Am J Physiol Endocrinol Metab 282: E222–E230, 2002;
10.1152/ajpendo.00189.2001.

Acetyl-coenzyme A synthetase is a lipogenic enzyme controlled by SREBP-1 and energy status

HIROHITO SONE,[1] HITOSHI SHIMANO,[1] YUKI SAKAKURA,[1] NORIYUKI INOUE,[1]
MICHIYO AMEMIYA-KUDO,[3] NAOYA YAHAGI,[3] MITSUJIRO OSAWA,[2] HIROAKI SUZUKI,[1]
TOMOTAKA YOKOO,[1] AKIMITSU TAKAHASHI,[1] KAORUKO IIDA,[1]
HIDEO TOYOSHIMA,[1] ATSUSHI IWAMA,[2] AND NOBUHIRO YAMADA[1]
[1]*Department of Internal Medicine, Institute of Clinical Medicine, and* [2]*Department of Immunology, Institute of Basic Medicine, University of Tsukuba, Tsukuba 305-8575; and* [3]*Department of Metabolic Disease, School of Medicine, University of Tokyo, Tokyo 113-8655, Japan*

Received 2 May 2001; accepted in final form 11 September 2001

Sone, Hirohito, Hitoshi Shimano, Yuki Sakakura, Noriyuki Inoue, Michiyo Amemiya-Kudo, Naoya Yahagi, Mitsujiro Osawa, Hiroaki Suzuki, Tomotaka Yokoo, Akimitsu Takahashi, Kaoruko Iida, Hideo Toyoshima, Atsushi Iwama, and Nobuhiro Yamada. Acetyl-coenzyme A synthetase is a lipogenic enzyme controlled by SREBP-1 and energy status. *Am J Physiol Endocrinol Metab* 282: E222–E230, 2002; 10.1152/ajpendo.00189.2001.—DNA microarray analysis on upregulated genes in the livers from transgenic mice overexpressing nuclear sterol regulatory element-binding protein (SREBP)-1a, identified an espressed sequence tag (EST) encoding a part of murine cytosolic acetyl-coenzyme A synthetase (ACAS). Northern blot analysis of the livers from transgenic mice demonstrated that this gene was highly induced by SREBP-1a, SREBP-1c, and SREBP-2. DNA sequencing of the 5′ flanking region of the murine ACAS gene identified a sterol regulatory element with an adjacent Sp1 site. This region was shown to be responsible for SREBP binding and activation of the ACAS gene by gel shift and luciferase reporter gene assays. Hepatic and adipose tissue ACAS mRNA levels in normal mice were suppressed at fasting and markedly induced by refeeding, and this dietary regulation was nearly abolished in SREBP-1 knockout mice, suggesting that the nutritional regulation of the ACAS gene is controlled by SREBP-1. The ACAS gene was downregulated in streptozotocin-induced diabetic mice and was restored after insulin replacement, suggesting that diabetic status and insulin also regulate this gene. When acetate was administered, hepatic ACAS mRNA was negatively regulated. These data on dietary regulation and SREBP-1 control of ACAS gene expression demonstrate that ACAS is a novel hepatic lipogenic enzyme, providing further evidence that SREBP-1 and insulin control the supply of acetyl-CoA directly from cellular acetate for lipogenesis. However, its high conservation among different species and the wide range of its tissue distribution suggest that this enzyme might also play an important role in basic cellular energy metabolism.

lipogenic enzyme; acetate; diabetes; insulin; transcription

INTRACELLULAR CHOLESTEROL and fatty acid synthesis are regulated at the transcriptional level, mainly by sterol regulatory element-binding proteins (SREBPs), transcription factors belonging to the basic-helix-loop-helix leucin zipper family (2–4, 28). SREBPs are synthesized in a membrane-bound form. Upon sterol deprivation, nuclear SREBPs are cleaved to enter the nucleus and activate the transcription of genes involved in cholesterol and fatty acid synthesis by binding to sterol regulatory elements (SREs) or to palindromic sequences called E boxes within their promoter regions (3, 5, 26, 28). SREBPs consist of three isoforms (SREBP-1a, SREBP-1c, and SREBP-2), where SREBP-1a and -1c are generated from a single gene through alternative splicing (14, 31).

Cumulative lines of evidence, including normal, transgenic, and knockout mice on diet studies, established that SREBP-1 plays a role in regulating the transcription of genes involved in fatty acid synthesis, whereas SREBP-2 is actively involved in the transcription of cholesterogenic enzymes (13, 24). SREBP-1a is a stronger activator than SREBP-1c because of a longer transactivation domain, and it has a wider range of target genes involved in both cholesterol and fatty acid synthesis (22, 23). Transgenic mice overexpressing nuclear SREBP-1a in the liver demonstrated a marked induction of cholesterogenesis and lipogenesis resulting in engorged fatty livers (22).

Lipogenic enzymes, which are involved in energy storage through synthesis of fatty acids and triglycerides, are coordinately regulated at the transcriptional level during different metabolic states (9, 11). Recent in vivo studies demonstrated that SREBP-1c plays a crucial role in the dietary regulation of most hepatic lipogenic genes. These include studies of the effects of the absence or overexpression of SREBP-1 on hepatic lipogenic gene expression (22–24), as well as physiological changes of SREBP-1c protein in normal mice after dietary manipulations, such as placement on high carbohydrate diets, polyunsaturated fatty acid-enriched diets, and fasting-refeeding regimens (12, 15,

Address for reprint requests and other correspondence: H. Shimano, Dept. of Internal Medicine, Institute of Clinical Medicine, Univ. of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan (E-mail: hshimano@md.tsukuba.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

25, 29, 30). Recent studies suggest that insulin or insulin-facilitated glucose uptake mediates lipogenesis through SREBP-1c induction (7, 10, 18).

Acetyl-CoA synthetase (ACAS) is an intracellular enzyme that catalyzes the formation of acetyl-coenzyme A (acetyl-CoA) from coenzyme A and acetate (17). ACAS is known to be involved in ethanol and acetate metabolism of bacteria, and its molecular characterization has been well described from a microbiological point of view (8, 27). ACAS activity has also been well known among researchers in ruminology to play a crucial role in energy production of ruminants, because volatile fatty acids (also known as short-chain fatty acids), produced through fermentation of cellulose and other fibers in rumen, are their main source of energy. Even in other mammals, including rodents and humans, acetate, a major component of volatile fatty acids, can contribute considerably as an energy source as a result of fermentation of dietary fibers (20).

Beyond this limited information, the molecular characterization of ACAS in mammals has not been well understood. Because ACAS produces acetyl-CoA, which is a key branching molecule for different metabolic pathways, it could play an important role in energy metabolism in mammals. In a search for new targets of SREBP-1, we cloned the murine ACAS cDNA and analyzed its gene promoter. Investigation of the tissue-specific expression profile and nutritional regulation demonstrated a new aspect of this gene as a lipogenic enzyme.

MATERIALS AND METHODS

Materials. Mice (C57BL/6NCrj) were obtained from Charles River Japan (Yokohama, Japan). All animal experiments had been approved by a review of the institutional board of animal welfare. Streptozotocin was purchased from Sigma (St. Louis, MO). TRIzol reagent (GIBCO-BRL, Rockville, MD) was used to isolate total RA. [α-^{32}P]dCTP and Hybond-N+ membrane were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The BcaBEST Labeling Kit (TaKaRa Biomedicals, Kyoto, Japan) was used to label radioactive probes. The BAS 2000 system (Fuji Photo Film, Tokyo, Japan) was used to detect the signals of Northern and dot blot analysis. NIH Image version 1.62 (free software distributed by the National Institutes of Health, Bethesda, MD) was used to quantify intensity of the detected bands.

cDNA cloning of the murine ACAS gene and its promoter region. Poly(A)$^+$ RNA samples were isolated from livers of male SREBP-1a transgenic and male normal control mice (C57BL/6NCrj) of 8 wk of age and applied to Gene Expression Microarray serviced by Incyte Genomics (St. Louis, MO). A 514-bp expressed sequence tag (EST) (AA537637), which encoded a part of the murine ACAS cDNA, was located, and homologs and other searches were performed in the National Center for Biotechnology Information (NCBI) database to collect the related ESTs. 5′-Rapid amplification of cDNA ends (RACE) was performed by using the 5′ RACE system version 2.0 (GIBCO-BRL) to extend a known sequence to get further upstream probes for more efficient screening of libraries. A probe was obtained by PCR with a forward primer, S39, GGACAAGGTGTTCGGAACTTG, and a reverse primer, AS209, ACAGAACGCCGGTGCAGCTC.

A liver cDNA library of SREBP-1a transgenic mice was prepared in the pCMV7 vector. The library was screened for full-length cDNA of ACAS using the probe just described. Obtained clones were sequenced three times by dye-terminator cycle sequencing using the Dye Primer Cycle Sequencing Kit (Perkin-Elmer, Wellesley, MA) and chemiluminescence sequencers (model 377, ABI 100, Perkin-Elmer) and were analyzed by ABI Prism software version 3.0 (Perkin-Elmer).

Luciferase promoter assay of ACAS gene. We obtained a clone containing the 5′ flanking region of the ACAS gene by screening the BAC library of mouse genomic DNA (Incyte Genomics). An *Eco*RI fragment (a 7.8-kb fragment) containing the promoter and 5′UTR of ACAS was subcloned into the pGEM3Zf vector (Promega, Madison, WI), was designated as pGEM3/ACAS/BAC/*Eco*RI 5′ flanking region of the ACAS gene, and was sequenced. Three DNA fragments (sizes from 676, 463, and 331 bp 5′ upstream of the ACAS coding region) of the ACAS gene promoter were obtained by PCR with forward primers pS676, ACTAGCTAGC GGAAGGTTCA TATTGGGGAT CTGTGC; pS436, ACTAGCTAGC GTAACCCAAC CCTTGTCACT CCAAG; and pS331, ACTAGCTAGC GCCTCCTCGC CTGTCACCTC TG, and a 3′ primer, pAS1, TCCGCTCGAG CGCATCAAGT TCCGAACACC TTGTC. They were ligated into *Xho*I-*Nhe*I sites of the pGL3-Basic vector (Promega) and designated as pGL3-ACAS676, ACAS463, and ACAS331, respectively. Transfection and luciferase assays were performed as previously described (1) except that pRL-SV40 (Tokyo Ink, Tokyo, Japan) was used as a reference plasmid instead of pSVβgal. Either an expression plasmid of SREBP-1a, -1c, or -2, under the regulation of the CMV early promoter (pCMV-SREBP-1a, -1c, -2) (0.2 μg) or an empty vehicle vector (pCMV7) (0.2 μg) as a control, was cotransfected with the indicated luciferase construct and pRL-SV40 (0.2 μg) into HepG2 cells by using SuperFect reagent (Qiagen, Hilden, Germany). The cells were incubated in DMEM with 10% FCS and cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) to suppress endogenous SREBP activity. The Dual Luciferase System (Picagene Dual Seapansy, Toyo Ink, Tokyo, Japan) was used to measure firefly and seapansy luciferase activities with a luminometer, Lumat LB9507 (Berthod, Berlin, Germany), according to the manufacturer's instruction.

Gel shift assay. The DNA probe was prepared by annealing both strands of the SRE (see Fig. 3) containing sequence of the mouse ACAS gene promoter, GGGCTACACCCCATCACTCCACGGGCC, and was labeled with [α-^{32}P]dCTP by the Klenow enzyme, followed by purification on G50 Sephadex columns. The labeled DNA was incubated with a recombinant SREBP-1 protein (100 ng) in a mixture containing 10 mM Tris·HCl, pH 7.6, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.5 μg/ml poly(dI-dC), 0.1% Triton X-100, and 1 mg/ml nonfat milk for 30 min on ice. The DNA-protein complexes were resolved on a 4.6% polyacrylamide gel.

ACAS gene expression profile in various tissues. Tissue survey of murine and human ACAS mRNA levels was performed using Poly(A)$^+$ RNA dot blots that were normalized by eight different housekeeping genes (RNA Master Blots and Human Multiple Tissue Expression Array; Clontech, Palo Alto, CA) according to the manufacturer's instructions. Probes used for murine and human blots were produced by PCR with a forward primer S39 and a reverse primer AS209 (for murine blots) and a forward primer, h3S, GACACTCTCGTGTGGGACAC, and a reverse primer, h2AS, CCTTGTTGTCTGTCCTGTGAGC (for human blots), respectively, which were set on the basis of reported ESTs (AW007194 and AW242634).

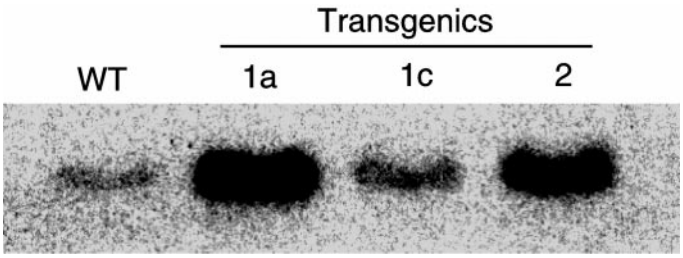


Fig. 1. Expression of the acetyl-CoA synthetase (ACAS) gene in livers from wild-type (WT) and transgenic mice overexpressing sterol regulatory element-binding protein (SREBP)-1a, -1c, and 2. Northern blot analysis of total RNA from livers of SREBP-1a, -1c, and -2 transgenic mice is shown. Mice were fed on a high-protein/low-carbohydrate diet for 2 wk to induce the phospho*enol*pyruvate carboxykinase (PEPCK) promoter used for transgene expression. This diet suppresses the basal level of the ACAS mRNA.

Animals and dietary manipulation. Mice were housed in colony cages with a 12:12-h light-dark cycle and were fed a regular chow diet until the dietary manipulations. C57BL/6 mice 8 wk of age were used for dietary and streptozotocin (STZ) studies. Mice homozygous for the disrupted SREBP-1 gene allele B (SREBP-$1^{-/-}$) were handled as previously described (24). Transgenic mice overexpressing human nuclear SREBP-1a, -1c, and -2 in the liver have been previously described (22). The fasting (24 h) and refeeding (12 h) protocol was as previously described (24). To prepare diabetic mice, STZ (100 mg/kg) or saline was administered by peritoneal injection of 8-wk-old C57BL/6 mice. Some of the diabetic mice received subcutaneous insulin administration (100–400 $U \cdot kg^{-1} \cdot day^{-1}$, Novolet N, Novo Nordisk, Bagsvaerd, Denmark) for 7 days to correct their blood glucose levels. The effect of acetate on the hepatic ACAS gene was estimated by giving water containing indicated concentrations of acetic acid for 24 h.

RESULTS

Cloning of mouse ACAS gene. Results of the microarray analysis identified an EST clone, expression of which was highly (10-fold) increased in the livers of transgenic mice overexpressing nuclear SREBP-1a (22) compared with that of wild-type mice. This clone (AA537637) had a high similarity to bacterial ACAS. The increase of this gene transcript by SREBP-1a was confirmed by Northern blot analysis of total RNA from livers of SREBP-1a transgenic mice (Fig. 1). The hepatic mRNA level of this gene was also elevated by SREBP-1c and SREBP-2, demonstrating that any member of the SREBP family can induce this gene. On the basis of the high similarity to bacterial homologs and the data we will present later, we assumed that this clone was a part of the murine ACAS gene. We cloned a whole ACAS cDNA by screening a mouse liver cDNA library using the 5′ RACE method. The murine cDNA sequence of ACAS open reading frame consisted of 2103 bp coding for 701 amino acids (Fig. 2). The sequence is

```
   1 atggggcttcccgaggagcggcgcaagagcggcagtggaagccgg
     M  G  L  P  E  E  R  R  K  S  G  S  G  S  R
  46 gctcgtgaggagaccggcgccgagggccgggtgcggggttggtcc
     A  R  E  E  T  G  A  E  G  R  V  R  G  W  S
  91 ccgccgccggaggtcagacgctcggcgcacgtcccctctctgcag
     P  P  P  E  V  R  R  S  A  H  V  P  S  L  Q
 136 cgctaccgcgagctgcaccggcgttctgtggaggagccacgggag
     R  Y  R  E  L  H  R  R  S  V  E  E  P  R  E
 181 ttttgggggaaacattgccaaggaattttactggaaaactgcatgc
     F  W  G  N  I  A  K  E  F  Y  W  K  T  A  C
 226 cctggcccattcctccagtacaacttttgatgtgactaaagggaaa
     P  G  P  F  L  Q  Y  N  F  D  V  T  K  G  K
 271 atattcactgagtggatgaaaggagcaactacaaacatctgctac
     I  F  T  E  W  M  K  G  A  T  T  N  I  C  Y
 316 aacgtgctggatcgaaatgtccatgagaaaaaacttggcgacaaa
     N  V  L  D  R  N  V  H  E  K  K  L  G  D  K
 361 gttgctttttactgggagggcaatgagccaggggagaccaccaag
     V  A  F  Y  W  E  G  N  E  P  G  E  T  T  K
 406 atcacatacgtgaactcctggtccaggtgtgtcagttcagcaat
     I  T  Y  R  E  L  L  V  Q  V  C  Q  F  S  N
 451 gttctccgtaaacagggcattcagaagggtgaccgagtggccatc
     V  L  R  K  Q  G  I  Q  K  G  D  R  V  A  I
 496 tacatgcctatgatcttggaacttgtggtggctatgctggcatgt
     Y  M  P  M  I  L  E  L  V  V  A  M  L  A  C
 541 gctcgccttggagctttgcactccattgtgtttgcaggcttctct
     A  R  L  G  A  L  H  S  I  V  F  A  G  F  S
 586 gcagagtctctctgtgaaaggatcttggattccagttgctgcctg
     A  E  S  L  C  E  R  I  L  D  S  S  C  C  L
 631 ctcatcactacagatgccttctacaggggggaaaaacttgtgaac
     L  I  T  T  D  A  F  Y  R  G  E  K  L  V  N
 676 ctgaaggagctggctgatgagtccttggagaagtgccgagagaag
     L  K  E  L  A  D  E  S  L  E  K  C  R  E  K
 721 ggcttcccagtgagatgctgcattgtggtcaaacatctggggcgg
     G  F  P  V  R  C  C  I  V  V  K  H  L  G  R
 766 gcagagctgggcatgaatgactccccccagccagtccccaccagtt
     A  E  L  G  M  N  D  S  P  S  Q  S  P  P  V
 811 aagaggccatgtccagatgtccagatctgctggaacgaaggggtt
     K  R  P  C  P  D  V  Q  I  C  W  N  E  G  V
 856 gacttatggtggcatgaactcatgcagcaggcaggagacgagtgt
     D  L  W  W  H  E  L  M  Q  Q  A  G  D  E  C
 901 gagcctgagtggtgtgatgctgaggacccactcttcatcttgtac
     E  P  E  W  C  D  A  E  D  P  L  F  I  L  Y
 946 accagtggctccacaggcaaacctaagggtgtggtgcacacaatt
     T  S  G  S  T  G  K  P  K  G  V  V  H  T  I
 991 ggaggctatacgctctatgtggctacaactttcaagtatgtgttt
     G  G  Y  T  L  Y  V  A  T  T  F  K  Y  V  F
1036 gatttccacccggaagatgtgttctggtgcacagcagacatcggc
     D  F  H  P  E  D  V  F  W  C  T  A  D  I  G
1081 tggatcactggtcattcctatgtcacctatgggccactggctaat
     W  I  T  G  H  S  Y  V  T  Y  G  P  L  A  N
1126 ggtgccactagtgttttgtttgaggggatccccacatacccagat
     G  A  T  S  V  L  F  E  G  I  P  T  Y  P  D
1171 gaagggcgcttgtggagcattgtggacaaatacaaggtgaccaag
     E  G  R  L  W  S  I  V  D  K  Y  K  V  T  K
1216 ttctacacggcaccaacagccatccggatgctcatgaagtttgga
     F  Y  T  A  P  T  A  I  R  M  L  M  K  F  G
1261 gatgatcctgtcaccaagcatagccgggcatccctgcaggtgctg
     D  D  P  V  T  K  H  S  R  A  S  L  Q  V  L
1306 ggcacagtaggtgaacccatcaaccctgaagcctggctatggtac
     G  T  V  G  E  P  I  N  P  E  A  W  L  W  Y
1351 caccgggtagtaggttcccagcgctgccccattgtagacaccttc
     H  R  V  V  G  S  Q  R  C  P  I  V  D  T  F
1396 tggcaaacagaaacaggtggccatatgctgacccctctccctggc
     W  Q  T  E  T  G  G  H  M  L  T  P  L  P  G
1441 gccacacccatgaaacctggttctgcttctttcccattcttcggt
     A  T  P  M  K  P  G  S  A  S  F  P  F  F  G
1486 gtagcgcctgcaatcctgaatgagtccggggaggagctggaaggg
     V  A  P  A  I  L  N  E  S  G  E  E  L  E  G
1531 gaagctgaaggttatctggtgttcaagcagccctggccagggatc
     E  A  E  G  Y  L  V  F  K  Q  P  W  P  G  I
1576 atgcgcacagtctatgggaaccacacacggtttgagaccacctac
     M  R  T  V  Y  G  N  H  T  R  F  E  T  T  Y
1621 tttaagaagttccctggctactatgtgaccggagatggctgccga
     F  K  K  F  P  G  Y  Y  V  T  G  D  G  C  R
1666 cgggatcaggatggctattactggatcacgggcaggattgatgac
     R  D  Q  D  G  Y  Y  W  I  T  G  R  I  D  D
1711 atgctcaatgtgtctggacatctcctgagtacagcagaggtggaa
     M  L  N  V  S  G  H  L  L  S  T  A  E  V  E
1756 tcggcacttgtggaacacgaggctgtcgcagaggcagctgtggtg
     S  A  L  V  E  H  E  A  V  A  E  A  A  V  V
1801 ggccaccctcatcctgtgaagggcgaatgcctctactgctttgtt
     G  H  P  H  P  V  K  G  E  C  L  Y  C  F  V
1846 accttgtgtgatggccataccttcagccccacactcacagaggaa
     T  L  C  D  G  H  T  F  S  P  T  L  T  E  E
1891 ctcaagaagcagattagagaaaagattggccccattgccacacca
     L  K  K  Q  I  R  E  K  I  G  P  I  A  T  P
1936 gactacatccagaatgcacctggcttgcctaaaacacgctcaggg
     D  Y  I  Q  N  A  P  G  L  P  K  T  R  S  G
1981 aaaatcatgaggcgagttctccggaagattgctcagaatgaccat
     K  I  M  R  R  V  L  R  K  I  A  Q  N  D  H
2026 gacctgggggatacatctacggtggctgacccatctgtcatcaac
     D  L  G  D  T  S  T  V  A  D  P  S  V  I  N
2071 catctcttcagtcaccgctgcctgaccacccagtga 2106
     H  L  F  S  H  R  C  L  T  T  Q  *
```

Fig. 2. cDNA and amino acid sequences of the mouse ACAS gene. This cDNA sequence was essentially identical to a clone that was registered by P. S. Haghighi and co-workers in the NCBI GenBank as a murine ACAS cDNA (AF216873) with four mismatched amino acids (amino acids 498–500; LQS instead of PAI; amino acid 655; L instead of P) and another reported sequence (19).

Fig. 3. DNA sequence of 5′ flanking region of the mouse ACAS gene. The promoter and a part of the 5′UTR sequence of the mouse ACAS gene is shown and numbered in relation to the putative translation initiation site. A sequence (ATCACTCCAC) highly similar to the sterol regulatory element (ATCACCCCAC) and inverted Sp1 site (CCCCGCCCC), two Nkx6.1 sites, and two C/EBP sites were found and underlined.

highly preserved among many species; i.e., 92% homologous with human, 74% with Drosophila, 63% with *Caenorhabditis elegans*, 58% with *Saccharomyces cerevisiae* (yeast), and 64% with *Escherichia coli* at the amino acid level. This cDNA was essentially identical to a clone that was registered by P. S. Haghighi and co-workers in the NCBI GenBank as a murine ACAS cDNA (AF216873). Very recently, Luong et al. (19) reported the amino acid sequences of human and murine ACAS and their regulation by SREBP and, furthermore, demonstrated that they have an ACAS activity when overexpressed in the cultured cells.

Promoter analysis of the ACAS gene. We obtained a clone containing the mouse ACAS gene from a mouse genomic DNA BAC library. DNA sequencing of the 5′ flanking region of the mouse ACAS gene is shown in Fig. 3. We identified a sequence (ATCACTCCAC at −350 bp) that is highly similar to a classic SRE (ATCACCCCAC). The only mismatched base (6th T instead of C) was at the position of a residue that separates two direct repeats of PyCAC in the consensus and is not conserved among SREs in different SREBP target genes. Downstream of this SRE, a binding site of Sp1 (CCCCGCCCC), an essential cofactor required for activation by SREBPs, was also found in an inverted orientation, which made this region a highly probable binding site for SREBPs. Upstream of the SRE, the computer-assisted search found two Nkx6.1 sites and two C/EBPα sites, suggesting that this gene could be expressed and participate in energy metabolism in pancreatic β-cells.

For the promoter analysis, the luciferase reporter gene containing this region (668 bp) was constructed and tested for SREBP activation in transfection studies in HepG2 cells, a hepatic cell line of human origin (Fig. 4). The marked increase in luciferase activity of the ACAS promoter was observed by expression of nuclear SREBP-1a by cotransfection. Deletion of the promoter sequence upstream of the SRE did not essentially change the luciferase activity, whereas further deletion of the SRE completely abolished the SREBP activation. The data demonstrate that the SRE is absolutely required for SREBP activation of this gene. Activation of the ACAS promoter was compared among isoforms of the SREBP family. Expression of nuclear SREBP-1a, -1c, and -2 resulted in 89-, 57-, and 45-fold relative increases in luciferase activity, respectively. The data demonstrate that every member of the SREBP family can activate this ACAS promoter, although SREBP-1a has the highest transcriptional activity. Similar activation by nuclear SREBP-1a, -1c, and -2 was also observed in transfection studies with human embryonic kidney 293 cells (27-, 13-, and 6.6-fold, respectively). Replacement of the tyrosine residue for arginine in the basic region of SREBP (YR mutation) has been shown to result in loss of its binding activity to an SRE (16). Transfection of the YR mutant SREBP-1a and -1c did not cause any significant acti-

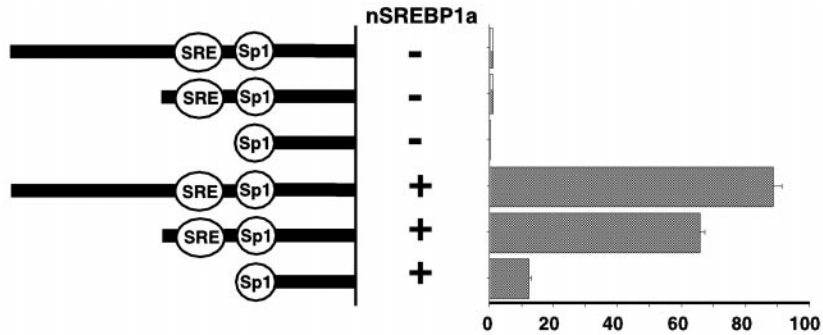


Fig. 4. Deletion studies with the SRE-containing promoter region of the ACAS gene. Luciferase reporter genes containing the mouse ACAS promoter region (668 bp) and its sequential deleted fragments in the context of presence or absence of the SRE were constructed and transfected with a CMV promoter expression vector of nuclear SREBP-1a or an empty control vector, and pRL-SV40 as a reference plasmid into HepG2 cells. Luciferase activity was measured and normalized to the sea urchin activity. Values are represented as degree of change compared with the empty control vector.

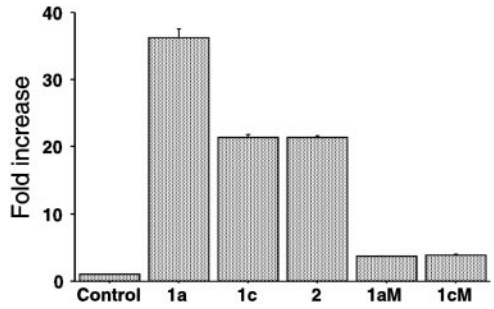


Fig. 5. Activation of the ACAS promoter luciferase gene by SREBPs. A firefly luciferase reporter gene containing the mouse ACAS promoter region (668 bp) was constructed and transfected with a CMV promoter expression vector of nuclear SREBP-1a, -1c, 2 or an empty control vector, and a reference plasmid into HepG2 cells. A mutant version of SREBP-1a (1aM) and -1c (1cM) expression plasmids,in which the tyrosine residue in the basic region of SREBP was replaced by arginine, was also tested. Firefly luciferase activity was measured and normalized to the seapansy luciferase activity. Values are represented as degree of change compared with the value from the empty control vector.

vation (Fig. 5), suggesting that SREBP activation of the ACAS promoter is mediated through its binding to the SRE. To confirm the direct binding of SREBP to the SRE in the ACAS promoter, gel mobility shift assays were conducted (Fig. 6). The labeled SRE probe was shifted by incubation with SREBP-1 protein, and the specific binding was confirmed by a supershift after addition of SREBP-1 antibody. These promoter studies confirmed that the SRE in the ACAS gene promoter is responsible for SREBP activation of ACAS gene expression.

ACAS gene expression profile in various tissues. The tissue survey of human and mouse ACAS gene expression is shown in Fig. 7, *A* and *B*, respectively. In both species, ACAS was ubiquitously expressed in almost every tissue tested. Particularly in mice, it was highly expressed in kidney, liver, submaxillary gland, epididymus, and testis.

Effects of fasting and refeeding on ACAS expression in mice. Recently, SREBP-1 has been reported to play a crucial role in hepatic expression of lipogenic enzyme genes, especially in nutritional regulation, as was observed in fasted and refed mice (24). As shown by Northern blot analysis in Fig. 8, ACAS expression was significantly downregulated in a fasted state and markedly upregulated by refeeding in both liver and adipose tissue. This nutritional change is similar to changes of other lipogenic enzymes that are controlled by SREBP-1 (24). This refeeding induction of the ACAS gene was nearly abolished in the livers and adipose tissue of SREBP-1 knockout (KO) mice (Fig. 8), indicating that lipogenic induction of the ACAS gene by refeeding is controlled mainly by SREBP-1. At the same time, a slight but significant increase in ACAS RNA was seen in the SREBP-1 KO mice as well as wild-type mice, suggesting that other factors contribute to expression to a lesser degree.

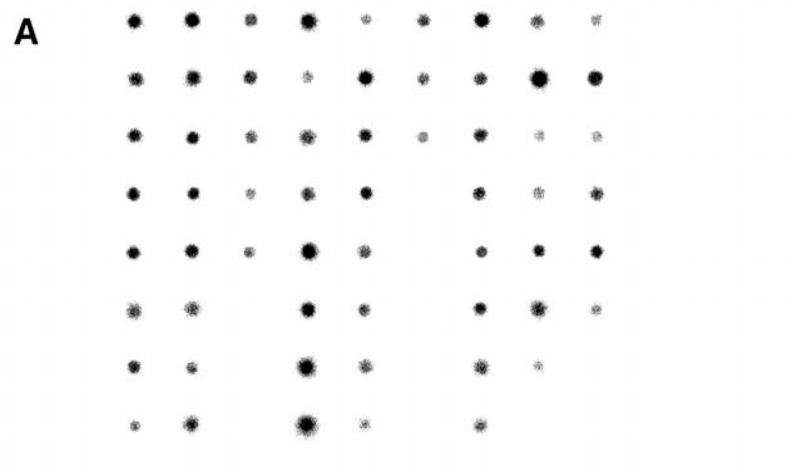
Effect of insulin-depleted diabetes on ACAS expression. Because insulin has also been known to be important for lipogenic enzyme expression, we estimated the effects of insulin depletion and its supplementation on the hepatic mRNA level of ACAS. As shown in Fig. 9, STZ-induced diabetic mice showed markedly decreased ACAS expression in the livers compared with normal control mice, expression that was totally restored by insulin administration.

We also investigated the consequence of ACAS expression after overloading the substrate of the enzyme in drinking water. Acetic acid loading resulted in a significant decrease of ACAS expression in both fasted and fed mice (Fig. 10). A dose-dependent suppression was observed for acetate concentrations between 0 and 10% in fasted animals. In fed animals, acetate overloading also suppressed ACAS expression. There was no significant difference in either food or water consumption that might have affected SREBP-1c expression.

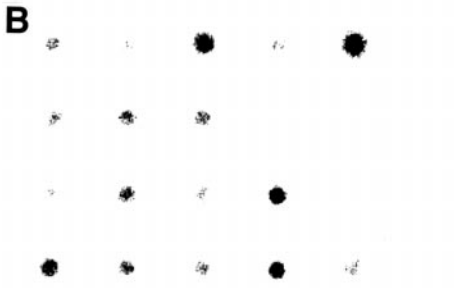
DISCUSSION

ACAS is a new member of the family of lipogenic enzymes. The current study clearly demonstrates that expression of the mouse acetyl-CoA synthetase gene is

Fig. 6. Gel mobility shift assay demonstrating a direct binding of SREBP to the SRE in the mouse ACAS gene promoter. A double-stranded DNA fragment containing the SRE in the mouse ACAS gene promoter was labeled with [α-^{32}P]dCTP and incubated in the reaction mixture with (*lanes 2–4*) or without (*lane 1*) recombinant nuclear SREBP-1c protein. The shifted band of the DNA probe and protein complex is indicated by the arrow (*lane 2*). In a competition assay, a 1,000-fold molar excess of an unlabeled SRE probe was added (*lane 3*). Specificity of SREBP-1 binding to the SRE probe was confirmed by a supershift after the addition of anti-human SREBP-1 monoclonal antibody (2A4, ATCC, 25 ng/ml; *lane 4*).



	1	2	3	4	5	6	7	8	9
A	whole brain	cerebellum, left	substantia nigara	heart	esophagus	colon, transverse	kidney	lung	liver
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorata	stomach	colon, descending	skeltal muscle	placenta	pancreas
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	recutum	spleen	bladder	adrenal
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid
E	occipital lobe	caudate nucleus	spinal cord	ventricular, left	ileum		peripheral blood leukocyte	prostate	salivary gland
F	temporal lobe	hippo-campus		ventricular, right	ilocacum		lymph node	testis	mammary gland
G	p.g. of cerebral cortex	meddula oblongata		inter-ventricular septum	appendix		bone mallow	ovary	
H	pons	putamen		apex of heart	colon, ascending		trachea		



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeltal muscle	smooth muscle cell		
C	pancreas	thyroid	thymus	submax. gland	spleen
D	testis	ovary	prostate	epididymus	uterus

Fig. 7. Tissue distribution of human and mouse ACAS gene expression. Human (*A*) and mouse (*B*) poly(A)$^+$ RNA dot blots (RNA Master Blots and Human Multiple Tissue Expression Array, Clontech) were hybridized with a radiolabeled mouse ACAS cDNA probe as described in MATERIALS AND METHODS.

nutritionally regulated in the same fashion as all other known lipogenic enzymes. Hepatic/adipose ACAS expression was suppressed by fasting and highly induced by refeeding, which is a typical feeding response of lipogenic genes. It was also suppressed in a state of insulin depletion by administration with STZ and was restored by insulin supplement, also a well known response of lipogenic enzymes in a diabetic state. Therefore, nutritional regulation of ACAS followed a lipogenic pattern. This is not surprising, as ACAS is one of the enzymes responsible for the production of acetyl-CoA, an initial substrate for lipogenesis. In a nutritional state favorable for lipogenesis, acetyl-CoA is produced from glycolysis and transported from mi-

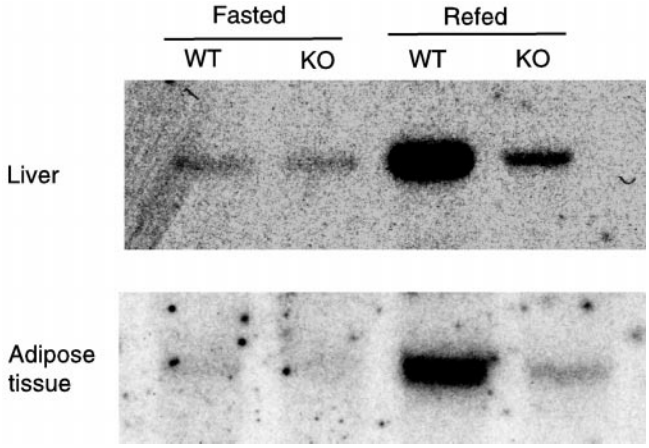


Fig. 8. Nutritional regulation of ACAS gene expression in livers and adipose tissue from WT and SREBP-1-deficient mice after fasting and refeeding treatment. Five male WT and SREBP-1-deficient knockout (KO) mice were fasted (24 h) and refed (12 h) with a high-carbohydrate diet. Total RNA was extracted from livers and adipose tissue of each group and subjected to Northern blot analysis using a radio-labeled mouse ACAS cDNA probe.

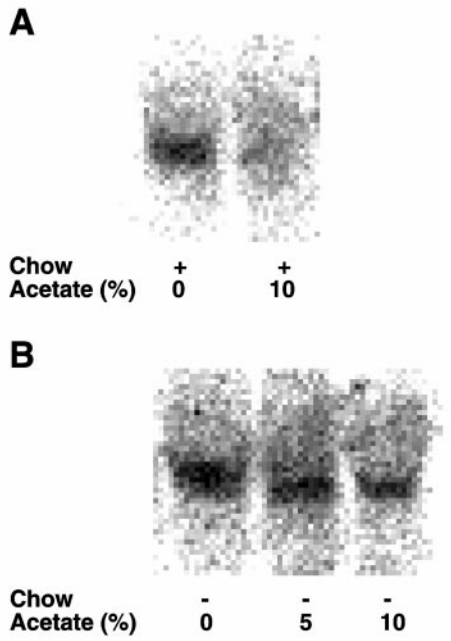


Fig. 10. Acetic acid loading resulted in a significant decrease of ACAS expression in both fasted and fed mice. Mice (C57BL6) were fed ad libitum (*A*) or fasted (*B*) and were given water containing the indicated concentration of acetic acid for 24 h. Total RNA was extracted from livers of each group and subjected to Northern blot analysis by use of a radiolabeled mouse ACAS cDNA probe. (Note that "fed" and "fasted" experiments were done separately so that basal signal levels of these separate studies cannot be compared directly.)

tochondria to the cytosol through a sequence of steps. However, lipogenic induction of cytosolic ACAS suggests that direct production of acetyl-CoA from free acetate in the cytosol might play a role in lipogenesis. The relative contribution of this pathway to lipogenesis remains unknown, and it awaits gene KO mice of this gene to estimate this. This enzyme could be more important in ruminants in which glycolytic activity is low and acetate is a main source of energy.

The ACAS gene is a target of SREBPs. The expression of the EST clone from the ACAS gene was upregulated by SREBP-1a, which led us to clone this gene. Recently, we reported that SREBP-1c is a dominant factor for the expression of most lipogenic genes in the liver (24). Absence of hepatic/adipocytic induction of the ACAS gene in refed SREBP-1 KO mice in the current study supports the notion that ACAS is another target of SREBP-1 as a lipogenic enzyme. Upregulation of the ACAS gene by SREBPs has already been shown in the first report of this gene (19). The SRE sequence was found in the promoter region of the ACAS gene and was confirmed to be responsible for SREBP activation by promoter analysis. Luciferase assays showed that the ACAS promoter was activated by SREBP-1a, -1c, or -2, consistent with the observation that the ACAS mRNA was increased in livers from transgenic mice overexpressing any of the SREBPs. The relative activity of each SREBP isoform for the ACAS SRE as estimated by Northern blot analysis of transgenic livers (Fig. 1) was similar to that of classic SRE: SREBP-1a > SREBP-2 > SREBP-1c (21). This is presumably due to a high similarity between the SRE in the ACAS promoter and the classic SRE originally found in the low-density lipoprotein receptor promoter.

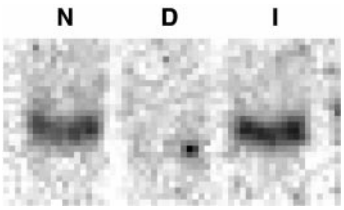


Fig. 9. Insulin dependency of ACAS gene expression in livers from treated diabetic mice. Streptozotocin (STZ)-treated diabetic mice (C57BL6) (D) and insulin-supplemented mice (I) were prepared as described in MATERIALS AND METHODS. N, normal control mice. Total RNA was extracted from livers of each group and subjected to Northern blot analysis using a radio-labeled mouse ACAS cDNA probe.

The current studies with STZ-induced diabetic mice demonstrated that insulin regulates ACAS gene expression. This is consistent with the previous report on changes in hepatic ACAS enzyme activity in STZ-induced rats (21). Because insulin is important for SREBP-1c expression, insulin-dependent ACAS expression can be explained at least partially by its activation of SREBP-1c.

Physiological roles of ACAS gene in mammals. The decreased ACAS expression in the mouse liver by oral administration of acetate is implicative. The suppression of the enzyme expression by excess substrate is a good contrast to the regular mechanism of lipogenic enzyme regulation, in which conversion of excess energy to lipids is free from a negative feedback control. There may be a regulatory system for cytosolic production of acetyl-CoA by excess exogenous acetate. In addition, this gene is highly expressed in many other

tissues, as well as in lipogenic organs. We also observed a considerable expression of this gene in cultured cells such as 293 cells (data not shown). The ACAS gene expression in the cultured cells is reported to be partially under sterol regulation, as predicted from the control by SREBPs (19). These observations suggest that ACAS might have some physiological roles other than in lipogenesis. From this standpoint, it is important to identify and clone a mitochondrial ACAS. This enzyme produces acetyl-CoA in mitochondria and would be involved in ketogenesis or ATP production in the tricarboxylic acid cycle and should be regulated in a different way from the cytosolic enzyme.

Crabtree et al. (6) have proposed futile cycling of acetate between free acetate and acetyl-CoA though cytoplasmic and mitochondrial pathways. One hypothesis of why such a pathway exists is to provide a means by which free acetate levels can be controlled (i.e., buffered). This hypothesis is attractive when one considers that ACAS is expressed in all tissues studied. There could be other functions for ACAS as well. Further studies are needed to clarify the physiological roles and regulation of both enzymes in cellular energy metabolism.

In the current studies, we cloned and identified the murine ACAS gene as a target of SREBPs and a new member of the lipogenic enzyme family. Acetyl-CoA plays a pivotal role in cellular fuel metabolism. Further studies on ACAS might open up a new aspect of glucose and fatty acid metabolism and have therapeutic implications, because acetate is known to be a better fuel source than glucose, especially for individuals with impaired glucose tolerance and diabetes.

We thank Hiroshi Kajikawa, PhD, Chief Researcher, Digestive Microbiology Laboratory of National Institute of Animal Industry (Tsukuba, Japan); Kenji Tayama, Ph.D., Manager, Central Research Institute, Mitsukan Group (Handa, Japan); and Hiromitsu Nakauchi, MD, PhD, Professor of Immunology, Institute of Basic Medicine, University of Tsukuba (Tsukuba, Japan) for fruitful discussion. We are also grateful to Alyssa H Hasty, Ph.D., Vanderbilt University Medical Center (Nashville, TN), for careful reading of the manuscript.

This work is supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR), Health Sciences Research Grants (Research on Human Genome and Gene Therapy) from Ministry of Health and Welfare, and a research project grant of the University of Tsukuba. H. Sone is the recipient of a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (no. 12770632).

REFERENCES

1. **Amemiya-Kudo M, Shimano H, Yoshikawa T, Yahagi N, Hasty AH, Okazaki H, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Osuga Ji Harada K, Gotoda T, Sato R, Kimura S, Ishibashi S, and Yamada N.** Promoter analysis of the mouse sterol regulatory element-binding protein (SREBP)-1c gene. *J Biol Chem* 275: 31078–31085, 2000.
2. **Briggs MR, Yokoyama C, Wang X, Brown MS, and Goldstein JL.** Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence. *J Biol Chem* 268: 14490–14496, 1993.
3. **Brown MS and Goldstein JL.** The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89: 331–340, 1997.
4. **Brown MS and Goldstein JL.** A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci USA* 96: 11041–11048, 1999.
5. **Brown MS, Ye J, Rawson RB, and Goldstein JL.** Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 100: 391–398, 2000.
6. **Crabtree B, Gordon MJ, and Christie SL.** Measurement of the rates of acetyl-CoA hydrolysis and synthesis from acetate in rat hepatocytes and the role of these fluxes in substrate cycling. *Biochem J* 270: 219–225, 1990.
7. **Foretz M, Pacot C, Dugail I, Lemarchand P, Guichard C, Le Liepvre X, Berthelier Lubrano C, Spiegelman B, Kim JB, Ferre P, and Foufelle F.** ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol Cell Biol* 19: 3760–3768, 1999.
8. **Garre V, Murillo FJ, and Torres-Martinez S.** Isolation of the facA (acetyl-CoA synthetase) gene of Phycomyces blakesleeanus. *Mol Gen Genet* 244: 278–286, 1994.
9. **Goodridge AG.** Dietary regulation of gene expression: enzymes involved in carbohydrate and lipid metabolism. *Annu Rev Nutr* 7: 157–185, 1987.
10. **Hasty AH, Shimano H, Yahagi N, Amemiya-Kudo M, Perrey S, Yoshikawa T, Osuga Ji Okazaki H, Tamura Y, Iizuka Y, Shionoiri F, Ohashi K, Harada K, Gotoda T, Nagai R, Ishibashi S, and Yamada N.** Sterol regulatory element-binding-1 protein is regulated by glucose at the transcriptional level. *J Biol Chem* 276: 37402–37408, 2000.
11. **Hillgartner FB, Salati LM, and Goodridge AG.** Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. *Physiol Rev* 75: 47–76, 1995.
12. **Horton JD, Bashmakov Y, Shimomura I, and Shimano H.** Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc Natl Acad Sci USA* 95: 5987–5992, 1998.
13. **Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, and Shimano H.** Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest* 101: 2331–2339, 1998.
14. **Hua X, Yokoyama C, Wu J, Briggs MR, Brown MS, Goldstein JL, and Wang X.** SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc Natl Acad Sci USA* 90: 11603–11607, 1993.
15. **Kim HJ, Takahashi M, and Ezaki O.** Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J Biol Chem* 274: 25892–25898, 1999.
16. **Kim JB, Spotts GD, Halvorsen YD, Shih HM, Ellenberger T, Towle HC, and Spiegelman BM.** Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. *Mol Cell Biol* 15: 2582–2588, 1995.
17. **Knowles SE, Jarrett IG, Filsell OH, and Ballard FJ.** Production and utilization of acetate in mammals. *Biochem J* 142: 401–411, 1974.
18. **Koo SH and Towle HC.** Glucose regulation of mouse S14 gene expression in hepatocytes. Involvement of a novel transcription factor complex. *J Biol Chem* 275: 5200–5207, 2000.
19. **Luong A, Hannah V, Brown M, and Goldstein J.** Molecular characterization of human acetyl-CoA synthetase, an enzyme regulated by sterol regulatory element-binding proteins. *J Biol Chem* 275: 458–466, 2000.
20. **McNeil NI.** The contribution of the large intestine to energy supplies in man. *Am J Clin Nutr* 39: 338–342, 1984.
21. **Murthy VK and Steiner G.** Hepatic acetate levels in relation to altered lipid metabolism. *Metabolism* 22: 81–84, 1973.
22. **Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, and Goldstein JL.** Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* 98: 1575–1584, 1996.

23. **Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, and Goldstein JL.** Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J Clin Invest* 99: 846–854, 1997.
24. **Shimano H, Yahagi N, Amemiya Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S, and Yamada N.** Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem* 274: 35832–35839, 1999.
25. **Thewke DP, Panini SR, and Sinensky M.** Oleate potentiates oxysterol inhibition of transcription from sterol regulatory element-1-regulated promoters and maturation of sterol regulatory element-binding proteins. *J Biol Chem* 273: 21402–21407, 1998.
26. **Tontonoz P, Kim JB, Graves RA, and Spiegelman BM.** ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol Cell Biol* 13: 4753–4759, 1993.
27. **Van den Berg MA, de Jong Gubbels P, Kortland CJ, van Dijken JP, Pronk JT, and Steensma HY.** The two acetyl-coenzyme A synthetases of Saccharomyces cerevisiae differ with respect to kinetic properties and transcriptional regulation. *J Biol Chem* 271: 28953–28959, 1996.
28. **Wang X, Briggs MR, Hua X, Yokoyama C, Goldstein JL, and Brown MS.** Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. *J Biol Chem* 268: 14497–14504, 1993.
29. **Worgall TS, Sturley SL, Seo T, Osborne TF, and Deckelbaum RJ.** Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem* 273: 25537–25540, 1998.
30. **Yahagi N, Shimano H, Hasty A, Amemiya-Kudo M, Okazaki H, Tamura Y, Iizuka Y, Shionoiri F, Ohashi K, Osuga J, Harada K, Gotoda T, Nagai R, Ishibashi S, and Yamada N.** A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. *J Biol Chem* 274: 35840–35844, 1999.
31. **Yokoyama C, Wang X, Briggs MR, Admon A, Wu J, Hua X, Goldstein JL, and Brown MS.** SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell* 75: 187–197, 1993.