < 0.05$.

Non-parametric Mann Whitney tests were performed to obtain *p* values.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed as described previously (25). Nuclear extracts were prepared from 293 cells cotransfected with LXRα and hRXRα or LXRβ and hRXR. Double-stranded oligonucleotides containing the DR4 element or its mutated version (see Fig. 7) were synthesized with overhangs and used at a final concentration of 0.1 pM (hABC1DR4) or 0.5 μM (competitors). An oligonucleotide corresponding to a canonical half-site sequence (AGGTCA) was added to each sample to reduce the background (1 μM). Polyclonal antibodies against peptides from LXRα (P20, sc-1202X), LXRα/β (C19, sc-2101X), RXRα (D20, sc-553X), and RORα (K-20, sc-6063X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

RESULTS

Increased ABC1 mRNA in Human Macrophages Treated with Sterols and/or Retinoic Acid—To investigate whether the endogenous *ABC1* gene can be activated by oxysterols and/or retinoic acid in macrophages, we performed Northern blot analysis of total RNA from human THP-1 macrophages. Fig. 1 shows a significant increase of ABC1 mRNA in cells treated with 22(*R*)-Hch (2-fold induction, $p < 0.05$) or 9CRA (2-fold, $p < 0.05$). An additive effect was obtained with combined treatment (4-fold, $p < 0.05$ when compared with separate treatments). These responses suggest possible activation of transcription by LXR/RXR (32).

Characterization of the 5′ Region of the hABC1 Gene—To identify the promoter of the human *ABC1* gene, we performed 5′-RACE PCR using poly(A)$^+$ mRNA from cholesterol-loaded THP-1 macrophages and HepG2 cells (Fig. 2*b*). In macrophages this revealed a single major transcript (transcript A) consisting of a first exon of 217 bp followed by a second exon of 160 bp, 73% identical to mouse exon 1 (GenBank™/EBI accession number X75926). This exon is then followed by the published human exons 2, 3, and 4 (21).

In HepG2 cells, 5′-RACE PCR revealed three different transcripts (Fig. 2*b*). Transcript B represents a truncated version of exon 2 found in THP-1 cells (only the last 29 bp) followed by the published exons 2, 3, and 4 (21). Transcript C contains one exon of 372 bp upstream of the published exon 2, which is different from the exons found in THP1 cells. Transcript D has the same 5′ structure as transcript C but lacks the published exon 3.

A BLAST search of the GenBank™/EBI Data Bank (htgs)

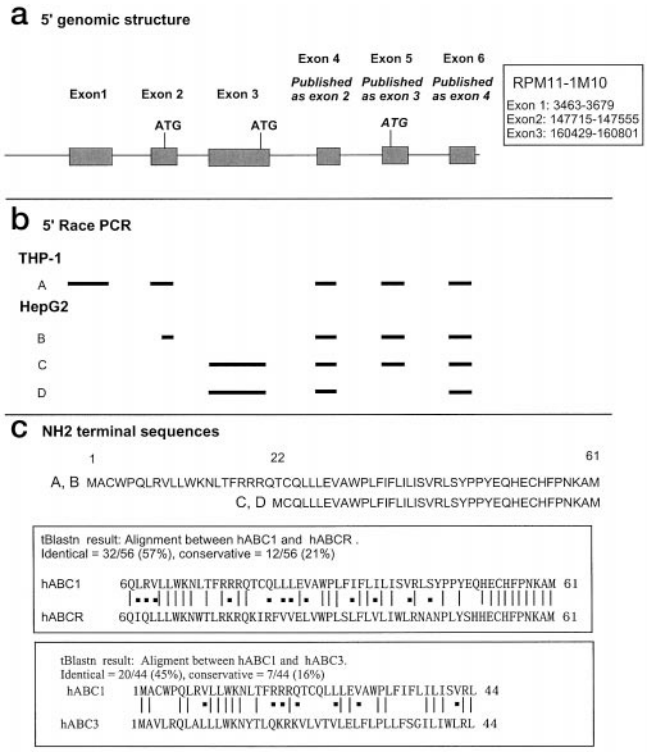

FIG. 2. **Analysis of *hABC1* 5′ sequence.** *a*, partial gene structure; *b*, results of 5′-RACE PCR; and *c,* amino-terminal sequence. *a*, the *hABC1* promoter was cloned from the human library RPCI-11, and the structure of the 5′-end of the gene was determined by sequence analysis. Positions of exons in RP11-M10 sequence are indicated in the *inset*. *b*, 5′-RACE PCR was performed on cholesterol-loaded THP-1 macrophages. These cells express exons 1 and 2 (transcript *A*). A 5′-RACE PCR was also conducted on HepG2 cells, which express a truncated version of exon 2 and also exon 3 (transcripts *B*, *C*, *D*). Two possible ATG start codons are present in exons 2 and 3. *c*, a comparison of the deduced amino-terminal sequence (*NH_2-terminal sequence*) of hABC1 with the nucleotide data base (tBlastn) revealed similarities with two members of the ABC1 family, ABCR and ABC3. |, identity; ■, conserved substitution.

revealed 100% homology of these exons (Fig. 2*a*, *exons 1–3*) with fragments of the human genomic clone RP11–1M10 (working draft sequence, GenBank™/EBI accession number AC012230). A comparison of the sequences from the published exon 2, the 5′-RACE PCR product, and RP11–1M10 revealed a C instead of a T at position +15 and a G instead of an A at position +17.

Conceptual translation of the transcripts revealed two new start codons in frame with the previously published ATG located in exon 5 (14) (Fig. 2, *a* and *c*). In the case of the transcript characteristic of THP1 cells, a new ATG located in exon 2 resulted in an extra 60 amino acids at the amino terminus. In the case of HepG2 cells, a new start codon at the 3′-end of exon 3 may be functional in transcript C and also transcript D, which lacks the previously published start codon. This results in an extra 39-amino acid fragment for transcript C.

A comparison of the putative amino-terminal amino acid sequences of ABC1 (transcripts A, B) with nucleotide data bases revealed strong homology to the amino-terminal sequences of two members of the ABC1 family (57% identity with ABCR and 45% identity with ABC3 (Fig. 1c). This strongly suggests that the amino-terminal sequence of hABC1 is authentic.

Sequence of the hABC1 Promoter—The promoter region upstream of exon 1 was responsive to sterols when transfected into cells (see below), whereas the 2.3-kilobase region upstream of transcript B was not responsive (data not shown). Thus, we focused our attention on the former region.

acctgagttttggccagaataaggtgacatttagtttgttggcttgatggatgacttaaatatttagacatatggtg

CREBP1CJUN

tgtaggcctgcattcctactcttgccttttttttgcccctccagtgttttgggtagttttgctcccctacagccaaagg

-928bp

*caaacaga*taagttggaggtctggagtggctacataatttttacacgactgcaattctctggctgcacttcacaa

atgtatacaaactaaatacaagtcctgtgtttttatcacagggaggctgatcaatataatgaaattaaaagggg

CEBPB

gctggtccatattgttctgtgtttttgtttgtttgttttttttgtttttgtggcctccttcctctcaatttatgaagagaagc

HNF3B IRF1

agtaagatgttcctctcgggtcctctgagggacctggggagctcaggctgggaatctccaaggcagtaggtc

NF-KB

gcctatcaaaaatcaaagtccaggtttgtgggggggaaaacaaaagcagcccattacccagaggactgtcc

gccttcccctcaccccagcctaggcctttgaaaggaaacaaaagacaagacaaaatgattggcgtcctgag

ggagattcagcctagagctctctctccccaatcccctccctccggctgaggaaactaacaaaggaaaaaaaa

Sac1

attgcggaaagcaggatttagaggaagcaaattccactggtgcccttggctgccgggaacgtggactagag

Stat1

agtctgcggcgcagccccgagcccagcgctttcccgcgcgtcttaggccggcgggcccgggcgggggaag

gggacgcagaccgcggaccctaagacacctgctgtaccctccacccccacccacccacctccccccaa

ctccctagatgtgtcgtgggcggctgaacgtcgcccgtttaaggggcgggccccggctccacgtgctttctgct

MYCMAX

gagtgactgaactacatataaacagaggccgggaacggggcggggaggagggagagcacaggctttgac

AP1 SP1

cgatagtaacctctgcgctcggtgcagccgaatcTATAAaaggaactagtcccggcaaaaaccccg**taa**

ttgcgagcgagagtgagtggggccgggacccgcagagccgagccgacccttctctcccgggctg

+101

cggcagggcagggcggggagctccgcgcaccaacagagc

FIG. 3. ***hABC1* promoter sequence.** This sequence is identical to the working draft sequence of the genomic clone RP11–1M10 (position 2335–3463 bp, GenBank™/EBI accession number AC012230) except for the italicized 5′-end which is new. The fragment used in transfection covers from −928 to +101 bp. The *arrowhead* at −469 bp shows the *Sac*I site that was used to generate a deleted promoter fragment. An analysis of the sequence (Matinspector) revealed numerous putative transcription binding sites (*underlined*). *Dashed lines* indicate a transcription binding site on the complementary strand. The *bold type* represents the 5′-end of exon 1.

Fig. 3 presents a partial sequence of genomic DNA with a fragment of exon 1 cloned from the human RPCI.11 BAC library. A potential TATA box is present at −32 bp and an Sp1 site at −101 bp. An analysis of this sequence revealed several potential transcription factor binding sites.

hABC1 Promoter Is Functional and Sterol-responsive in Macrophages—To investigate the function of the potential *hABC1* promoter, we transfected the macrophage-like RAW 264.7 cell line with a promoter-luciferase construct (Fig. 4*a*). To test for activation by sterols we used 22(*R*)-Hch, a potent activator of LXR·RXR (36) but a poor activator of SF1 (27) and 9CRA, to activate endogenous RXR. Compared with basal conditions, transfected cells treated with 22(*R*)-Hch or 9CRA exhibited 7- and 8-fold higher promoter activity, respectively ($p < 0.001$) (Fig. 4*a*). When both compounds were added together, there was a synergistic 37-fold induction ($p < 0.001$). A similar response was obtained with promoter fragments containing 928 bp (Fig. 4*a*) or 469 bp (data not shown) of upstream sequence.

Next we compared the response of the *ABC1* promoter to different sterols (Fig. 4*b*). We treated the transfected cells with 25-Hch, which is a good activator of SF1 and a weak activator of LXR (27). Cells were also treated with 7-Kch, which is relatively abundant in human arterial foam cells (37). 25-Hch is a poor inducer of the *hABC1* promoter compared with 22(*R*)-Hch (1.5-fold activation, $p < 0.05$). No significant effect of 7-Kch was detected. However, when added in combination with 9CRA, a significant additive effect was detected for 7-Kch (2-fold when compared with 9CRA alone, $p < 0.01$). This pattern of sterol responsiveness is consistent with a transcriptional mechanism involving LXR.

LXRα and LXRβ Are Both Expressed in Macrophages—To further investigate the potential role of LXRα and LXRβ in the sterol-mediated up-regulation of hABC1, we verified that the mRNA of these nuclear receptors was present in RAW264.7

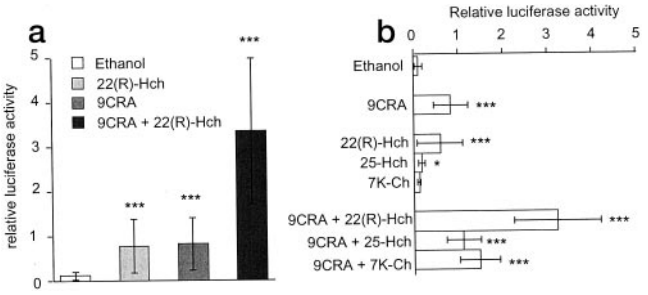

FIG. 4. **Activation of the hABC1 promoter by oxysterols and retinoic acid in RAW 264.7 cell.** *a*, a fragment of the *hABC1* promoter (from −828 to +101 bp) was linked to the firefly luciferase reporter gene. The resulting plasmid was cotransfected with a control reporter plasmid (*Renilla* luciferase) in the mouse macrophage-like RAW 264.7 cells. Four independent transfection experiments (each in triplicate) were performed. The results are expressed as a ratio between the Firefly and *Renilla* luciferase activities. Cells were treated with vehicle (ethanol) or 22(*R*)-Hch (10 μM) or 9CRA (10 μM) or 22(*R*)-Hch (10 μM) and 9CRA (10 μM) for 24 h in fetal bovine serum medium complemented with 10% lipoprotein-deficient serum. *b*, activation of the *hABC1* promoter by various oxysterols and/or 9CRA. Similar experiments to those performed in *a* were done using 22(*R*)-Hch (10 μM), 25-Hch (10 μM), 7-Kch (10 μM), and 9CRA (10 μM). Three to four independent experiments in duplicate or triplicate were performed. *Bars* indicate mean ± S.D. Significance of treatment *versus* ethanol is indicated by ***, $p < 0.001$; *, $p < 0.05$.

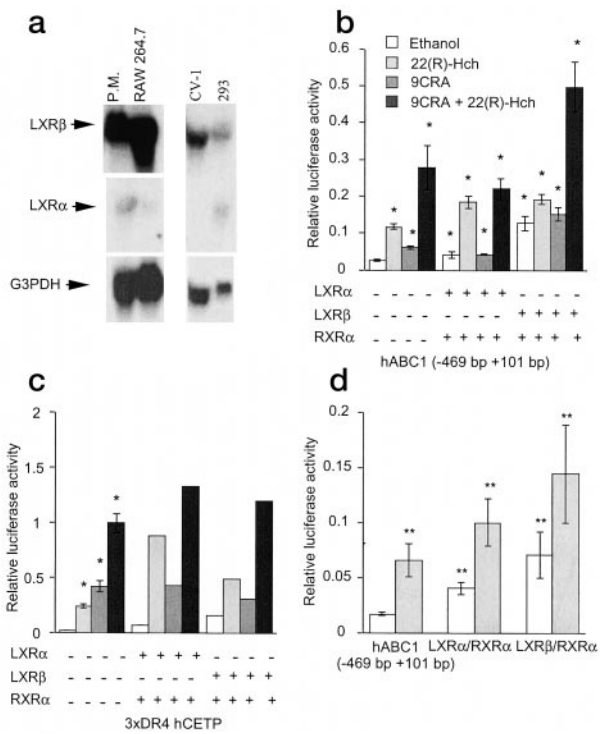

FIG. 5. **Expression of LXRα and LXRβ in various cell lines.** Sterol transactivation by LXR/RXR of the *hABC1* promoter is shown. *a*, cells were isolated and cultured as described under "Material and Methods." A Northern blot was performed with 35 μg of total RNA for each cell line. Hybridizations were performed using probes of similar specific activities for hLXRα, mLXRβ, and mouse glycerol-3-phosphate dehydrogenase (*G3PDH*) as an internal standard. *h*, human; *m*, mouse. *b*, 293 cells were transfected with the *hABC1* promoter (from −469 to +101 bp) or a construct containing three copies of a sterol-responsive element of the *CETP* promoter (*c*) (25). These constructs were cotransfected with the *Renilla* luciferase reporter gene and hLXRα, mLXRβ, and/or hRXRα as designated. The cells were treated 24 h with vehicle alone or 22(*R*)-Hch (5 μM) and/or 9CRA (10 μM) in fetal bovine serum medium with 10% lipoprotein-deficient serum. The results represent 2 independent experiments in duplicate for the transfection using the *hABC1* promoter and 1–2 experiments in duplicates for the transfection using the *hCETP* promoter. *d*, CV-1 cells were transfected and treated according to the protocols described in *b*. 2–3 independent experiments in duplicates were performed. *Bars* indicate mean ± S.D. Significance of treatment *versus* ethanol is indicated by **, $p < 0.01$; *, $p < 0.05$.

cells (Fig. 5*a*). We also analyzed their expression *in vivo*, using thioglycolate-elicited peritoneal macrophages from mice. Both receptors were detected in macrophages by Northern blot of total RNA, with a much stronger signal for LXRβ. We also selected two cell lines for our transactivation experiments, CV-1 and 293 cells. As shown in Fig. 5, both LXRα and LXRβ could be detected in 293 cells but only LXRβ in CV-1 cells. Because the quantities of mRNA for LXRs are relatively low in 293 cells and CV-1 cells, we chose to perform transactivation experiments in these cell lines.

LXR/RXR Transactivates the hABC1 Promoter in 293 and CV-1 Cells—To define the involvement of hRXRα and/or LXRα/β in the sterol up-regulation of hABC1, we cotransfected 293 cells with the human *ABC1* promoter and with these receptors (Fig. 5*b*). We used the shorter promoter (deletion −460 bp), which was sterol-responsive in macrophages (data not shown). In 293 cells without cotransfected LXR/hRXR we observed up-regulation of the promoter by 22(*R*)-Hch (4.5-fold, $p < 0.05$) and 9CRA (3.5-fold, $p < 0.05$) alone. The combination of 9CRA and 22(*R*)-Hch resulted in an additive effect (10-fold, $p < 0.05$). Basal activity was slightly increased (1.5-fold) when LXRα/hRXRα were cotransfected, as was the sterol response, but there was no additional effect by 9CRA or 9CRA and 22(*R*)-Hch. However, cotransfection of LXRβ/RXRα caused a 5-fold increase in basal expression and a synergistic effect of 9CRA and 22(*R*)-Hch (19-fold induction compared with ethanol and 3-fold induction compared with 9CRA alone, $p < 0.05$).

As a positive control for these experiments we used a construct containing three copies of the LXR·hRXRα binding site of the *CETP* promoter (25) (Fig. 5*c*). Even in the absence of transfected receptors, this construct was highly sterol- and retinoic acid-responsive in 293 cells (14- and 24-fold increase in luciferase activity, respectively, $p < 0.05$), and an additive or synergistic effect (58-fold) was obtained with cotreatment. LXR transfection resulted in increased basal activity and increased induction by sterols. However, 9CRA provided no further increase in activity compared with non-transfected cells. These results suggest that endogenous LXRs in 293 cells play a role in the response of both *ABC1* and *CETP* promoters with a further increase in sterol-dependent promoter activity when LXR and RXR are cotransfected.

In CV-1 cells a significant sterol-activation of the *ABC1* promoter was detected without transfected receptors (4-fold, $p < 0.01$, Fig. 5*d*). Cotransfection with LXRα/hRXRα or LXRβ/hRXRα increased the basal activity of the promoter (2- and 4-fold, respectively, both $p < 0.01$). Exposure to 22(*R*)-Hch resulted in increased transactivation of the promoter (6- and 8-fold, respectively), compared with the control with no receptor.

Deletional Analysis of hABC1 Promoter—To define the region of the *hABC1* promoter involved in its sterol-mediated activation we carried out further deletional analysis (Fig. 6). Similar results to Fig. 4*a* were obtained with fragments of the promoter from −160 bp or −100 bp to +101 bp. Deletion of exon sequences (from +3 bp to +101 bp) reduced the basal activity, but the response to 22(*R*)-Hch and 9CRA was maintained. Notably, deletion of sequences between −101 and −36 bp reduced the basal activity and abolished the response to sterol and/or retinoic acid. Interestingly, the region covering −100 to +36 bp contains an almost perfect DR4 element located on the non-coding strand between −70 and −55 bp (Fig. 6, *inset*).

Mutational Analysis of the DR4 Element—To test the hypothesis that the DR4 element is responsible for sterol activation, we introduced a mutated version of this element in the longer version of the *hABC1* promoter (from −928 to +101 bp).

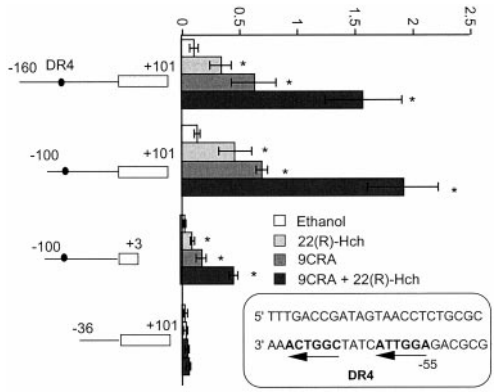

FIG. 6. **Deletional analysis of *hABC1* promoter.** Deletions were performed by enzymatic digestion or PCR amplification of the *hABC1* promoter. The results represent two independent experiments of duplicates. *Bars* indicate mean ± S.D. Significance of treatment *versus* ethanol is indicated by ***, $p < 0.001$; *, $p < 0.05$.

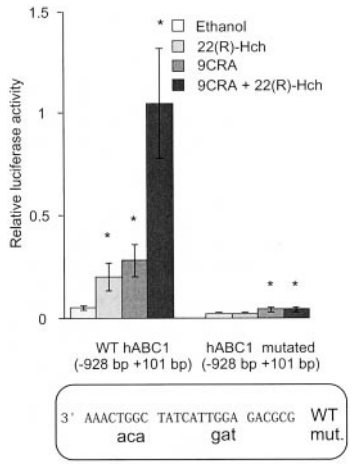

FIG. 7. **Mutational analysis of *hABC1* promoter.** Raw 264.7 cells were transfected with wild-type (*WT*) *hABC1* promoter (from −928 to +101 bp) or its mutated version. The mutations are presented in Fig. 7. The results represent two independent experiments of duplicates. *Bars* indicate mean ± S.D. Significance of treatment *versus* ethanol is indicated by ***, $p < 0.001$; *, $p < 0.05$.

Both half-sites were mutated by changing nucleotides away from the nuclear hormone binding consensus sequence, as shown in Fig. 7. This mutation reduced the basal expression (2.5-fold), but the activity was still readily detectable. Importantly, the mutation abolished the response to 22(*R*)-Hch alone or in combination with 9CRA.

The DR4 Element Binds LXR/RXR—To determine whether the DR4 element binds LXR/RXR, we used oligonucleotides containing the wild-type DR4 or the mutated version (as in the functional assay, Fig. 7) to perform electrophoretic mobility shift assays using nuclear extracts from 293 cells cotransfected with LXRβ/hRXRα (Fig. 8, *top*) or LXRα/hRXRα (Fig. 8, *bottom*). When the *hABC1* wild-type DR4 element is used alone, a single major shift in activity is detected for both types of nuclear extracts (Fig. 8, *lane 1*), which disappears with excess of cold competitor indicating specificity (Fig. 8, *lane 2*). Because the mutated version is unable to compete with the intact oligonucleotide (Fig. 8, *lane 3*), the integrity of the DR4 itself is necessary for transcription factor binding. In cells cotransfected with LXRβ/RXR, antibodies recognizing the LXRα or LXRβ/α common region and anti-RXR antibodies markedly reduced binding activity and produced supershifted bands (Fig. 8, *top*). In cells cotransfected with LXRα/RXR, antibodies to LXRα, LXR β/α, and RXRα showed similar effects except that the LXRα antibody did not produce a supershifted complex (25). Antibodies specific for RORα had no (or a minimal) effect as expected. These results show that LXRα/RXRα or LXRβ/RXRα bind this DR4 element.

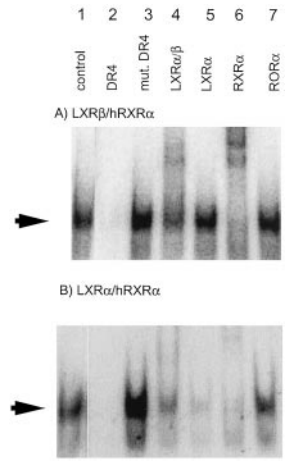

FIG. 8. **Electrophoretic mobility shift assay of human *ABC1* promoter fragment.** Oligonucleotides containing the DR4 element (see Fig. 6) that was identified as a potential binding site for LXR/RXR or a mutated version (*mut. DR4*) were ^{32}P-radiolabeled. Competitors (*lanes 2* and *3*) correspond to cold wild-type or mutant oligonucleotides. After incubation with these oligonucleotides and nuclear extracts from 293 cells transfected with LXRα/hRXRα (*B*) or LXRβ/hRXRα (*A*), some samples were incubated with polyclonal antibodies for LXRα/β, LXRα, RXRα, or RORα (negative control) or with a monoclonal antibody targeting LXRβ. Three different experiments were performed with similar results. The *arrow* indicates the position of the retarded complex.

DISCUSSION

In this study we have identified a region of the human *ABC1* promoter, which is active in macrophages and is induced by 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid. Our characterization of the major transcript in cholesterol-loaded THP1 macrophages led to the identification of this promoter and also showed that most of the potential upstream initiation codons (ATG) in the previously published cDNA (21) are unlikely to represent the authentic translation initiation site. LXRα/RXRα or LXRβ/RXRα binds to a DR4 element in the *hABC1* promoter and mediates its activation by oxysterol and retinoic acid. Thus, LXR and/or RXR agonists could be useful drugs to reverse foam cell formation and atherogenesis.

A unique DR4 element mediates the sterol up-regulation of the *hABC1* gene through LXR/RXR, and mutational analysis suggests that this is the only site involved. Unlike previously identified DR4 elements (25, 36), the element in the *ABC1* promoter is found in an inverse orientation on the non-coding strand (Fig. 6). The almost canonical sequence of this element might explain its high efficiency. Mutations in the DR4 result in a decrease of the basal expression of the promoter, but significant activity is still detectable. A weak activation of the mutated promoter by 9CRA (Fig. 7) suggests the presence of another binding site for RXR. However, the DR4 element we identified is responsible for the entire sterol-mediated activation of the 1-kilobase *hABC1* promoter (Fig. 3).

The pattern of activation of the *ABC1* promoter by sterols suggests that its expression may be suboptimal in atherosclerotic lesions. Thus, 7-KCh is relatively abundant in oxidized-LDL and in atheroma foam cells (37) and is a poor activator of the *hABC1* promoter (Fig. 4*b*). 27-Hch, also abundant in foam cells, is a relatively poor activator of LXR (32, 36). Thus, the accumulation of oxysterols in atherosclerotic lesions probably does not result in optimal activation of hABC1. This suggests that small molecules that are optimal LXR activators might be

effective drugs at reversing foam cell formation and that they might be useful as a treatment for atherosclerosis. The activation of the *hABC1* promoter by 9CRA is increased 2-fold when given with 7-Kch (Fig. 4*b*). This further suggests that with regard to the induction of ABC1 by oxysterols, an unfavourable foam cell environment could also be switched to a more favorable one by delivery of ligands for RXR.

The inability of LXRβ to compensate for the lack of LXRα in LXR$\alpha^{-/-}$ mice (24) suggests that these receptors have different targets. *In vitro*, both LXRα and LXRβ are able to up-regulate hABC1 (Fig. 5, *b–d*) or CETP (25), but LXRβ is clearly more effective than LXRα in mediating the sterol response of hABC1 (Fig. 5*b*). This is also consistent with the fact that LXRβ appears to be more highly expressed than LXRα in macrophages (Fig. 5*a*).

To conclude, we have shown that hABC1 is up-regulated at the transcriptional level by oxysterols and 9CRA acting though LXR/RXR which binds a proximal DR4 element located on the non-coding strand. These results provide strong support for the idea that LXRs act directly to coordinate the activities of molecules mediating reverse cholesterol transport (25). This may lead to a functional coordination of different steps of reverse cholesterol transport. For example, CETP activity results in the remodeling of HDL into small particles and liberates free apoA-I from HDL (38). Free apoA-I appears to be the optimal substrate for ABC1 (39, 40). Thus, coordinate induction of CETP and ABC1 by LXR/RXR might act synergistically to enhance cholesterol efflux from macrophage foam cells.

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Sterol-dependent Transactivation of the*ABC1* Promoter by the Liver X Receptor/Retinoid X Receptor

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