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Human ABCA1 BAC transgenic mice show increased HDL-C and ApoAI dependent efflux stimulated by an internal promoter containing Liver X Receptor Response elements in intron 1

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Abstract

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 HDL-C 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 LXREs is functional in vivo and directly contributes to regulation of the human ABCA1 gene in multiple tissues and to raised HDL-C, 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 principle that increased human ABCA1 efflux activity is associated with an increase in HDL levels in vivo.
**Introduction**

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 hypolipoproteinemia associated with reduced efflux (1-5). These and further investigation and characterization of the biochemical phenotype of heterozygotes for *ABCA1* deficiency (6), have demonstrated that lipidation of the nascent ApoA-I rich HDL 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 approximately 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 upregulation 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 4 nucleotides, and this element has been shown to be activated by both ligands of RXR (rexinoids) and LXR (oxysterols) separately and together (11). These data (10-12) have clearly shown that the LXRE in the promoter influences *ABCA1* regulation *in vitro*. However, thus far this has been the only LXRE described in the *ABCA1* gene, and there has been no *in vivo* validation of the sterol responsiveness of the human *ABCA1* protein.

Levels of mRNA may be poor predictors of protein expression (12), as mRNA levels can vary almost 20X 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, while decreases in cellular cholesterol efflux secondary to either antisense *in vitro* inhibition of the gene (1) or *in vivo* mutations (1-5) are associated...
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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 which 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 LXREs in intron 1. Activation of this functional internal promoter in human intron 1 by oxysterols 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 principle for therapeutic strategies directed toward the activation of ABCA1 expression and activity.

**Experimental procedures**

**Transient transfection assay**

Cells were transfected for 3 hours by lipofection using ExGen 500 (Euromedex) in OPTIMEM 1. Medium was then replaced with Dulbecco’s Modified Eagle’s Medium (DMEM) containing 0.2% fetal calf serum and cells were incubated for 48 hours. Cell extracts were prepared and assayed for luciferase activity as described (15). Twenty-four hours before transfection, HepG2, HUH7, CaCo2, Cos-1 and RK13 cells were plated in 24-well plates in DMEM supplemented with 10% fetal calf serum at 5 x 10⁵ cells/well. Transfection mixes contained 100 ng of tkpGL3 reporter vector or pGL3 containing an 8 kb fragment from ABCA1 intron 1 (pGL3-8kb). Transfection mixes contained 50 ng of reporter plasmid (tkpGL3) containing multiple copies of the putative LXREs and 25 ng of LXRα and RXR expression plasmids, in the presence of the internal control β-galactosidase expression vector. After transfection, cells were treated for 48 hours with 1 μM 22(R)-hydroxycholesterol (Sigma).
Gel Mobility Shift Assay

LXRα and RXR were transcribed and translated in vitro using pCDNA3-LXRα and pSG5-mRXRα as templates and the TNT coupled transcription/translation system (Promega). Gel mobility shift assays (20 μl) contained 10 mM Tris (pH 8), 40 mM KCl, 0.1% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 0.2 μg of poly (dIdC), 1 μg herring sperm DNA, and 2.5 μl each of in vitro synthesized LXRα 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 minute incubation on ice, 1 ng of 32P-labeled oligonucleotide was added, and the incubation continued for an additional 10 minutes. DNA-protein complexes were resolved on a 6% polyacrylamide gel in 0.5 X TBE. Gels were dried and subjected to autoradiography at -80°C.

Multicopy cloning

250 picomoles of each oligonucleotide to which half sites for BamHI and BglII restriction enzymes had been added, were phosphorylated using PNK kinase (Roche), incubated for 5 minutes at 95°C, then 10 min at 65°C and cooled to room temperature. Multimeric copies were then generated using T4 ligase, cloned in TKpG13 vector and verified by sequencing.

Generation of BAC transgenic mice

BAC's 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 previously described (1,6). Version 1.7 of Clustal W 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 BAC's were purified for injection using the Qiagen Maxi Prep kit, followed by cesium chloride purification (16) and dialysis overnight. BACs were quantified using agarose gel electrophoresis, and sets of 300 C57BL/6xCBA eggs were injected with 30ng 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 chow diet for 7 days. The diets were purchased from Harlan Teklad with the high fat/high cholesterol diet (TD 90221) containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate. This diet has previously been shown to result in upregulation of ABCA1 mRNA levels in mouse liver, assessed at 7 days after feeding (17). The control diet contained 0.5% sodium cholate (TD 99057).
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Quantification of human and mouse ABCA1 transcripts

RNA from mouse liver and peritoneal macrophages were isolated using Trizol (Gibco BRL), and 3µg of total RNA was reverse transcribed using Superscript II (Gibco BRL). Human and mouse specific primers were used along with 18S primers (Ambion Inc.) to quantitate transcript abundance. Human specific primers are as follows:

Ex3F: CAAACATGTCAGCTGTTACTGGAA G
Ex4R: GAGCCTCCCCAGGAGTG

Mouse specific primers are as follows:

Ex5F: CATTAAGGACATGCACAAGGTC C
Ex6R: CAGAAAATCCTGCAGCTTCAATT

Standard cycling conditions of denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 1 minute were used. PCR products were separated on 2% agarose gels and images captured using BioRad multianalyst software using a gelpdoc system. Bands were quantified using NIH image Version 1.6. All values are ratios with the corresponding 18S 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:

Human ABC1 Forward Primer (5’CCTGACCAGGTTTCC3’);
Human ABC1 Reverse Primer (5’TCTGATGTTGCTTC3’);
Human ABC1 TaqMan probe (5’TACATCTGGAGAACATTGCTCTGA3’),
Mouse ABC1 Forward Primer (5'TCCGAGCGAATGTCCTTC3’),
Mouse ABC1 Reverse Primer (5’CGCCTCAACTTTACGAAGGC 3’),
Mouse ABC1 Taqman probe (5’CCCAACTTCCTGCAGGCTCATC3’).

The RT-PCR reaction was carried out on ABI P... 7700 in a final volume of 50 µl, containing 40ng of total RNA, 200 µM primers and 600 µM probe in 1x TaqMan One-Step RT-PCR Master mix (PE Biosystems, CA), according to the manufacturer’s instruction. The primers and probe for 18s 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 minutes. After activation of the AmpliTaq Gold at 95°C for 10 minutes, the PCR reaction was carried out for 40 cycles (denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute). 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 cDNA was used to amplify putative transcripts using the predicted transcript primers and an *ABCA1* exon 3 reverse primer, following manufacturer's instructions. Positive transcripts were confirmed using nested PCR, and sequenced. In addition, RNA was isolated from BAC transgenic and control littermate tissue using Trizol (Gibco BRL), and 5' RACE was performed using previously described primers (18) following manufacturer's instructions (Clontech). All products were TA cloned (Invitrogen) and sequenced using an ABI 3100 automated DNA sequencer. Primers used for transcript amplification were:

- Exon 1bF: GTTGTCATCTTTGAACAAACTG
- Exon 1cF: GAGAAGGGAACTACATTGCTTG
- Exon 1dF: CACGGTAGAACTTTCTACTG
- Ex3R: CATTCATGTTGTTCATAGGGT

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 littermates were sacrificed by CO₂ inhalation, various tissues were isolated and placed in 500µl of low salt lysis buffer containing complete protease inhibitor tablets (Boehringer Mannheim) on ice. The tissues were homogenized and sonicated. The resulting homogenate was centrifuged at 14000rpm for 10 minutes at 4°C, and the supernatant was aliquoted into tubes. Protein levels were quantified using the Lowry assay. 100 - 150µg of protein was separated on 7.5% polyacrylamide gels, and was transferred to PVDF membranes (Millipore). Membranes were probed with *ABCA1*PEP4 polyclonal rabbit antibody (directed against residues 2236 to 2259 in *ABCA1*) (Wellington et al, manuscript in preparation) or monoclonal anti-glyceraldehyde phosphate dehydrogenase (Chemicon) as a control. The membrane was dipped in ECL (Amersham), and exposed to X-OMAT blue film (Kodak). Protein levels were quantitated using NIH image software.

*ABCA1* immunocytochemistry

To further compare the *in vivo* cellular expression pattern of *ABCA1* protein in both *ABCA1* BAC transgenic mice and their wild-type littermates, immunocytochemistry was performed on a variety of fixed tissues using the polyclonal *ABCA1*PEP4 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 cold 3%
paraformaldehyde in 0.1M phosphate buffered saline (PBS). The brain and liver were removed from each mouse and post-fixed for 24-48 hours in the same fixative. For each organ 30-50 mm sections were cut on a vibrating microtome (Vibratome). Sections were collected in sterile PBS at 4°C, rinsed in 0.1 M PBS with 0.3% Tween 20, and incubated in blocking solution (0.1% PBS with 0.3% Tween 20, 3% whole goat serum, and 5% bovine serum albumin) for 2 hours at room temperature.

Sections of liver were incubated for 48 hours with ABCA1PEP4. Brains from each mouse were processed for combined immunocytochemistry with a neuron-specific (NeuN, Chemicon) antibody, and ABCA1PEP4. Sections were sequentially placed into primary antisera against ABCA1 (diluted 1:2500 in block solution) and NeuN (dilution 1:50 in block solution) for 48 hours at 4°C. Following incubation with the primary antibody, sections were washed several times in blocking solution and incubated in secondary antibody for 48 hours 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 primary at a 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 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 5μl of 0.1M EDTA. For the measurement of HDL-C, the plasma was mixed 1:1 with 20% PEG20, vortexed, incubated at room temperature for 10 minutes, and spun at maximum speed for 5 minutes at room temperature (19). 20μl of the resultant supernatant was added to 96 well maxisorp plates (Millipore), and 200μl of Infinity cholesterol reagent (Sigma) was added to the wells. The plates were quantified in an ELISA reader at 492nm. For the measurement of total cholesterol, 5μl of plasma was added to the same plates, 200μl of Infinity cholesterol reagent was added, and the plate quantified as above. Triglycerides were measured by adding 10μl of the plasma to a 96 well plate, followed by the addition of 100μl of solutions from a triglyceride kit (Boehringer mannheim). FPLC separation of plasma lipoproteins was performed using two Superose™ 6 (Pharmacia) columns in series as previously described (19). Equal volumes of plasma (40μl) from mice (n=8) in each group were pooled for the analysis. The cholesterol and TG content in each 0.5ml fraction was
assessed using commercially available enzymatic kits (Boehringer Mannheim). Apoproteins were measured as previously described (20,21).

**Establishment of primary fibroblast and macrophage cultures**

For the isolation of macrophages, mice were injected intraperitoneally with 2ml of 3% thioglycollate, and 3 days later were sacrificed by CO₂ inhalation. 5ml of DMEM containing 10% FBS, L-glutamine, and penicillin/streptomycin (all Gibco BRL) was injected into the body cavity. The mouse was gently massaged, and the media was withdrawn and placed in tubes on ice. The cell pellet was resuspended in 1 ml of the above media, and plated at a density of 5x10⁶ cells/ml in a volume of 300μl. The cells were incubated in a humidified atmosphere of 5% CO₂, at 37°C until used. Fibroblasts were isolated by dissecting the femurs of the mice, and triturating the above media through the bone to remove all bone marrow. The media was added to tubes on ice, spun at 1200rpm, for 5 minutes at 4°C, and the pellet was resuspended in 10 ml of media. The cells were plated on 10cm tissue culture plates (Corning) and left in a humidified atmosphere with 5% CO₂ at 37°C.

**Measurement of efflux in fibroblast and macrophage cells**

24 hours post plating of the macrophage cells, [3H] cholesterol (2μCi/ml) (NE N Dupont), and 50μg/ml AcLDL (Intracel) were preincubated at 37°C for 30 minutes. Media was made containing DMEM, 1%FBS, pen/strep, L-glutamine, 1μM ACAT inhibitor (CI-976, a kind gift from Dr. Minghan Wang) and the preincubated 50μg/ml AcLDL and [3H] cholesterol. The media in the 24 well plates were replaced with 300μl of this cholesterol containing media, and the plates were incubated for 24 hours. The labeled media was then replaced with 0.2% defatted BSA (Sigma) or 10% delipidated serum (Sigma) containing media for about 24 hours. The media was again replaced with 300μl of DMEM, pen/strep, L-glutamine, either with or without 20μg/ml ApoA1 (Calbiochem), and the treatment compounds (9)cis-retinoic acid (Sigma) and 22(R)-hydroxy cholesterol (Steraloids). 24 hours later, the media was withdrawn and centrifuged at maximum speed for 5 minutes at room temperature. 100μl of the supernatant was added to scintillation vials, and radioactivity was quantitated. 200μl of 0.1N NaOH was added to each well containing the cells and incubated for 20 minutes at room temperature. 100μl 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 count in the medium plus the cell lysate.

**Statistical analyses**

All statistical analyses were performed using one way anova followed by the Newman-Keuls post test, except for the protein quantification and the analyses of the statistical significance between the means and standard deviations of the data provided in the footnote of Table 4. 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 to transfection of the empty pGI3 vector alone (Figure 2A).

In order to detect the presence of regulatory elements, we scanned the human ABCA1 intron 1 from position -1 to -24156 and discovered several putative regulatory elements. Among these,

![Diagram of LXRE elements in the ABCA1 5' region](image)

Figure 1. Localization of LXRE elements in the ABCA1 5' region.
A schematic diagram of the putative LXRE elements discovered in intron 1 is shown. ABCA1 genomic organization at the 5' end is shown above, and the ABCA1 BAC269 is shown below. The BAC contains 13.5kb 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 -7656bp, -7174bp and -4686bp in the ABCA1 genomic DNA and are also contained within the BAC.
we discovered several possible LXRE's containing imperfect direct repeats of the nuclear receptor half site AGGTCA separated by four nucleotides (DR4) (Figure 1, Table 1) (22). LXRE's are regulated by oxysterols (23,24) and are important transcription control points in cholesterol metabolism (25). An LXRE in exon 1 of ABCA1 had previously been shown 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 bind LXR-RXR heterodimers, gel retardation assays were performed (Figure 2B). As shown before when LXRα 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 previously described in ABCA1 exon 1 (+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 Figure 2B, all three constructs competed for binding of the CYP7A probe in a dose dependant manner, although with seemingly different efficiencies.

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 LXRα and RXR in cos-1 cells (Figure 3). As previously described, 3 copies of the consensus LXRE (3XLXRE), 5 copies of the CYP7 LXRE (5X CYP7-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-fold and 6-fold induction, respectively. In contrast, 2 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 LXRE's in intron 1 that we identified by bioinformatic and in vitro methods are functional in vivo, we generated mouse lines transgenic for the ABCA1 gene in

ABC11 BAC transgenic mice show increased cholesterol efflux

Table 1. LXR elements in the ABCA1 gene

<table>
<thead>
<tr>
<th>Target Sequence AGGTCA</th>
<th>Percent Match</th>
<th>Ratio</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGGTCA TAT CGGTCA</td>
<td>83</td>
<td>10/12</td>
<td>+</td>
</tr>
<tr>
<td>GGATCA CTG AGGTCA</td>
<td>83</td>
<td>10/12</td>
<td>-</td>
</tr>
<tr>
<td>AGATCA CTTG AGGTCA</td>
<td>92</td>
<td>11/12</td>
<td>+</td>
</tr>
<tr>
<td>AGGTCA CTGA AGGCCA</td>
<td>83</td>
<td>10/12</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 2. Intron 1 of ABCA1 has promoter activity.

(A) HepG2 (liver), HuH7 (hepatoma), CaCo2 (intestinal) and RK13 (kidney) cell lines were cotransfected with empty pGI3 vector or pGI3 containing an 8kb fragment upstream of exon 2 of ABCA1 intron1 (pGI3-8kb). Cells were then incubated for 48h. Luciferase activity was determined and plotted as fold activation relative to empty pGI3-transfected cells. (B) Gel mobility shift assays are shown in which LXRα and RXR were incubated as indicated with the radiolabeled probe corresponding to CYP7-LXRE. Binding of the LXRα-RXR heterodimer was tested by competition, by adding 5-, 25-, or 50-fold molar excess of each unlabeled oligonucleotide corresponding to the putative LXREs shown in Figure 1 and Table 1.
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**Figure 3.** Functional assessment of LXREs by LXRα transactivation.
Cos-1 cells were cotransfected with multiple copies of the putative LXREs in the reporter plasmid TKpGL3 and expression plasmids for LXRα and RXR. Cells were then treated for 48 h with 1 μM 22(R)-hydroxycholesterol. Luciferase activity was determined and plotted as fold activation relative to vehicle-treated cells.

which the promoter and exon 1 regions had been deleted. BAC 269 was used to aid in the identification of novel elements differing from the previously known active LXR element (10) in the promoter. Protein expressed from this BAC would be predicted to be full-length as the translation initiation site is in exon 2. We obtained several BAC founder lines, and all analyses were performed on two individual founder lines.

These two founder lines had different copy numbers of human BACs. Southern blot analysis revealed founder XA with 3-4 BACs and founder XB with 1-2 BACs (data not shown). In order to elucidate the transcript generated by the BAC transgenic mice lacking exon 1 of the ABCA1
Figure 4. Characterization of the alternative splice variants containing ABCA1 intron 1. (A) Schematic diagram indicating the location of the splice variants discovered in the BAC transgenic mice, and also confirmed in wild type mice and humans. Exon 1b is 120bp in length and contains a TA rich region approximately 2.5kb upstream. Exon 1c is 136bp in length, and exon 1d 178bp in length. A CAAT box is located upstream of exon 1b and CAAT and TATA boxes are found immediately upstream of exon 1c. (B) Identification of the alternative transcript involving exon 1b, 1c and 1d in wild type mouse and human liver tissue. Duplex RT-PCR was performed on mouse RNA with primers generating an exon 1b transcript fragment of ~360bp, and a fragment corresponding to the previously characterized transcript of ~250bp. Both transcripts were found in wild type and transgenic mice, with the alternative transcript being highly upregulated in the BAC transgenic mice. Different levels of each transcript were seen in liver from humans compared to wild type mice. RT-PCR generated fragments of ~380bp, and 450bp for exon 1c and exon 1d transcripts respectively. These transcripts were found to be present in both mouse and human liver RNA.
ABCA1 transgenic mice show increased cholesterol efflux

Human specific ABCA1 mRNA is upregulated 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 upregulated 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 18S RNA (Figure 5A and C). Human ABCA1 specific primers indicated the presence of 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 (Figure 5C). In the same samples, the endogenous mouse transcript is also increased in both wild type and BAC mice by 1.2-1.8 fold. (Figure 5C). In the conventional RT-PCR assay, the human ABCA1 band showed an increase of ~38% when the BAC mice were fed an atherogenic diet (Figure 5B). In the same assay, the endogenous mouse transcript in both the wild type and BAC mice were increased by ~17% in response to feeding of the atherogenic diet (Figure 5B). There was no difference observed in the amount of the endogenous mouse transcript in BAC mice when compared to wild type mice under chow or atherogenic diet conditions in both assays. Similar increases in both the human and the endogenous mouse ABCA1 transcripts was observed in peritoneal macrophage cells isolated from mice on atherogenic diets (data not shown). This clearly shows that the human ABCA1 gene contained within the BAC is regulated in response to dietary cholesterol in vivo. This induction is most likely due to the oxysterol dependent activation of LXRα or LXRβ from the LXREs present within the first intron of the ABCA1 gene.

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 to nontransgenic mice, that was distinguishable by our anti-ABCA1 antibody (Figure 6A). When the mice were fed an atherogenic diet, of the various tissues tested, the levels of ABCA1 protein were further induced in the liver (Figure 6C), giving us our first indication that ABCA1 expression levels could be up regulated by elements separate from those found in its promoter and exon 1 regions, and that LXRE's
Figure 5. Regulation of human and mouse ABCA1 transcripts

(A) Quantitative human and mouse ABCA1 specific RT-PCR was performed on wild type and BAC mouse tissue, both on chow and an atherogenic diet. An 18S primer control was included with each sample. The PCRs were separated on agarose gels and quantified using NIH image. Lanes 1, 4, 7 and 10 were amplified using mouse specific ABCA1 primers, lanes 2, 5, 8 and 11 were amplified using human specific primers and lanes 3, 6, 9 and 12 were amplified using 18S specific primers. Mouse and 18S specific transcripts were amplified in all the four mice. Human specific transcripts were only amplified in the BAC transgenic mice.

(B) The transcripts were quantitated using NIH image, and the ratio between the transcripts and the corresponding 18S bands were obtained. The human transcript was upregulated by 38% (p<0.001) in the BAC mice fed atherogenic diet compared to those fed chow diet. The endogenous mouse transcript was upregulated by 17% (p<0.001) in both the wild type and BAC mice fed an atherogenic diet when compared to those fed a chow diet. There was no significant difference observed in endogenous mouse transcript levels when comparing wild type and BAC mice both on chow diet or when comparing wild type and BAC mice both on an atherogenic diet.

(C) Quantitative real time PCR was performed on RNA isolated from the liver of wild type and BAC transgenic mice, both on an atherogenic diet and a control chow diet. Two sets of mice were used in this analysis (solid bars representing one set, and the mottled bars representing the next set), each analysed in two separate experiments in triplicate. As observed with the conventional RT-PCR method, both the human and the endogenous mouse transcript showed induction in response to the atherogenic diet. The human ABCA1 gene was upregulated by 1.4-2.3 fold in response to the high cholesterol diet and endogenous mouse ABCA1 transcript showed an upregulation of 1.2-1.8 fold in response to the same diet.

identified in intron 1 are likely to be functional in vivo. This alternative promoter is also active in macrophages and fibroblasts as determined by the increase in ABCA1 protein in these tissues in BAC transgenic mice and their response to oxysterol stimulation (Figure 6B).

Protein expression in tissues does not necessarily give any indication of the cellular distribution of a protein and can lead to misinterpretation of the expression level of a protein in a particular cell. We performed further immunohistochemical analysis on liver and brain (Figures 7A to M) of wildtype littermates and BAC transgenic mice. We observed qualitative increases in ABCA1 expression in the liver in the transgenic mice (Figures 7B and D) compared with wild type littermates (Figures 7A 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 (Figures 7K-M) and wildtype mice (Figures 7H-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 (Figure 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 to control littermates, was seen both on chow and atherogenic diet (n=4) (p=0.005 and 0.007 respectively) (Table 2 and Figure 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 wildtype littermates, the HDL-C levels increased significantly.
ABCA1 BAC transgenic mice show increased cholesterol efflux

A

Primer

Wt Chow Diet | Wt Atherogenic Diet | BAC Chow Diet | BAC Atherogenic Diet

Primers
mouse | human | 18S | mouse | human | 18S | mouse | human | 18S

500bp

B

% change in transcript

Human transcript
BAC chow vs. BAC atherogenic

Mouse transcript
Wt chow vs. Wt atherogenic

Mouse transcript
BAC chow vs. BAC atherogenic

Mouse transcript
Wt chow vs. BAC chow

Mouse transcript
Wt atherogenic vs. BAC atherogenic

* p<0.001
** p>0.05

C

Mouse ABCA1

Human ABCA1

wt chow | wt fat | BAC chow | BAC fat | wt chow | wt fat | BAC chow | BAC fat | wt chow | wt fat | BAC chow | BAC fat

4.00 | 3.50 | 3.00 | 2.50 | 2.00 | 1.50 | 1.00 | 0.50 | 0.00 | 23000.00 | 18000.00 | 13000.00 | 8000.00 | 3000.00

49
upon feeding of a cholesterol-rich diet \((n=4, p<0.001\) and \(p=0.002\) respectively) (Table 2), consistent with upregulation of the ABCA1 protein. The level of upregulation 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 upregulated upon stimulation through the LXR pathway.

Apoproteins A1 and AII were also significantly increased in the BAC transgenic compared to wildtype mice on a chow diet \((n=16, p<0.05\) and \(p<0.0001\) respectively) (Table 3).

To assess for qualitative differences in lipoprotein particles between the human ABCA1 BAC transgenics and their littermate controls, FPLC analysis was performed (Figure 8B). HDL-C levels, as indicated by the total area of the HDL peak (fractions 30-38), were increased in the transgenic mice, compared to the non-transgenic controls. The size distribution of the HDL particles appears slightly different, as the peak appears in fraction 34 in the wildtype and 35 in transgenic mice, indicating an increase in slightly smaller HDL particles, with increased

### Table 2. Analysis of plasma lipid profiles in ABCA1 BAC transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Wt Chow diet</th>
<th>BAC Chow diet</th>
<th>Wt Atherogenic Diet</th>
<th>BAC Atherogenic Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (mg/dl)</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>65.29 ± 5.8</td>
<td>92.65 ± 4.6</td>
<td>112.84 ± 14.2</td>
<td>180.68±15.5</td>
</tr>
<tr>
<td>HDL-C</td>
<td>36.98 ± 1.8</td>
<td>61.18 ± 11.0</td>
<td>77.84 ± 8.8</td>
<td>137.30±27.9</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>42.66 ± 7.55</td>
<td>49.95 ± 3.62</td>
<td>50.60 ± 2.33</td>
<td>57.30 ± 4.43</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td>28.31 ± 3.77</td>
<td>31.47 ± 4.80</td>
<td>35.00 ± 4.50</td>
<td>43.38 ± 4.68</td>
</tr>
</tbody>
</table>

Figure 6. Expression of ABCA1 protein in BAC transgenic mouse tissue.

(A) Western blot analysis of various mouse tissues from BAC transgenic mice and control littermates on chow diet. Tissue from two different founder lines were analyzed and showed similar results. ABCA1 was detected in liver, brain, small intestine, testis, lung and stomach, with highest level of upregulation in the BAC mouse liver when compared to control. (B) Increase of ABCA1 protein levels in liver in response to ad libitum feeding of an atherogenic 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 probed with an anti GAPDH antibody (Sigma) to ensure equal protein loading levels, and the corresponding GAPDH lanes are shown below the western blots. (C) Western blot analysis was also 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.
ABCA1 BAC transgenic mice show increased cholesterol efflux

<table>
<thead>
<tr>
<th>Wt chow vs. Wt atherogenic</th>
<th>Wt chow vs. BAC atherogenic</th>
<th>BAC chow vs. Wt atherogenic vs. BAC atherogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>P value</td>
<td>P value</td>
</tr>
<tr>
<td>0.0003</td>
<td>0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0.005</td>
<td>&lt;0.0001</td>
<td>0.002</td>
</tr>
<tr>
<td>0.17</td>
<td>0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>0.34</td>
<td>0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 3. Quantitation of Apoprotein levels in BAC transgenic mice

<table>
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<tr>
<th>Wt chow diet</th>
<th>BAC chow diet</th>
<th>Wt chow Vs. BAC chow</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 16 Mean ± SD</td>
<td>n = 16 Mean ± SD</td>
<td>P value</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>0.43 ± 0.018</td>
<td>0.49 ± 0.018</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>0.35 ± 0.022</td>
<td>0.57 ± 0.032</td>
</tr>
<tr>
<td>Apo B</td>
<td>0.15 ± 0.0099</td>
<td>0.13 ± 0.0059</td>
</tr>
<tr>
<td>Apo C-III</td>
<td>0.39 ± 0.027</td>
<td>0.47 ± 0.032</td>
</tr>
</tbody>
</table>

A

![Image](image_url)

B

![Image](image_url)

C

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

Localization of ABCA1 protein was determined by immunocytochemical analysis using an ABCA1 specific polyclonal antibody (ABCPEP4) in liver and brain tissues. Endogenous levels of ABCA1 are identified in sections from wild type liver (low power, A and high power C). Elevated ABCA1 levels are seen in liver tissues from BAC transgenics (low power, B and high power, D). The tissue distribution of ABCA1 is similar in sections from cerebral cortex from both wild type and BAC transgenic brains (F and G). ABCA1 was predominantly neuronal as shown by co-localization with the neuronal marker NeuN in wild type (H, I, J) and BAC transgenic brains (K, L, M). A primary antibody omitted control showing no staining is shown in panel E. The scale bars are panel A-B 40mm, C-E 25mm, F-G 25mm, H-M 20 μm.
ABCA1 BAC transgenic mice show increased cholesterol efflux

**Figure 8.** Analysis of plasma lipid levels in transgenic mice. (A) BAC transgenic mice show a 65% increase in HDL-C levels compared to control littermates on a chow diet. Furthermore, HDL-C levels in both BAC transgenic and wild type mice were increased by >100% in response to feeding of the atherogenic diet, with the BAC transgenic mice having close to 2x the HDL-C seen in the nontransgenic littermates. (B) While quantitative changes are apparent, there are no major qualitative changes in HDL-C in transgenic versus nontransgenic mice, either on chow (A) or atherogenic diets (B).

expression of ABCA1. This is in keeping with the role of ABCA1 in the initial lipidation of ApoA1 and not its subsequent enlargement. Remnant lipoproteins and LDL-C (fractions 12-20, 24-28, respectively) were not readily different between transgenics and controls. HDL-C levels were further increased on feeding with the atherogenic diet (Figure 8B, panel B) 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.
Chapter 2

**ABCA1 BAC transgenic mice show increased efflux**

A defect in cholesterol and phospholipid removal mediated by apolipoproteins has been previously observed 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 (Figure 9A) (Table 4) and fibroblast (Figure 9B) (Table 4) cultures obtained from the transgenic mice when compared to wildtype littermates. These efflux levels were further significantly increased when the mice were fed the atherogenic diet (Figure 9A and 9B). 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)-hydroxy cholesterol which are specific activators of the LXR/RXR pathway also significantly upregulated the efflux levels from both the macrophage (figure 9A) and fibroblast cells (Figure 9B) (Table 4). BAC mice on atherogenic versus chow diets showed higher levels of upregulation of efflux when compared to wildtype 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 difference in upregulation of efflux between stimulated BACs and stimulated wildtype 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 cholesterol efflux and the decrease in plasma HDL-

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**Figure 9.** Analysis of efflux levels in primary macrophage and fibroblast cells.

**(A)** Primary macrophage cultures were established from mice as described. Efflux was measured 24 hours after the addition of ApoAI and after stimulation by 9(cis) retinoic acid and 22(R)-hydroxy cholesterol (n=4, see Table 4). We observed a significant increase (46%) in efflux levels of BAC transgenic macrophages when compared to macrophages from wild type (wt) littermates on the same chow diet (p<0.001). Both sets of animals showed an increase in efflux upon stimulation of the cultures with 9(cis) retinoic acid and 22(R)-hydroxy cholesterol. BAC transgenic mice on the atherogenic diet showed an increase in efflux when compared to BAC and wt mice on the control chow diet (p<0.0001 and p=0.0004 respectively). This efflux rate was further increased when the cultures were stimulated with 9(cis) retinoic acid and 22(R)-hydroxy cholesterol (p<0.01). **(B)** Efflux was also performed on fibroblast cultures established from BAC transgenic and control mice (n = 4, see Table 4). An increase in efflux was seen in BAC transgenic mice when compared to wt littermates on chow diet (p=0.03). This level is further increased in both transgenic and wt mice by 55.8% in response to stimulation by 9(cis) retinoic acid and 22(R)-hydroxy cholesterol. BAC transgenic mice fed the atherogenic diet showed a significant (>100%, p<0.0001) increase in efflux levels when compared to chow fed transgenic animals. These levels were mildly increased (by 11.2%) when the cultures were stimulated with 9(cis) retinoic acid and 22(R)-hydroxy cholesterol.
ABCA1 BAC transgenic mice show increased cholesterol efflux

A

untreated 22(R)-hydroxy cholesterol 9(cis)retinoic acid

%Efflux

0 25 50 75

Wt-chow diet Wt-atherogenic diet Wt-chow diet Wt-atherogenic diet Wt-chow diet Wt-atherogenic diet Wt-chow diet Wt-atherogenic diet

* P<0.01
** P<0.001

B

untreated 22(R)-hydroxy cholesterol 9(cis)retinoic acid

%Efflux

0 10 20 30 40

wt-chow diet wt-atherogenic diet wt-chow diet wt-atherogenic diet wt-chow diet wt-atherogenic diet wt-chow diet wt-atherogenic diet

* P<0.05
** P<0.001
Table 4. Analysis of [3H]-cholesterol efflux to ApoAI in ABCA1 BAC transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Wt chow diet</th>
<th>BAC chow diet</th>
<th>Wt atherogenic diet</th>
<th>BAC atherogenic diet</th>
<th>Wt chow VS BAC chow</th>
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<tr>
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<td>n=4</td>
<td>n=4</td>
<td>n=4</td>
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</tr>
<tr>
<td>Mean ± SD</td>
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<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>0.31 ±0.014</td>
<td>0.45 ±0.0033</td>
<td>0.40 ±0.022</td>
<td>0.54 ±0.0077</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Macrophage [22(R)-OH chol.] 9(cis) RA</td>
<td>0.46 ±0.0085</td>
<td>0.49 ±0.0067</td>
<td>0.52 ±0.0054</td>
<td>0.59 ±0.015</td>
<td>0.002</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>0.12 ±0.011</td>
<td>0.14 ±0.017</td>
<td>0.16 ±0.014</td>
<td>0.29 ±0.011</td>
<td>0.03</td>
</tr>
<tr>
<td>Fibroblast [22(R)-OH chol.] 9(cis) RA</td>
<td>0.19 ±0.014</td>
<td>0.22 ±0.015</td>
<td>0.27 ±0.014</td>
<td>0.32 ±0.0033</td>
<td>0.01</td>
</tr>
</tbody>
</table>

P values for uninduced vs. induced in each condition: a,b,c,g: p<0.0001, d: p<0.001, e: p<0.0002, f: p<0.0004, h: p<0.05

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 (r) 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 ABCA1 protein (r²=0.98, p=0.001), showing that any approach which 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 BAC transgenic mice containing sequence from all of the introns, including intron 1 without promoter sequence, has allowed for the identification of three novel transcripts initiating in intron 1 and demonstrated that an internal promoter containing LXREs in intron 1 contributes to the normal regulation of ABCA1 and its responsiveness to oxysterol stimulation.

It could be argued that the increase in ABCA1 protein and HDL levels in this study is due to in vivo regulation of the endogenous mouse ABCA1 protein alone. Numerous findings argue against this. Firstly, the baseline 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 which was more than 2x greater than the increase in endogenous mouse mRNA in the same experiments. This provides formal proof that transcription of the human ABCA1 with only intron1 shows cholesterol responsive regulation in vivo.

The human ABCA1 gene comprises 50 exons spanning 149kb genomic DNA (28). Translation begins in exon 2 and transcription had previously been shown to be initiated at a 303bp exon
located 24,459bp upstream of exon 2 (18). Here we have shown three other transcription initiation sites utilizing sequence from intron 1 and giving rise to three new ABCA1 transcripts. At the present time, approximately 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 which 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 which 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 theses 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 to the promoter LXRE's, remains to be determined. However, it is clear that alternate transcript decisions in regard to intron 1 sequence are influenced by specific factors which 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 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 ApoA1 gene in rats, whilst in humans this clearly results in activation of ApoA1 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 intron1 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 LXR/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 LXR-α, β and RXR-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 exon1c 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 subspecies may also influence the ability to observe ABCA1-mediated increases in HDL-C.

The availability of mice described in this manuscript will now allow us to address the question 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, provides additional support for the development of therapeutics influencing ABCA1 protein expression.

Acknowledgements
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References
Chapter 2


ABCA1 BAC transgenic mice show increased cholesterol efflux


Chapter 2


