Transcription Factor Sterol Regulatory Element–Binding Protein 2 Regulates Scavenger Receptor Cla-1 Gene Expression

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Objective—The human scavenger receptor class B type I (Cla-1) plays a key role in cellular cholesterol movement in facilitating transport of cholesterol between cells and lipoproteins. Indirect evidence has suggested that Cla-1 gene expression is under the feedback control of cellular cholesterol content. To define the molecular mechanisms underlying such putative regulation, we evaluated whether Cla-1 is a target gene of the sterol regulatory element–binding protein (SREBP) transcription factor family.

Methods and Results—Transient transfections demonstrated that SREBP factors induce Cla-1 promoter activity and that SREBP-2 is a more potent inducer than the SREBP-1a isoform. The 5′-deletion analysis of 3 kb of the 5′-flanking sequence of the Cla-1 gene, combined with site-directed mutagenesis and electrophoretic mobility shift assay, allowed identification of a unique sterol responsive element. SREBP-mediated Cla-1 regulation was confirmed in stably transfected human embryonic kidney 293 cells expressing the active form of SREBP-2 at incremental levels. In these cell lines, Cla-1 mRNA and protein levels were increased in direct proportion to the level of SREBP-2 expression.

Conclusions—These findings provide evidence that SREBP-2, a key regulator of cellular cholesterol uptake through modulation of the expression of the low-density lipoprotein receptor gene, may influence cellular cholesterol homeostasis via regulation of Cla-1 gene expression. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: SR-BI ■ SREBP ■ cholesterol homeostasis ■ regulation of gene expression ■ transcription factor
separate gene. All 3 are produced as precursors inserted into the membrane of the endoplasmic reticulum (ER). Proteolytic activation of the precursor form and subsequent release of the active Nter domain of the SREBP (nSREBP) is tightly regulated and occurs when the ER membrane becomes depleted in cholesterol. The soluble active nSREBP then enters the nucleus and activates transcription by binding either to 10-bp repeats known as sterol response elements (SREs), or to E-box sequences located in the promoter region of target genes. Use of transgenic mice overexpressing either of the active nuclear SREBP isoforms has demonstrated that the SREBP-1 isoforms are more relevant to fatty acid metabolism, whereas SREBP-2 is more selective in regulating cholesterol metabolism.7,8

Expression of the LDL-receptor, a key component of the cellular pathway for cholesterol uptake, is modulated as a function of cellular cholesterol content through SREBP-mediated transcriptional regulation. Indirect evidence suggests that SR-B1, which may modulate membrane cholesterol content and cellular cholesterol pool size through a pathway distinct from that of the LDL-receptor, may also be a target gene for SREBP factors. Indeed, SREBP-1a can induce the transcriptional activity of the rat SR-BI promoter through 2 SRE-binding sites in vitro.9 Feedback control of SR-BI expression dependent on cellular cholesterol status has also been suggested in vitro in human keratinocytes10 and in vivo in mouse adrenal glands11 and rat ovaries.12 However, in contrast, microarray analyses of RNA from livers of transgenic mice overexpressing SREBP isoforms 1a and 2 did not reveal changes in SR-BI mRNA levels.13

In this study, we evaluated the question of whether expression of the human SR-BI/Cla-1 gene is regulated via SREBP factors. Analysis of the 5’-proximal region of the Cla-1 gene allowed identification of a functional SRE-binding site through which SREBP-2 modulates Cla-1 promoter activity. Moreover, stable overexpression of nSREBP-2 in a human cell line is associated with significant upregulation of both levels of Cla-1 endogenous mRNA and protein. These findings strongly suggest that Cla-1 is a target gene for SREBP-mediated transcriptional regulation and imply that intracellular cholesterol status is implicated in regulation of its expression.

**Methods**

**Plasmid Constructs and Site-Directed Mutagenesis**

The Cla-1 promoter luciferase reporter plasmid p-2913 cloned into pGL3 basic vector (Promega), the corresponding 5’-deletion constructs p-258 and p-58 and the luciferase reporter plasmid for the mouse SR-BI promoter were described previously.14 The expression vectors pCMV5-SREBP-1a and pCMV5-SREBP-2 were provided by K. Schoonjans (IGBMC, Illkirch, France). To generate the luciferase reporter plasmid for the LDL-receptor promoter, the proximal region (~171 to +57) of the gene was amplified by polymerase chain reaction (PCR) using human genomic DNA as template and cloned into pGL3 basic vector. The sequence was then verified in the final construct by DNA sequencing.

Site-directed mutants were prepared from construct p-258 using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). The synthetic complementary oligonucleotides SRE1 (5’-CCC GCC CCG TGG CCC GCC CGC CCC GCC CGC-3’), and the E-box sequence (5’-ACC ACT GCT GTG CTG CCG GCC GTC TGT-3’) containing mismatched bases (underlined) were used to mutate the respective putative binding sites in the Cla-1 promoter sequence. The presence of the mutation was confirmed by sequencing.

**Stable Transfection of Human Embryonic Kidney 293 Cells With nSREBP-2**

The cDNA encoding for nSREBP-2 (amino acids 1–481) was subcloned into the pcDNA3.1 expression vector (Invitrogen) containing the neomycin resistance gene. Human embryonic kidney 293 (HEK293) cells were grown for 24 hours in serum-free medium and then incubated with 4 μg plasmid DNA and 5 μg Lipofectamine (Invitrogen). After 6 hours of incubation, cells were trypsinized, and a serial dilution in complete medium was performed. Selective medium (complete medium supplemented with 0.5 g/L G418-sulfate) was applied 2 days later. After 2 weeks, wells containing 1 surviving colony were selected, and HEK–nSREBP-2 clones were expanded in selective medium.

**Transient Transfections, Electrophoretic Mobility Shift Assay, Immunoblot Analysis, RNA Preparation, and Real-Time Quantitative RT-PCR**

Please see the expanded Methods section, available online at http://atvb.ahajournals.org/

**Results**

**Statin and SREBP-Mediated Induction of Cla-1 Promoter Activity**

Because an earlier report demonstrated that rat SR-BI promoter activity was upregulated in vitro by statin treatment and the SREBP-1a transcription factor,9 we investigated whether the Cla-1 gene was regulated in a similar manner. The possibility that Cla-1 promoter activity could be modulated by intracellular sterol content was initially examined by transient transfection studies performed in HepG2 cells cultured in the absence or presence of cerivastatin, a potent 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor. Figure 1 shows that addition of cerivastatin for 24 hours resulted in differential activation of Cla-1 promoter/reporter constructs carrying ~3 kb (p-2913), ~0.4 kb (p-258), or ~0.2 kb (p-58) of 5’-flanking sequence of the Cla-1 gene. Whereas p-2913 and p-258 promoter activities were similarly and significantly increased 1.8-fold and 1.7-fold, respectively, by statin treatment, the activity of the p-58 construct remained unchanged. To ascertain whether the increase in Cla-1 promoter activity of p-2913 and p-258 induced by cerivastatin treatment was the result of SREBP-dependent promoter activation after cellular cholesterol depletion and not the result of a cholesterol-independent effect of statin, we performed cotransfection studies of Cla-1 promoter constructs and expression vectors coding for either nSREBP-1a or nSREBP-2 (Figure 1). As seen for the cerivastatin experiments, a statistically significant upregulation of promoter activity was observed with the p-2913 and p-258 constructs with either of the SREBP isoforms, whereas no activation occurred with the shortest p-58 construct. These data indicated that the Cla-1 promoter is a target for SREBP-dependent transactivation.
Differential Activation of mSR-BI and Cla-1 Promoter Activities by SREBP-1a and SREBP-2

It has been reported that SREBP-1a, SREBP-1c, and SREBP-2 may exhibit distinct efficacy in promoting transcriptional induction of target promoters. Comparison of transcriptional activation of 5'-deletion Cla-1 promoter constructs by statin or by cotransfection of SREBP-1a or SREBP-2 plasmids in HepG2 cells. The 5'-deletion Cla-1 constructs p-2913, p-258, or p-58 were cotransfected with pSVGal as reference plasmid. Statin effect (white columns), Cerivastatin was added to a concentration of 100 nM in serum-free DMEM 16 hours after transfection. Luciferase and β-galactosidase activities were assayed 24 hours later. SREBP cotransfection experiments, Cla-1 promoter constructs were transfected with expression vectors (20 ng) for SREBP-1a (striped columns) or SREBP-2 (black columns). Results are expressed as fold induction relative to normalized luciferase activities obtained with the respective control cells (no statin treatment or cotransfection with pCMV5 empty vector). Statistically significant differences from the controls ***P < 0.001; **P < 0.01; *P < 0.02.

Identification and Characterization of a Functional SREBP-2 Binding Site in the Promoter of the Cla-1 Gene

Data in Figure 1 revealed that p-2913 and p-258 responded in a similar manner to cerivastatin and to SREBP-mediated increase of luciferase activity. These data suggested that the 5'-deleted region between positions −2913 and −258 was not involved in the SREBP-specific response. Additionally, the lack of response of the p-58 plasmid to statin or SREBPs indicated that the DNA motif(s) required for the SREBP-mediated response lies in the region between positions −258 and −58. Analysis of this region revealed 3 putative SREs (SRE1, SRE2, and SRE3), identified according to their homology with the consensus SRE sequence (ATC ACC CCA C). The functionality of these DNA sequences as an SRE was evaluated by site-directed mutagenesis. Whereas the induction of p-258 promoter activity by SREBP-2 was not affected by mutation of the putative SRE1 and SRE3, mutation of the SRE2 sequence resulted in the complete loss of SREBP-2 activity (Figure I, available online at http://atvb.ahajournals.org).

SREBP-2 affinity for the SRE2 sequence was evaluated by electrophoretic mobility shift assay (EMSA) analysis (Figure 2A). Similar results, with an identical fold increase in Cla-1 promoter activity were obtained with 20 ng of the SREBP-2 plasmid (dose-dependent promoter induction was maintained up to 100 ng of SREBP-2 vector; data not shown), no further increase was observed when similar amounts of SREBP-1a vector were used (plateau induction at ~1.6-fold; Figure 2A). Similar results, with an identical fold increase, were obtained using the full-length p-2913 construct in place of p-258 (data not shown). To validate the capacity of SREBP-1a and SREBP-2 to transactivate target promoters in our luciferase assay, we evaluated the SREBP-mediated induction of the activity of a control reporter construct containing the proximal promoter (~2.1 kb of 5'-flanking sequence). Surprisingly, a marked increase in promoter activity was observed with both SREBP forms; however, as seen for the human promoter constructs, there was a clear differential activation of transcriptional activity of the mouse SR-BI promoter by cotransfection of either pCMV5–SREBP-1a or SREBP-2 (3.2-fold and 17.6-fold increase with SREBP-1a and SREBP-2, respectively; Figure 2C). Together, these data demonstrate that under the experimental conditions tested, SREBP-2 activates human and mouse SR-BI promoters with greater efficiency than SREBP-1a.

Figure 1. Comparison of transcriptional activation of 5'-deletion Cla-1 promoter constructs by statin or by cotransfection of SREBP-1a or SREBP-2 plasmids in HepG2 cells. The 5'-deletion Cla-1 constructs p-2913, p-258, or p-58 were cotransfected with pSVGal as reference plasmid. Statin effect (white columns), Cerivastatin was added to a concentration of 100 nM in serum-free DMEM 16 hours after transfection. Luciferase and β-galactosidase activities were assayed 24 hours later. SREBP cotransfection experiments, Cla-1 promoter constructs were transfected with expression vectors (20 ng) for SREBP-1a (striped columns) or SREBP-2 (black columns). Results are expressed as fold induction relative to normalized luciferase activities obtained with the respective control cells (no statin treatment or cotransfection with pCMV5 empty vector). Statistically significant differences from the controls ***P < 0.001; **P < 0.01; *P < 0.02.

Figure 2. Dose-dependent activation of Cla-1 and LDL-receptor promoters by SREBP-1a and SREBP-2. The Cla-1 promoter construct p-258 (A) or the human LDL-receptor promoter construct (B) were transiently transfected in HepG2 cells in the presence of increasing amounts (1, 5, and 20 ng) of SREBP-1a and SREBP-2 expression vectors. C, Transactivation of the mouse SR-BI promoter by SREBP-1a and SREBP-2. The mouse SR-BI promoter construct was cotransfected in HepG2 cells with SREBP-1a or SREBP-2 expression vectors (20 ng). Results were expressed as fold induction relative to normalized luciferase activities in control cells.
3) using either in vitro–translated nSREBP-2 or nuclear extracts prepared from HEK293 cells overexpressing nSREBP-2 (see below). A retarded protein–DNA complex was detected in the presence of recombinant nSREBP-2 and HEK293 nuclear extracts (complex I, lanes 1 and 5). This complex appeared to be specific because the addition of an excess of unlabeled SRE2 probe prevented its formation (lanes 2 and 6), whereas an unlabeled mutated SRE2 oligonucleotide did not (lanes 3 and 7). The consensus SRE sequence identified in the promoter of the LDL-receptor gene was used as a control. The addition of an excess of unlabeled LDL-receptor SRE specifically competed with the SRE2–protein complex I (lanes 4 and 8). We equally observed that incubation of the SRE2 probe with nuclear extracts from HEK293 cells overexpressing nSREBP-2 produced another DNA–protein complex (complex II, lane 5). Because the SRE2 oligonucleotide is GC-rich, we tested the possibility that the protein involved in this second complex could be Sp1, a transcription factor that binds to such DNA motifs. Addition of a molar excess of an unlabeled oligomer containing an Sp1 binding sequence specifically prevented formation of complex II but not that of complex I (lane 9). Complex II was also competed out by the addition of an excess of unlabeled wild-type (lane 6) or mutated (lane 7) SRE2 oligonucleotides but not by that of cold LDL-receptor SRE (lane 8). These results suggest that Sp1 binds to a site adjacent to that of SREBP-2 on the SRE2 probe and may act as an SREBP cofactor. Further confirmation of the implication of Sp1 in this Cla-1 SRE2/protein complex II was provided by the disappearance of this complex and the formation of a supershifted band after addition of anti-Sp1 antibody (lane 10).

Cla-1 Gene Expression Is Activated as a Function of nSREBP-2 Levels in Stably Transfected HEK293 Cells

To further evaluate whether Cla-1 gene expression is regulated by SREBP-2, we established stably transfected HEK293 cells expressing the human nuclear active form nSREBP-2. Stable HEK293 clones were assayed for the level of recombinant nSREBP-2 (rec.nSREBP-2) mRNA expression by quantitative RT-PCR using primer pairs that discriminate rec.nSREBP-2 cDNA from endogenous SREBP-2 cDNA (Table I, available online at http://atvb.ahajournals.org). Figure 4A shows the results of 4 clones that express very low (clone 1), moderate (clone 2), or high levels (clones 3 and 4) of rec.nSREBP-2. As reported previously in transgenic SREBP mouse models or in vitro cell systems, graded overexpression of rec.nSREBP-2 (rec.nSREBP-2) mRNA expression by quantitative RT-PCR using primer pairs that discriminate rec.nSREBP-2 cDNA from endogenous SREBP-2 cDNA (Table I, available online at http://atvb.ahajournals.org). Cla-1 mRNA levels were equally increased incrementally as a function of the level of expression of rec.nSREBP-2 in the cell clone (Figure 4B, 1.5-fold, 3.4-fold, and 4.1-fold changes for clone 2, 3, and 4, respectively). Changes in protein levels were assessed by densitometric analysis of Western blots (Figure 4C). Although HEK293 cells normally express Cla-1 protein at low levels, comparison of Cla-1 protein expression between HEK–nSREBP-2 high expressor clones (clones 3 and 4) and 2 clones expressing nSREBP-2 at very low levels (clones 1 and 5) revealed greater Cla-1 protein levels in both high expressor clones (1.5-fold and 1.9-fold increase for clones 3 and 4, respectively; P<0.0002; Figure 4C). It is noteworthy that the fold changes measured at the mRNA level were
have been identified in the rat promoter, analysis of 3 kb of 5'-flanking sequence of the Cla-1 gene revealed the presence of a unique functional SRE (at least 2 SREBP responsive regions could be detected in the mouse SR-BI promoter; data not shown). Based on site-directed mutation and EMSA analyses, this SRE element is most likely 5'-ATCACGCACCC-3', thereby resembling the human LDL-receptor SRE-1 (5'-ATCACCCCCAC-3') and the SRE identified in the rat SR-BI proximal promoter (5'-AGCAGCGGCC-3'). Preference activation of different target promoters by either of the SREBP isoforms has been reported; 15-17 indeed, such a mechanism has been proposed to partially account for the differential in vivo regulation of genes involved in fatty acid metabolism and cholesterol biosynthesis by SREBP-1 and SREBP-2, respectively. To date, no specific base change in SREBP recognition sites has been firmly identified that provides greater specificity in binding to individual SREBPs. However, it has been postulated that SREBP-2 could possibly display higher affinity for DNA motifs typified by the LDL-receptor SRE-1. 18 Conversely, it has also been suggested that differences in the first 2 5' nucleotides of the LDL-receptor SRE-1 could explain the exclusive binding of SREBP-2 to the human squalene synthase SRE (8/10) 5'-TCCACCCACC-3'. 17,22 The Cla-1 SRE2 site, as presently identified shares an identical 5' end but diverges in its 3' end from that of the LDL-receptor SRE-1, and on the basis of the aforementioned hypotheses, would not therefore correspond to a preferential target of the SREBP-2 form. Greater promoter activation by SREBP-2 than by SREBP-1a, as observed in the present study for human and mouse SR-BI promoters, has rarely been reported previously. Therefore, the Cla-1 SRE2 site may constitute an interesting sequence to analyze, along with the SREBP-responsive element(s) of the mouse SR-BI promoter; such analysis may lead to identification of key nucleotides in SRE motifs for preferential binding specificity/affinity of SREBP-2 versus SREBP-1a.

The general transcription factors NF-Y and Sp1 are coregulatory transcription factors that are critical to high levels of SREBP-mediated activation of promoters. 22,23 In this study, we observed that the GC-box 3' of the Cla-1 SRE2 sequence interacts with the Sp1 transcription factor. The presence of this functional Sp1-binding site adjacent to the Cla-1 SRE2 site further supports the notion that Cla-1 is a member of the gene family activated by SREBP factors. It is relevant that an 11-bp deletion in the Cla-1 gene, which corresponds to this Sp1-binding sequence, has been identified recently in a Taiwanese Chinese population. 24 It would be of interest to evaluate whether this deletion, which was shown to be associated with reduced basal Cla-1 promoter activity in vitro but also with increased HDL-cholesterol levels in the aforementioned population, may affect SREBP-2-mediated regulation of Cla-1 gene expression.

A recent analysis of microarrays hybridized with RNA from livers of SREBP-1a and SREBP-2 transgenic mice failed to identify SR-BI as a target gene for SREBP. 13 One explanation for this apparent discrepancy with the current study resides in the fact that changes in SR-BI gene expression in the livers of these SREBP transgenic mice may be modest and therefore could not meet the criteria (>2.5-fold change) required to qualify SR-BI as an SREBP target.

**Discussion**

We established that the promoter regions of the mouse SR-BI gene and of its human homolog Cla-1 are transactivated by SREBP factors and preferentially by SREBP-2 compared with SREBP-1a. The significance of such regulation was highlighted by the analysis of HEK293 cells expressing the nuclear active nSREBP-2 at incremental levels, in which Cla-1 gene expression was increased as a function of that of nSREBP-2. The demonstration that SREBP-2 regulates Cla-1 gene expression, a receptor that mediates the bidirectional regulation of cellular cholesterol homeostasis, suggests a potential control of Cla-1 expression at a post-transcriptional level. In this context, it is relevant that PDZK1, a PDZ domain containing protein essential for SR-BI protein expression, 19 could not be detected by quantitative RT-PCR in HEK293 cells; therefore, PDZK1 does not appear to be implicated in this potential post-transcriptional effect.

As a control for the Western blots, the use of an anti-LDL-receptor antibody revealed, as expected, a greater expression of LDL-receptor in high expressor clones than in very low expressor clones.

For this cell type, and are modulated in a similar manner, as demon-strated for the present work, mouse and human SR-BI promoters were shown to respond to SREBP-1a and SREBP-2. These data corroborate those of Lopez et al 10 on the induction of rat SR-BI promoter activity by SREBP-1a. Whereas 2 SREs
Absence or moderate transcriptional activation of SR-BI expression may also underpin the complex regulation of this gene in hepatic tissue. Indeed, whereas SR-BI expression has been reported to be reduced in liver parenchymal cells of rats fed a high-cholesterol diet,25 thereby suggesting cholesterol-mediated feedback control of SR-BI expression, concomitant increased expression was reported in liver Kupffer cells.25 Alternatively, minor changes in the transcription levels of the cholesterol 7α-hydroxylase gene, as reported in SREBP-2 transgenic mice,13 may increase bile acid biosynthesis in the hepatocyte. On binding to bile acids, FXR activates transcription of the Shp gene, a repressor of the orphan nuclear receptor LRH-1, which we identified recently as a positively acting transcription factor of SR-BI/Cla-1 expression in mouse liver.14 Whether such regulation of the SR-BI gene is relevant when SREBP action is maintained constant as in SREBP transgenic mouse models remains indeterminate.

Long-term treatment of hypercholesterolemic patients with statins has proven efficacious in reducing plasma LDL-cholesterol levels (up to 35%), whereas baseline values of HDL-cholesterol generally remain unchanged or may be slightly increased (≤10%). However, early and transitory reduction of HDL-cholesterol levels after high-dose atorvastatin has been reported in patients with heterozygous26 or homozygous familial hypercholesterolemia (HFH; up to 21% reduction after 4 weeks treatment).27 Such an effect could be partially related to an early response to statin-induced cellular cholesterol deprivation, which may result in SREBP-mediated upregulation of hepatic Cla-1 expression and subsequent increase in selective uptake of HDL-cholesterol with concomitant reduction in plasma HDL-cholesterol levels. In HFH patients, the transient effect of atorvastatin on HDL-cholesterol levels was 2-fold greater in patients with total LDL-receptor deficiency compared with receptor-defective HFH subjects, thereby suggesting a direct link to the absence of LDL-receptor deficiency.22 This observation emphasizes the fact that metabolic processes influencing HDL-cholesterol levels are responsive to statin treatment and that these processes may be dependent on the presence of functional LDL-receptors. Indeed, atorvastatin therapy has been shown to decrease the mass and activity of the CE transfer protein28,29 and also to decrease hepatic lipase activity;30 both effects favor elevation of HDL-cholesterol and may be directly influenced by plasma concentrations of very LDL and LDL particles. Thus, in HFH patients treated with high-dose atorvastatin, intravascular remodeling of HDL particles could be partially altered because of LDL-receptor deficiency, revealing a statin-induced upregulation of Cla-1 expression with a subsequent lowering impact on HDL-cholesterol levels. This mechanistic hypothesis requires further studies to confirm it but suggests that 1 additional beneficial action of statins may involve minor changes in the hepatic expression of Cla-1, a low-affinity but high-capacity receptor that may positively influence the dynamic flux of the reverse cholesterol transport pathway.

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References

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EXPANDED METHODS SECTION

Cell culture

HepG2 and HEK293 cells were maintained in culture in a humidified 5% CO₂ atmosphere at 37°C in complete medium consisting of DMEM supplemented with 10% foetal bovine serum, 2mM L-glutamine and penicillin-streptomycin.

Transient transfections

Transient transfections were carried out with the Lipofectin Liposomal reagent (Invitrogen). HepG2 cells were seeded in 6-well plates at 2 x 10⁵ cells per well, grown for 24h and then incubated for 16h with 1ml of serum-free medium containing 0.64 – 1 µg of luciferase promoter construct, 0.3 µg of a β-galactosidase expression plasmid (pSV-gal, Promega) and 5µg Lipofectin (Invitrogen). When indicated, Cla-1 promoter constructs were co-transfected with 1 to 20 ng of pCMV5-SREBP-1a or pCMV5-SREBP-2 expression vectors. Control cells were co-transfected with corresponding amounts of empty pCMV5 vector. After incubation for 16h, the medium was replaced by fresh medium containing 10% foetal calf serum for 24h. Cell extracts were prepared in lysis buffer (Promega) and β-galactosidase and luciferase activities were determined as described previously ¹. Luciferase values were normalized to β-galactosidase activities. Transfection data represent the mean (+/- standard deviation) of three to six independent experiments each performed in triplicate.

Electrophoretic Mobility Shift Assay

EMSA using HEK293 nuclear extracts, prepared from confluent 75-mm flasks by the method described by Dignam et al.², and in vitro-synthesized SREBP-2, obtained using the TNT T7-coupled reticulocyte lysate system (Promega), were performed as described previously ¹. When indicated, 1.6µg of rabbit affinity-purified polyclonal antibody raised against Sp1
(Santa Cruz Biotechnology) was incubated with nuclear extracts for 30 minutes on ice before the addition of the probe. The sequences of the oligonucleotides were as follows: Wild type Cla-1 SRE2 (5’-CTGCCCGTCCGATCAGCGCCCCGCCCCGTC-3’), mutated Cla-1 SRE2 (5’-CTGCCCGTCCGTAGTGCGCCCCGCCCCGTC-3’), Sp1 binding site (5’-ATTTCGATCGGGGCGGGGCGAGC-3’), human LDL-receptor SRE (5’-ATCACCCCACGTGCAAACACTCTCCCTCCCTGC-3’).

Immunoblot analysis
Cellular extracts (40 µg of protein) from HEK-nSREBP-2 clones and HepG2 cells were separated by electrophoresis on 8% SDS-polyacrylamide gel under reducing conditions, and then blotted onto a Hybond C-super nitrocellulose membrane (Amersham). Cla-1 protein was detected using a rabbit polyclonal antibody raised against a peptide corresponding to amino acids 496-509 of the murine SR-BI sequence. Following washing of the membranes with PBS-Tween, detection of the LDL receptor and β-actin was performed with a rabbit polyclonal antibody against the human LDL receptor (Progen) and with a mouse monoclonal antibody raised against β-actin (clone AC15-Sigma), respectively. Primary antibodies were detected using a peroxydase-conjugated anti-rabbit or anti-mouse IgG and revealed by chemiluminescence (ECL-plus reagent, Amersham). Quantification of Western blots was performed using the Scion Image program.

RNA Preparation and Real Time Quantitative RT-PCR
DNA-free total RNA was prepared from HEK-nSREBP-2 clones and HEK293 control cells using NucleoSpin RNA II (Macherey-Nagel) according to the manufacturer's instructions. RT-PCR analysis was performed as follows: 1.5 µg of RNA was hybridized with 75 ng of random hexamer (Promega) and reverse-transcribed using 200 units of M-MLV reverse
transcriptase (Invitrogen). Real time quantitative PCR was performed using a LightCycler PCR System (Roche). The specific primers are described in Table I. The reaction contained 25 ng of reverse transcribed total RNA, 300 nM forward and reverse primers, 3 mM MgCl₂, and 2 µl of LightCycler - FastStart DNA Master SYBR-GreenI, in a final volume of 20 µl. RT-PCR products were analyzed on ethidium bromide-stained agarose to ensure that a single amplicon of the expected size was obtained. All reactions were performed at least in triplicate and ALA (Delta Aminolevulinate Synthase) RNA amplification was used as the reference to account for variability in the initial quantities of cDNA. In all PCR assays and for each primer set, expression of a control cDNA (pool of reverse transcribed HEK RNA) was included and used as inter-run calibrator. Expression data were based on the crossing points calculated by the LightCycler data analysis software and corrected for PCR efficiencies of the target and the reference gene. Results were confirmed with a second independent isolation of RNAs.
**SUPPLEMENTAL FIGURE I**

*Mutation analysis of putative SRE sites present in the proximal region of the Cla-1 promoter.*

HepG2 cells were transfected with the pCMV5 expression vector for the human mature form of SREBP-2 (20 ng) or the empty pCMV5 vector as control together with the Cla-1 p-258 reporter construct (-258 to +135) containing either the wild-type 5’-flanking sequence of the Cla-1 gene or the corresponding region mutated for putative SRE sites (-204 to -195, -157 to -148 and -132 to -123 for SRE3, SRE2 and SRE1, respectively). An E-Box sequence (-20 to -15), conserved between the human, mouse and rat promoter sequences, located downstream of the -258/-58 region and present in the p-58 construct, was also mutated and used as a control mutant. pSVGal was included in all experiments as reference plasmid. Luciferase activities were measured 48h after transfection, and normalized for βGal activities in order to control for transfection efficiency. Results were expressed as fold induction relative to normalized luciferase activities obtained with control cells. The cross depicts the introduction of the site-directed mutations in the putative SREs or E-box site. * indicates the statistical difference (p=0.0004) from wild type p-258 construct.
**SUPPLEMENTAL FIGURE II**

*Expression levels of relevant SREBP responsive genes in HEK-SREBP-2 clones.*

For each HEK-SREBP-2 clone (#1 to #4; see figure 4), mRNA levels were assessed by quantitative RT-PCR using specific primers. For each gene, data (± SD) represent the amount of mRNA relative to that measured in HEK-SREBP-2 clone #1 which was set arbitrarily to 1. Graded overexpression of rec.nSREBP-2 was associated with a concomitant increase in the levels of mRNA of genes known to be regulated by SREBPs. For the LDL-receptor gene, fold changes were of 14, 22 and 26 for clones #2, 3 and 4, respectively when compared to clone #1. 3-Hydroxy-3-methylglutaryl Coenzyme A reductase (HMGCR), an enzyme of the cholesterol biosynthesis pathway, or the ATP citrate lyase (ACLY), an enzyme that generates
Acetyl-CoA which is essential for both cholesterol and fatty acid synthesis were also strongly activated in the HEK-nSREBP-2 clones, but less significantly than that of the LDL-receptor. The mRNA levels of the Glucose-6-phosphate dehydrogenase (G6PD) gene, which participates in the generation of NADPH used for fatty acid synthesis, have been shown to be dramatically increased in livers of transgenic mice overexpressing nSREBP-1a, but modestly elevated in livers of nSREBP-2 transgenic mice. Preferential activation of the G6PD promoter by SREBP-1a was also reported in HepG2 cells. We observed a modest and graded elevation in levels of G6PD mRNA in the HEK-nSREBP-2 clones with the highest fold increase (x 2.1) measured in clone #4. As SREBP-1 and -2 genes themselves are target genes for SREBP activation, we evaluated the influence of rec. nSREBP-2 overexpression on endogenous SREBP-1 and SREBP-2 mRNA levels in the HEK-nSREBP-2 clones. The expression of both genes was found to be moderately increased as compared to their respective expression in clone #1 (2.4, 1.7 and 1.9-fold change for SREBP-2 and 3.3, 3, and 2-fold change for SREBP-1 in clone #4, #3 and #2, respectively). As a control, we assessed hypoxanthine phosphorybosyltransferase 1 (HPRT) gene expression, for which no SREBP regulation has been described. The HPRT mRNA levels were found unchanged among HEK-nSREBP-2 clones. Statistical differences from clone #1 are indicated (Student’s t test; ***p<0.001, **p<0.01, *p<0.05).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Amplicon</th>
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<tr>
<td>ACLY</td>
<td>ATP Citrate Lyase</td>
<td>5'-CAC CGA GTG AAG TCG ATA A-3'</td>
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<td>5'-CTG CAA GCA AAT GCC CTT TC-3'</td>
<td>5'-CCC TCC ATC GGT TTT CAC AC-3'</td>
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<td>Cla-1</td>
<td>Human Scavenger Receptor Class B Type I</td>
<td>5'-GAG CTT TGG CCT TGG TCT ACC T-3'</td>
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<td>G6PD</td>
<td>Glucose-6-Phosphate dehydrogenase</td>
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<td>5'-GGC CAG CCA CAT AGG AGT T-3'</td>
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<td>HMG coenzyme A reductase</td>
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<td>5'-GAC AAT TCC CCA GCC ATT AC-3'</td>
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<td>HPRT</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
<td>5'-TGA CAC TGG CAA AAC AAT GC-3'</td>
<td>5'-AAC ACT TCG TGG GGT CCT TT-3'</td>
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<td>LDLr</td>
<td>Low-Density Lipoprotein Receptor</td>
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<td>5'-CTC CAG GCA GAT GTT CAG G-3'</td>
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<td>PDZK1</td>
<td>PDZ domain-containing 1</td>
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<td>Recombinant SREBP-2</td>
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<td>5'-AGC ATC TCG TCG ATG TCT CC-3'</td>
<td>148bp</td>
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REFERENCES


