Sterol Regulatory Element-binding Protein-2- and Liver X Receptor-driven Dual Promoter Regulation of Hepatic ABC Transporter A1 Gene Expression

**MECHANISM UNDERLYING THE UNIQUE RESPONSE TO CELLULAR CHOLESTEROL STATUS**

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ABC transporter A1 (ABCA1) mediates and rate-limits biogenesis of high density lipoprotein (HDL), and hepatic ABCA1 plays a major role in regulating plasma HDL levels. HDL generation is also responsible for release of cellular cholesterol. In peripheral cells ABCA1 is up-regulated by the liver X receptor (LXR) system when cell cholesterol increases. However, cholesterol feeding has failed to show a significant increase in hepatic ABCA1 gene expression, and its expression is up-regulated by statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors), suggesting distinct regulation. In this study we investigated the mechanism of regulation of the rat hepatic ABCA1 gene and identified two major ABCA1 transcripts and two corresponding promoter regions. Compactin activated the novel liver-type promoter in rat hepatoma McARH7777 cells by binding the sterol regulatory element-binding protein-2 (SREBP-2). In contrast, compactin repressed the previously identified peripheral-type promoter in an LXR-responsive element-dependent but not E-box-dependent manner. Thus, compactin increased the liver-type transcript and decreased the peripheral-type transcript. The same two transcripts were also dominant in human and mouse livers, whereas the intestine contains only the peripheral-type transcript. Treatment of rats with pravastatin and a bile acid binding resin (cholestimide), which is known to activate SREBP-2 in the liver, caused a reduction in the hepatic cholesterol level and the same differential responses in vivo, leading to increases in hepatic ABCA1 mRNA and protein and plasma HDL levels. We conclude that the dual promoter system driven by SREBP-2 and LXR regulates hepatic ABCA1 expression and may mediate the unique response of hepatic ABCA1 gene expression to cellular cholesterol status.

High density lipoprotein (HDL) is thought to be antiatherogenic because plasma levels are negatively correlated with cardiovascular risk and because it removes cholesterol accumulated in cells in vitro (1, 2). HDL is assembled from a helical apolipoprotein and cellular cholesterol and phospholipid by a reaction mediated by ABC transporter A1 (ABCA1) (3, 4). The absence of functional ABCA1 in Tangier disease and in knock-out mice results in deficiency of plasma HDL, indicating the essential role of ABCA1 in HDL biogenesis (5, 6).

Recent studies have shown the most important role of hepatic ABCA1 in maintaining plasma HDL levels. Selective knockdown of hepatic ABCA1 in mice causes a substantial (up to 80%) decrease in plasma HDL cholesterol (7, 8), and overexpression of hepatic ABCA1 raises HDL cholesterol levels (9, 10).

In peripheral cells, such as macrophages and fibroblasts, ABCA1 gene expression is up-regulated by loading cholesterol (11, 12). This response is mediated by cholesterol-sensing nuclear receptor liver X receptor (LXR), which directly enhances ABCA1 gene expression, facilitating the release of excess cellular cholesterol by increasing the biogenesis of HDL particles (13, 14). In contrast, cholesterol feeding of mice or rats has failed to show a significant increase in hepatic ABCA1 mRNA expression (15, 16). In the traditional model of reverse cholesterol transport, cholesterol released by peripheral tissues to HDL is transported to the liver, and the liver plays a role in converting cholesterol to bile acids for bile excretion (17). It is unclear how cholesterol regulates hepatic ABCA1 gene expression.

Several lines of evidence have suggested distinct regulation of hepatic ABCA1 expression. Statins (3-hydroxy-3-methylglutaryl (HMG-CoA) reductase inhibitors) that inhibit the mevalonate pathway and subsequent formation of endogenous oxysterol LXR agonists down-regulate ABCA1 expression in macrophages and fibroblasts (18–20) but up-regulate its expression in HepG2 cells (18, 21). In addition, hepatic ABCA1 mRNA expression in mice is far less sensitive to synthetic LXR agonists than extrahepatic ABCA1 expression (22–24). Moreover, in mice containing a human BAC clone that lacks the previously described ABCA1 pro-

The abbreviations used are: HDL, high density lipoprotein; ABCA1, ABC transporter A1; SREBP, sterol regulatory element (SRE)-binding protein; LXR, liver X receptor; LXRE, LXR-responsive element; HMG-CoA, 3-hydroxy-3-methylglutaryl-Coenzyme A; 5′ RACE, rapid amplification of cDNA ends; LDLR, low density lipoprotein receptor; siRNA, small interfering RNA; RT, reverse transcription; HPLC, high performance liquid chromatography.

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moter, human ABCA1 is expressed only in the liver (25, 26), suggesting the presence of a liver-specific promoter.

Our preliminary experiments showed that ABCA1 mRNA expression in the rat liver was increased by the treatment with a statin (pravastatin) and a bile acid binding resin (colestipol). The combination of a statin and bile acid binding resin has been shown to potently deplete hepatic cholesterol and increase the amount of mature nuclear SREBP-2 but not of SREBP-1c (27, 28). SREBP-2 is a transcription factor that regulates many genes involved in cholesterol synthesis and uptake in response to cellular cholesterol levels. Rat hepatoma McARH7777 cells have been shown to mimic the behavior of SREBPs in the liver, and they have been used in an *in vitro* model (29). Because our preliminary data also showed that ABCA1 mRNA expression was decreased by sterols in McARH7777 cells, we investigated the molecular mechanism underlying these unique responses. As a result, we succeeded in identifying a novel promoter region that mediates the liver-specific cholesterol response of ABCA1.

**EXPERIMENTAL PROCEDURES**

**Rapid Amplification of cDNA Ends (5′-RACE)—**The 5′ RACE was carried out with the GeneRacer kit (Invitrogen), which amplifies only full-length transcripts via elimination of truncated messages, and total RNAs from rat liver and intestine, McARH7777 cells, mouse liver, or human liver (Clontech, #64022-1) were used as templates. The double-strand cDNA was reverse-transcribed with random primers. To obtain 5′ ends, the first-strand cDNA was amplified using the GeneRacer™ 5′ primer and a reverse gene-specific primer, which is located at exon 4/5 (gagaattccccggaccaaatgcggtt for rat ABCA1, gagaatccgcgagccaggtt for mouse ABCA1, or gagaagacgtagcttcActgcttctcatcctggtg for human ABCA1). The amplified products were subcloned and sequenced.

**Plasmid—**The type P (−1040/+18) and type L (−950/+123) ABCA1 promoter fragments were amplified by using rat tail genomic DNA as a template. The primers for the PCR amplifications were designed based on the nucleotide sequences (type P forward, aaagcttctctctccgcaataagtgtt for rat ABCA1, gagaagcagtagcttcActgcttctcatcctggtg for mouse ABCA1, or gagaacgtagcttcActgcttctcatcctggtg for human ABCA1). The amplified products were subcloned and sequenced.

**Cell Culture and mRNA Analysis—**The rat hepatoma cell line McARH7777 and the rat fibroblast cell line Rat2 were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 10% horse serum or 10% fetal calf serum, respectively. To evaluate the effect of various reagents on mRNA expression, cells were treated in medium containing 10% delipidated serum and the appropriate additions for 16 h.

Gene-specific mRNA quantitation was performed by real-time PCR on an ABI Prism 7300 sequence detection system (Applied Biosystems). Total RNA was extracted from cells with the RNeasy Mini kit (Qiagen) or from the liver with the RNeasy Lipid Tissue mini kit (Qiagen) and treated with DNase to eliminate genomic DNA contamination according to the manufacturer’s instructions (Qiagen). The relative expression levels of mRNA were determined with the TaqMan one-step RT-PCR Master Mix Reagent Kit. The primer/probe sequences used were:

- rat total ABCA1 forward (ccgcgactgtagtaagag), reverse (agaggct-gaaaccaaaagac), probe (6-carboxyfluorescein (FAM)-cttttgtgctgcaagcgtt-TAMRA); rat type P ABCA1 forward (tttgtgctctgttcActg), reverse (cccaaccta-cacactgac), probe (FAM-ctcttttttttctccggtt-TAMRA); rat HMG-CoA synthase forward (tgcattctgccgatttactacctg), reverse (tcgccatctgcggcata), probe (FAM-tgcctcactacac-acagactgactcctc-TAMRA).
- rat SREBP-1c forward (ggagccatggattgcacatt), reverse (ctgtctcactacacagac), probe (FAM-cagctcataac-acagactgactcctc-TAMRA).

To determine the relative abundance of total and type P ABCA1, we used a standard curve based on serial dilution of an ABCA1 cDNA containing two target sequences for the TaqMan primer probe. The 4447-bp standard cDNA was prepared by PCR amplification using the RT transcripts of rat mRNA as a template. The primer sequences were designed based on exon 1 (forward, gagaacctctctggtgcttctcctggtc) and exon 29 (reverse, actgctcggcttctggtc).

**Cell Fractionation and Immunoblot Analysis—**Immunoblot analysis of ABCA1 was performed on the membrane fraction prepared from cells or tissues as described previously (19). The anti-ABCA1 antibodies used were gifts from Dr. Shinji Yokoyama (Nagoya City University Graduate School of Medical Sciences) or Dr. Michael L. Fitzgerald and Dr. Mason W. Freeman (Massachusetts General Hospital and Harvard Medical School). The membranes were reprobed with anti-transferrin receptor antibody (Zymed Laboratories Inc.) or anti-calnexin antibody (StressGen Biotechnologies) for the loading controls. For analysis of SREBP, cells were fractionated into nuclear extract and membrane pellet fractions as described (29). Aliquots of extracts were subjected to SDS-PAGE and immunoblot analysis with monoclonal antibody against SREBP-1 (2A4, MBL, Japan) or SREBP-2 (1D2, MBL, Japan). The signals were detected using horseradish peroxidase-conjugated secondary antibodies and Supersignal West Femto substrate system (Pierce), and the intensities were quantified with a film and an image analyzer LAS3000 (Fujifilm).

**Transient Transfections and Reporter Gene Assays—**McARH7777 cells were seeded in 24-well plates 24 h before transfection. For basal promoter assays, cells were transfected with 61 ng of firefly luciferase reporter DNA and 6.1 ng of Renilla luciferase vector (phRL-TK) (Promega) with Effectene (Qiagen). Six hours after transfection, cells were exposed to 16 h to the appropriate reagents in the medium containing 10%
delipidated serum. Cells were lysed, and luciferase activity was determined. Firefly luciferase activity was normalized to the Renilla luciferase activity in each well.

**RNA Interference against SREBP-2**—McARH7777 cells were transiently transfected with the mixture of three different StealthRNAs (Invitrogen) specific for rat SREBP-2 gene (sense1 (ccauucugaccaacagcccaaau), antisense1 (aaucaggcgguggggsgguagg)); sense2 (ggccucucugcguacacucuuua), antisense2 (uuacuacugauccagcaggg); sense3 (ggucucuuggcagcagacagg)), or StealthRNAi negative control medium GC (Invitrogen) by using lipofectamine2000 reagent (Invitrogen), and they were incubated for 24 h. Transfection efficiency was monitored with a fluorescent oligonucleotide (BLOCK-IT fluorescent oligo; Invitrogen) and estimated to be 80–90%.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed with the DIG Gel Shift Kit 2nd Generation (Roche Applied Science). The DIG-3'-end-labeled ABCA1 SRE fragment (gggtgccttaagag) or human low density lipoprotein receptor (LDLR) SRE fragment (aaatctccccactgc) was incubated with the nuclear form of human SREBP-2 protein (1–418). The recombinant SREBP-2 was expressed in *Escherichia coli* with the pET-28b/SREBP-2 vector and purified as described previously (32). In competition experiments, a 30- or 300-fold excess of unlabeled oligonucleotides, such as intact or mutated (gggtGcggCCGcagg) ABCA1-SRE or LDLR-SRE, was added to the reaction mixture. SREBP-2-DNA complex was separated from free probe by 4% native polyacrylamide gel electrophoresis and blotted to Hybond-N+ (Amersham Biosciences). The shifted bands were visualized with AP-conjugated DIG antibody by using the chemiluminescence substrate.

**Animals and Lipoprotein Analysis**—Male Wistar rats (8 weeks of age, Charles River, Japan) were fed either standard rodent chow (MF diet; Oriental Yeast) or the same diet containing powdered 0.1% pravastatin (Sankyo Co., Japan) plus 0.4% rodent chow (MF diet; Oriental Yeast) for 8 days. Rats in the pravastatin/colestimide-supplemented group were given pravastatin (10 mg/kg) orally 6 h before the end of the treatment period. Rats had free access to diet and water throughout the period of the experiment. All animals were treated in accordance with the laboratory animal care guidelines of the National Institute of Health Sciences at Tokyo.

**Other Procedures and Statistics**—The cholesterol content of plasma lipoproteins was analyzed with a dual detection HPLC system consisting of two tandem connected TSKgel Lipopropak XL columns (300 × 7.8-mm; Tosoh, Japan) at Skylight Biotech Inc (Akita, Japan). Cholesterol in the liver was extracted with chloroform/methanol (2:1), and total cholesterol was quantitatively determined by an enzymatic method (33).

All results are representative of between two and five separate experiments. Data were analyzed for statistical significance by two-tailed Student’s *t* test or analysis of variance with Student-Newman-Keuls test as a post hoc test.

**RESULTS**

**Unique Response of Hepatic ABCA1 mRNA Expression to Cellular Cholesterol Status**—Studies have shown differential regulation of hepatic and extrahepatic ABCA1 mRNA expression (18–24). Consistent with the studies using fibroblasts and macrophages (11, 12, 18–20), the ABCA1 mRNA level in Rat2 fibroblasts was raised by incubating cells with sterols and reduced by treating cells with compactin (Fig. 1A, bottom). However, compactin did not alter the ABCA1 mRNA level in rat hepatoma McARH7777 cells (Fig. 1A, top). Notably, steroids (1 µg/ml 25-hydroxycholesterol and 10 µg/ml cholesterol) reduced the ABCA1 mRNA level (by 40%) in this cell line, but a synthetic LXR agonist, TO901317, increased it. Immunoblot analysis shows that ABCA1 mRNA and protein expression were similarly affected by these treatments (Fig. 1B).

**Liver Contains Two Types of ABCA1 mRNA**—To investigate the mechanism of the unexpected response of hepatic ABCA1 mRNA expression to cellular cholesterol status, we performed 5' RACE on the total RNA extracted from rat liver, intestine, and McARH7777 cells. The single major transcript present in rat intestine (Fig. 2A, left) contained a first exon of 222 bp followed by a second exon of 166 bp (Fig. 2, B and C), and they were 91% identical to mouse exon 1 and 2 (NM_013454). This also corresponded to a previously described ABCA1 transcript in human fibroblasts and macrophages (13, 14) and was designated the peripheral type (type P). 5' RACE PCR revealed three major transcripts in rat liver (Fig. 2A, left) and also in McARH7777 cells (Fig. 2A, bottom). One transcript was identical to the intestinal transcript (type P). The second transcript (liver type; type L) lacked exon 1 but contained all of exon 2. The third transcript (type L') in rat liver and McARH7777 cells also lacked exon 1 but contained a truncated 95 bp exon 2 that included the ATG translation start site (Fig. 2C and supplemental Fig. 1, A and D). Two minor bands were detected in addition...
to these major bands. Mouse and human liver RNAs also contained two major ABCA1 transcripts corresponding to type P and type L, respectively (Fig. 2A, middle and right) (supplemental Fig. 1, B–D). The mouse L type transcript was identical to the previously described mouse transcript mExon 1b (34). We did not detect transcripts corresponding to previously described variants mouse Exon 1d and human Exon 1d (26, 34) (supplemental Fig. 1, B–D).

We used a real-time RT-PCR to quantitatively determine the relative expression levels of the two transcripts in different rat tissues (Fig. 2D). Total and type P ABCA1 transcripts were quantitated using two sets of specific TaqMan primer-probe targeting sequences in exons 28 and 29 (for total ABCA1 mRNA) and in exon 1 (for type P ABCA1 mRNA) (Fig. 2C), respectively, and a standard ABCA1 cDNA containing two target sequences. Type P transcript was present in all tissues but in relatively smaller amounts compared with the total ABCA1 transcript in the liver and kidney, suggesting an abundance of transcript(s) other than type P in these tissues. Based on the assumption that RT-PCR efficiencies were the same for the two sets of primer probes, the ratio of type P to total ABCA1 mRNA in the liver was calculated as 0.27.

Compactin Diminishes Peripheral-type but Increases Liver-type ABCA1 mRNA Expression—5’ RACE PCR revealed that the treatment of McARH7777 cells with compactin diminished the type P transcript and increased the type L transcript (Fig. 2A, right). We used real-time RT-PCR to confirm these changes and to analyze the levels of the two transcripts quantitatively. As shown in Fig. 2A, compactin reduced the type P ABCA1 mRNA level by 97% but did not affect the total ABCA1 mRNA levels. The data represent the means ± S.D. of a typical experiment performed in triplicate. *, p < 0.05, versus vehicle-treated cells. Similar data were obtained in two separate experiments.
Dual Promoter Regulation of Hepatic ABCA1 Gene Expression

level. The ratio of type P to total transcripts in the control cells was 0.25 \pm 0.05, and the increase in type L mRNA was assumed to be 30 \pm 9%. Compacting treatment of McARH7777 cells also led to an increase in the mRNA level of the SREBP-2 target gene HMG-CoA synthase. SREBP-2 is a transcriptional factor that is cleaved to an active form in response to depletion of cellular cholesterol (35). Treatment of cells with sterols (1 \mu g/ml 25-hydroxycholesterol and 10 \mu g/ml cholesterol) reduced the total and type P ABCA1 mRNA levels by 43 and 58%, respectively. In contrast, the level of type P ABCA1 mRNA was increased by TO901317 in McARH7777 cells, and the profile of the response was similar to that of an LXR-target gene, SREBP-1c (Fig. 3A).

The response of type P ABCA1 mRNA expression to both compactin and TO901317 was exactly the same as that of total ABCA1 in rat fibroblasts (Fig. 3B). The ratio of the type P to total ABCA1 in this cell line was 0.77 \pm 0.07.

Liver-type ABCA1 Promoter Is Functional and Activated by Compacting—We subcloned the -950 to +123 region of exon 2 of the rat ABCA1 gene (Fig. 2B) into luciferase reporter plasmid pGL3-basic. This region, designated liver-type (type L), yielded substantial promoter activity but was unresponsive to LXR agonist TO901317 when transfected into McARH7777 cells (Fig. 4A, top). The activity of this type L promoter was increased by treating the cells with compactin and decreased by sterols. The compactin-elicted increase was prevented by adding either 2 mM mevalonate (Fig. 4A, top), the product of the HMG-CoA reductase reaction, or sterols (Fig. 5A) to the medium. This pattern of response was consistent with a transcriptional mechanism involving sterol-responsive element (SRE) and SRE-binding protein (SREBP)-2. Statins are known to increase, whereas sterols inhibit SREBP-2 activation (29). Immunoblot analysis shows treatment of cells with compactin caused a marked increase in nuclear SREBP-2 but a decrease in nuclear SREBP-1c (Fig. 4B).

Liver-type Promoter Is Activated by SREBP-2—To evaluate the involvement of SREBP-2 in activation of the type L promoter, we modulated the SREBP-2 level by overexpressing it or by means of RNA interference. Co-transfection with an expression plasmid of the mature form of SREBP-2 resulted in marked activation of the promoter (Fig. 4C, top), whereas the compactin-mediated activation was abolished in the cells treated with siRNA against SREBP-2 (Fig. 4D, top).

We performed a deletional analysis of the gene to identify the region of the type L promoter involved in its sterol-mediated response. The response to sterols was preserved when the promoter insert was sequentually deleted down to position -224 (Fig. 5A), and the remaining region contained two potential SRE-like motifs (Fig. 5B). We then carried out a mutational analysis to determine whether these elements mediate the above-observed response to sterols (Fig. 5C, left). Mutation of the SRE site resulted in complete loss of the sterol-mediated repression, whereas mutation in the SREa site had no effect. Consistent with these findings, enhancement of the reporter gene transcription by overexpressing mature SREBP-2 was abolished by mutating SREb but not SREa (Fig. 5C, right).

The direct binding of SREBP-2 to SREb was confirmed by electrophoretic mobility shift assay, and the SREb probe was shifted by adding recombinant SREBP-2 protein (Fig. 5D). This shift was diminished by the presence of an excess amount of either unlabeled SRE probe or LDLR-SRE probe but not by the mutated SREb probe. Furthermore, the formation of SREBP-2-LDLR-SRE complex was also diminished by the presence of excess amount of unlabeled SRE (Fig. 5E). These data indicate the specific binding of SREBP-2 to the SREb motif.

Compacting-induced Repression of the Peripheral Type Promoter Is Mediated by LXR—The type P promoter region (from -1040 to +18 of exon 1), which corresponds to the previously established ABCA1 proximal promoter regions in the mouse and human gene (13, 14, 36), contains LXR (Fig. 2B). The reporter gene activity was diminished by compaction (Fig. 4A, 224).
bottom). Statins have been shown to decrease type P promoter activity (37), and this effect is attributable to either inhibition of oxysterol endogenous LXR ligand formation via the mevalonate pathway or repression through SREBP-2 binding to an E-box element (37, 38). Overexpression of SREBP-2 in McARH7777 cells did not affect either wild-type or LXRE-mutated promoter activity (Fig. 4C, bottom, and Fig. 6, right), suggesting that SREBP-2 does not act as an E-box repressor in this cell line. Compaction-elicted repression was prevented by introducing a mutation into the LXRE in the promoter but not by mutating the E-box element (Fig. 6, left). Also, compactin diminished the activity of the luciferase-reporter driven by four tandem repeats of LXRE (Fig. 6, left). These findings suggest that compactin reduced the type P promoter activity in McARH7777 cells by depleting the endogenous LXR ligand level, not by repressing the E-box element.

The type P promoter activity was increased by TO901317 but diminished by sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) (Fig. 4A). Notably, sterols increased the type P promoter activity when endogenous ligands were depleted by compactin. Knockdown of SREBP-2 with siRNA decreased the basal promoter activity to the level present in compactin-treated cells, and compactin did not further decrease the activity (Fig. 4D).

**Cholesterol Depletion Up-regulates Hepatic ABCA1 Expression and Raises Plasma HDL Cholesterol**—To evaluate the in vivo effect of cholesterol depletion on ABCA1 mRNA expression in the liver, rats were fed a diet containing a mixture of pravastatin and colestimide. This treatment is known to increase the active nuclear form of SREBP-2 but decrease that of SREBP-1 in the liver (27, 28). Pravastatin/colestimide treatment caused a reduction in cholesterol content in the liver...
Hepatic ABCA1 is responsible for maintaining the majority of plasma HDL and also plays a discrete role in HDL biogenesis (7–10). Data have accumulated suggesting that hepatic ABCA1 expression is distinctly regulated by cellular cholesterol status in contrast to peripheral ABCA1 expression. Pravastatin/colestimide treatment of rats raised ABCA1 mRNA and protein levels in the liver but lowered their levels in the intestine (Fig. 7 A and B). The addition of sterols to the medium decreased ABCA1 mRNA expression in hepatoma McARH7777 cells but increased it in fibroblasts, whereas compactin had the opposite effect (Fig. 1A), consistent with the results of previous studies (18–21, 37).

In this study we identified novel and highly expressed ABCA1 transcripts and corresponding promoter regions in addition to the previously described peripheral-type transcript (13, 14) in rat liver, whereas only the peripheral-type transcript was present in the intestine (Fig. 2A). Our findings support the concept that the novel SREBP-2- and LXR-driven dual-promoter system mediates the unique response of hepatic ABCA1 mRNA expression to cellular cholesterol status (Fig. 8).

The same two types of ABCA1 transcript were also dominant in mouse and human livers and in rat hepatoma McARH7777 cells (Fig. 2A), and cholesterol depletion with statin caused the same direction of changes in total and type P ABCA1 mRNA levels both in rat liver and McARH7777 cells. These findings indicate the relevance of this cell line as an in vitro model. McARH7777 cells, but not HepG2 cells (Figs. 3A and 7A), are known to reflect the physiological sterol regulation of SREBPs in the liver (29).

Each Promoter Exhibits a Distinct Response to Depletion of Cellular Cholesterol with Statins—Treatment of McARH7777 cells with compactin increased the type L promoter activity but

in treated rats) (Fig. 7C). The amount of cholesterol in the very low density lipoprotein fraction was markedly decreased (5.8 ± 3.1 mg/dl in control versus 1.9 ± 0.5 mg/dl in treated rats), and the plasma total cholesterol was unchanged (44.0 ± 3.1 mg/dl in control versus 49.9 ± 4.1 mg/dl in treated rats).

FIGURE 6. Compactin-induced repression of the peripheral type promoter is mediated by LXR. McARH7777 cells were transfected with either the type P ABCA1 promoter or LXREx4-Luc along with control phRL-TK and treated with vehicle-alone, compactin (50 μM) or 4 μM TO901317 for 16 h (left panel). The same promoter plasmid was co-transfected with control phRL-SV40 and either an empty or nuclear SREBP-2 expression plasmid (right panel). The cells were incubated with growth medium for 16 h. The values are the means ± S.D. (n = 3–6) relative to vehicle-treated wild-type activity.

FIGURE 7. Cholesterol depletion in the liver by statins and bile acid binding resin up-regulates hepatic ABCA1 mRNA and protein expression and raises plasma HDL levels. A, the mRNA levels of total and type P ABCA1, HMG-CoA synthase, SREBP-1c in the liver and intestine of rats fed normal chow (Con) or pravastatin/colestimide (P/C) for 8 days. Data were normalized by using the 18 S rRNA levels. B, hepatic and intestinal ABCA1 protein expression. Membrane fractions from the liver and intestine were prepared, and aliquots (liver, 35 μg of protein per lane; intestine, 40 μg of protein per lane) were subjected to immunoblot analysis for ABCA1 and the loading control calnexin. C, plasma total cholesterol and HDL cholesterol levels and lipoprotein profile were analyzed with the HPLC system. VLDL, very low density lipoprotein. The values are the means ± S.E. (n = 3 control, n = 4 P/C treated rats), *, p < 0.05, versus control mice.

(1.20 ± 0.02 mg/g in control versus 0.96 ± 0.07 mg/g in treated rats, p < 0.01) and a marked increase in mRNA expression of the typical SREBP-2 target gene HMG-CoA synthetase, whereas mRNA expression of the LXR target gene SREBP-1c was reduced (Fig. 7A). Moreover, total ABCA1 mRNA was increased and type P ABCA1 mRNA was decreased, indicating an increase in type L ABCA1 mRNA. In contrast, the same treatment reduced both the total and type P ABCA1 mRNA levels in the small intestine. Immunoblot analysis showed that ABCA1 protein level was increased in the liver of treated rats but decreased in the intestine, consistent with the total mRNA levels in these organs.

These changes were accompanied by an increase in HDL cholesterol (25.1 ± 2.3 mg/dl in control versus 35.0 ± 3.3 mg/dl in treated rats) (Fig. 7C). The amount of cholesterol in the very low density lipoprotein fraction was markedly decreased (5.8 ± 3.1 mg/dl in control versus 1.9 ± 0.5 mg/dl in treated rats), and the plasma total cholesterol was unchanged (44.0 ± 3.1 mg/dl in control versus 49.9 ± 4.1 mg/dl in treated rats).

DISCUSSION

Each Promoter Exhibits a Distinct Response to Depletion of Cellular Cholesterol with Statins—Treatment of McARH7777 cells with compactin increased the type L promoter activity but
Dual Promoter Regulation of Hepatic ABCA1 Gene Expression

**Figure 8. Dual promoter regulation of hepatic ABCA1 by SREBP-2 and LXR.** When cells were treated with statins, an increase in the active form of SREBP-2 activated the type L promoter through the SRE complex, whereas the type P promoter was repressed, presumably by a decrease in endogenous LXR ligand synthesis but not by SREBP-2-mediated repression of the E-box element.

decreased the type P promoter activity. These changes explain unaltered levels of total ABCA1 mRNA by compactin in McARH7777 cells, whereas ABCA1 expression in Rat2 fibroblasts was decreased.

The type L promoter we identified in this study contains SRE and is directly activated by binding an active form of SREBP-2 in response to depletion of cellular cholesterol. The type L promoter activity was, therefore, increased by compactin (Fig. 4A, top), and the increase was prevented by sterols (Fig. 5A). In contrast, compactin markedly diminished the type P promoter activity in an LXRE-dependent, but not E-box-dependent manner (Fig. 6, left). The reduction was fully restored by mevalonate and partially restored by sterols (Fig. 4A). These findings are consistent with the idea that the basal activity of the type P promoter in McARH7777 cells is mainly regulated by the level of endogenous sterol LXR ligands and that statins inhibit the ligand synthesis.

Endogenous LXR ligand production has been reported to be increased by SREBP-2 overexpression in Chinese hamster ovary cells (37). However, the type P promoter activity in McARH7777 cells was not enhanced by overexpressing SREBP-2 (Figs. 4C and 6). Instead, SREBP-2-siRNA treatment decreased the promoter activity to the level present in compactin-treated cells (Fig. 4D, bottom), suggesting that the basal activity of the type P promoter in McARH7777 cells is sustained by SREBP-2 through the supply of endogenous ligands for LXR.

In contrast to vascular endothelial cells (38), overexpression of SREBP-2 in McARH7777 cells did not diminish the type P promoter activity, regardless of the mutation in LXRE (Figs. 4C and 6, right), suggesting that SREBP-2 does not act as an E-box repressor in this cell line. Mutation of the E-box element resulted in up-regulation of the type P promoter activity (Fig. 6), indicating that the promoter was already repressed in McARH7777 cells by the E-box element-mediated mechanism under the basal conditions. Binding of a complex of USF1, USF2, and Fra2 to the E-box element has been shown to repress the promoter activity in macrophages (39).

Our findings in vivo suggest that SREBP regulation of the type L promoter is functional in rat liver. Pravastatin/colestimide treatment caused an increase in total ABCA1 mRNA as well as in other SREBP-target, HMG-CoA synthase mRNA, but diminished type P mRNA indicated an increase in type L ABCA1 mRNA level (Fig. 7A). The decrease in type P ABCA1 mRNA level may be explained by the reduction in endogenous LXR ligand level caused by pravastatin.

**Type P Promoter in Hepatic Cells Exhibits a Unique Response to Sterols**—The effect of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) on ABCA1 expression was found to be complex. Sterols decreased both total and type P ABCA1 mRNA levels in McARH7777 cells but raised them in Rat2 fibroblasts (Figs. 1 and 3). Cholesterol loading of fibroblasts and macrophages has been shown to increase ABCA1 expression through activating LXR (11–14).

Exposure of McARH7777 cells to sterols decreased the amount of nuclear SREBP-2 and the type L promoter activity in an SRE-dependent manner (Figs. 4A and 5C). It has been well established that treating cells with a mixture of 25-hydroxycholesterol and cholesterol inhibits SREBP-2 processing, thereby decreasing nuclear SREBP-2 (29). Exposure of cells to cholesterol alone had no effect on either type L or P promoter activity (supplemental Fig. 2), consistent with a study showing that cholesterol is insufficient to decrease nuclear SREBP-2 in McARH7777 cells (29).

The type P promoter activity and mRNA level were unexpectedly decreased by sterols in McARH7777 cells but were increased by TO901317 (Figs. 3A and 4A). Another study has also shown that sterols (25-hydroxycholesterol plus cholesterol) decrease the mRNA expression of an LXR-target gene SREBP-1c in hepatic cells (including McARH7777 cells) (29). Our findings on the regulation of the type P promoter are entirely consistent with the mechanism proposed for SREBP-1c mRNA expression (29). The basal activity of type P promoter in McARH7777 cells as well as SREBP-1c mRNA expression is maintained by tonic activation of LXR by endogenously synthesized sterols. 25-Hydroxycholesterol is known to be a partial LXR agonist and to exhibit very low activity compared with the activity of the endogenous ligand 24(S)-25-epoxycholesterol (40). In intact cells, 25-hydroxycholesterol might compete with endogenous ligands for LXR activation, thereby decreasing the basal type P promoter activity, and thus, the weak LXR activation might become evident when endogenous ligands were depleted with compactin (Fig. 4A, bottom). We have also observed similar competitive inhibition of endogenous ligand activity by an LXR partial agonist in hepatic cells (41).
**Dual Promoter Regulation of Hepatic ABCA1 Gene Expression**

*Possible Role of the Dual Promoter System*—In the model of reverse cholesterol transport, the liver plays a role in converting cholesterol derived from HDL to bile acids and excreting them (2, 17). This process is facilitated by oxysterol-activated LXR, which up-regulates expression of cholesterol 7α-hydroxylase, the rate-limiting enzyme for the conversion of cholesterol to bile acids, and of ABCG5/ABCG8, transporters involved in cholesterol/sterol excretion into the bile (42, 43). The activation of LXR may also up-regulate ABCA1 expression through activating the type P promoter. Excess ABCA1 expression in the liver may cause re-transport of cholesterol to peripheral tissues. However, our findings suggest that the rise in the type P promoter activity must be counterbalanced by reduced activation of SREBP-2-driven type L promoter. It is possible that the SREBP- and LXR-driven dual promoter systems function to prevent overshooting of the LXR-mediated ABCA1 expression. Indeed, ABCA1 mRNA has been shown to be unaffected by feeding cholesterol to mice, whereas other LXR target ABCG5/ABCG8 mRNAs were markedly induced (15).

The liver is the major site of production of apoA-I, the major apolipoprotein of HDL (44). The biogenesis of HDL with apoA-I in the liver seems to be mediated by ABCA1 in an autocrine-like manner (45). Some investigators have proposed that the liver produces HDL mainly in the form of relatively cholesterol-poor phospholipid-apoA-I complex particles, providing an efficient cholesterol acceptor for the reactions mediated by ABCA1, scavenger receptor class B type I, and ABCG1 in peripheral cells (7, 46, 47). A recent study has suggested that hepatic ABCA1 generates cholesterol-poor nascent HDL particles, whereas ABCA1 in peripheral tissues is critical in transferring cellular cholesterol to the early HDL particles (46).

Our findings may be consistent with this proposed tissue-selective function of ABCA1. Under cholesterol-depleted conditions, HDL biogenesis in the liver is enhanced by the increase in hepatic ABCA1 expression by SREBP-2-driven L-type promoter activation, which may result in an increase in the transport of peripheral cholesterol to the liver. The L-type promoter may also ensure constant ABCA1 expression and HDL production even when endogenous LXR agonists are depleted in the liver.

A single promoter containing both SRE and LXRE has been found in the genes encoding fatty acid synthase and SREBP-1c (48, 49). Cooperative regulation of these genes by SREBP and LXR has been suggested to serve to balance their expression under fluctuating sterol conditions (50). In contrast, our results established regulation by the dual promoter system containing SRE and LXRE in hepatic ABCA1 gene expression. Further investigation is needed to fully understand the physiological relevance of this dual regulation system.

Our findings indicate that cholesterol depletions with pravastatin and colestimide decreased ABCA1 mRNA and protein levels in the intestine but increase their levels in the liver, resulting in elevation of the plasma HDL concentration (Fig. 7). Statins are widely used for the treatment of hypercholesterolemia and are thought to raise plasma HDL cholesterol by 5–15% in addition to reducing low density lipoprotein cholesterol (51, 52). Our findings in this study suggest that statins may raise plasma HDL levels by stimulating type L promoter activity in the liver. Because hepatic ABCA1 is the most promising therapeutic target for raising HDL levels, our findings also provide information important to the design and development of a new therapeutic strategy to prevent and treat atherosclerosis.

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