Human ABCA1 BAC Transgenic Mice Show Increased High Density Lipoprotein Cholesterol and ApoAI-dependent Efflux Stimulated by an Internal Promoter Containing Liver X Receptor Response Elements in Intron 1*

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By using BAC transgenic mice, we have shown that increased human ABCA1 protein expression results in a significant increase in cholesterol efflux in different tissues and marked elevation in high density lipoprotein (HDL)-cholesterol levels associated with increases in apoAI and apoAII. Three novel ABCA1 transcripts containing three different transcription initiation sites that utilize sequences in intron 1 have been identified. In BAC transgenic mice there is an increased expression of ABCA1 protein, but the distribution of the ABCA1 product in different cells remains similar to wild type mice. An internal promoter in human intron 1 containing liver X response elements is functional in vivo and directly contributes to regulation of the human ABCA1 gene in multiple tissues and to raised HDL cholesterol, apoAI, and apoAII levels. A highly significant relationship between raised protein levels, increased efflux, and level of HDL elevation is evident. These data provide proof of the principle that increased human ABCA1 efflux activity is associated with an increase in HDL levels in vivo.

A significant step in the elucidation of mechanisms of reverse cholesterol transport resulted from the identification of mutations in ABCA1 underlying Tangier disease, as well as familial hyperalphaproteinemia associated with reduced efflux (1–5). These and further investigations and characterizations of the biochemical phenotype of heterozygotes for ABCA1 deficiency (6) have demonstrated that lipidation of the nascent apoAI-rich HDL1 particle is a rate-limiting step in the maintenance and regulation of HDL cholesterol (HDL-C) levels in humans. The ABCA1 gene is also rate-limiting for cholesterol efflux and HDL-C levels in different species, including mouse (7, 8) and chicken (9), demonstrating conservation of this pathway in cholesterol metabolism over at least 400 million years.

Studies of heterozygotes for ABCA1 deficiency have also demonstrated a very strong relationship between levels of cellular cholesterol efflux and HDL-C levels in plasma, with ∼82% of the variation in HDL-C levels in these patients being accounted for by the decrease in cellular cholesterol efflux. This clearly has demonstrated in these patients that ABCA1 is the major but not the only contributor to cellular cholesterol efflux in humans (6).

There have been recent significant additional advances with regard to understanding regulation of ABCA1 expression. A direct mechanism of sterol-mediated up-regulation of gene expression of ABCA1 has been shown to be due to transactivation of the ABCA1 promoter by LXR and RXR (10–12), two members of the nuclear receptor superfamily. This sterol-mediated activation has been shown to be dependent on the binding of RXR/LXR heterodimers to a DR4 element in the promoter of the ABCA1 gene. Transcriptional sequences representing LXR response elements (or LXREs) are composed of direct repeats of the motif AGGTCA separated by four nucleotides, and this motif serves as the binding site for RXR/RXR or RXR/LXR complexes.

These data (10–12) have clearly shown that the LXRE in the ABCA1 gene, transcriptional sequences representing LXREs in different species, and cellular cholesterol efflux are directly dependent on LXREs (13, 14). Therefore, even though the LXRE in the ABCA1 gene is not the only factor involved in the regulation of cellular cholesterol efflux, it accounts for the majority of the variation in HDL-C levels in these patients being accounted for by the decrease in cellular cholesterol efflux. This clearly has demonstrated in these patients that ABCA1 is the major but not the only contributor to cellular cholesterol efflux in humans (6).

Levels of mRNA may be poor predictors of protein expression (12), as mRNA levels can vary almost 20 times and still yield the same level of gene product. Alternatively, the same level of expression of an mRNA can result in vastly different levels of a protein (13, 14). Therefore, even though in vitro studies have shown an increase of ABCA1 mRNA on oxysterol stimulation (10, 11), it is most important to determine whether there is an increase in ABCA1 protein associated with raised ABCA1 mRNA expression. Furthermore, whereas decreases in cellular
cholesterol efflux secondary to either antisense in vitro inhibition of the gene (1) or in vivo mutations (1–5) are associated with decreased efflux and decreased HDL-C levels, it is currently unknown whether overexpression of ABCA1 in vivo is associated with increased HDL-C levels and an increase in tissue-specific cholesterol efflux.

The use of transgenic technologies using BACs offers important advantages for generating mice expressing human ABCA1. The inclusion of endogenous regulatory elements within the transgene allows for assessment of normal temporal, tissue-, and cell-specific expression of human ABCA1. Furthermore, inclusion of selected endogenous promoter sequences allows for dissection of the contribution of different sequences to the normal regulation of the ABCA1 gene. Such information is not possible using cDNA transgenic approaches that often result in poorly expressed genes that are not physiologically regulated.

Here we demonstrate, both in vitro and in vivo, that the ABCA1 gene has an internal promoter containing LXR{\textregistered} in intron 1. Activation of this functional internal promoter in human intron 1 by oxyesters in vivo directly contributes to an increase in human-specific mRNA in tissue and leads to increased protein expression. These experiments have led to the identification of three novel ABCA1 transcripts with different transcription initiation sites that utilize sequences in intron 1. In addition, increased human ABCA1 expression results in a remarkable and significant increase in cholesterol efflux and HDL-C levels. These studies provide important proof of the principle for therapeutic strategies directed toward the activation of ABCA1 expression and activity.

**EXPERIMENTAL PROCEDURES**

**Transient Transfection Assay**—Cells were transfected for 3 h by lipofection using ExGen 500 (Euromedex) in Opti-MEM I. Medium was then replaced with Dulbecco's modified Eagle's medium (DMEM) containing 0.2% fetal calf serum, and cells were incubated for 48 h. Cell extracts were prepared and assayed for luciferase activity as described (15). Twenty-four hours before transfection, HepG2, Huh7, CanCo2, Cos-1, and RK13 cells were plated in 24-well plates in DMEM supplemented with 10% fetal calf serum at 5 × 10{\textsuperscript{4}} cells/well. Transfection mixtures contained 100 ng of tkpGl3 reporter vector or pGl3 containing an 8-kb fragment from ABCA1 intron 1 (pGl3–8kb). Transfection mixtures contained 50 ng of reporter plasmid (pGl3) containing multiple copies of the putative LXR{\textregistered} and 25 ng of LXR{\textregistered} and RXR expression plasmids, in the presence of the internal control β-galactosidase expression vector. After transfection, cells were treated for 48 h with 1 μM 22R-hydroxysterol (Sigma).

**Gel Mobility Shift Assay**—LXR{\textregistered} and RXR were transcribed and translated in vitro using pCDNA3-LXR{\textregistered} and pSG5-RXR{\textregistered} as templates and the TNT-coupled transcription/translation system (Promega). Gel mobility shift assays (20 μl) contained 10 μM Tris (pH 8), 40 μM KCl, 0.1% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 0.2 μg of poly(dI-dC), 1 μg of herring sperm DNA, and 2.5 μg each of in vitro synthesized LXR{\textregistered} and RXR proteins. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μl) through the addition of unprogrammed lysate. After a 10-min incubation on ice, 1 ng of 32P-labeled oligonucleotide was added, and the incubation was continued for an additional 10 min. DNA-protein complexes were resolved on a 6% polyacrylamide gel in 0.5× TBE. Gels were dried and subjected to autoradiography at ~80 °C.

**Multicopy Cloning**—250 pcmols of each oligonucleotide to which half-sites for both BAC and BpII restriction enzymes had been added were phosphorylated using T4 polynucleotide kinase (Roche Molecular Biochemicals), incubated for 5 min at 95 °C and then 10 min at 65 °C, and cooled to room temperature. Multimeric copies were then generated using T4 ligase, annealed in T4Gl3 vector, and verified by sequencing. 

**Detection of BAC Transgenic Mice**—BACs containing the ABCA1 gene were identified by screening high density BAC grid filters from a human BAC library. Four BACs containing ABCA1 were sequenced as described previously (1, 6). Version 1.7 of ClustalW with modifications was used for multiple sequence alignments with Boxshade for graphical enhancement. The 5′ end of BAC 269 is at position 13491 in intron 1 (i.e. 13491 nucleotides from the 5′ end of exon 2). This BAC was chosen for further purification as it alone contained intron 1 sequence without the human ABCA1 promoter, allowing us to test for functionality of the putative intronic regulatory elements. The BACs were purified for injection using the Qiagen Maxi Prep kit, followed by cesium chloride purification (16) and dialysis overnight. BACs were quantified using absorbance at 260 nm and electrophoresis, and sets of 300 C57BL/6 mice were injected with 30 ng of the purified BAC DNA. Founders were genotyped with DNA extracted from tail pieces, followed by subsequent PCR amplification of exon 2, exon 26, and exon 49 of the ABCA1 gene.

**Feeding of High Cholesterol Diets**—BAC mice and control littermates were provided free access to water and a high fat/high cholesterol or a control diet for 7 days. The diets were purchased from Harlan Teklad with the high fat/high cholesterol diet (TD 90021) containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate. This diet has been shown previously to result in up-regulation of ABCA1 mRNA levels in mouse liver, assessed at 7 days after feeding (17). The control diet contained 0.5% sodium cholate (TD 99057).

**Quantification of Human and Mouse ABCA1 Transcripts**—RNA from mouse liver and porcine macrophages were isolated using Trizol (Life Technologies, Inc.), and 3 μg of total RNA was reverse-transcribed using Superscript II (Life Technologies, Inc.). Human- and mouse-specific primers were used along with 18 S primers (Ambion Inc.) to calculate transcript abundance. Human-specific primers are as follows: Ex0F, CCAACAATGTGACCTGAATCAGGAG, and Ex4R, GAGC- GCACTGATGTCCTGCG. Mouse-specific primers are as follows: Ex5F, CATTAAAGGACATGCAAGGTCG, and Ex6R, CAGAAAATCTCGCA-GCTTCATATT. The standard cycling conditions of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min were used. PCR products were separated on 2% agarose gels, and images were captured using Bio-Rad multianalyst software using a gelsoc system. Bands were quantified using NIH Image version 1.6. All values are ratios with the corresponding 18 S bands.

**Quantitative Real Time PCR for Human and Mouse ABCA1 Levels**—The human ABCA1 primers, mouse ABCA1 primers, and their Taqman probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). The TaqMan probe contains a reporter dye at the 5′ end and a quencher dye at the 3′ end. The sequences of the primers and the probes are as follows: human ABCA1 forward primer, 5′-CTGACCGCGGTGTTCCTCC-3′, and human ABCA1 reverse primer, 5′-TTCCTGCAGGGATGTCCTC-3′; human ABCA1 TaqMan probe, 5′-ACATCTGGGAAAAGACTTGGTCCTGTA-3′; mouse ABCA1 forward primer, 5′-TCCGACGAAGTTCCTCCTC-3′, and mouse ABCA1 reverse primer, 5′-GGCTCTAATTCCTTACAGAAGGC-3′; mouse ABCA1 TaqMan probe, 5′-CCCCAATCTGCGACCGGCTCAGATC-3′. The RT-PCR was carried out on ABI PRISM 7700 in a final volume of 50 μl containing 40 ng of total RNA, 200 μM primers, and 600 μM probe in 1× TaqMan One-step RT-PCR Master mix (PE Biosystems, CA), according to the manufacturer’s instructions. The primers and probe for 18 S or rodent GAPDH were used as the internal controls for human ABCA1 and mouse ABCA1, respectively. The reverse transcription reaction was run at 48 °C for 30 min. After activation of the AmpliTaq Gold at 95 °C for 5 min, the RT-PCR was carried out for 40 cycles (94 °C for 15 s and annealing and extension at 60 °C for 1 min). Data quantification and analysis were performed according to the manufacturer’s protocol (PE Biosystems). Values were calculated relative to the level of the control. Each sample was assayed in triplicate during two independent experiments.

**Detection of Alternate Transcripts Involving Intron 1 Sequence Arising from Three Different Transcription Start Sites**—In order to identify the transcript generated in the BAC mice lacking exon 1, ABCA1 intron 1 sequence was searched by ProScan for putative transcription sites, and several likely sites were determined. Primers were synthesized, and CLONTECH marathon-ready mouse and human liver cDNAs were used to amplify putative transcripts using the predicted transcript primer and an ABCA1 exon 3 reverse primer, following the manufacturer’s instructions. Positive transcripts were confirmed using nested PCR and sequenced. In addition, RNA was isolated from BAC transgenic and control liver tissue using Trizol (Life Technologies, Inc.), and 5′-rapid amplification of cDNA ends was performed using primers described previously (18) and following the manufacturer’s instructions (CLONTECH). All products were TTA-cloned (Invitrogen) and sequenced. The number of PCR cycles required for specific amplification are as follows: exon 1F, GTTGGCTACCTTTGAGGAAA; exon 1E, GAGAGGGAACTCACCAGTTGTT; exon 1D, CCAGTAGACATTCTACTGGT; and Ex3R, CCTATCGCTGTTCA-TAGGGT. Standard cycling conditions were used for PCR amplification of all three transcripts.

**Western Blot Analysis of the Distribution of ABCA1 BAC transgenic**
mice and control littersmates were sacrificed by CO₂ inhalation, and various tissues were isolated and placed in 500 µl of low salt lysis buffer containing complete protease inhibitor tablets (Roche Molecular Biochemicals) on ice. The tissues were homogenized and sonicated. The resulting homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was aliquoted in 1 ml of this cholesterol-containing buffer and transferred to polyvinylidene difluoro membranes (Millipore). Membranes were probed with ABC1PEP4 polyclonal rabbit antibody (directed against residues 2236–2259 in ABCA1) or monoclonal anti-glycerolaldehyde phosphate dehydrogenase (Chemicon) as a control. The membrane was dipped in ECL (Amersham Pharmacia Biotech) and exposed to X-Omat blue film (Eastman Kodak Co.). Protein levels were quantitated using NIH image software.

ABCA1 Immunocytochemistry—To compare further the in vivo cellular expression pattern of ABCA1 protein in both ABCA1 BAC transgenic mice and their wild type littersmates, immunocytochemistry was performed on a variety of fixed tissues using the polyclonal ABC1PEP4 antibody described above. Transgenic and wild type mice were deeply anesthetized with pentobarbital, injected intraperitoneally with 100 units of heparin in sterile water, and then transcardially perfused with 0.1 M PBS containing 5% dimethylsulfoxide (Sigma) and 1% Triton X-100. Tissues removed included the heart, liver, kidney, brain, and sciatic nerve, and were incubated in 4% paraformaldehyde (Polysciences) in PBS with 0.25% sucrose, postfixed for 1 h with the same fixative, embedded in paraffin, cut on a vibrating microtome (Vibratome). Sections were collected in 0.1 M PBS with 0.2% Triton X-100 and incubated in blocking solution (0.1% PBS with 0.3% Tween 20, 3% normal goat serum, and 5% bovine serum albumin) for 2 h at room temperature.

Sections of liver were incubated for 48 h with ABC1PEP4. Brains from each mouse were processed for combined immunocytochemistry with a neuron-specific (NeuN, Chemicon) antibody and ABC1PEP4. Sections were sequentially placed into primary antisera against ABCA1 (1:500, 12 h), followed by the secondary antibody, sections were then washed several times in blocking solution and incubated in secondary antibody for 48 h at 4 °C. Secondary antibodies (Molecular probes) were used as follows: goat anti-mouse Alexa 488 with NeuN at a dilution of 1:200, and goat anti-rabbit CY-3 with ABCA1 at a primary dilution of 1:200.

Following further washes with 0.1 M PBS the sections were dry-mounted on gelatin-coated slides, dehydrated by serial ethanol washes, and permanently mounted with Fluoromount (Gurr). Sections were analyzed using an upright fluorescence microscope (Zeiss), and digital images were captured on a CCD camera (Princeton Instrument Inc.). Combined and NeuN/ABCA1-stained sections were processed into double immunofluorescence figures using Northern exposure image program.

Measurement of Plasma Lipid and Apoprotein Levels—Mice were either bled by saphenous vein withdrawal or by cardiac puncture, and the collected blood was added to tubes containing 5 µl of 0.1 M EDTA. For the measurement of HDL-C, the plasma was mixed 1:1 with 20% PEG20, vortexed, incubated at room temperature for 10 min, and spun at maximum speed for 5 min at room temperature (19). 20 µl of the resultant supernatant was added to 96-well maxisorp plates (Millipore), and 200 µl of Infinity cholesterol reagent (Sigma) was added to the wells. The plates were quantitated in an enzyme-linked immunosorbent assay reader at 492 nm. For the measurement of total cholesterol, 5 µl of plasma was added to the same plates; 200 µl of Infinity cholesterol reagent was added, and the plate was quantitated as above. Triglycerides were measured by adding 10 µl of the plasma to a 96-well plate, followed by the addition of 100 µl of solutions from a triglyceride kit (Roche Molecular Biochemicals). PFLC separation of plasma lipoproteins was performed using BioPore™ 6 (Amersham Pharmacia Biotech) columns in series as described previously (19). Equal volumes of plasma (40 µl) from mice (n = 8) in each group were pooled for the analysis. The cholesterol and triglyceride content in each 0.5-ml fraction was measured by adding commercially available enzymatic kits (Roche Molecular Biochemicals). Apoproteins were measured as described previously.

Establishment of Primary Fibroblast and Macrophage Cultures—For the isolation of macrophages, mice were injected intraperitoneally with 2 ml of 3% thioglycollate and 3 days later were sacrificed by CO₂ injection. 5 ml of DMEM containing 10% fetal bovine serum, 1-glutamine, and penicillin/streptomycin (all from Life Technologies, Inc.) was injected into the body cavity. The mice was gently massaged, and the media were withdrawn and placed in tubes on ice. The cell pellet was resuspended using 25 ml of DMEM and plated at a density of 5 × 10⁵ cells/ml in a volume of 300 µl. The cells were incubated in a humidified atmosphere of 5% CO₂ at 37 °C until used. Fibroblasts were isolated by digesting the femurs of the mice and triturating the above media through the bone to remove all bone marrow. The media were added to tubes on ice and spun at 1200 rpm for 5 min at 4 °C, and the procedure was repeated three times. The supernatant was decanted in 0.2% delipidated serum (Sigma) containing media for about 24 h. The media were again replaced with 30 µl of DMEM, penicillin/streptomycin, 1-glutamine, either with or without 20 µg/ml apoAI (Calbiochem), and the treatment compounds 9-cis-retinoic acid (Sigma) and 22(R)-hydroxycholesterol (Steraloids). 24 h later, the media were washed and centrifuged at maximum speed for 5 min at room temperature. 100 µl of the supernatant was added to scintillation vials, and radioactivity was quantitated. 200 µl of 0.1 N NaOH was added to each well containing the cells and incubated for 20 min at room temperature. 100 µl of this lysate was added to scintillation vials and quantified. Efflux was calculated as the total counts in the medium divided by the sum of the counts in the medium plus the cell lysate.

Statistical Analyses—All statistical analyses were performed using one-way analysis of variance followed by the Newman-Keuls post-test, except for the protein quantification and the analyses of the statistical significance between the means and S.D. of the data provided in the footnotes of Table IV. The statistical analyses of these two data sets were performed using unpaired t tests.

RESULTS

Intron 1 Contains a Functional Promoter—To investigate whether the internal intron 1 fragment could drive transcription of a reporter gene, we transfected an 8-kb fragment of intron 1 upstream of exon 2 into different cell types, including several hepatic (HepG2 and HuH7), intestinal (CaCo2), and renal (RK13) cell lines. Indeed, in these cell lines, a significant activation of the reporter gene was observed as compared with transfection of the empty pG3 vector alone (Fig. 2A).

In order to detect the presence of regulatory elements, we scanned the human ABCA1 intron 1 from position −1 to −24,156 and discovered several putative regulatory elements. Among these, we discovered several possible LXREs containing imperfect direct repeats of the nuclear receptor half-site AGGTCA separated by four nucleotides (DR4) (Fig. 1 and Table I) (22). LXREs are regulated by oxygenos (23, 24) and are important transcription control points in cholesterol metabolism (25). An LXRE in exon 1 of ABCA1 had been shown previously to be active in the regulation of the gene in vitro (10–12). All the putative LXREs are contained within the 8-kb fragment. To investigate whether these DR4 elements were indeed able to activate LXRE-RXR heterodimers, gel retardation assays were performed (Fig. 2B). As shown before when LXREs and RXR proteins were incubated with the labeled CYP7-LXRE oligonucleotide in vitro, a complex was observed in the presence of the LXR-RXR heterodimer (24). An excess of unlabeled CYP7 competed efficiently for binding to the probe, but no competition for binding was observed with a DR-2 oligonucleotide. As a control, the unlabeled LXRE described previously in ABCA1 exon 1
positions occurring in exon 2. Novel putative LXR elements were identified at above, and the ABCA1 schematic diagram of the putative LXRE elements discovered in DNA and are also contained within the BAC.

cloned in front of a luciferase reporter gene were assayed by dependent manner, although with seemingly different structs competed for binding of the CYP7A probe in a dose-

signal was then competed with increasing quantities of each unlabeled putative LXRE. As shown in Fig. 2B, all three con-

(4-LXRE) (10, 11) also competed efficiently for binding. The signal was then competed with increasing quantities of each unlabeled putative LXRE. As shown in Fig. 2B, all three con-

To determine whether these potential LXREs also possessed functional relevance, multiple copies of these oligonucleotides cloned in front of a luciferase reporter gene were assayed by cotransfection with the expression plasmids for LXRa and RXR in COS-1 cells (Fig. 3). As described previously, three copies of the consensus LXRE (5XLXRE), 5 copies of the CYP7 LXRE (5XCYPT-LXRE), 2 copies of the +4 LXRE in ABCA1 exon 1 (2X+4-LXRE) showed a strong activation in the presence of cotransfected LXR and RXR plasmids (10, 24). The 3 copies of the putative 4686 LXRE and 7656 LXRE of ABCA1 intron 1 showed a 2- and 6-fold induction, respectively. In contrast, two copies of the 7174 LXRE showed a weaker activation by the LXR-RXR heterodimer.

Detection of Alternate Transcripts in Intron 1 —In order to determine if the LXREs in intron 1 that we identified by bioin-

This induction is most likely due to the oxysterol-de-

These approaches followed by sequencing, we identified three novel transcripts containing published exon 2 and exon 3 se-

Human-specific ABCA1 mRNA Is Up-regulated in BAC Transgenic Mice in Response to Cholesterol Feeding—In order to determine if the human-specific transcript is present in BAC transgenic mice, and further up-regulated upon stimulation when mice are fed a high cholesterol-containing atherogenic diet, we performed human- and mouse-specific quantitative RT-PCR and quantitative real time PCR on mouse liver RNA, normalized to 18 S RNA (Fig. 5, A and C). Human ABCA1-

transcript only in the BAC transgenic lines. Quantitation of mRNA levels by real time PCR resulted in a 1.4–2.3-fold induction of the human ABCA1 transcript when the BAC mice were fed an atherogenic diet (Fig. 5C). In the same samples, the endoge-

FIG. 1. Localization of LXRE elements in the ABCA1 5‘ region.

Man BACs. Southern blot analysis revealed founder XA with 13.5 kb of intron 1 sequence followed by the rest of the gene, with the ATG occurring in exon 2. Novel putative LXR elements were identified at positions −7656 bp, −7174 bp, and −4686 bp in the ABCA1 genomic DNA and are also contained within the BAC.

(+4-LXRE) (10, 11) also competed efficiently for binding. The signal was then competed with increasing quantities of each unlabeled putative LXRE. As shown in Fig. 2B, all three con-

Protein expressed from this BAC would be predicted to be full-length as the translation initiation site is in exon 2. We

these transcripts were seen at different levels in hepatic tissue. For example, the transcript with exon 1b was expressed at the highest level in livers from chow-fed BAC transgenics compared with wild type mice fed the same diet. Furthermore, all three transcripts occur in wild type mice and humans (Fig. 4B).

Human ABCA1 Protein Is Increased in BAC Transgenic Mice—In order to determine if the ABCA1 protein is expressed in the absence of its upstream promoter and exon 1 sequences, we first performed Western blot analysis of several different tissues in the mice. We observed that there was indeed an increase in ABCA1 protein levels in the liver, small intestine, testis, stomach, and brain compared with nontransgenic mice that was distinguishable by our anti-ABCA1 antibody (Fig. 6A). When the mice were fed an atherogenic diet, of the various tissues tested, the levels of ABCA1 protein were further in-

The signal was then competed with increasing quantities of each unlabeled putative LXRE. As shown in Fig. 2B, all three con-

In order to elucidate the transcript generated by the BAC transgenic mice lacking exon 1 of the ABCA1 gene, we performed RT-PCR and 5′-rapid amplification of cDNA ends. By utilizing these approaches followed by sequencing, we identified three novel transcripts containing published exon 2 and exon 3 se-

increases in ABCA1 expression in the liver in the transgenic mice (Fig. 7, B and D) compared with wild type littermates (Fig. 7, A and C). There was no observable alteration in the subcellular
distribution of ABCA1. For example, in the cortex, ABCA1 is predominantly located in the nucleus of neurons in both transgenic (Fig. 7, K–M) and wild type mice (Fig. 7, H–J). This is the first indication of ABCA1 protein expression in different tissues including brain. There was virtually no staining observed in the primary antibody-omitted control (Fig. 7E).

ABCA1 BAC Transgenic Mice Show Increased HDL-C Apoprotein Levels—We next determined if the increase in ABCA1 protein in the BAC mice resulted in an increase in its activity by measuring the plasma lipid levels in these mice. A significant increase in HDL-C levels in the ABCA1 BAC transgenic mice compared with control littermates was seen both on chow and atherogenic diet (n = 110054, p = 0.005 and 0.007, respectively) (Table II and Fig. 8A). These data show that the alternate promoter in intron 1 is important and sufficiently functional to result in increased expression of ABCA1 protein and increased HDL-C levels. Furthermore, in both the BAC transgenic mice and wild type littermates, the HDL-C levels increased significantly upon feeding of a cholesterol-rich diet (n = 110054, p = 0.001 and 0.002, respectively) (Table II), consistent with up-regulation of the ABCA1 protein. The level of up-regulation of HDL-C in the BAC mice on atherogenic compared with chow diet was higher than the level of HDL-C increase in the wild type littermates on atherogenic versus chow diet, providing additional proof that the human ABCA1 transcript is indeed up-regulated upon stimulation through the LXR pathway.

Apoproteins AI and AII were also significantly increased in the BAC transgenic compared with wild type mice on a chow diet (n = 1100516, p = 0.05 and 0.0001, respectively) (Table III).

To assess for qualitative differences in lipoprotein particles...
between the human *ABCA1* BAC transgenics and their littermate controls, FPLC analysis was performed (Fig. 8B).

HDL-C levels, as indicated by the total area of the HDL peak (fractions 30–38), were increased in the transgenic mice, compared with the non-transgenic controls. The size distribution of the HDL particles appears slightly different, as the peak appears in fraction 34 in the wild type and fraction 35 in transgenic mice, indicating an increase in slightly smaller HDL particles, with increased expression of *ABCA1*. This is in keeping with the role of ABCA1 in the initial lipidation of apoAI and not its subsequent enlargement. Remnant lipoproteins and low density lipoprotein cholesterol (fractions 12–20, 24–28, respectively) levels were not readily different between transgenics and controls. HDL-C levels were further increased on feeding of the atherogenic diet (Fig. 8B) with peaks occurring in the same fraction. Thus, the increased HDL-C concentration in *ABCA1* BAC transgenic mice likely reflects an increased number of HDL particles and not the presence of larger HDL particles.

*ABCA1* BAC Transgenic Mice Show Increased HDL-C and Cholesterol Efflux  
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defect in cholesterol and phospholipid removal mediated by apolipoproteins has been observed previously in *ABCA1*-defective Tangier disease fibroblasts (26), and *ABCA1* has been shown to mediate cholesterol efflux to apoAI or HDL from cells (4, 27). In order to determine if there was an increase in efflux of cholesterol in the mice expressing high levels of *ABCA1*, we established primary peritoneal macrophage and fibroblast cultures from these mice. We observed increased efflux of [3H] cholesterol to apoAI from both peritoneal macrophage (Fig. 9A) (Table IV) and fibroblast (Fig. 9B) (Table IV) cultures obtained from the transgenic mice when compared with wild type littermates. These efflux levels were further significantly increased when the mice were fed the atherogenic diet (Fig. 9A and B). We observed that there was no increase in efflux when apoAI was omitted as the efflux acceptor (data not shown). In addition, we observed that stimulation of cultures with 9-cis-retinoic acid and 22(R)-hydroxycholesterol, which are specific activators of the LXR/RXR pathway, also significantly up-regulated the efflux levels from both the macrophage (Fig. 9A) and fibroblast cells (Fig. 9B and Table IV). BAC mice on athero-

genic versus chow diets showed higher levels of up-regulation of efflux when compared with wild type littermates on atherogenic and chow diets, indicating a larger induction of efflux, and a larger response to LXR/RXR activation in the presence of the human *ABCA1* gene, especially in fibroblasts where the
ABCA1 BAC Mice Show Increased HDL-C and Cholesterol Efflux

Here we show that increasing human ABCA1 protein expression results in a significant increase in HDL-C, apoAI, and apoAII levels in vivo. No major change in the distribution of HDL particles is seen, suggesting that this increase in ABCA1 protein predominantly results in an increase in the number of HDL particles. We have previously shown, based on families with low HDL-C, a strong correlation between the reduction in plasma HDL-C and raised apoAI and apoAII levels. The relationship between the increase in efflux and increase in HDL-C appears to be linear, with a correlation coefficient (r²) of 0.87 (p = 0.007) showing that raised efflux levels are associated directly with a proportionate increase in cholesterol efflux and the decrease in plasma HDL-C in these patients (6). Here we demonstrate that increasing efflux is associated with a proportionate and predictable increase in HDL-C and raised apoAI and apoAII levels. The relationship between the increase in efflux and increase in HDL-C appears to be linear, with a correlation coefficient (r²) of 0.87 (p = 0.007) showing that raised efflux levels are associated directly with a proportionate increase in HDL-C in vivo. Furthermore, the rate of efflux was almost completely correlated with the level of apoAII, showing that any approach that results in an increase in net functional ABCA1 protein levels in the cell could be expected to have a proportionate increase in cholesterol efflux. Moreover, the establishment of a high cholesterol diet for 7 days. There was a graded increase in ABCA1 protein levels, with liver from wild type chow-fed animals showing the lowest levels and transgenic animals fed the atherogenic diet showing the highest levels. All Western blots were performed on cultured macrophage and fibroblast cells that were used for efflux assays. ABCA1 protein was detected in both peritoneal macrophage and fibroblast cells, with the transgenic animals showing higher levels of protein that the control littermates. The protein levels in these tissues were also increased in response to feeding of an atherogenic diet.

difference in up-regulation of efflux between stimulated BACs and stimulated wild type mice was 73%.

DISCUSSION

Here we show that increasing human ABCA1 protein expression results in a significant increase in HDL-C, apoAI, and apoAII levels in vivo. No major change in the distribution of HDL particles is seen, suggesting that this increase in ABCA1 protein predominantly results in an increase in the number of HDL particles. We have previously shown, based on families with low HDL-C, a strong correlation between the reduction in plasma HDL-C and raised apoAI and apoAII levels. The relationship between the increase in efflux and increase in HDL-C appears to be linear, with a correlation coefficient (r²) of 0.87 (p = 0.007) showing that raised efflux levels are associated directly with a proportionate increase in cholesterol efflux and the decrease in plasma HDL-C in these patients (6). Here we demonstrate that increasing efflux is associated with a proportionate and predictable increase in HDL-C and raised apoAI and apoAII levels. The relationship between the increase in efflux and increase in HDL-C appears to be linear, with a correlation coefficient (r²) of 0.87 (p = 0.007) showing that raised efflux levels are associated directly with a proportionate increase in cholesterol efflux. Moreover, the establishment of a high cholesterol diet for 7 days. There was a graded increase in ABCA1 protein levels, with liver from wild type chow-fed animals showing the lowest levels and transgenic animals fed the atherogenic diet showing the highest levels. All Western blots were performed on cultured macrophage and fibroblast cells that were used for efflux assays. ABCA1 protein was detected in both peritoneal macrophage and fibroblast cells, with the transgenic animals showing higher levels of protein that the control littermates. The protein levels in these tissues were also increased in response to feeding of an atherogenic diet.

difference in up-regulation of efflux between stimulated BACs and stimulated wild type mice was 73%.

DISCUSSION

Here we show that increasing human ABCA1 protein expression results in a significant increase in HDL-C, apoAI, and apoAII levels in vivo. No major change in the distribution of HDL particles is seen, suggesting that this increase in ABCA1 protein predominantly results in an increase in the number of HDL particles. We have previously shown, based on families with low HDL-C, a strong correlation between the reduction in plasma HDL-C and raised apoAI and apoAII levels. The relationship between the increase in efflux and increase in HDL-C appears to be linear, with a correlation coefficient (r²) of 0.87 (p = 0.007) showing that raised efflux levels are associated directly with a proportionate increase in cholesterol efflux and the decrease in plasma HDL-C in these patients (6). Here we demonstrate that increasing efflux is associated with a proportionate and predictable increase in HDL-C and raised apoAI and apoAII levels. The relationship between the increase in efflux and increase in HDL-C appears to be linear, with a correlation coefficient (r²) of 0.87 (p = 0.007) showing that raised efflux levels are associated directly with a proportionate increase in cholesterol efflux. Moreover, the establishment of a high cholesterol diet for 7 days. There was a graded increase in ABCA1 protein levels, with liver from wild type chow-fed animals showing the lowest levels and transgenic animals fed the atherogenic diet showing the highest levels. All Western blots were performed on cultured macrophage and fibroblast cells that were used for efflux assays. ABCA1 protein was detected in both peritoneal macrophage and fibroblast cells, with the transgenic animals showing higher levels of protein that the control littermates. The protein levels in these tissues were also increased in response to feeding of an atherogenic diet.

difference in up-regulation of efflux between stimulated BACs and stimulated wild type mice was 73%.
argue against this. First, the base line and increase in ABCA1 protein in the BAC transgenic mice was significantly greater than seen in the control littermate mice (\(p < 0.049, n = 3\)). This could only reasonably be ascribed to the effects of the human ABCA1 protein. In addition, quantitative PCR using mouse- and human-specific primers clearly has shown an increase after feeding (38\%) of human \(ABCA1\) mRNA that was more than 2× greater than the increase in endogenous mouse mRNA in the same experiments. This provides formal proof that transcription of the human \(ABCA1\) with only intron 1 shows cholesterol-responsive regulation \textit{in vivo}.

The human \(ABCA1\) gene consists of 50 exons spanning 149-kb genomic DNA (28). Translation begins in exon 2, and transcription had been shown previously to be initiated at a 303-bp exon located 24,459 bp upstream of exon 2 (18). Here we have shown three other transcription initiation sites utilizing

![Figure 7: Distribution of ABC1 protein in wild type and ABC1 BAC transgenic mice.](image)

**Table II**

|                      | Wt chow diet | BAC chow diet | Wt atherogenic diet | BAC atherogenic diet | Wt chow vs. BAC chow | Wt chow vs. Wt atherogenic | BAC chow vs. BAC atherogenic | Wt atherogenic vs. BAC atherogenic |
|----------------------|--------------|---------------|---------------------|----------------------|----------------------|-----------------------------|-----------------------------|-----------------------------|
| n = 4, Mean ± S.D.   |              |               |                     |                      |                      |                             |                             |                             |
| Total cholesterol    | 65.29 ± 5.78 | 92.65 ± 4.59  | 112.84 ± 14.2       | 180.68 ± 15.46       | 0.0003               | 0.01                        | <0.0001                    | 0.0007                      |
| HDL-C                | 36.88 ± 1.76 | 61.18 ± 11.02 | 77.84 ± 8.89        | 137.30 ± 27.91       | 0.005                | <0.0001                    | 0.002                      | 0.007                       |
| Triglycerides        | 42.66 ± 7.55 | 49.95 ± 3.62  | 50.60 ± 2.33        | 57.30 ± 4.43         | 0.17                 | 0.09                        | 0.04                       | 0.04                        |
| Non-HDL cholesterol  | 28.31 ± 3.77 | 31.47 ± 4.80  | 35.00 ± 4.50        | 43.38 ± 4.68         | 0.34                 | 0.07                        | 0.028                      | 0.04                        |
ABCA1 BAC Mice Show Increased HDL-C and Cholesterol Efflux

sequence from intron 1 and giving rise to three new ABCA1 transcripts. At the present time, ~35 mutations have been described in the ABCA1 gene (1–6). However, in our own studies, in some patients in whom mutations have been mapped to this particular gene, no DNA sequence variation in the coding region or splice donor/acceptor sites has been detected that could account for the phenotype observed. The approach to assessing the mutations had been to look at each splice site and exon, as well as the regular promoter in an effort to identify potential DNA variants that could account for the disruption of protein function (1, 6). The failure to detect mutations in some of these patients, together with the finding of the importance of these alternate transcripts in the regulation of the ABCA1 gene, may explain how expression could be compromised in some patients with defects in efflux that map to this gene but in whom no mutations have yet been described. One example of mutations disrupting the ratios of alternative protein isoforms implicated as the cause of abnormal phenotype is that affecting urogenital development in Denys-Drash syndrome (29). Further analysis and comparison of the sequence of the different ABCA1 transcripts may help to identify missing mutations and confirm the functional significance of these sequences.

It is apparent that different transcription start sites using an alternate promoter involving sequence in intron 1 can be used to enhance the information contained with the ABCA1 gene. Alternate splicing of nuclear pre-mRNA is a general mechanism for controlling gene expression leading to various RNA isoforms from a single primary transcript (30–32). What is unusual here is that the splicing event involves intronic sequence, which in contrast to alternate splicing of exonic sequence has only been described infrequently (33–35). The specific capacities of these sequences in intron 1 for protein
interactions and the importance of the contribution of these specific sequences in modulating cellular responses to physiological signals, such as oxysterol stimulation, when compared with the promoter LXREs, remains to be determined. However, it is clear that alternate transcript decisions in regard to intron 1 sequence are influenced by specific factors that may vary in different cell types, suggesting this event is of primary importance.

These newly discovered alternate transcripts are not seen equally in all tissues and, therefore, may provide further insights into the complex tissue-specific regulation of this gene, with certain transcripts likely to play a more major role in certain tissues. The presence of these alternate transcripts is also seen in endogenous mouse tissues, but there appears to be species-specific regulation of ABCA1, with these transcripts not being detected in all tissues at the same level as they are seen in humans.

Species-specific regulation of other genes involved in HDL metabolism has been reported. Fibrates, as an example, decrease the transcription of the ApoAI gene in rats, whereas in humans this clearly results in activation of ApoAI gene expression (20, 21). The availability of human ABCA1 transgenic mice further allows the investigation of the role of other transcription factors influencing the responsiveness of the intron 1 promoter to oxysterol stimulation. The breeding of these mice to others where various transcription factors are no longer present will help to determine their role in influencing the responsiveness of this promoter to oxysterol stimulation.

We have shown that intron 1 of the ABCA1 gene contains an internal promoter that is sufficient to drive ABCA1 protein expression and can regulate responsiveness to LXRE/RXR stimulation in vitro and in vivo. These LXREs, which are more than 15 kb away from the previously identified promoter, clearly identify the importance of intragenic sequences for the regulation of ABCA1. The LXREs we identified appear functional in vivo, resulting in significantly raised HDL-C levels and increased ABCA1 protein expression, particularly in liver, brain, small intestine, macrophages, and fibroblasts. Our experiments also demonstrate cross-species functional complementarity with murine LXRx-, Rrx-α-sufficient to transactivate the human ABCA1 gene.

Cavalier et al. (36) have recently described a similar line of human BAC transgenic mice lacking exon 1 of the ABCA1 gene, although its effects on plasma lipid levels and cholesterol efflux are not reported. Interestingly, they only describe one transcript in these mice, which is equivalent to our exon 1c transcript. They also describe a line of transgenic mice containing a full-length BAC but were unable to demonstrate differences in cholesterol efflux and plasma lipid levels in these mice.

The mice described by Cavalier et al. (36) were created on the FVB background. Our mice are C57BL/6xCBA/J hybrids. Strain differences in HDL and its metabolism and response to a high fat diet are well documented (37–47). As we are unaware of any studies comparing lipid metabolism in FVB mice to other strains, a direct comparison cannot be made. However, there are numerous ways these strain differences might affect HDL-C levels in transgenics created in them. For example, strain differences may contribute to factors such as apoAI acceptor levels which may, in turn, influence the ability of increased ABCA1 to increase plasma HDL-C concentrations. Similarly, factors influencing cholesterol removal from HDL or the turnover of various HDL subtypes may also influence the ability to observe ABCA1-mediated increases in HDL-C.

The availability of mice described in this paper will now allow us to add questions as to how effectively these animals can resist experimental atherosclerosis. Since the first description of the cellular defect in Tangier disease, where decreased HDL levels appeared to be associated with a decrease in cholesterol efflux (48, 49), the question as to whether increasing efflux would result in an increase in HDL-C levels and decreased atherosclerosis has been present. This challenge assumed greater importance with the discovery of the ABCA1 gene as the gene mutated in Tangier disease (1–6). The discovery and demonstration that increasing cholesterol efflux can indeed be associated with an increase in HDL-C levels provide additional support for the development of therapeutics influencing ABCA1 protein expression.

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### Table IV

|                      | Wt chow diet | BAC chow diet | Wt atherogenic diet | BAC atherogenic diet | Wt chow vs. BAC chow diet | Wt chow vs. Wt atherogenic diet | BAC chow vs. BAC atherogenic diet | Wt atherogenic vs. BAC atherogenic diet | n = 4, mean ± S.D. | p value |
|----------------------|--------------|---------------|---------------------|----------------------|---------------------------|--------------------------------|----------------------------------|--------------------------------------|-------------------------------|---------|
| **Macrophage**       |              |               |                     |                      |                           |                                 |                                  |                                      |                               |         |
| 22(R)-OH cholesterol  | 0.31 ± 0.014a | 0.45 ± 0.0033b | 0.40 ± 0.022c       | 0.54 ± 0.0077d      | <0.001                    | 0.004                          | <0.0001                          | <0.0001                              |                               |         |
| 9-cis-retinoic acid   | 0.46 ± 0.0085 | 0.49 ± 0.0067  | 0.52 ± 0.0054       | 0.59 ± 0.015         | 0.002                     | <0.0001                        | <0.0001                          | 0.0002                               |                               |         |
| **Fibroblast**       |              |               |                     |                      |                           |                                 |                                  |                                      |                               |         |
| 22(R)-OH cholesterol  | 0.12 ± 0.011b | 0.14 ± 0.017f  | 0.16 ± 0.014g       | 0.29 ± 0.011h        | 0.03                      | 0.002                          | <0.0001                          | <0.0001                              |                               |         |
| 9-cis-retinoic acid   | 0.19 ± 0.014c | 0.22 ± 0.015   | 0.27 ± 0.014        | 0.32 ± 0.0033        | 0.01                      | 0.0002                         | <0.0001                          | 0.0003                               |                               |         |

* The p value for the uninduced versus induced condition is p < 0.0001.
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Human ABCA1 BAC Transgenic Mice Show Increased High Density Lipoprotein Cholesterol and ApoAI-dependent Efflux Stimulated by an Internal Promoter Containing Liver X Receptor Response Elements in Intron 1

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