Cooperative transcriptional activation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 genes by nuclear receptors including Liver-X-Receptor

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The ATP-binding cassette transporters ABCG5 and ABCG8 form heterodimers that limit absorption of dietary sterols in the intestine and promote cholesterol elimination from the body through hepatobiliary secretion. To identify cis-regulatory elements of the two genes, we have cloned and analyzed twenty-three evolutionary conserved region (ECR) fragments using the CMV-luciferase reporter system in HepG2 cells. Two ECRs were found to be responsive to the Liver-X-Receptor (LXR). Through elaborate deletion studies, regions containing putative LXREs were identified and the binding of LXRα was demonstrated by EMSA and ChIP assay. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated by EMSA and ChIP assay. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated by EMSA and ChIP assay. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated by EMSA and ChIP assay.

INTRODUCTION

Factors that modulate circulating and tissue cholesterol levels have major impacts on cardiovascular disease (CVD). The ATP-binding cassette (ABC) transporters ABCG5 and ABCG8 form heterodimers that limit absorption of dietary sterols in the intestine and promote cholesterol elimination from the body through hepatobiliary secretion. To identify cis-regulatory elements of the two genes, we have cloned and analyzed twenty-three evolutionary conserved region (ECR) fragments using the CMV-luciferase reporter system in HepG2 cells. Two ECRs were found to be responsive to the Liver-X-Receptor (LXR). Through elaborate deletion studies, regions containing putative LXREs were identified and the binding of LXRα was demonstrated by EMSA and ChIP assay. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated by EMSA and ChIP assay. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated by EMSA and ChIP assay. When the LXREs were inserted upstream of the intergenic promoter, synergistic activation by LXRα was demonstrated by EMSA and ChIP assay.

Although it had been demonstrated that the ABCG5 and ABCG8 genes are positively regulated by LXRα, the LXRE response element (LXRE) has yet to be identified in these genes. The human ABCG5 and ABCG8 genes, each with 13 exons, are located next to each other in a head-to-head configuration on chromosome 2p21. Their start codons are separated by a 374-bp intergenic region (8). Recent studies have demonstrated that the orphan nuclear receptor liver receptor homolog-1 (LRH-1), hepatocyte nuclear factor 4α (HNF4α), and GATA transcription factors positively regulate transcription of the two genes and binding sites for these factors were identified in the divergently transcribed intergenic promoter region (9, 10). The ABCG5 gene is 29 kb in length, and the ABCG8 gene is 46 kb in length (8). The full-length copies of both genes span approximately 75 kb, where some important cis-regulatory elements must be present. To identify the functional LXRE of the ABCG5/ABCG8 gene, we focused on conserved regions determined by comparing the corresponding sequences of human and mouse genes using the evolutionary conserved region (ECR) browser (http://ecrbrowser.dcode.org). Twenty-three ECRs were selected and corresponding DNA fragments have been cloned. The ECRs have been analyzed using the CMV-luciferase reporter.
system in HepG2 cells. As a result, we have identified two LXREs in ABCG5/ABCG8 genes for the first time and propose that these LXREs, especially in the ECR20, play major roles in regulating these genes in combination with GATA4, HNF4α, and LRH-1, which had been shown to bind to the intergenic region.

RESULTS AND DISCUSSION

Evolutionary conserved regions (ECRs) of ABCG5 and ABCG8 genes

In order to focus on evolutionary meaningful regions of the two genes, genomic DNA sequence of the human ABCG5 and ABCG8 genes was compared with that of corresponding mouse genes using the ECR browser (http://ecrbrowser.dcode.org). Evolutionary conserved regions (ECRs), with a minimum length of 200 bp and a minimum identity of 60%, were searched, resulting in forty-nine ECRs. We have selected twenty-three out of forty-nine by filtering out ECRs where intron sequences take up less than 80% and where transposons or simple repeats are found. Their positions in ABCG5 and ABCG8 genes are summarized in Supplementary Table S1.

Two ECRs respond to LXR nuclear receptor

Although it had been demonstrated that the ABCG5 and ABCG8 genes are positively regulated by LXRα, no LXRE has been identified in these genes. To find out which ECRs contain LXREs, we first constructed eighteen pE-CMV-LUC reporter plasmids by inserting each of the ECR fragments, amplified by the PCR reaction, into the p-T-CMV-LUC plasmid (11). Then, we performed transient cotransfection assay by introducing each of the eighteen expression plasmids and their ligands, T0901317, into HepG2 cells. DNA fragments containing the upstream region (pE10,11-1-CMV-LUC; 1-141) resulted in little change in reporter activity, while the same truncation of the downstream region up to (pE10,11-3-CMV-LUC; 1-141) did not alter responsiveness to the LXRα, while the same truncation including mutated putative LXRE (pE10,11-1-mt3-CMV-LUC; 1-141) obliterated responsiveness to the LXRα as expected. When a 22-bp DNA fragment containing either the putative LXRE or mutant LXRE was inserted into the pCMV-LUC reporter plasmid with 50 ng of an internal control pCMV-LacZ, 10 ng of nuclear receptor expression plasmid and 400 ng of carrier DNA (pGemB). Twenty-four hours later, cells were incubated for an additional 24 h in the presence of appropriate agonists (RA: 10 μM 9-cis-retinoic acid; T: 10 μM T0901317). The fold activation values were calculated by dividing the luciferase activity in each experiment by that of control cells treated with a vehicle for the agonists. Values are expressed as the mean ± S.E. (3 < n < 9).

Identification of a putative LXRE response element (LXRE) in ECR10

LXRα/RXRα was shown to activate ECR10,11 by approximately three fold. To map the LXRE response element (LXRE) in the ECR10,11, we made a series of truncations in the 1,043-bp ECR10,11. Each of these plasmids, with LXRα or RXRα expression plasmids, was cotransfected into HepG2 cells. DNA fragment containing the upstream region (pE10,11-1-CMV-LUC; 1-617) resulted in little change in reporter activity, while the downstream region (pE10,11-2-CMV-LUC; 394-1,043) resulted in a drastic decrease in LXRE-dependent induction, suggesting that there is a LXRE within the upstream 617-bp region containing the ECR10 (Fig. 2A). The LXRE consists of direct repeats of the core sequence AGGTCA separated by four nucleotides (DR4). By applying computational analysis to the upstream 617-bp region, we discovered a putative LXRE (TGACCTGAGGTCACC; 20-41 from the 5'-terminus of the E10,11). Further truncation of the downstream region up to (pE10,11-3-CMV-LUC; 1-141) did not alter responsiveness to the LXRα, while the same truncation including mutated putative LXRE (pE10,11-1-mt3-CMV-LUC; 1-141) obliterated responsiveness to the LXRα as expected. When a 22-bp DNA fragment containing either the putative LXRE or mutant LXRE was inserted into the pCMV-LUC reporter plasmid (pE10,11-DR4-CMV-LUC and pE10,11-mtDR4-CMV-LUC; 20-41) and cotransfected along with LXRα, there is an LXRE-dependent induction of reporter activity only with the pE10,11-DR4-CMV-LUC plasmid as expected. Moreover, when the short oligonucleotide containing DR4 was dimerized (pE10,11-2XDR4-CMV-LUC), much stronger LXRE-dependent induction was observed. Taken together, we have identified a putative LXRE in the ECR10 of ABCG5 and ABCG8 genes.

Identification of a putative LXRE response element (LXRE) in the ECR20

LXRα/RXRα was shown to activate ECR19,20 by about five fold. To map the LXRE response element (LXRE) in the ECR19,20, we made a series of truncations in the 1,389-bp ECR19,20. Each of these plasmids, with LXRα and RXRα expression plasmids, was cotransfected into HepG2 cells. DNA fragments containing the
Identification of LXRx response elements (LXREs) in ECR10 and ECR20. (A) Fine mapping of an LXRE in ECR10,11 by transfection of HepG2 cells. At the left side is the schematic representation of the deletion series of the pE10,11-CMV-LUC reporter plasmid. The right side depicts the fold of activation calculated from normalized firefly luciferase activity for each construct. Values are expressed as the mean ± S.E. (3 < n < 9). Agonists were used at the concentration of 10 μM (RA : 9-cis-retinoic acid, T : T0901317). (B) EMSA was performed for a putative DR4 element in the ECR10 region. A 22-bp DNA fragment containing the LXRE was labeled with 32P and used as a probe. The labeled probe was incubated with in vitro translated LXRα/RXRα protein with or without a competitor. As a competitive inhibitor, 100-fold excess amounts of the following unlabeled fragments were used. DR4: DR4 consensus, CTCTTC TGACCT CCTG TGACCT; LXRE: DR4-ECR10, TCC TGACCT CAGG TTACCC ACC; mtLXRE: mutant DR4-ECR10, TCC GTCAAA CTGG TAACCC CTC. (C) Chromatin immunoprecipitation assays to detect binding of RXR and H3K4me1 to putative LXRE. After immunoprecipitation, a 142 bp DNA fragment containing the LXRE was amplified by PCR. (D) Fine mapping of an LXRE in ECR19,20 by transfection of HepG2 cells. At the left side is the schematic representation of the deletion series of the pE19,20-CMV-LUC reporter plasmid. The right side depicts the fold of activation calculated from normalized firefly luciferase activity for each construct. Agonists were used at the concentration of 10 μM (RA : 9-cis-retinoic acid, T : T0901317). (E) EMSA demonstrating the binding of the LXRE probe to HepG2 nuclear extract proteins. Specific binding of LXRx to the LXRE in ECR19,20 was demonstrated. Proteins (LXRx/RXRx) produced in vitro were incubated with the 32P-labeled LXRE (ECR19,20 oligonucleotide probe. LXRE: DR4-ECR19, ACT GTGTAACCCCTC; mtLXRE: mutant DR4-ECR19, ACT GTGTAACCCCTC. (F) Chromatin immunoprecipitation assays to detect binding of LXRx, RXRx, H3K4me1 and p300 to putative LXRE. Immunoprecipitated chromatin was amplified by PCR using primers designed to amplify 254 bp of the LXRE.
CMV-LUC; 974-998) obliterated responsiveness to the LXRα. On the other hand, a 22-bp region from 954 to 975 demonstrated moderate responsiveness to LXR (pE19,20-DR4-CMV-LUC, Fig. 2D). This minimal 22-bp region contains a putative LXRE (ACTGGACTTCTGGTAACCCCTC). When the putative LXRE were mutated (pE19,20-mtDR4-CMV-LUC; ACTTtag TCTGGTAACCCCTC), there was no responsiveness to LXR (Fig. 2D). A differently mutated LXRE (ACTGtcaaa CTGGTAACCCCTC) obliterated responsiveness to the LXR as well (data not shown). Taken together, we have identified a putative 22-bp LXRE which requires neighboring sequences for maximal responsiveness to LXR in the ECR20 of ABCG5 and ABCG8 genes.

Specific binding of nuclear receptors to the LXREs in ECR10 and ECR20

To demonstrate that the DR4 element of human LXREs in ECR10 binds LXR/RXR heterodimer, we performed an electrophoretic mobility shift assay (EMSA) with LXRα and RXRα proteins produced by in vitro transcription/translation reaction and a 32P-labeled DNA probe. A strong shifted band was evident when the LXRE/RXRα heterodimer was allowed to bind (Fig. 2B). The shifted band was efficiently competed by the unlabeled consensus DR4 oligonucleotide and unlabeled probe (LXRE), but not even by a 100-fold molar excess of the unlabeled mutant oligonucleotide (mtLXRE), indicating that the LXRα/RXRα heterodimer binds to this response element in a specific manner in vitro. To demonstrate that the DR4 element of human LXRE in ECR20 binds the LXR/RXR heterodimer, we performed an electrophoretic mobility shift assay (EMSA) using nuclear extract from HepG2 cells and a 32P-labeled DNA probe (Fig. 2E). A strongly shifted band was evident when the LXRE/RXRα heterodimer was allowed to bind (Fig. 2E). The shifted band was efficiently competed by the unlabeled consensus DR4 oligonucleotide and unlabeled probe (LXRE), but not even by a 300-fold molar excess of the unlabeled mutant oligonucleotide (mtLXRE). Similar results were obtained when LXRα and RXRα proteins produced by in vitro transcription/translation reaction were used (data not shown). These results indicate that the LXRE/RXRα heterodimer binds to this response element in a specific manner in vitro.

Chromatin immunoprecipitation assays were performed to determine whether LXR and RXR are bound to the LXRE regions in the nuclei of HepG2 cells. As shown in Fig. 2C, ECR10 LXRE-containing chromatin fragment from agonist-treated HepG2 cells were enriched by immunoprecipitation with anti-RXR. This result provides evidence that RXR binds to the LXRE in ECR10 of the ABCG8 gene in living HepG2 cells. However, no LXRα occupancy was observed at the LXRE in ECR10 (data not shown), which may have resulted from the low affinity of LXRα to the LXRE or due to the sub-optimal anti-LXR antibody.

We also tested the presence of H3K4me1 at the LXRE because H3K4me1 is a dominant mark for active and potentially active distal regulatory regions (12). As expected, specific binding of H3K4me1 at the LXRE was observed (Fig. 2F). Taken together, the DR4 in ECR20 of the ABCG5 and ABCG8 genes appears to be a functional LXRE.

Synergistic transcriptional activation of ABCG5 and ABCG8 genes

Previous studies have demonstrated that several transcription factors are responsible for the regulation of ABCG5 and ABCG8 genes. For example, liver receptor homolog 1 (LRH-1), hepcoty nuclear factor 4α (HNF4α), and GATA transcription factors have been shown to regulate these genes through the intergenic promoter regions. The LXREs, bound to the response elements identified in this study, is very likely to interact with other transcription factors which are bound to the intergenic promoter regions. To test this possibility, we have constructed reporter plasmids which contain the 144-bp intergenic promoter region and LXREs in the ECRs 10 and 20. Moderate synergistic tran-
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Expression plasmids were constructed by inserting appropriate ECR fragments, by the PCR amplification of a BAC clone (RPCI11-128C5), into the pT-CMV-LUC plasmid (11). Primer pairs for the PCR amplification of ECRs are summarized in Supplementary Table S2. Detailed strategies used for constructing expression and reporter plasmids are available upon request.

Searching for the evolutionary conserved regions
The evolutionary conserved regions (ECRs) in ABCG5 and ABCG8 genes, with a minimum length of 200 bp and a minimum identity of 60%, were searched by aligning human and mouse sequences using the ECR browser (http://ecrbrowser.dcode.org).

Cell culture and transient cotransfection experiments
HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were grown at 37°C under 5% CO2. Twenty-four hours before transfection, HepG2 cells were plated at a density of 1.2 × 10^5 cells per well in a 24-well plate. Each transfection contained 1 to 10 ng of the transcription factor expression plasmids as described in the figure legends, 20 ng of a CMV-luciferase reporter plasmid, 50 ng of a control plasmid pCMV-lacZ, and 400 ng of pGem1 carrier using 1.2 μl of 0.45% PEI (Aldrich). Twenty-four hours after transfection, cells were stimulated with appropriate agonists (9-cis-retinoic acid and T0901317) at a final concentration of 10 μM. Twenty-four hours after treatment, the ratio of luciferase/β-galactosidase activities were determined.

Electrophoretic mobility-shift analysis (EMSA)
Cultured cells in 60 mm dishes were lysed in a 200 μl lysis buffer (0.35 M sucrose, 10 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 1 mM DTT, 0.5 mM PMSF) and put on ice for 10 min. Five μl of 10% Nonidet P-40 was added, and nuclei were pelleted by centrifugation at 1,000 rpm for 1 min. Nuclear pellets were lysed in 40 μl of salt buffer (20 mM HEPES (pH 7.9), 1 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF), and incubated on ice for 30 min. The nuclear extract (supernatant) was retained after centrifugation at maximal speed for 25 min at 4°C. The LXRα and RXRα proteins were also synthesized using TNT Quick Coupled Transcription/Translation Systems (Promega). The strand sequences for human ABCG8 LXR (Ecr10) were 5'-TCCTGATCTCACCTGACCC-3' and 5'-GGTGAGGAACCTATGCTGAC-3'. Mutated fragments (mutant DR4-ECR10) were 5'-TCCTGATCTCACCTGACCC-3' and 5'-GGTGAGGAACCTATGCTGAC-3'. The sequences for human ABCG8 LXRE (Ecr20) were 5'-ACTTACCCACTGTCAGCAG-3' and 5'-GGTGAGGAACCTATGCTGAC-3'. The sequences for human ABCG8 LXRE (Ecr20) were 5'-ACTTACCCACTGTCAGCAG-3' and 5'-GGTGAGGAACCTATGCTGAC-3'. Double-stranded oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (NEB). Binding
reaction mixtures (10 μl final volume) contained either 5 μg of nuclear extract or 3 μl of in vitro-translated LXRα/RRα, as well as 20 mM HEPES (pH 8.0), 25% glycerol, 1.5 mM MgCl₂, 1 μg of poly (dI/dC), 10 pmol of poly (dA/dT), and 0.02 pmole of the labeled oligonucleotide probe. Samples were incubated for 30 min at room temperature. For competition, unlabeled double-stranded oligonucleotides in molar excess over the labeled probe were added. The samples were loaded onto 4% non-denaturing polyacrylamide gel in a 0.5% TBE buffer and electrophoresed at 90V. The gel was dried and exposed for 1 day at room temperature for autoradiography.

**Chromatin immunoprecipitation (ChIP) assay**

HepG2 cells in a 60-mm dish were treated with 1 μM T0901 317 plus 1 μM cis-retinoic acid for 4 hours. Cells were cross-linked with 1.42% formaldehyde in medium for 15 min at room temperature. Cross-linking was stopped with 125 mM glycine for 5 min at room temperature. Cells were harvested and washed twice with cold PBS. The collected cells were lysed with a 500 μl IP buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin) by resuspending the pellet and pipetting up and down several times in a microcentrifuge tube. The nuclei were collected by centrifugation, and resuspended in a 0.5 ml IP buffer containing inhibitors. The washed pellet was resuspended in 0.5 ml IP buffer containing inhibitors and was sonicated on ice, and centrifuged at 12,000 g for 20 min at 4°C. Twenty μl of the supernatant was stored at 4°C to be used as the input, and the remaining volume was divided into two aliquots. One aliquot was immunoprecipitated with antibodies against RXRα, LXRα, p300 (Santa Cruz Biotechnology) or H3K4me1 (Cell Signaling) by incubating overnight at 4°C. The other one without antibodies was used as a negative control. The immunoprecipitates were analyzed by PCR using the following primers for ECR10, 5'-ACTTGACTTCTGGTAACCCCT-3' and 5'-CTATGATGG-3' For ECR20, 5'-CCTCCTAGTGTCCCAAGTT-3' and 5'-CATGTTGGTCAGGCTCAAAC-3' and 5'-GTGAGCACATTT-

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