

Transcriptional Regulation of the ATP Citrate-lyase Gene by Sterol Regulatory Element-binding Proteins*

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In an attempt to identify unknown target genes for SREBP-1, total RNA from a stable Chinese hamster ovary cell line (CHO-487) expressing a mature form of human SREBP-1a (amino acids 1–487) with a LacSwitch Inducible Mammalian Expression System was subjected to a polymerase chain reaction subtraction method. One of the fragments was found to have 90 and 86% homology with rat and human ATP citrate-lyase (ACL) cDNA, respectively. When Hep G2 cells are cultured under either sterol-loaded or -depleted conditions, expression of the gene is induced approximately 2–3-fold by sterol depletion. To investigate the direct effect of SREBP-1a on transcription, luciferase assays using the promoter of the human ACL gene were performed. These deletion studies indicated that a minimum 160-base pair segment contains the information required for the transcriptional regulation brought about by enforced expression of SREBP-1a. Luciferase assays using mutant reporter genes revealed that SREBP-dependent transcriptional regulation is mediated by two nearby motifs, the SREBP-binding site (a TCAGGCTAG sequence) and the NF-Y-binding site (a CCAAT box). It was confirmed by gel mobility shift assays that recombinant SREBP-1a binds to the sequence. Data from studies with transgenic mice and reporter assays show that the ACL gene promoter is activated by SREBP-1a more strongly than SREBP-2 in contrast to the HMG CoA synthase and LDL receptor gene promoters, which exhibit the same preference for the two factors. Therefore, SREBPs transcriptionally regulates ACL enzyme activity, which generates the cytosolic acetyl CoA required for both cholesterol and fatty acid synthesis.

Two SREBPs,¹ designated SREBP-1 and -2, regulate the transcription of a number of genes encoding enzymes and proteins involved in cholesterol or fatty acid metabolism (1). SREBPs are synthesized as 125-kDa membrane-bound precursors

and are activated by releasing the transcriptionally active NH₂-terminal portion in a two step proteolysis (2–4). Studies using transgenic mice overexpressing either a mature form of SREBP-1 or SREBP-2 have revealed that SREBP-1 mainly regulates lipogenic gene expression and that SREBP-2 regulates cholesterologenic gene expression (5, 6). This characterization is further supported by our previous findings that SREBP-2 gene expression is controlled by the intracellular cholesterol level and that this transcription is directly regulated by SREBPs through the SREBP-binding site in the promoter region (7).

Unlike SREBP-2, two isoforms of SREBP-1 (SREBP-1a and SREBP-1c) are produced by alternate promoters (8). SREBP-1a has a longer and therefore more potent transactivation domain at the extreme NH₂ terminus than SREBP-1c. RNA protection assays have revealed that the SREBP-1c is more abundant than SREBP-1a in most mouse and human tissues (9). However, SREBP-1c has been reported to be weaker than SREBP-1a in stimulating the transcription of target genes in *in vitro* assays because of its shorter transactivation domain (10, 11).

In the current study we have established a CHO cell line transiently expressing a more potent transcription factor, human SREBP-1a, to identify unknown target genes for SREBP-1. In these cells expression of exogenous SREBP-1a is repressed until an inducer, isopropyl β-D-thiogalactopyranoside (IPTG), is added to the medium. When the cells are cultured with a medium containing sterols and IPTG, this system enables genes induced in response to transient expression of SREBP-1a to be picked up, independent of the effects of endogenous SREBPs inactivated by sterols in the medium. Using a subtraction PCR method, we have subsequently managed to obtain several gene fragments induced by SREBP-1a. We have characterized the 5'-flanking region of one of these genes, the human ACL gene, and defined the region responsible for SREBP-dependent regulation. Furthermore, we have examined with reporter assays and transgenic mice expressing either SREBP-1a or SREBP-2 which isoform, SREBP-1 or -2, is preponderantly involved in regulation.

EXPERIMENTAL PROCEDURES

Materials—Hygromycin B, G418, and lipoprotein-deficient serum were purchased from Sigma. Restriction enzymes were obtained from New England Biolabs.

Construction of an Expression Plasmid for Human SREBP-1a—To generate a construct of human SREBP-1a containing amino acids 1–487, designated pOPI3SREBP1, a 1.5-kilobase pair fragment was made by PCR including *NotI* restriction sites at both ends. The *NotI*-*NotI* PCR-generated fragment was ligated into the pOPI3CAT vector (Stratagene).

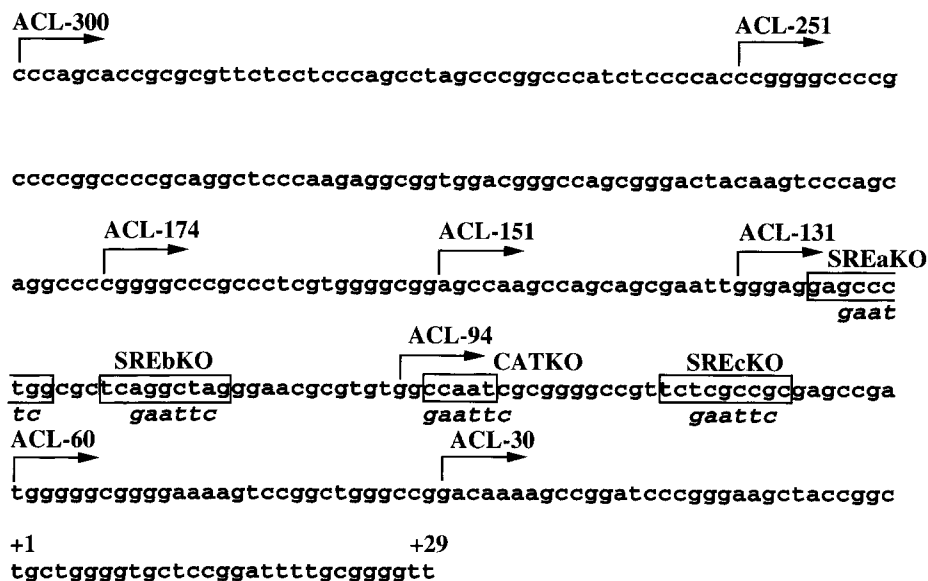
Stable Cell Lines—A *lac* repressor expression plasmid, p3'SS (Stratagene), was stably transfected into CHO cells. On day 0, 1.5 × 10⁵ CHO

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¹ The abbreviations used are: SREBP, sterol regulatory element-binding protein; ACL, ATP citrate-lyase; HMG CoA, 3-hydroxy-3-methylglutaryl Coenzyme A; IPTG, isopropyl β-D-thiogalactopyranoside; LDL, low density lipoprotein; PCR, polymerase chain reaction; SRE, sterol regulatory element; CHO, Chinese hamster ovary; bp, base pair(s).

FIG. 1. The human ACL promoter sequence (13). The transcription start site is +1. Three putative SRE sites and a CCAAT sequence are boxed. The sites used for preparation of truncated reporter gene constructs are indicated by arrows. The mutant sequence in either the SRE sites or the CCAAT box is shown by *italic letters* under the individual original sequence.



cells were seeded into each 60-mm dish with medium A (Dulbecco's modified Eagle's medium/Ham's F12 medium, 100 units/ml penicillin, and 100 μ g/ml streptomycin) supplemented with 7% fetal calf serum. On day 1, each monolayer was transfected with 3 μ g of the plasmid using a calcium phosphate precipitation method. On day 2, the cells were trypsinized and reseeded into three 100-mm dishes. On day 4, the cells were refed with medium A supplemented with 7% fetal calf serum and hygromycin (400 μ g/ml). Visible clones were picked up, subcloned, and maintained in the same medium. One of the clones expressing the *lac* repressor was established and designated CHO-Lac. The CHO-Lac cells were further transfected with pOPI3SREBP1 and cultured with a medium containing hygromycin and G418 (500 μ g/ml) to obtain the CHO-487 cell line as described above.

PCR Subtraction—On day 0, 7×10^5 cells (CHO-Lac or CHO-481) were seeded into 100-mm dishes with medium A supplemented with 7% fetal calf serum. On day 3, the cells were refed with the same medium containing 1 μ g/ml 25-hydroxycholesterol, 10 μ g/ml cholesterol, and 1 mM IPTG. After 19 h of incubation, the cells were harvested and total RNA was prepared. Poly(A) RNA was purified with an oligo(dT) column (Amersham Pharmacia Biotech). According to the procedures described in a previous paper (12), PCR fragments induced by SREBP-1a were subcloned into a Bluescript vector. To pick up real positive fragments, Northern blot analyses on CHO-487 were carried out using each PCR fragment as a probe, and nucleotide sequences for inducible fragments were determined.

Construction of the Reporter Genes for Luciferase Assays—The luciferase reporter plasmids were constructed by cloning the *Bg/II-HindIII* PCR fragments coding the 5'-flanking region of human ACL gene (13) into the same restriction sites of a pGL3 basic vector (Promega). To generate ACL-300, ACL-251, ACL-174, ACL-151, ACL-131, ACL-94, ACL-60, and ACL-30, PCR primers were designed to hybridize at the corresponding position (Fig. 1) and were coupled with the common downstream primer from nucleotide +29. To disrupt one of three putative SREBP-binding sites within ACL-131 (the sites, SREa, SREb, and SREc), the megaprimers were amplified with the upstream primer with four to six mutations (GCCCTG \rightarrow GAATTC at the SREa, AGGCTA \rightarrow GAATTC at the SREb, TCGCCG \rightarrow GAATTC at the SREc) and the common downstream primer, and further extended in the 2nd PCR as described previously (14). The expression plasmids, pSREBP1(1–487) and pSREBP2(1–481), and reporter plasmids, pHMG S containing a 0.5-kilobase human HMG CoA synthase promoter and pLDLR containing a 1.5-kilobase human LDL receptor promoter, have been described previously (15, 16).

Tissue Cultures and Cell Transfection—HEK 293 cells were cultured, and cell transfection, luciferase, and β -galactosidase assays were performed as described previously (14–16).

Northern Blot Analysis for Stable CHO Cells and Hep G2 Cells—Total RNA from CHO-Lac and CHO-487 cells were prepared as described above. Hep G2 cells were set up on day 0 (1.8×10^6 cells/100-mm dish) in medium B (Dulbecco's modified Eagle's medium, 100 units/ml penicillin, and 100 μ g/ml streptomycin) supplemented with 7% fetal calf serum. On day 1, the medium was removed, and the cells were

then washed with phosphate-buffered saline and refed with medium B containing 5% lipoprotein-deficient serum supplemented either with 1 μ g/ml 25-hydroxycholesterol plus 10 μ g/ml cholesterol or with a 50 μ M concentration of a HMG CoA reductase inhibitor (pravastatin) plus 50 μ M sodium mevalonate. After 48 h of culture, total RNA was extracted and fractionated on formaldehyde/agarose gels and then transferred to Nylon membranes (Roche Molecular Biochemicals). Riboprobes were prepared using CHO ACL cDNA, human S17 cDNA (from 1 to 370 bp) (17) and human HMG CoA synthase (from 576 to 867 bp) (18) with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals). Hybridization signals were quantified with AttoPhos (Amersham Pharmacia Biotech) using a FluorImager 595 (Molecular Dynamics).

Gel Mobility Shift Assay—A double-stranded DNA fragment corresponding to nucleotides –118 to –102 of the ACL gene was 3' end-labeled with a digoxigenin-11-ddUTP using a digoxigenin gel shift kit (Roche Molecular Biochemicals). The digoxigenin-labeled probe was incubated with 50 μ g of recombinant SREBP-1a in binding buffer. The reaction conditions have been described previously (15). In competition assays, an excess amount of an unlabeled 17-bp fragment containing SREb in the human ACL promoter or the mutant SREb was added prior to addition of the labeled probe. Recombinant SREBP-1a (1–487) containing six consecutive histidines were expressed using a pET28 vector (Novagen) in *Escherichia coli* and purified by nickel-nitrilotriacetic acid agarose affinity chromatography (Qiagen).

Transgenic Mice Study—Transgenic mice were as described previously (19, 20). Animals of each group ($n = 3$) were fed a high protein/low carbohydrate diet for 2 weeks and fasted overnight prior to sacrifice. An equal aliquot of liver total RNA was pooled (15 μ g) and subjected to Northern blot analysis using cDNA probes for mouse HMG CoA Synthase, LDL receptor and ACL (21, 22). The resulting bands were quantified by exposure of the filters to BAS2000 with BASTation software (Fuji Photo Film, Co., Ltd, Japan), and the results were normalized to the signal generated from 36B4 mRNA.

RESULTS

Expression of ACL mRNA Is Highly Up-regulated by SREBP-1a in CHO-487 Cells—For the establishment of CHO-487, an expression plasmid containing human SREBP-1a (1–487) under the control of the Rous sarcoma virus promoter and *lac* operator was introduced into CHO cells constitutively expressing the Lac repressor (CHO-Lac). After 19 h of incubation of the cells with 1 mM IPTG, we examined the SREBP-1a inducibility by RNase protection assays. In CHO-487 cells SREBP-1a mRNA was slightly detectable even in the absence of IPTG but was increased approximately 10-fold by IPTG (data not shown). To carry out the subtraction PCR, we prepared total RNA from CHO-487 and CHO-Lac cells cultured with sterols plus 1 mM IPTG. It should be noted that sterols decrease the amount of the endogenous nuclear form of

SREBPs in both cell lines and that IPTG increases exogenous SREBP-1a (1–487) in CHO-487 cells. We obtained several PCR fragments induced by SREBP-1a including fatty acid synthase, HMG CoA synthase, and stearoyl CoA desaturase-2 cDNA. One of these fragments was found to have 90 and 86% homology with rat and human ACL cDNA, respectively (data not shown), suggesting that this fragment encodes CHO ACL. Fig. 2 shows that expression of the mRNA for HMG CoA synthase was completely down-regulated in CHO-Lac cells and was induced in CHO-487 cells only by exogenous SREBP-1a. Expression of ACL mRNA was approximately 7-fold higher in CHO-487 cells. Unlike HMG CoA synthase, expression of ACL mRNA was not completely suppressed even in the presence of the high concentration of sterols.

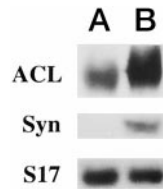


FIG. 2. Northern blot analysis for the ACL and HMG CoA synthase gene in CHO-Lac and CHO-487 cells. CHO-Lac (A) and CHO-487 (B) cells were cultured with 1 $\mu\text{g}/\text{ml}$ of 25-hydroxycholesterol, 10 $\mu\text{g}/\text{ml}$ of cholesterol, and 1 mM IPTG for 19 h. 20- μg total RNA samples were fractionated on 1% agarose gel, transferred to nylon membrane, and hybridized with a digoxigenin-labeled riboprobe for ACL, HMG CoA synthase, or S17. The fold change in ACL mRNA, relative to that in CHO-Lac cells, was calculated after correction for loading differences with S17. Signals were quantified with a FluorImager 595. In three separate experiments the same relative mRNA levels were obtained.

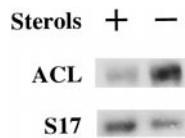


FIG. 3. ACL mRNA expression is regulated by cellular cholesterol levels. Hep G2 cells were cultured with medium containing 5% lipoprotein-deficient serum supplemented with either 1 $\mu\text{g}/\text{ml}$ of 25-hydroxycholesterol plus 10 $\mu\text{g}/\text{ml}$ of cholesterol (sterol-loaded conditions) or 50 μM of a HMG CoA reductase inhibitor, pravastatin plus 50 μM of sodium mevalonate (sterol-depleted conditions) for 48 h. Northern blot analysis was carried out as described in the legend to Fig. 2.

Expression of ACL mRNA Is Regulated by Sterols in Hep G2 Cells—To determine whether expression of ACL mRNA is regulated by the intracellular cholesterol level, human hepatoma cells, *i.e.* Hep G2 cells, were cultured under either sterol-depleted or -loaded conditions, and Northern blot analysis was performed. Under the sterol-depleted conditions, expression of ACL mRNA was up-regulated 2–3-fold (Fig. 3), indicating that the induction is not restricted in CHO-487 cells. Taken together, it is likely that the expression of the ACL gene is controlled by the intracellular cholesterol level through the action of SREBPs.

SREBP-1a Directly Regulates the Transcription of Human ACL Gene—To determine whether SREBP-1a directly regulates the transcription of ACL gene, we carried out luciferase assays using reporter genes including various deletion versions of the human ACL 5'-flanking region. HEK 293 cells were cotransfected with one of the reporter genes and either an expression plasmid for human SREBP-1a (1–487), pSREBP1(1–487), or an empty expression vector. Because a previous report demonstrated that all the minimal response elements for the ACL gene expression are localized within the human ACL promoter –300-bp region (13), we focused on the promoter activity of the first 300 bp 5' to the transcription start site. Fig. 4 clearly shows that enforced expression of SREBP-1a enhances the luciferase activities from 5- to 15-fold as long as the reporter gene contained the segment from –131 to +29 bp (the transcription start site is position +1). We also obtained the same results when the cells were cultured under either sterol-depleted or -loaded conditions (data not shown). Although it was found that expression of ACL mRNA in transgenic mice overexpressing either SREBP-1 or SREBP-2 was accelerated, it has been remained unclear whether this elevation is a direct effect of SREBPs action. This is, therefore, the first evidence demonstrating that SREBP-1 directly regulates the transcription of the ACL gene.

The Elements Responsible for the SREBP-mediated Transcriptional Regulation of the ACL Gene—To identify the sequence motifs in the region (from –131 to +29 bp) responsible for the SREBP-mediated transcriptional regulation of the ACL gene, we further carried out luciferase assays using various mutant versions of reporter genes. We found three putative SREBP-binding sites in the region (SREa, SREb, and SREc in Fig. 1) and mutated each of them to generate mutant versions of reporter genes. Furthermore, we mutated a CCAAT se-

FIG. 4. Regulation of ACL promoter-luciferase reporter genes by SREBP-1a(1–487). HEK 293 cells were transfected with one of human ACL promoter reporter genes (200 ng), a plasmid encoding β -galactosidase (100 ng), and an expression plasmid (10 ng), pSREBP1(1–487) for 4 h. The cells were incubated for 48 h and then lysed, and enzyme activities were determined. The ratio of luciferase activity in relative light units is divided by the β -galactosidase activity (U, units) to give a normalized luciferase value (relative light units/unit). The values given are the averages of data from more than three experiments performed in triplicate.

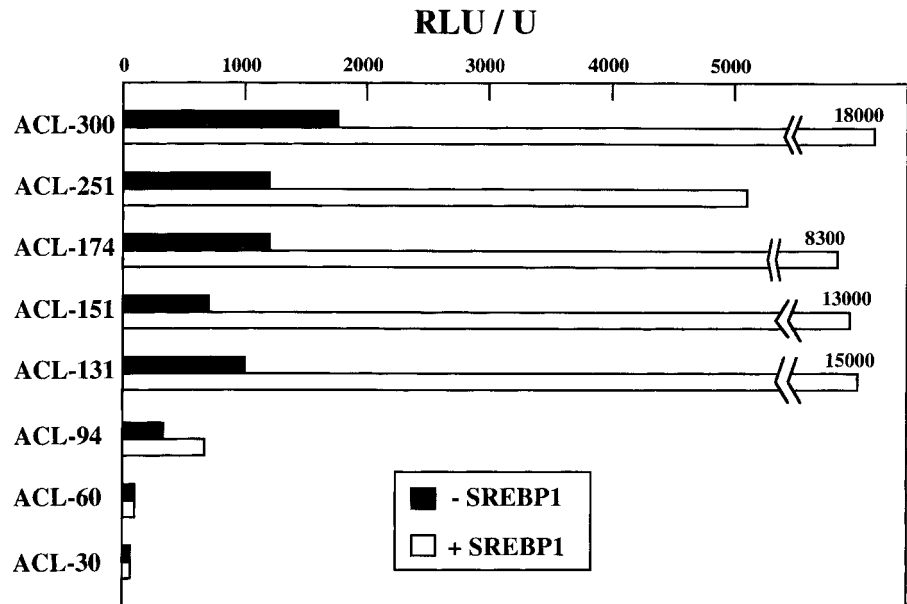


FIG. 7. Differential sensitivity of the ACL, HMG CoA synthase, and LDL receptor promoters to overexpressed SREBP-1a or SREBP-2. HEK 293 cells were transfected with one of reporter genes (ACL-131, pHMG S, and pLDLE; 200 ng), a plasmid encoding β -galactosidase (100 ng), and an indicated amount of expression plasmid, pSREBP1a(1-487) or pSREBP2(1-481), for 4 h. The cells were incubated for 48 h and then lysed, and enzyme activities were determined. The fold activation (luciferase activity in the presence of SREBP-1a or SREBP-2 versus in the absence) is shown. The values given are the averages of data from three experiments performed in triplicate.

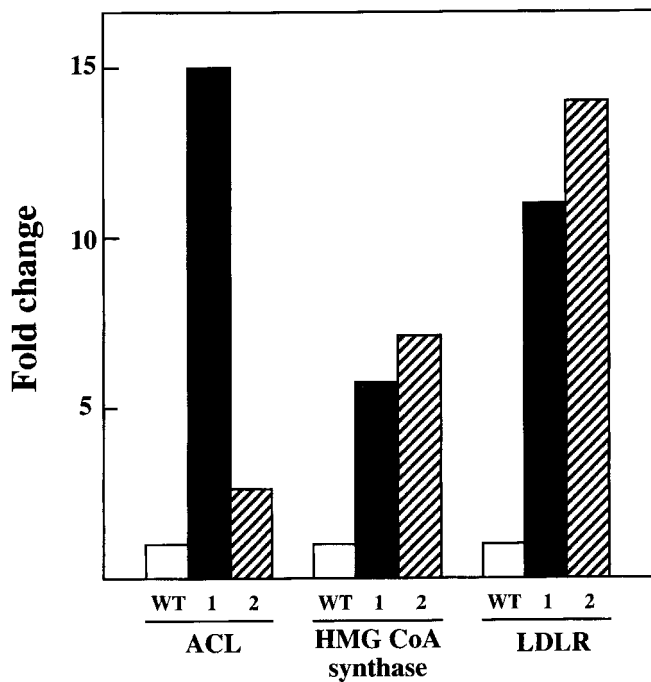
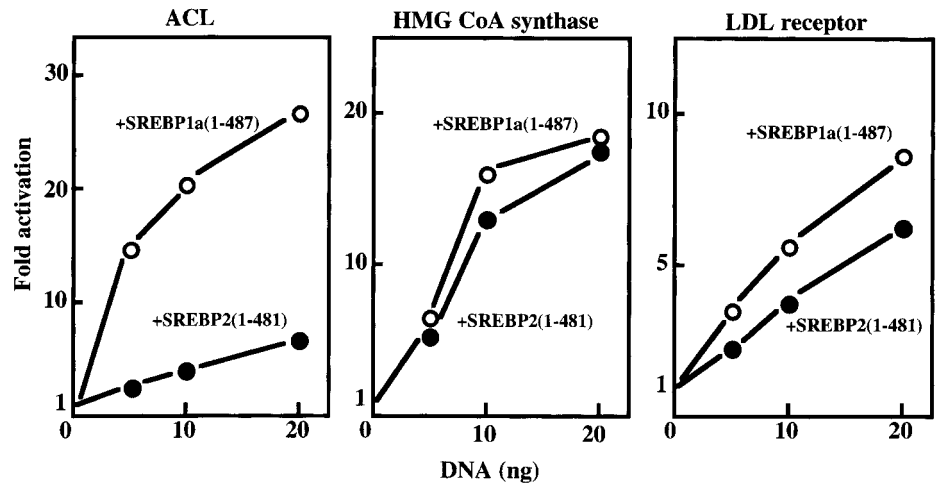


FIG. 8. Quantification of mRNA levels of ACL, HMG CoA synthase, and LDL receptor in the livers from wild-type (WT), TgBP-1a, and TgBP-2 mice as measured by Northern blot analysis. The values are fold changes versus corresponding wild-type signals that are arbitrarily set at 1. Animals of each group ($n = 3$) were fed a high protein/low carbohydrate diet for 2 weeks and fasted overnight prior to sacrifice. An equal aliquot of liver total RNA was pooled (15 μ g) and subjected to Northern blot analysis using a 32 P-labeled cDNA probe for mouse HMG CoA synthase, LDL receptor, and ACL. The resulting bands were quantified by exposure of the filters to BAS2000 with BASstation software, and the results were normalized to the signal generated from 36B4 mRNA (21, 22).

compared with that of the human gene, both the SREb sequence and the CCAAT box with the 13-bp spacing are highly conserved. The current study shows that ACL gene expression is enhanced by SREBP-1 in CHO cells and the livers of transgenic mice as well as human Hep G2 cells. Taken together, it is likely that transcription of ACL gene is regulated by a combination of SREBPs and NF-Y across species. Several genes, including SREBP-2, farnesyl pyrophosphate synthase, squalene synthase, and HMG CoA synthase have been shown to be regulated by the same combination of transcription factors (14, 15, 23). The distances between the two binding sites for SREBP and NF-Y in the promoter of these genes are all in the 15–21-bp spacing range that has been shown to give the highest induc-

tion of transcription by SREBP-2 in *in vitro* reporter assays (14). It may be possible that SREBP-2 requires this 15–21-bp spacing for the regulation of these genes involved in cholesterol synthesis but that SREBP-1 has a shorter necessary or ideal spacing for the induction of genes involved in fatty acid metabolism, including ACL and glycerol-3-phosphate acyltransferase with their 13- and 9-bp spacing, respectively. Interestingly, the SREb sequence (TCAGGCTAG) in the human ACL gene resembles the SRE motif (TCAGCCTAG) observed in the mouse glycerol-3-phosphate acyltransferase gene most closely among known SRE sequences (25).

ACL as well as acetyl CoA carboxylase and fatty acid synthase has been demonstrated to be induced during lipogenesis in the liver and belongs to a class of lipogenic enzymes. Recent studies with SREBP-1 knockout mice demonstrated that SREBP-1 plays a crucial role in nutritional induction of lipogenic enzymes including ACL (21, 22). In the absence of SREBP-1, the ACL mRNA level was profoundly suppressed despite concomitant activation of SREBP-2. This suggests that SREBP-1 is a major physiological regulator for ACL in the liver. It is consistent with our obtained result that SREBP-1 rather than SREBP-2 predominantly stimulates ACL gene expression in the transfection studies. However, residual ACL mRNA level was still found in the livers of SREBP-1 knockout mice that had been refed after fasting. In contrast, in a fasted state with both SREBP-1 and -2 suppressed, ACL mRNA was completely absent irrespective of the presence of SREBP-1, suggesting some significant contribution of SREBP-2 to ACL gene transcription in a nonfasted state. Because ACL is a cytosolic enzyme that generates and provides acetyl CoA for both cholesterol and fatty acid synthesis *de novo*, it might be reasonable that ACL gene transcription can be maintained in a sterol-regulatory fashion under the control of SREBP-2, even though the main regulation of ACL is mediated by SREBP-1 in a lipogenic regulatory manner. When CHO-Lac cells were cultured with sterols, there was a marked decline in HMG CoA synthase mRNA, but a much smaller decline in ACL mRNA (Fig. 2). This finding also supports the concept that basal transcription of the ACL gene is driven primarily by factors other than SREBPs, whereas the HMG CoA synthase gene is more dependent on SREBPs, presumably on SREBP-2 in particular.

It has been reported that insulin activates expression of mRNA for ACL as well as other lipogenic enzymes. Recently, several investigations have demonstrated that SREBP-1c expression is transcriptionally stimulated by insulin (26, 27) and that the insulin-dependent hepatic expression of lipogenesis-related genes including fatty acid synthase, acetyl CoA carboxylase, S14, and L-pyruvate kinase is mediated by SREBP-1c

(28). It is therefore probable that hepatic ACL gene expression is under the control of SREBP-1c, a predominant form in the liver and mediator of insulin action. Intensive analyses of the rat ACL promoter sequence revealed that the region from -104 to -20 of the gene is responsible for regulation because of insulin (29, 30). However, this region does not contain the corresponding SREb site required for sterol-mediated regulation. It therefore remains unclear whether the SREBP action through the SREb motif might be involved in the insulin-dependent stimulation of transcription. In our recent study we found that a putative insulin response element in the human microsomal triglyceride transfer protein gene promoter contains a functional SRE sequence (16). Because both insulin and SREBPs negatively regulate microsomal triglyceride transfer protein gene expression, SREBP-1c, which is believed to respond to insulin, might partly mediate insulin action under physiological conditions. Further work is required to elucidate the mechanism by which ACL gene expression is regulated by the insulin cascade and the SREBPs.

In this study we have demonstrated that ACL is a new target gene for SREBP-1 using CHO-487 cells transiently expressing an active form of SREBP-1a. It has been reported that SREBPs activate the transcription of a number of genes encoding enzymes involved in cholesterol and fatty acid synthesis. It is noteworthy that the SREBPs, especially SREBP-1, can further regulate these two pathways by governing the synthesis of an initial common substrate, acetyl CoA, by a regulation of ACL gene expression.

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