The E-box motif in the proximal ABCA1 promoter mediates transcriptional repression of the ABCA1 gene

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Abstract **To identify regulatory elements in the proximal human ATP-binding cassette transporter A1 (hABCA1) gene promoter we transfected RAW cells with plasmids containing mutations in the E-box, AP1, and liver X receptor (LXR) elements as well as the two Sp1 motifs. Point mutations in either Sp1 site or in the AP1 site had only a minor effect whereas mutation of the LXR element decreased promoter activity. In contrast, mutation or deletion of the E-box motif caused a 3-fold increase in transcriptional activity under basal conditions. Gel shift and DNaseI footprint analysis showed binding of a protein or protein complex to this region. Preincubation of nuclear extracts with antibodies established that USF1, USF2, and fos related antigen (Fra) 2 bind to DNA sequences in the human ABCA1 promoter that contains the intact E-box but not the mutant or deleted E-box. Co-transfection of USF1 and USF2 enhanced, but Fra2 repressed, ABCA1 promoter activity. Thus, a complex consisting of USF1, USF2, and Fra2 binds the E-box motif 147 bp upstream of the transcriptional start site and facilitates repression of the human ABCA1 promoter. These combined studies identify a novel site in the human ABCA1 promoter involved in the regulation of ABCA1 gene expression.—** Yang, X-P., L. A. Freeman, C. L. Knapper, M. J. A. Amar, A. Remaley, H. B. Brewer, Jr., and S. Santamarina-Fojo. **The E-box motif in the proximal ABCA1 promoter mediates transcriptional repression of the ABCA1 gene. *J. Lipid Res.* 2002.** 43: **297–306.**

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Maintaining cholesterol homeostasis is a critical task for higher eukaryotes. Whereas appropriate levels of cholesterol are essential for maintaining membrane fluidity and sterol levels, inappropriate levels can lead to disease and even death. The balance of cholesterol synthesis, uptake, metabolism, efflux, transport, and elimination is thus exquisitely regulated. The metabolic pathways leading to cholesterol biosynthesis and uptake through the LDL and HDL pathways have been extensively investigated. However, the molecular processes involved in eliminating excess cholesterol from cells have only just begun to be elucidated.

A key player in cholesterol efflux appears to be the ATP-binding cassette transporter A1 (ABCA1) protein. The ABCA1 gene, a member of the ATP-binding cassette transporter family, encodes a membrane protein that facilitates the cellular efflux of cholesterol and phospholipids to apolipoprotein A-I (apoA-I) or poorly lipidated HDL from a variety of cells (1–3). Mutations in both copies of the ABCA1 gene lead to Tangier disease (4–10), characterized by accumulation of cholesterol ester in the tonsils, liver, spleen, and intestinal mucosa, and low levels of serum HDL and familial hypoalphalipoproteinemia.

Given the key role of the ABCA1 gene in facilitating cholesterol efflux, it is of interest to determine how the ABCA1 gene itself is regulated. Initial studies indicate that transcription of the native ABCA1 gene is regulated by a number of metabolites. ABCA1 expression is highly regulated by cAMP and sterols (3, 8, 11–17). Oxysterols and 9-*cis*-retinoic acid (RA) stimulate ABCA1 transcription through LXR/retinoid X receptor (RXR) heterodimers (18–20). In addition, a Zn finger protein that maps to a hypoalphalipoproteinemia locus has been reported to repress basal and induced expression of the hABCA1 gene (21–24). Finally, interferon γ down-regulates ABCA1 (25).

Earlier, we reported that the 200 bp region upstream of the human ABCA1 gene promoter confers cholesterol regulation of the hABCA1 gene (14). This region contains multiple binding motifs potentially involved in the regulation of ABCA1 gene transcription (14, 15, 18). In order to identify transcription factors involved in regulation of ABCA1 gene expression, we examined the activity of the

Abbreviations: ABC, ATP binding cassette; CRA, 9-*cis*-retinoic acid; Fra, fos related antigen; hABCA1, human ATP-binding cassette transporter A1; 22(*R*)-Hch, 22-*R*-hydroxycholesterol; LRP, LDL receptor related protein; LXR, liver X receptor; RA, retinoic acid; RXR, retinoid X receptor.

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proximal 200 bp wild-type and mutant ABCA1 promoters in transient transfection assays. In the present study we report that the E-box motif 147 bp upstream of the transcriptional start site of the hABCA1 gene promoter binds the helix-loop-helix transcription factors USF1 and USF2, as well as fos related antigen (Fra) 2, leading to repression of the ABCA1 gene. These results identify a new pathway for the repression of ABCA1 gene transcription.

MATERIALS AND METHODS

Construction of reporter plasmids for luciferase assay

Plasmids containing mutant Sp1, AP1, E-box, LXR, and deleted E-box fragments were constructed by site-directed mutagenesis using the overlap PCR method and the PXP1 −995 to +120 bp construct (14) as template. The primers listed below were used to amplify −200 to +44 bp (p200-L) of the human ABCA1 promoter. Upper-case letters represent wild-type sequence whereas lower-case letters represent mutant sequence.

MDistal Sp1F: 5′TCGCCCGTTTAgGcttgGGcgCCCGGCTC3′
MDistal Sp1R: 5′GAGCCGGGcgCCcaagCcTAAACGGGCGA3′
Mproximal Sp1F: 5′CAGAGGCCGGGAgGcttgGGcgGGAGGGA3′
Mproximal Sp1R: 5′TCCCTCCcgCCcaagCcTCCCGGCCTCTG3′
MAP1F: 5′CGTGCTTTCTGCTGAGgatgcGAACTAC3′
MAP1R: 5′GTAGTTCgcatcCTCAGCAGAAAGCACG3′
MEBoxF: 5′CGGCTCCtcacggCTTTCTGCTGAGT3′
MEBoxR: 5′ACTCAGCAGAAAGccgtgaGGAGCCG3′
DEboxF: 5′GCCTCCTTTCTGCTGAGTGACTGA3′
DEboxR: 5′GAAAGGAGCCGGGGCCC GCCCCA3′
MLXRF: 5′CTTTGtgtGATAGTAAActaCTGCGCTCGGTGCA
MLXRR 5′TGCACCGAGCGCAGtagTTACTATCacaCAAAG
S224-*Hind*III: 5′ACTCCCAAGCTTTGTCGTGG3′
44-*Hind*III: 5′GAGAAGCTTCGGCTCGGCTCTG3′

S224-*Hind*III and 44-*Hind*III were the upstream and downstream primers used for overlap PCR. The *Hind*III sites are underlined. The resulting fragments that spanned −200 to +44 of the hABCA1 gene were ligated into the *Hind*III site of the PXP1 luciferase reporter plasmid (26). For co-transfection studies pCMV-USF1, pCMV-USF2 (27) (generous gifts from Dr. Michele Sawadogo), and pCMV-Fra2 plasmids were utilized. The pCMV-Fra2 expression vector was generated by PCR from cDNA clone 947,694 (ATCC) using forward (5′-TAA CAG AGC GGC CGC ATC ATG TAC CAG GAT TAT CCC-3′) and reverse (5′-AGG TGC GGC CGC TTA CAG AGC CAG CAG AGT GGG GGA GTT CAA GGA GTC TGA-3′) primers. The resulting PCR fragment containing the Fra2 coding sequence was digested with *Not*I and cloned into the *Not*I site of a pUC18-derived vector containing the cytomegalovirus immediate early promoter and the polyadenylation site of SV40 (28). All constructs were confirmed by sequencing.

Cell culture and transfection

Murine RAW 264.7 cells and human embryonic kidney 293 cells (American Type Culture Collection, Rockville, MD) were grown in DMEM with 10% FBS. Approximately 1.5×10^5 cells were plated in 12-well plates (Costar, Corning, NY), grown to 50–70% confluency and cotransfected with 1.5 μg of the ABCA1 promoter-luciferase plasmid and 0.5 μg of the β-galactosidase vector (pCMVβ; Clontech, Palo Alto, CA) by using the Superfectin Reagent Kit (Qiagen, Valencia, CA). For cotransfection experiments, 0.8 μg of the ABCA1 promoter-luciferase construct (wild-type or E-box deleted) was cotransfected with 0.8 μg of either pCMV-USF1, pCMV-USF2, pCMV-Fra2, or the corresponding empty vectors. USF overexpression plasmids were CsCl-purified and Fra2 overexpression plasmids were purified using an EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). Three hours after addition of DNA, the cells were refed with fresh media containing 10% FCS. Sixteen hours later, cells were washed with PBS and refed with DMEM containing 0.1% BSA and either 50 μg/ml cholesterol, 2 μg/ml 22-(*R*)-hydroxycholesterol [22(*R*)-Hch], 10 μM 9-*cis*-retinoic acid (9CRA), 10–100 nmol estradiol (Sigma, St. Louis, MO), 10–100 nM regular insulin (Sigma), or 0.1% ethanol for 24 h. After harvesting, 10 μl of cell extracts were used for luciferase and β-galactosidase assays by using the Promega dual luciferase assay system (Promega, Madison, WI) or the Tropix Galacto-Light Plus Kits, respectively (Tropix, Bedford, MA). In a single transfection study each plasmid was transfected in quadruplicates. A total of at least three independent transfections were performed for each study. The ratio of luciferase activity in relative light units was divided by the β-galactosidase activity to give a normalized luciferase value.

Gel mobility shift assay

Three double-stranded hABCA1 promoter fragments (Fragment A spanning −171 to −71 bp, Fragment EB spanning −156 to −130 bp, and Fragment EB spanning −156 to −130 bp) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Lofstrand, Gaithersburg, MD). Nuclear extracts were isolated from unstimulated RAW 264.7 cells, and HepG2 cells as well as RAW 264.7 cells after stimulation with the same concentrations of cholesterol and 22-*R*-hydroxycholesterol [22(*R*)-Hch] shown above (Paragon Bioservices Inc., Baltimore, MD). One nanogram (10,000 cpm) of radiolabeled probe was added to 2.5 μg nuclear extract in 20 μl of a 20 mM TRIS gel shift buffer (pH 7.9) containing 60 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 1.3 mM $MgCl_2$, 10% glycerol, 3% Ficoll and 3 μg of double-stranded poly (dIdC) as described (29), and incubated for 10 min on ice followed by 10 min at room temperature. The incubated mixture was loaded on a 6% polyacrylamide gel in 0.25 × tris-boric acid-EDTA (TBE) buffer and electrophoresed at 100 V for 90 min followed by autoradiography. For competition assays, nuclear extracts were preincubated for 10 min on ice in a 20 μl reaction mixture in the presence or absence of a 100–200-fold excess of double-stranded DNA competitors for Sp1 (−173 to −155 bp), AP1 (−135 to −155 bp), LXR (−54 to −69 bp) and E-box (−158 to −136 bp) before addition of probe. For supershift assays, nuclear extracts were preincubated with antibodies against different E-box binding proteins including Mad1, Mad2, Mad3, Max, c-Myc, MyoD, USF1, and USF2, as well as Fra2 (Santa Cruz Biotechnology, Santa Cruz, CA), on ice for 30 min before addition of probe.

DNaseI protection assay

End-labeled Fragment A was digested with AspHI. The 94 bp fragment was gel-purified from a 10% acrylamide TBE gel (Novex). One microliter of probe (10,000 cpm) was added to 14 μl of RAW cell nuclear extract (5.2 μg/μl protein) in gel shift buffer (see above) and incubated on ice for 10 min. After 10 min at RT, 20 μl DNaseI digestion buffer (10 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, 5.0 mM $MgCl_2$) was added, then 15 s later DNaseI was added and incubated for 1 min 45 s. DNaseI stop buffer (10 mM Tris-HCl pH 8.0, 0.6 M sodium acetate pH 7.0, 0.5% SDS, 100 mM EDTA) was then added. Two microliters of protease K at 20 mg/ml were added and the samples were incubated at 37°C for 30 min. Ten microliters of 3 M NaOAc and 4.0 μl tRNA (10 mg/ml) were added. Samples were phenol-chloroform extracted and

the aqueous phase was precipitated with 2.5 volumes of 100% ethanol. After a 70% ethanol wash, pellets were dissolved in sequencing gel loading buffer, heated and run on an 8% sequencing gel. Naked DNA was digested with DNaseI as described above except that nuclear extract addition and protease K treatment were omitted. Maxam-Gilbert sequencing was performed as described (30).

Western analysis

RAW cell nuclear extracts (35 μg protein per lane) from cells stimulated with cholesterol or 22-*R*-hydroxycholesterol were loaded onto NuPage Bis-Tris 4–12% gradient gels (Invitrogen, Carlsbad, CA) and run according to manufacturer's specifications. Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corp, Bedford, MA). Antibodies (2 mg/ml stock) against USF1 (sc-229X and sc8983X) and USF2 (sc862X and sc861X) and Fra2 (sc-604X) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and were used according to manufacturer's specifications.

RESULTS

Analysis of binding motifs in the proximal hABCA1 gene promoter

The proximal −200 bp of the hABCA1 promoter contains multiple binding motifs, including Sp1 (−100 and −166 bp), AP1 (−131 bp), LXR (−69bp), and E-box (−147bp) (14, 15, 18). Transcription factors that bind to Sp1, AP1, LXR, and E-box motifs have been implicated in the transcriptional regulation of other genes involved in lipid metabolism including apolipoprotein A-II (apoA-II) (31), apolipoprotein C-III (apoC-III) (32), apolipoprotein E (apoE) (33), chicken vitellogenin II (34), fatty acid synthase (35–37), stearoyl-CoA desaturase 1 (38), the LDL receptor (39), the LDL receptor related protein (LRP) (40), CYP7a (41, 42), CETP (43), ABCG5/ABCG8 (44), ABCG1 (45), and SREBP-1c (46, 47). To investigate the potential role of the transcription factor binding motifs in this region of the hABCA1 gene promoter, we generated luciferase reporter constructs under the control of the −200 bp hABCA1 promoter, either wild-type (p200-L) or mutated. **Figure 1A** shows the locations of point mutations introduced into the −200 bp promoter region of the hABCA1 gene.

The effect of these point mutations on the transcriptional activity of the hABCA1 gene in unstimulated RAW cells is illustrated in Fig. 1B. Under the conditions utilized, mutations in the distal Sp1 site, the AP1 site, and the proximal Sp1 sites had only a minor effect on promoter activity. In contrast, mutation of the E-box caused a strong and significant increase in promoter activity, and mutation of the LXR element caused a strong and significant decrease in promoter activity. These data are consistent with binding of a transcriptional repressor to the hABCA1 E-box and binding of a transcriptional activator to the LXR element. Whereas previous studies have implicated the LXR element as the hABCA1 promoter motif responsive to oxysterols (15, 18, 20), the repressive effect of the E-box on hABCA1 promoter activity is a novel finding.

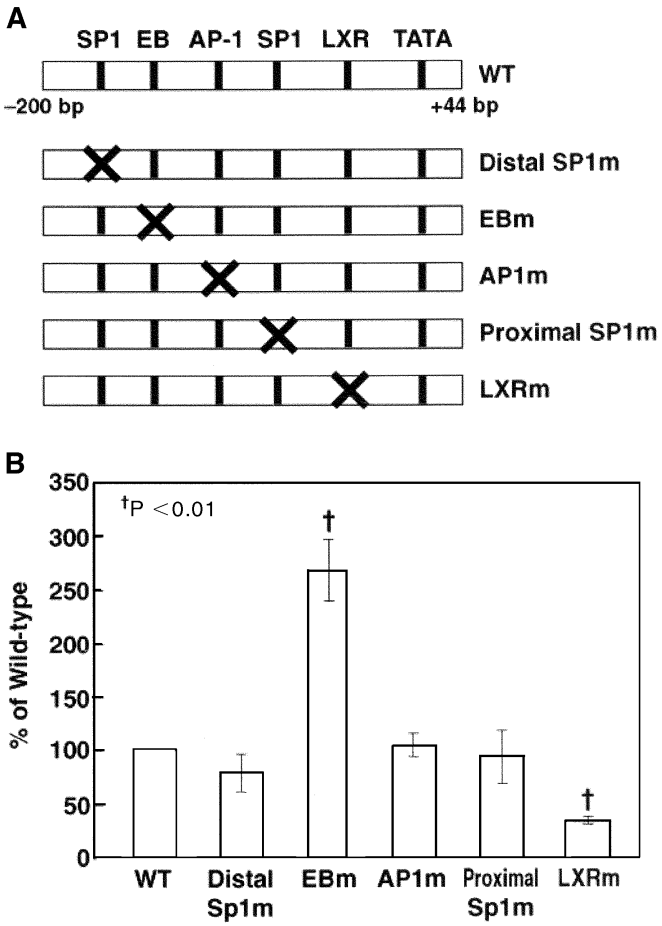

Fig. 1. Effect of point mutations on human ATP-binding cassette transporter 1 (hABCA1) promoter activity. A: Schematic illustration that shows the location of the point mutations introduced into the −200 bp promoter region of the hABCA1 gene. B: Summarizes the luciferase activity in RAW cells after transfection with wild-type and mutant constructs. The data shown represent the mean of three independent transfection studies. Values are expressed relative to the wild-type construct.

Mutation of the E-box increases transcription of the hABCA1 gene

Figure 2 demonstrates that mutation or deletion of the E-box motif increases transcription of the human ABCA1 gene in unstimulated RAW cells by approximately 3-fold. Compared with the wild-type promoter activity in unstimulated cells, the promoter activity of RAW cells transfected with either reporter constructs and stimulated with cholesterol, *cis*-retinoic acid (CRA), and oxysterol increased by up to 40-fold. Furthermore, mutation or deletion of the E-box in the proximal hABCA1 promoter had no effect on the stimulatory effect of either CRA or oxysterols. Similar findings were demonstrated for unstimulated (Fig. 2, inset) and stimulated human embryonal kidney 293 cells (data not shown). These results indicate that the LXR-mediated activation of ABCA1 gene transcription by CRA and oxysterols does not require an intact E-box motif and are consistent with binding of a transcriptional repressor to the E-box in the human ABCA1 gene.

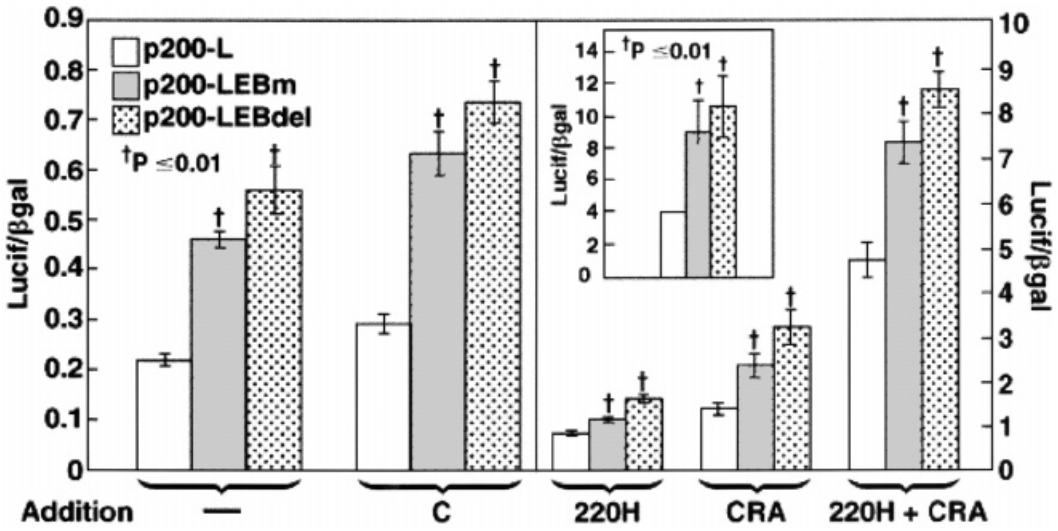

Fig. 2. Mutation of the E-box increases transcription of the hABCA1 gene. RAW 264.7 cells were transfected with wild-type construct (p200-L), mutant E-box construct (p200-EBm), or the deleted E-box construct (p200-EBdel) along with a β-galactosidase expression plasmid. Three hours after transfection, cells were refed with fresh media containing 10% FCS. Sixteen hours later, cells were washed with PBS and refed with DMEM media containing 0.1% BSA and either 50 μg/ml cholesterol, 2 μg/ml 22-*R*-hydroxycholesterol [22(*R*)-Hch], 10 μM 9-*cis*-retinoic acid (CRA), or 2 μg/ml 22(*R*)-Hch plus 10 μM 9CRA for 24 h. Cell lysates were analyzed for luciferase and β-galactosidase activity. Luciferase values were normalized to β-galactosidase activity and expressed as mean ± SEM. Data from a representative experiment out of a total of three independent transfections are shown. Inset: As above but with human embryonal kidney 293 cells.

Binding of transcription factors to the E-box has been implicated in the regulation of gene expression by insulin and estrogen. In separate studies we demonstrated that incubation of RAW and 293 cells with either 10–100 nM estradiol or 10–100 nM insulin had no consistent effect on transcription of the wild-type p200-L (data not shown).

Nuclear transcription factors bind the E-box in the hABCA1 gene promoter

DNaseI footprint analysis of the ABCA1 proximal promoter revealed protection of the E-box in the presence of RAW nuclear extracts (**Fig. 3**), indicative of a protein binding to this region. To further demonstrate binding of nuclear transcription factors to the E-box motif, we performed gel-shift analysis of the hABCA1 promoter (**Fig. 4**). The probe utilized in Fig. 4B (left) was a 100 bp double-stranded fragment spanning −171 through −71 of the ABCA1 promoter (Fig. 4A, Fragment A). Incubation of the radiolabeled probe with nuclear extract isolated from unstimulated RAW cells resulted in a gel shift (Fig. 4B). The shift was abolished when either unlabeled Fragment A (A) or a double-stranded oligonucleotide spanning the E-box (EB) were used as competitors. Competition with the 27 bp fragment encoding a scrambled E-box (EBm) did not abolish the gel shift band. Similar results were observed when nuclear extracts from unstimulated 293 cells were utilized (data not shown).

Gel-shift analysis of the human ABCA1 promoter was also performed using either a 27 bp double-stranded probe spanning the E-box (Fig. 4A, Fragment EB, right panel) or an alternative probe containing a scrambled mutant E-box sequence as described in Materials and Methods (designated Fragment EBm; schematic not illustrated). Incubation of the wild-type E-box probe with unstimulated RAW cell nuclear extract resulted in a gel shift, indicating binding of a protein to this probe (Fig. 4B, middle panel). Addition of unlabeled wild-type competitor (EB) eliminated binding. In contrast, competition with the mutant E-box fragment (EBm) did not significantly affect binding to the wild-type E-box. Moreover, using the mutant E-box as a target probe for the binding of cellular nuclear extracts did not result in the formation of a gel shift band (Fig. 4B, right panel). These experiments therefore demonstrate specific binding of a protein to the wild-type E-box motif of the hABCA1 gene.

USF and Fra2 bind the E-box in the hABCA1 gene promoter

In the human ABCA1 promoter, the E-box is flanked by two Cs, leading to a sequence of CCACGTGC. This is a perfect match to the consensus motif for the transcription factor USF. To establish that USF is in fact the transcription factor that binds to the E-box in the hABCA1 gene promoter, we performed gel shift analysis utilizing USF-specific antibodies (Fig. 4C). Using the 100-bp fragment as a probe (Fig. 4C, left panel), we again demonstrated that the E-box gel-shifts some protein in the RAW cell nuclear extract. Addition of anti-USF antibodies against either the amino (N) or carboxy (C) terminus of USF1 or USF2 caused a supershift of the gel-shifted probe, confirming the identity of the E-box binding proteins as USF1 and USF2. Antibodies against other E-box binding proteins including Mad1, Mad2, Mad3, c-Myc, MyoD, and Max (data not shown) did not compete or supershift the DNA-

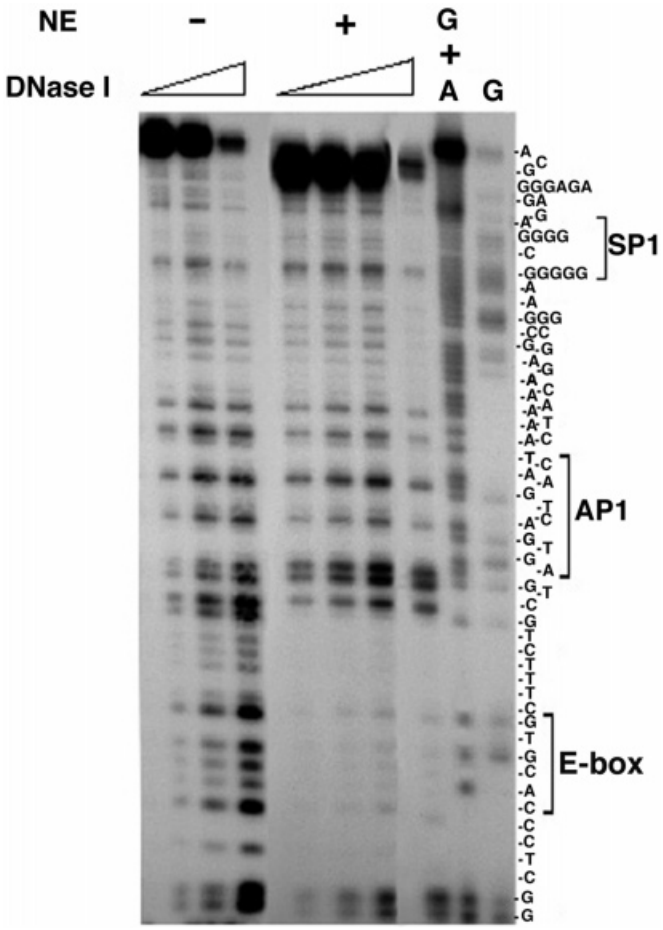

Fig. 3. Footprint analysis of the hABCA1 gene promoter. A radiolabeled fragment (−171 to −77 bp) of the human ABCA1 promoter was digested with different concentrations of DNaseI in the presence (+) or absence (−) of RAW cell nuclear extract (NE). The G and G+A ladders from Maxam and Gilbert sequence of the radiolabeled fragment are shown. The location of the Sp1, AP1, and E-Box motifs in the hABCA1 promoter are indicated on the right.

protein gel shift band. Fra, a member of the c-fos family of transcription factors that binds AP1 motifs, has been reported to interact with USF (48–50). Incubation of RAW cell (Fig. 4C) or 293 cell nuclear extracts (data not shown) with Fra2 but not Fra1 antibodies prevented the formation of the larger molecular weight DNA-protein complex but also resulted in the formation of a smaller molecular weight complex. This finding is consistent with binding of Fra2 to the 100 bp Fragment A but in addition, it suggests that other proteins, not displaced by the Fra2 antibody, are still associated with Fragment A.

Similar results were obtained by preincubating the 27 bp double-stranded fragment spanning the E-box (EB) with anti-USF (Fig. 4C, right panel). Incubation of the 27 bp probe with Fra2 antibody prevented the formation of the DNA-protein complex. Since the 27 bp probe lacks the AP1 DNA binding motif, these data indicate specific binding of Fra2 to the E-box-USF1-USF2 complex.

As with the 100 bp gel shift fragment, antibodies specific to other members of the helix-loop-helix family of transcription factors known to also bind the E-box motif including Mad1, Mad2, Mad3, c-Myc, MyoD, and Max did not alter the gel shift band obtained with the 27 bp EB probe (Fig. 4C, right panel). Separate studies demonstrated no differences in the gel shift banding patterns obtained when nuclear extracts isolated from unstimulated RAW cells and RAW cells stimulated with cholesterol or oxysterols were incubated with the EB probe (data not shown).

These combined data identify USF1, USF2, and Fra2 as the transcription factors that bind to the E-box in the proximal ABCA1 promoter and indicate that their binding is not modulated by known activators of ABCA1 gene expression.

USF1, USF2, and Fra2 are expressed in RAW cells

To establish the presence of USF1, USF2, and Fra2 in RAW cell and 293 cell nuclear extracts, Western blot hybridization analyses were performed utilizing antibodies specific to USF1 (N- and C-terminus), USF2 (N- and C-terminus), and Fra2. Our studies identified the two major USF immunoreactive bands of approximately 43 and 44 kDa in size, and one major Fra2 immunoreactive band 46 kDa in size (data not shown). Importantly, expression of the 18 kDa mini-USF2 isoform which lacks the carboxy-terminus transcriptional activating domain (51, 52) was not detected in either unstimulated RAW cells or RAW cells incubated for 24 h with cholesterol or oxysterols.

USF1 and USF2 enhance, but Fra2 represses, transcription of the hABCA1 promoter

To evaluate the effect of USF1, USF2, and Fra2 on hABCA1 gene transcription, the p200-L construct was co-transfected with pUSF1, pUSF2, or pFra2 in RAW cells (**Fig. 5**) and 293 cells (data not shown). USF1 and USF2 overexpression enhanced activity of the wild-type but not the E-box-deleted proximal hABCA1 promoter by up to 2.5-fold (Fig. 5A). In contrast, Fra2 overexpression decreased activity of the wild-type but not the E-box-deleted hABCA1 promoter (Fig. 5B). These findings identify Fra2 as a transcription factor that represses the proximal hABCA1 promoter.

DISCUSSION

In the present study we have identified an E-box motif 147 bp upstream of the transcriptional start site that exerts a repressive effect on the hABCA1 promoter. Mutation or deletion of the E-box increases promoter transcriptional activity in RAW cells and human embryonal kidney 293 cells under basal conditions by 3-fold and after stimulation with cholesterol, 9-CRA, oxysterols, and CRA plus oxysterols by as much as 40-fold. We demonstrate that E-box mediated repression of the hABCA1 gene involves binding of a protein complex that includes the ubiquitous transcription factors USF1, USF2, and Fra2 known to either enhance or decrease gene transcription. We show that overexpression of USF1 and USF2 activate, but Fra2

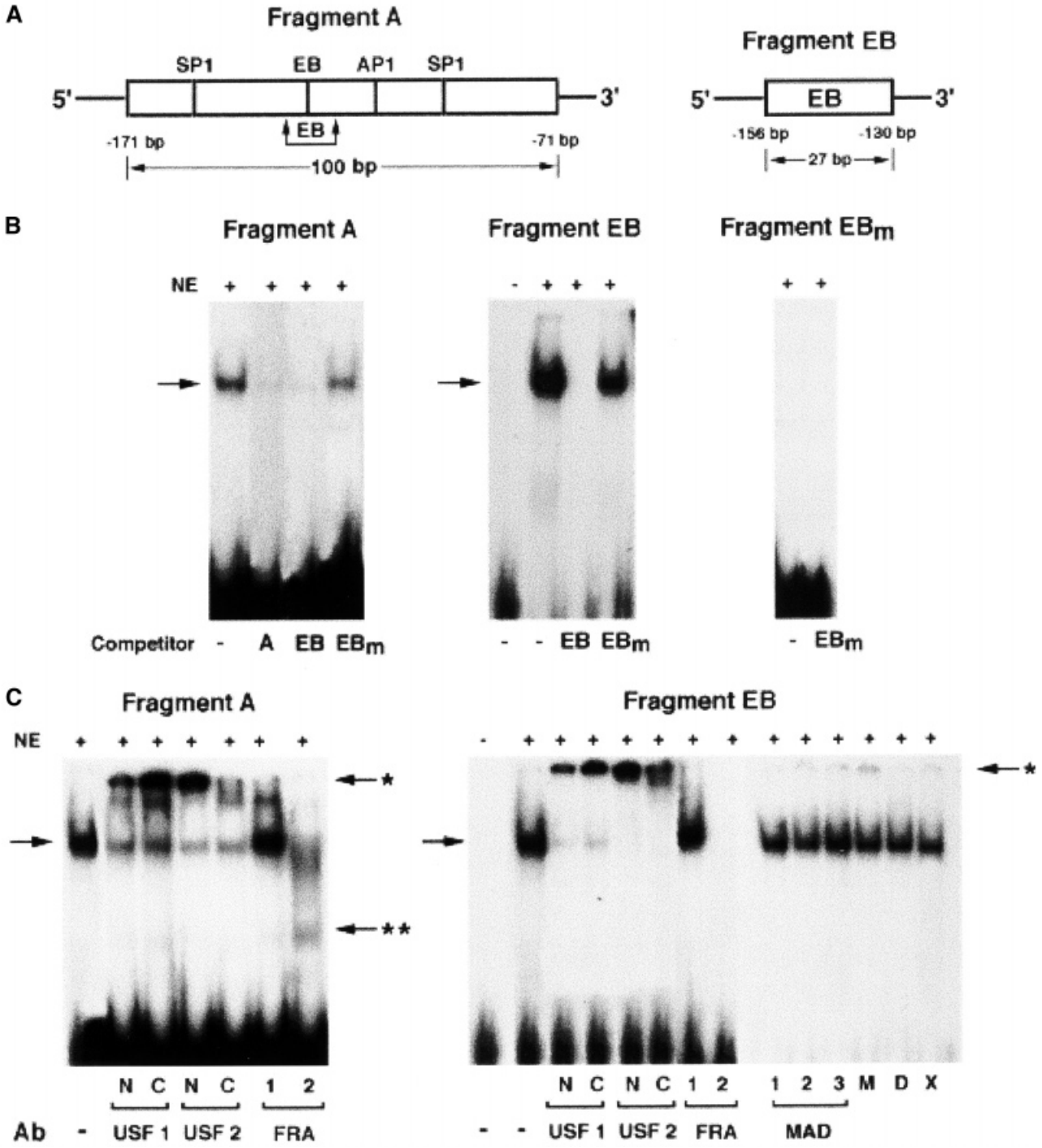

Fig. 4. Gel shift analysis of the hABCA1 gene promoter. A: Illustrates the probes used for the gel shift analysis. Fragment A (100 bp) includes binding motifs for Sp1, AP1, and the E-box. Fragment EB (27 bp) contains the E-box and Fragment EBm (not shown) contains a mutated E-box. B and C: The labeled fragments (Fragments A, EB, or EBm) used for the gel shift study are shown on top of each gel. Incubation of the radiolabeled probe with RAW cell nuclear extract (NE) is indicated (+). B: Shows the gel-shift analysis performed by incubating RAW cell nuclear extracts with radiolabeled Fragment A (left), EB (middle), or EBm (right) in the presence or absence of specific competitors (unlabeled Fragment A, EB, or EBm). C: Shows supershift analysis of Fragments A or EB with antibodies specific to the amino (N) or carboxyl (C) ends of USF1 and USF2 as well as Fra1 and Fra2. Arrows indicate the position of probe complexed with protein. Arrows with a single asterisk indicate the position of antibody-supershifted complex, and arrows with two asterisks indicate the position of the smaller molecular weight DNA-protein complex generated by incubation of nuclear extracts with the Fra2 antibody. Antibodies against other E-box binding proteins including Mad1 (1), Mad2 (2), Mad3 (3), c-Myc (M), MyoD (D), and Max (X) had no effect on the DNA-protein complex.

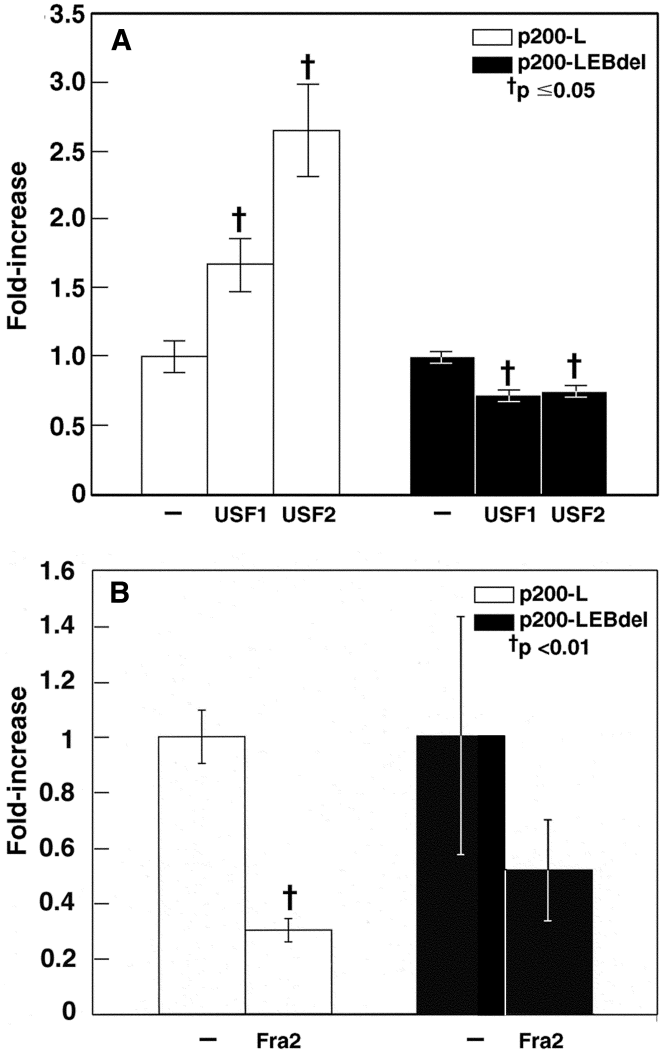

Fig. 5. USF1 and USF2 enhance, and Fra2 represses, transcription of the proximal hABCA1 gene promoter. RAW 264.7 cells were co-transfected with wild-type construct (p200-L) or E-Box-deleted construct (p200-LEBdel) and either empty plasmids as control, pCMV-USF1, pCMV-USF2 (A), or pCMV-Fra2 (B), along with a β-galactosidase expression plasmid. Cell lysates were analyzed for luciferase and β-galactosidase activity. Luciferase values were normalized to β-galactosidase activity and expressed as mean ± SEM. Data from a representative experiment out of a total of three independent transfections are shown.

represses, the proximal hABCA1 promoter, indicating that the USF1-USF2-Fra2 complex modulates E-box mediated regulation of ABCA1 gene transcription. These findings identify a novel mechanism as well as a potential new target for the modulation of ABCA1 gene expression that may be useful for drug development.

USF belongs to the helix-loop-helix family of transcription factors that bind the E-box motif (CACGTG) and include Myc, Mad1, Max, MyoD, and others (53). Although it has a well-established role as a transcription activator (53, 54), it is clear that in some promoter contexts USF does have the capacity to act as a transcription repressor (31, 32, 48, 51, 55–63). In our studies of the hABCA1 promoter, we found no evidence of transactivation via the E-box. On the contrary, transfection of EBm and EBdel constructs in RAW and 293 cells consistently led to increased rather than decreased ABCA1 promoter activity, indicative of E-box mediated gene repression. We thus expected the E-box binding transcription factors USF1 and USF2 to repress the hABCA1 promoter. However, co-transfection of USF1 and USF2 alone or in combination enhanced transcription of the proximal ABCA1 promoter indicating that USF1 and USF2 transactivate the hABCA1 gene.

The processes by which USF modulates gene transcription are not completely understood. However, several mechanisms have been postulated. USF can activate or inhibit gene transcription by competing for binding to the E-box with transcriptional repressors or activators (56–58). However, gel shift assays using antibodies specific to different E-box binding proteins failed to demonstrate their binding to the E-box motif. We also utilized Western blot analysis to search for changes in the expression of truncated and splice variants of USF2 that lack the transactivation domain (51, 52, 60, 61) and lead to gene repression. Under the conditions utilized in our studies, these USF variants were not detected in RAW cell or 293 cell nuclear extracts. USF1 and USF2 homodimers have been reported to inhibit, and USF1/USF2 heterodimers to enhance, gene transcription (54, 64). However, expression of USF1 and USF2 alone or in combination led to transactivation of the hABCA1 gene. USF can also modulate gene transcription through specific protein-protein interaction with transcriptional activators that bind DNA motifs distinct from the E-box (48, 49, 62). In the human ABCA1 promoter, the E-box motif is flanked by two Sp1 sites and an AP1 motif (14, 15, 18). Transcription factors which bind to Sp1 and AP1 have been implicated in the transcriptional regulation of other genes involved in lipid metabolism including apoA-II (31), apoC-III (32), chicken vitellogenin II (34), fatty acid synthase (35–37), stearoyl-CoA desaturase 1 (38), the LDL receptor (39), and the LDL receptor related protein (LRP) (40). Nevertheless, site-directed mutagenesis of the Sp1 and AP1 motifs failed to demonstrate a direct role for these factors in ABCA1 gene activation in RAW and 293 cells under our study conditions. Similar lack of transcriptional effects were reported by Porsch-Ozcurumez et al. (21) who studied ABCA1 promoter constructs with mutations in the two Sp1 motifs in HepG2 cells. Because our data demonstrate USF transactivation of the hABCA1 proximal promoter, we hypothesized that the observed E-box mediated repression of the hABCA1 promoter might be achieved by interaction of USF with other proteins that modify its transcriptional effect.

Review of the literature identified two transcription factors, the fos related antigens Fra1 and Fra2, known to interact with USF and modulate its transcriptional activity (48–50). Fra1 and Fra2 are members of the fos gene family that efficiently form dimers with Jun proteins. In some studies, interaction of Fra1 and Fra2 with specific members of the Jun family led to enhanced gene transcription

(65–67). However, because they lack transactivating domains (67), Fra1 and Fra2 are weak activators and can suppress transactivation by c-Fos and c-Jun (65–67).

In the hABCA1 gene, co-transfection studies demonstrated that Fra2 repressed gene transcription. In most contexts, Fra1 and Fra2 appear to exert their transcriptional effects by interacting with other members of the AP1 family of transcription factors and binding to the AP1 motif (65–67). However, specific cross-family interaction between USF and Fra has been reported (48–50). In the chicken αA-crystallin gene promoter (49), as in the hABCA1 promoter, interaction between USF and a protein complex containing Fra2 resulted in gene repression. Our gel shift studies confirmed binding of Fra2 to the E-box-USF1/USF2 complex in the hABCA1 gene promoter. Furthermore, by using a 27 bp fragment that contained only the E-box binding sequence, we demonstrated this interaction occurred in the absence of the flanking AP1 binding motif present in the hABCA1 promoter. The binding of AP1 proteins to sites outside the designated AP1 binding sequence has been reported (50). Our combined studies are consistent with the concept that a protein complex consisting of USF1, USF2, and Fra2 modulates transcription of the hABCA1 gene by binding to the E-box motif.

Our results indicate that an intact E-box binding site imparts a 3-fold repression on promoter activity of the human ABCA1 gene. This raises the question as to whether the E-box mediated repression of the ABCA1 promoter is constitutive or regulatory. We found that E-box-mediated repression persisted in the presence of various compounds known to stimulate transcription of the hABCA1 gene, including cholesterol, *cis*-RA, and 22-*R*-hydroxycholesterol. Also, although insulin and estrogen have both been proposed to act through USF (36, 68–70), we found that E-box-mediated repression persisted in cells treated with insulin and estrogen. Thus, although the overall effect of the E-box binding proteins is to repress hABCA1 promoter activity, none of the agents known to regulate transcription of the hABCA1 gene do so by countering repression through the hABCA1 E-box. These data indicate that the USF1-USF2-Fra2 complex is important for transcription of the hABCA1 gene, in that mutating their binding site significantly increases transcription. However, the role of these proteins as actual regulatory molecules remains to be demonstrated.

In summary, our data indicate that the human ABCA1 gene is highly regulated. The proximal 200 bp of the ABCA1 gene promoter is rich in binding sites for transcription factors with a potential role in modulating hABCA1 gene expression. The prominent transcription factor binding motifs in this region include Sp1, AP1, LXR, and an E-box motif. Our studies show that the E-box motif 147 bp upstream of the transcriptional start site mediates repression of the hABCA1 gene. USF1, USF2, and Fra2 bind to the E-box motif and facilitate repression of the human ABCA1 promoter. These combined studies identify a novel site in the human ABCA1 promoter involved in the regulation of ABCA1 gene expression.

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