Molecular Characterization of Human Acetyl-CoA Synthetase, an Enzyme Regulated by Sterol Regulatory Element-binding Proteins*

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Through suppressive subtractive hybridization, we identified a new gene whose transcription is induced by sterol regulatory element-binding proteins (SREBPs). The gene encodes acetyl-CoA synthetase (ACS), the cytosolic enzyme that activates acetate so that it can be used for lipid synthesis or for energy generation. ACS genes were isolated previously from yeast, but not from animal cells. Recombinant human ACS was produced by expressing the cloned cDNA transiently in human cells. After purification by nickel chromatography, the 70-kDa amino acid cytosolic enzyme was shown to function as a monomer. The recombinant enzyme produced acetyl-CoA from acetate in a reaction that required ATP. As expected for a gene controlled by SREBPs, ACS mRNA was induced when cultured cells were deprived of sterols and repressed by sterol addition. The pattern of regulation resembled the regulation of enzymes of fatty acid synthesis. ACS mRNA was also elevated in livers of transgenic mice that express dominant-positive versions of all three isoforms of SREBP. We conclude that ACS mRNA, and hence the ability of cells to activate acetate, is regulated by SREBPs in parallel with fatty acid synthesis in animal cells.

Sterol regulatory element-binding proteins (SREBPs)1 are transcription factors that activate more than 20 genes that produce enzymes required for the synthesis of cholesterol and unsaturated fatty acids in animal cells (1–3). The SREBPs differ from other transcription factors because they are synthesized as membrane-bound proteins whose active fragments must be released by proteolysis in order to enter the nucleus and activate transcription. The proteolytic release of the nuclear fragments is controlled by the cholesterol content of the cell; release is rapid when cells are depleted of cholesterol, and it is blocked when cholesterol overaccumulates (1).

In tissue culture cells and in livers of transgenic mice, nuclear SREBPs (nSREBPs) increase the levels of mRNAs encoding multiple enzymes in the cholesterol biosynthetic pathway, including 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase), HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, lanosterol demethylase, and others. In the fatty acid biosynthetic pathway, SREBPs increase the mRNAs encoding acetyl-CoA carboxylase, fatty acid synthetase, and stearoyl-CoA desaturase. As a result of these changes, there is a massive increase in the content of unsaturated fatty acids and cholesterol in livers of transgenic mice that overexpress either of two nuclear isoforms of SREBP (nSREBP-1a and nSREBP-2) (4, 5).

In addition to increasing enzymes that participate directly in lipid biosynthesis, the SREBPs increase the mRNA encoding ATP-citrate lyase, which is the major source of the acetyl-CoA that is the ultimate building block for lipid synthesis (6, 7). The SREBPs also increase the mRNAs for three enzymes that supply the NADPH that is needed for lipogenesis (malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) (8).

In the current studies, we used the technique of suppressive subtractive hybridization to identify additional genes whose mRNAs are regulated by SREBPs. As a source of SREBP-induced mRNAs, we used a genetically engineered cell line, designated N-BP1a cells, that produces graded amounts of nSREBP-1a in response to induction with ponasterone, a type of steroid hormone (9). We performed a subtractive hybridization designed to identify genes expressed at high levels in cells expressing nSREBP-1a but not in cells lacking nSREBPs. Among the differentially expressed genes, we identified a cDNA encoding a protein that resembles the enzyme acetyl-CoA synthetase (ACS), whose cDNA was isolated previously from the yeast Saccharomyces cerevisiae (10, 11). ACS catalyzes the activation of acetate, a reaction that is essential for all of the metabolic pathways that utilize this substance. The reaction requires ATP and proceeds through the formation of an acetyl adenylate (12, 13). ACS activity has been studied biochemically in animal cells (14), and the enzyme has been partially purified from rat liver (15), but the gene has not been cloned, and the molecular structure of the animal cell enzyme is unknown. Here, we provide the first molecular characterization of animal ACS, and we demonstrate that its mRNA is controlled by SREBPs.

EXPERIMENTAL PROCEDURES

Materials—We obtained anti-FLAG M2 monoclonal antibody from Kodak; anti-Xpress epitope monoclonal antibody, muristerone A, and ponasterone A from Invitrogen; [1,2-14C]acetic acid, sodium salt (112

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body epoxide (DLYDDDDK), and amino acids 2-701 of human ACS. Expression is driven by the cytomegalovirus (CMV) promoter/enhancer in the pcDNA 3.1/HisA vector (Invitrogen). pCMV-His-ACS was constructed as follows. PCR primers 5'-ATGGGGCTTCCTGAAG-3' and 3'-GGAATCTAGGTAAAGGTCAGG-9

specific and insert-specific primers. The full-length cDNA was amplified by PCR using vector-specific sequences of the full-length cDNA were obtained by PCR using vector-based primers ACS1 (5'-CATCTGCTACAATGTGCTGGATCG-3') and ACS2 (5'-GTGGAGCCACTGGTATACAAGATG-3') based on the 5' and 3' sequences, respectively, of the hamster ACS fragment. The 5' and 3' sequences, respectively, of the hamster ACS fragment. The 5'-GTGGAGCCACTGGTATACAAGATG-3' and 5'-GGAATCTAGGTAAAGGTCAGG-9

and hybridization of these pools with M19 "driver" cDNA, the two tester cDNA pools were digested with SmaI. The digested N-BP1a "tester" cDNA was divided into two groups; one group was ligated to the supplied adapter vector (Invitrogen), and transformed cells were plated onto LB-agarose lines using the RNA Stat-60 Kit (Tel-Test, Inc.; Friendswood, TX) and total RNA obtained from these lines using the Total RNA Kit (Qiagen, Inc.). Poly(A)+ RNA was isolated from total RNA using oligo(dT)-cellulose columns (Amersham Pharmacia Biotech). Delipidated serum (16) and lipoprotein-deficient serum (17) were prepared as described in the indicated references.

Cell Cultures—N-BP1a cells are a mutant line of CHO-K1 cells that is lacking in nuclear SREBPs, due to a deletion in the site 2 protease gene (18). N-BP1a and N-BP2 cells are lines of transfected M19 cells that express graded amounts of nSREBP-1a and nSREBP-2, respectively, under control of an edysone-inducible nuclear receptor system (9). Monolayers of M19, N-BP1a, and N-BP2 cells were set up on day 0 (7-8 x 10^6 cells/100-mm dish) and cultured in either 8-9% CO_2 (Hepa1c1c7 cells) or 5% CO_2 (5-BP1a cells) based on 5'-initiator methionine, six consecutive histidines, an anti-Xpress antibody (19), and ACS2 described above. A cDNA probe for mouse ACS was prepared by DIG-labelling the PCR product with a terminal deoxynucleotidyl transferase (TDT) and using the Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, DNA (3 μg/dish) was incubated at room temperature with serum-free medium containing twice the volume of Fugene 6 reagent as the amount of DNA. Sixteen hours after the transfection, the cells were scraped from the dish, collected by centrifugation, and washed once with ice-cold phosphate-buffered saline containing 0.5 mM phenylmethylsulfonyl fluoride. All subsequent operations were carried out at 4°C. Cells were lysed by passage through a 22-gauge needle 20 times in buffer A (60 mM potassium phosphate buffer (pH 7.5) containing 4 mM MgCl_2, 0.5 mM dithiothreitol (DTT), 150 mM NaCl, and 0.5 mM EDTA (pH 7.5)) supplemented with 5 mM imidazole (pH 7.5). The lysate was centrifuged at 1000 g for 5 min, after which the supernatant was centrifuged at 10^5 × g for 30 min. The 10^5 × g supernatant was loaded onto a 1-mL Ni²⁺-nitrilotriacetic acid-agarose column (Qiagen, Inc.) equilibrated with 10 μl of buffer A supplemented with 5 mM imidazole (pH 7.5). Chromatography was performed via gravity at a flow rate of ~100 μl/h. The column was washed with 16 μl of buffer A plus 10 mM imidazole (pH 7.5) followed by 16 μl of buffer A plus 20 mM imidazole (pH 7.5). Elution was achieved with a step gradient of imidazole in buffer A (50 mM steps beginning with 50 mM imidazole). The 100 mM imidazole fraction, containing ACS activity, was dialyzed at 4°C for 12 h against buffer B (60 mM potassium phosphate buffer (pH 7.5) containing 4 mM MgCl_2, 1 mM DTT, and 150 mM NaCl). This fraction was then concentrated with a Centriprep 30 filter (Amicon, Inc.), mixed with an equal volume of 100% glycerol, and stored in multiple aliquots at −20°C without loss of activity for at least 2 weeks.

Expression of pCMV-His-ACS in HeLa Cells—To express ACS, we utilized the CLONTECH PCR-Select Differential Subtractive Hybridization and Identification of ACS cDNA—Suppressive subtractive hybridization (19) was performed using the PCR-Select cDNA Subtraction Kit (CLONTECH) according to the manufacturer's protocol. Briefly, cDNA was prepared from 2 μg of poly(A)+ RNA obtained from uninduced M19 cells and also from N-BP1a cells that had been induced for 16 h in medium containing 5% delipidated FCS and 0.5 μM mursitronone and isolated as described under "Materials." Both the uninduced and induced cDNA pools were digested with RsaI. The digested N-BP1a "tester" cDNA was divided into two groups; one group was ligated to the supplied adapter set 1, and the other was ligated to the adapter set 2. After separation melting and hybridization of these pools with M19 "driver" cDNA, the two tester pools were annealed and subjected to adapter-specific polymerase chain reaction (PCR) using "forward"-subtracted cDNA probes with a signal intensity of >5-fold relative to the reverse-subtracted probes were then sequenced in their entirety. A partial 662-bp cDNA of hamster ACS (an internal fragment) was identified. A human HeLa cDNA library prepared in the pAX10 vector (20) was screened for ACS by PCR using primers ACS1 (5'-CATCTGCTACAATGTGCTGGATCG-3') and ACS2 (5'-GTGGAGCCACTGGTATACAAGATG-3') based on the 5' and 3' sequences, respectively, of the hamster ACS fragment. The 5' and 3' sequences of the full-length cDNA were obtained by PCR using vector-specific and insert-specific primers. The full-length cDNA was amplified from the HeLa cDNA library using primers 5'-GAACATTACATGATAGGCGCTCCG-3' and 5'-GGAATCTAGGTAAAGGTCAGG-9

 marketed as "Materials") was subjected to Northern blot analysis with various cDNA probes (9). cDNA probes were radiolabeled with [α-32P]dCTP (3,000 Ci/μmol) using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). The filters were exposed to X-AR5 film (Eastman Kodak Co.) with DuPont NEN Reflection screens for the indicated time at −80°C. The resulting bands were quantified by exposure of the filter to a Bioimage Analyzer with BAS1000 MacBus software (Fuji Medical System, Standish, ME), and the results were normalized to the signal generated from the reference GAPDH or cyclophilin mRNA.

Assay of Acetyl-CoA Synthetase Activity—ACS activity was determined by a modification of a previously described biochemical assay (23). Unless otherwise stated, each reaction mixture contained the following components in a final volume of 250 μl: 60 mM potassium phosphate (pH 7.5), 3 mM ATP, 0.1 mM CoA, 4 mM MgCl_2, 1 mM DTT, 0.24 mM sodium [14C]acetate (209-221 dpm/μmol), and the indicated amount of recombinant human ACS or cytosolic protein. Following incubation for 20 min at 30°C, the reaction was stopped with 50 μl of glacial acetic acid, after which a 10-μl aliquot of the reaction mixture was spotted onto a DEAE-cellulose membrane. The membrane was washed twice with a 7.1 ether/water mixture to remove [14C]acetate, leaving [14C]acetil-CoA bound to the membrane. The amount of [14C]acetil-CoA formed was determined by counting the washed membrane in a scintillation counter. The identity of the product as [14C]acetil-CoA was confirmed by thin layer chromatography.

Assay of Lipid Synthesis—Intact monolayers of Hepa1c1c7 and HTB-38 cells were incubated with sodium [1,2-14C]acetate or sodium

Expression Plusmid—pCMV-His-ACS encodes a 731-aa acid endo protein that contains full-length human ACS. It consists of an initiator methionine, six consecutive histidines, an anti-Xpress anti-
To identify novel target genes that are activated by SREBPs in mammalian cells, we used the technique of suppressive subtractive hybridization. The mRNAs were isolated from N-BP1a cells, which produce graded amounts of each of the SREBPs, we studied N-BP1a cells and N-BP2 cells, which express the nuclear forms of each SREBP under control of a steroid-responsive promoter that is activated by ponasterone. To permit such activation, the cells were co-transfected with a cDNA encoding the nuclear receptor under control of a constitutive promoter. We isolated permanent clones of cells that produce graded amounts of each of the nSREBPs in response to graded concentrations of ponasterone in the culture medium (9).

From the cDNA subtractive screen between uninduced M19 cells and induced N-BP1a cells, we identified 1630 clones. Dot blot analysis confirmed that 173 of these mRNAs were expressed more than 5-fold higher in N-BP1a cells as compared with M19 cells. Of these 173 mRNAs, 50 showed clear cut differential expression when tested by Northern blot analysis of mRNA from uninduced versus ponasterone-induced N-BP1a cells. DNA sequence determination revealed that 23 of these 50 clones corresponded to mRNAs that are already known to be induced by SREBP-1a. These included stearoyl-CoA desaturase (three clones), and lanosterol synthase (three clones). Of the remaining 26 differentially expressed clones, three were derived from a novel mRNA that showed sequence homology to yeast ACS.

To confirm that the putative ACS mRNA is activated by ponasterone, we used SREBP-2 cells, which express the nuclear forms of each SREBP under control of ponasterone. We used ponasterone concentrations that induced expression of nSREBP-2 (Fig. 1A). In the same experiments, we performed Northern blot analysis on total RNA extracted from these cells. The filters were probed with a [32P]-labeled cDNA fragment derived from one of the putative ACS clones that we identified in the differential subtractive hybridization (Fig. 1B). In the absence of nSREBP-1 or nSREBP-2 proteins (lanes 1 and 3), very little of the putative ACS mRNA was detectable (lanes 5 and 7). When nSREBP-1a and nSREBP-2 were induced by ponasterone (lanes 2 and 4), the amount of the putative ACS mRNA increased by 3–6-fold activated by ponasterone.

FIG. 1. Amounts of nuclear SREBP proteins (A) and ACS mRNA (B) in ponasterone-inducible N-BP cell lines. On day 0, N-BP cells were set up at 8 × 10⁵ cells/100-mm dish in medium A supplemented with 5% FCS, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μM sodium olate. On day 2, the N-BP1a and N-BP2 cell lines were switched to medium A supplemented with 5% lipotein-deficient FCS and the indicated amount of ponasterone. After incubation for 16 h at 37 °C, one dish of cells in each group was used to prepare whole cell lysates for immunoblot analysis (A), and five dishes were pooled for preparation of total RNA (B). A, intact cell monolayers were washed twice with ice-cold phosphate-buffered saline containing 1 mM sodium EDTA and 1 mM sodium EGTA, followed by passage through a 25-gauge needle 10 times. Aliquots of the whole cell lysate (100 μg/lane) were subjected to SDS-PAGE and immunoblot analysis, which was carried out with 4 μg/ml mouse antiFLAG M2 antibody. The filter was exposed to film for 1 h. The asterisk denotes an irrelevant cross-reacting protein. B, total RNA (15 μg/lane) was subjected to electrophoresis and blot hybridization with a hamster ACS-specific 32P-labeled probe (2 × 10⁶ cpm/ml), corresponding to amino acids 108–314 of the human sequence (Fig. 3), and a control 32P-labeled probe (2 × 10⁶ cpm/ml) directed against rat GAPDH. The filters in B were exposed to film at ~80 °C for 24 h (ACS) and 3 h (GAPDH), respectively. The amount of radioactivity in each band was quantified as described under “Experimental Procedures.” The fold increase for each mRNA of ponasterone-treated N-BP cell lines, relative to the respective untreated N-BP cell line (arbitrarily set at a value of 1), was calculated after correction for loading differences with GAPDH. These values are shown below each blot.

[2-¹⁴C]Pyruvate as described in the figure legends. Incorporation into lipids was determined by thin layer chromatography as described (17, 24). Saponification, lipid extractions, and quantification were carried out as described (8). The data are expressed as nmol of [¹⁴C]acetate or nmol of [¹⁴C]pyruvate incorporated into cholesterol or fatty acids per 2 h per mg of cell protein.

Immunoblot Analysis—Immunoblot analysis was carried out as described previously (25). For whole cell lysates, protein concentration was measured with a Coomasie Plus Protein Assay Reagent (Pierce). The filters were exposed for the indicated time to X-Omat Blue film (Kodak).

RESULTS

The mRNAs were isolated from N-BP1a cells, which produce graded amounts of SREBP-1a in response to ponasterone (9). These cells were derived from M19 cells, a line of CHO cells that lack nSREBPs as a result of mutations in the gene that encodes one of the proteases necessary to release the nuclear fragments from cell membranes (18). Because of the deficiency of nSREBPs, the M19 cells are unable to synthesize cholesterol and unsaturated fatty acids and thus will not grow in culture unless these substances are added to the culture medium. We transfected the M19 cells with cDNAs encoding the nuclear fragments of each of the SREBPs under control of a steroid-responsive promoter that was designed by No et al. (26). Transcription from this promoter requires the action of a nuclear hormone receptor that is

FIG. 2. Northern blots of ACS mRNA in livers from mice expressing SREBP-1a, -1c, and -2 transgenes (A) and in multiple tissues from wild-type mice (B). A, total liver RNA isolated from two male mice from each indicated group was pooled, and 15-μg aliquots were subjected to electrophoresis and blot hybridization for 2 h at ~65 °C with a hamster ACS-specific 32P-labeled probe (2 × 10⁶ cpm/ml) and a control 32P-labeled probe directed against GAPDH (2 × 10⁶ cpm/ml) as described under “Experimental Procedures.” The filters were exposed to film at ~80 °C for 8 h (ACS) and 3 h (GAPDH). The fold increase for each transgenic mRNA relative to wild-type mRNA (arbitrarily set at a value of 1) was calculated after correction for loading differences with GAPDH. B, a hamster-specific 32P-labeled probe (2 × 10⁶ cpm/ml) was hybridized for 2 h at ~65 °C to poly(A)+ RNA (2 μg/lane) from the indicated mouse tissues using a CLONTECH Multiple Tissue Northern blot. The filter was exposed to film at ~80 °C for 7 h. The same filter was subsequently hybridized with 32P-labeled probes for GAPDH and cyclophilin (2 × 10⁶ cpm/ml for each probe). Both filters were exposed to film for 3 h at ~80 °C.
The SREBP-mediated increase in mRNA for the putative ACS was confirmed in vivo by Northern blot analysis of total RNA obtained from the livers of transgenic mice that constitutively overexpress each of the three nuclear forms of SREBP, i.e. nSREBP-1a, nSREBP-1c, or nSREBP-2 (4, 5, 27). Fig. 2A shows that expression of the ACS homolog mRNA was increased 3.3–7.3-fold in the three SREBP transgenic livers as compared with that in wild-type liver.

To determine the tissue distribution of the putative ACS mRNA, we probed a commercially prepared filter containing poly(A⁺) RNA from various mouse tissues using the same 32P-labeled cDNA fragment that was used in Fig. 1B. We identified a single species of mRNA (~3.0 kilobases in length) that was most abundant in the liver and kidney. Lower levels were found in the heart, brain, and testis. The mRNA was not detectable in spleen, lung, and skeletal muscle.

The hamster cDNA sequence encoding the putative ACS was used to design primers to clone the full-length human homolog from a HeLa cDNA library (Fig. 3). The resulting human cDNA included an in-frame stop codon 15 nucleotides upstream of the first methionine. The open reading frame encodes a protein of 701 amino acids with a calculated molecular weight of 78,588 and a predicted isoelectric point of 6.27. A search of DNA databases revealed genes encoding similar proteins in mouse, yeast, and fruit flies. The sequence of the human protein is 93% identical to that of the mouse, 66% identical to that of Drosophila, and 45% identical to that of S. cerevisiae, the only one of these proteins that has been shown to have ACS activity (11, 28). All of the ACS sequences in Fig. 3 contained a conserved signature sequence (denoted by the overbar over residues 313–322 in the human sequence) that identifies a superfamily of enzymes that form adenylate intermediates (29, 30).

The coding region of the putative human ACS was cloned into a pCMV mammalian expression vector and introduced into HEK-293 cells by transfection. The vector was designed so that the NH₂ terminus of the protein contained six consecutive histidines followed by an Xpress epitope tag. The histidines allowed the protein to be purified by nickel column chromatography, and the Xpress tag permitted detection by immunoblotting. Fig. 4 shows a Coomassie-stained SDS-PAGE gel containing fractions collected from a nickel column loaded with cytosol from the transfected HEK-293 cells. The fractions eluted with 50 and 100 mM imidazole contained ACS activity as...
measured by a radiochemical assay. These fractions also contained an 83-kDa band that corresponded to the expected molecular weight of the ACS fusion protein. This band stained with an antibody against the Xpress tag when the proteins were transferred to a nitrocellulose filter and subjected to immunoblotting (Fig. 4B). The nickel column gave a purification of ~30-fold with a recovery of 14%. We obtained 80 μg of purified human ACS from 19 mg of starting cytosolic protein obtained from 75 dishes of cells. The specific activity of the purified protein was 1.5 × 10³ nmol/min/mg. This purified protein was used in the experiments described in Figs. 5–7.

To determine the molecular mass of the functionally active epitope-tagged ACS, we subjected the affinity-purified enzyme to gel filtration on a Superdex 200 column (Fig. 5). The ACS activity eluted from the Superdex 200 column as a single peak at a position that corresponded to an apparent molecular mass of ~80 kDa, as determined from the behavior of markers of known molecular weight (Fig. 5B). This value was similar to that obtained when the enzyme was denatured with SDS and subjected to PAGE and immunoblot analysis (Fig. 5A). It is also consistent with the molecular mass predicted from the protein sequence (78 kDa) plus the epitope tags (4 kDa). Considered together, these data suggest that the active form of ACS is a monomer of 78 kDa.

To characterize the activity of the purified human ACS, we varied the concentration of each of the three ACS substrates (acetate, CoA, and ATP), while keeping the other substrates at saturating levels (Fig. 6). The average $V_{max}$ for these three experiments was 265 ± 62 pmol/min/tube. The apparent $K_m$ values for acetate, CoA, and ATP were 73, 11, and 245 μM, respectively, as calculated from Lineweaver-Burke plots. These values are similar to the published apparent $K_m$ values for ACS that was partially purified from rat liver (15) except that the apparent affinity for CoA was 4-fold higher for the recombinant enzyme.

To determine whether the enzyme was active on other substrates besides acetate, we performed competition studies with unlabeled carboxylates in the presence of [14C]acetate (Fig. 7). Whereas unlabeled acetate competed efficiently with the [14C]acetate, we observed very little competition with β-hydroxybutyrate and β-ketoglutarate and weak but detectable competition with acetoacetate and propionate. In other experiments, we observed no competition by malate, palmitate, butyrate, or 3-hydroxy-3-methylglutarate (data not shown).

The data of Figs. 1 and 2 demonstrated that forced overexpression of nSREBPsl causes an elevation in ACS mRNA. To determine whether this mRNA varies when endogenous SREBPsl are regulated physiologically, we studied wild-type CHO cells that were deprived of sterols (−Sterols), or treated with a mixture of 25-hydroxysterols plus cholesterol (+Sterols) (Fig. 8). In the absence of sterols, the mature forms of SREBP-1 and SREBP-2 were found in nuclear extracts, as measured by immunoblotting (Fig. 8A). As expected from previous data (1), the addition of sterols caused a marked reduction in the nuclear contents of both SREBPsl. The amount of ACS mRNA was high in the absence of sterols and suppressed when sterols were added (Fig. 8B). The suppression was not as complete as the suppression of the mRNA encoding HMG-CoA synthase, an enzyme of cholesterol biosynthesis whose expression has been shown to depend completely on nSREBPsl (9). These data indicate that higher levels of ACS mRNA are induced by SREBPsl but that transcription can be maintained at a significant basal level even when SREBPsl are absent from the nucleus. This conclusion is supported by measurements of ACS activity in cell-free extracts, which showed a partial reduction (50%) when sterols were added (Fig. 8C).
We next carried out a series of experiments designed to reveal whether ACS expression can be rate-limiting for the incorporation of acetate into cellular lipids. For this purpose, we incubated cells from 95 different cell lines (mouse, rat, and human) with \(^{14}C\)acetate and measured the incorporation into \(^{14}C\)cholesterol and \(^{14}C\)fatty acids. The rates of such incorporation varied over a wide range (up to 80-fold). For further study, we selected two cell lines that represented the extremes of this variation: Hepa1c1c7 cells, which were derived from a mouse hepatoma, and HTB-38 cells, which were derived from a human adenocarcinoma of the colon. As shown in experiment A of Table I, the HTB-38 cells incorporated \(^{14}C\)acetate into \(^{14}C\)fatty acids and \(^{14}C\)cholesterol at rates that were 40- and 75-fold higher, respectively, than the rates in the Hepa1c1c7 cells. This correlated with a 25-fold higher ACS activity in the HTB-38 cells as measured in cell-free extracts. Similar results were obtained in an independent study, shown in experiment B of Table I. Despite the difference in ACS activity of the Hepa1c1c7 and HTB-38 cells, the two cell lines incorporated similar amounts of radioactivity from \(^{14}C\)pyruvate into \(^{14}C\)fatty acids as well as \(^{14}C\)cholesterol. This reflects the well known fact that \(^{14}C\)pyruvate is converted directly to \(^{14}C\)acetyl-CoA in mitochondria through the action of pyruvate dehydrogenase, bypassing any requirement for ACS.

As shown in Fig. 9, the mRNA for ACS was 6-7-fold more abundant in the human HTB-38 cells as compared with the mouse Hepa1c1c7 cells, irrespective of whether a mouse or human cDNA probe was used to perform the Northern blots.

To demonstrate directly that the low rate of \(^{14}C\)acetate incorporation in the Hepa1c1c7 cells is secondary to the low level of ACS expression, we transfected these cells with a cDNA encoding the His/Xpress-ACS fusion protein under control of the strong CMV promoter (Table II). Expression of this cDNA increased ACS activity by 66-fold as measured in cell-free extracts. The rate of \(^{14}C\)acetate incorporation into \(^{14}C\)fatty acids and \(^{14}C\)cholesterol increased 6.6-fold and 6.2-fold, respectively. There was no change in \(^{14}C\)pyruvate incorporation, as expected. Despite the fact that the ACS activity in the transfected Hepa1c1c7 cells was higher than the activity in the untransfected HTB-38 cells (2265 pmol/min/mg (Table II) versus 786 pmol/min/mg (Table I)), the rate of \(^{14}C\)acetate incorporation into \(^{14}C\)lipids remained substantially lower. This was true although the incorporation of radioactivity from \(^{14}C\)pyruvate into lipids was higher in the transfected Hepa1c1c7 cells than in the HTB-38 cells. This difference may be due to the fact that only a small percentage of the cells were transfected. The transfected cells may produce a vast excess of ACS, accounting for the high total ACS activity in the pooled cell extracts. However, a substantial fraction of the \(^{14}C\)acetate incorporation may be taking place in the nontransfected cells. These cells maintain their low rate of incorporation, thereby lowering the average value.

**DISCUSSION**

The experiments reported in this paper add ACS to the growing list of genes that are regulated by SREBPs. A partial...
Aspergillus, and the yeast, *S. cerevisiae*

- Pulse-labeled with 1.5 mm sodium [1,2-14C]acetate (69 dpm/pmol), and the other set was pulse-labeled for 2 h with 1.5 mm sodium [2-14C]pyruvate (4.4 dpm/pmol), after which three dishes in each set were harvested for measurement of 14C-lipid content. A blank value (obtained in two dishes per set and representing the amount of either [14C]acetate or [14C]pyruvate incorporated into [14C]fatty acids and [14C]cholesterol in cells incubated for 2 h at 4°C) was subtracted from each value.

- In the second group of cells, the cytosol (105 × g supernatant fraction) was assayed for ACS activity as described in the legend to Fig. 5 except that 25, 50, and 100 μg of cytosolic protein were used. Each value for ACS activity is the average of the three reactions assaying different amounts of cytosolic proteins. Values in parentheses denote relative difference between Hepalclc7 cells (set at a value of 1) and HTB-38 cells. Exp., experiment.

### Table I
Comparison of [14C]acetate and [14C]pyruvate incorporation into cellular lipids in two cell lines with markedly different ACS activities

| Cell line | Acetyl-CoA synthetase activity | [14C]Acetate incorporation | [14C]Pyruvate incorporation |
|-----------|-------------------------------|---------------------------|----------------------------|
|           | Fatty acids | Cholesterol          | Fatty acids | Cholesterol       |
|           | pmol/min/mg | 14C | pmol/2 h/mg | 14C | pmol/2 h/mg | 14C |
| Exp. A    | Hepalclc7   | 32 (1) | 0.35 (1) | 0.02 (1) | 11 (1) | 0.73 (1) |
|           | HTB-38      | 786 (25) | 13.9 (40) | 1.5 (75) | 8.4 (0.8) | 3.5 (4.8) |
| Exp. B    | Hepalclc7   | 24 (1) | 0.24 (1) | 0.01 (1) | 11 (1) | 0.85 (1) |
|           | HTB-38      | 965 (40) | 23 (96) | 1.6 (160) | 9.3 (0.8) | 2.5 (2.9) |

**FIG. 9.** Northern blots of ACS mRNA in two cell lines with markedly different ACS activities. Total RNA from the indicated cell line (mouse (Mo) and human (Hu)) was prepared from the same experiment shown as experiment A in Table I. Aliquots (15 μg/lane) were subjected to electrophoresis and blot hybridization with a human ACS-specific 32P-labeled probe (2 × 106 cpm/ml), a mouse ACS-specific 32P-labeled probe (2 × 106 cpm/ml), and a control 32P-labeled probe (2 × 106 cpm/ml) directed against rat cyclophilin. The filters were exposed to film at −80°C for 8 h (human ACS), 19 h (mouse ACS), and 0.5 h (rat cyclophilin). The amount of radioactivity in each band was quantified as described under “Experimental Procedures.”

The pattern of regulation of ACS mRNA by SREBPs in tissue culture cells more closely resembles that of fatty acid synthesis than of cholesterol synthesis. Previous studies have shown that cholesterol synthesis absolutely requires SREBPs, and it becomes undetectable when SREBPs disappear from the nucleus (9). Under the same conditions of SREBP deprivation, fatty acid synthesis declines only partially, indicating that fatty acid synthesis can be maintained at an appreciable basal level in the absence of SREBPs (9). Similarly, the mRNA encoding ACS was reduced only partially when SREBPs were reduced by sterol treatment, whereas the mRNA for the cholesterol biosynthetic enzyme HMG-CoA synthase was essentially eliminated (Fig. 8).

Whereas yeast requires ACS for growth, the same is not expected to be true for animal cells. Animal cells derive their essential acetyl-CoA from two mechanisms that do not require ACS: 1) pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA without generating free acetate, and 2) β-oxidation of fatty acids, which also yields acetyl-CoA as a direct end product. Both of these reactions occur predominantly in mitochondria. Under aerobic conditions, most of the acetyl-CoA is...
 oxidized within mitochondria through the citric acid cycle to produce energy. In order to be used for lipid synthesis, the acetyl-CoA must be transported to the cytoplasm. This transport is effected by the condensation of acetyl-CoA with oxaloacetate within mitochondria to form citrate, which is then transported to the cytosol where it is broken down by ATP-citrate lyase to yield acetyl-CoA directly. Importantly, ATP-citrate lyase mRNA has recently been shown to be induced by SREBPs (7), presumably to produce cytosolic acetyl-CoA for lipid synthesis.

Although acetate is not an essential source of acetyl-CoA in animals, there are several conditions in which relatively large amounts of acetate must be activated by ACS in order to be metabolized. 1) Acetate is generated in the colon by bacterial fermentation, and this production is increased by a high fiber diet (33, 34). The acetate is transported via the portal vein to the liver, where it is activated by ACS. 2) Ingested ethanol is oxidized in liver, first to acetaldehyde and then to acetate, which must be converted to acetyl-CoA. 3) An appreciable amount of acetate is generated from acetyl-CoA within cells by acetyl-CoA hydrolase, a ubiquitous cytosolic enzyme (35). ACS can salvage this acetate, reactivating it so that it can reenter metabolism. 4) In the nervous system, the neurotransmitter acetycholine is broken down by acetylcholinesterase, generating acetyl-CoA that must be converted to acetyl-CoA. 5) Acetate is generated within the nucleus of all cells by histone deacetylases (37). This acetate must be reactivated before it can be oxidized or reused.

Previous biochemical studies have revealed that mammalian livers contain two forms of ACS, one intramitochondrial and the other cytosolic (38). The intramitochondrial ACS activity appears to be constitutive, and it does not vary in response to metabolic manipulations. Cytosolic ACS activity in liver varies in parallel with variations in the rates of fatty acid synthesis (39, 40). Thus, cytosolic ACS activity falls when animals are starved, an event that leads to a decline in plasma insulin and a decline in fatty acid synthesis. On the other hand, ACS activity rises with insulin in response to the refeding of a high carbohydrate diet, which increases fatty acid synthesis. The insulin-mediated changes in fatty acid synthesis are elicited by post-transcriptional as well as transcriptional mechanisms (41). Transcriptional regulation is mediated by nuclear SREBP-1c, whose level rises in response to insulin (42). The current findings strongly suggest that the insulin-mediated changes in ACS activity are attributable to the changes in the nuclear content of SREBP-1c.

The ACS gene identified in these studies clearly produces the regulated cytosolic form of the enzyme. The source of the mitochondrial ACS activity is not yet clear. We have been unable to find another closely related gene in the data bases of animal cell protein and DNA sequences. It is possible that mitochondrial ACS is encoded by a gene that is related distantly, if at all, to the gene encoding cytosolic ACS.

Now that the gene encoding cytosolic ACS has been identified, it will be of interest to explore the physiologic role of the enzyme in liver and brain in response to ethanol and other sources of acetate.

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