THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 52, Issue of December 25, pp. 35299–35306, 1998

*Printed in U.S.A.*

# Nuclear Sterol Regulatory Element-binding Proteins Activate Genes Responsible for the Entire Program of Unsaturated Fatty Acid Biosynthesis in Transgenic Mouse Liver*

(Received for publication, August 3, 1998, and in revised form, September 15, 1998)

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**Previous studies have shown that the rate of fatty acid synthesis is elevated by more than 20-fold in livers of transgenic mice that express truncated nuclear forms of sterol regulatory element-binding proteins (SREBPs). This was explained in part by an increase in the levels of mRNA for the two major enzymes of fatty acid synthesis, acetyl-CoA carboxylase and fatty acid synthase, whose transcription is stimulated by SREBPs. Fatty acid synthesis also requires a source of acetyl-CoA and NADPH. In the current studies we show that the levels of mRNA for ATP citrate lyase, the enzyme that produces acetyl-CoA, are also elevated in the transgenic livers. In addition, we found marked elevations in the mRNAs for malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, all of which produce NADPH. Finally, we found that overexpressing two of the SREBPs (1a and 2) led to elevated mRNAs for stearoyl-CoA desaturase 1 (SCD1), an isoform that is detectable in nontransgenic livers, and SCD2, an isoform that is not detected in nontransgenic livers. This stimulation led to an increase in total SCD activity in liver microsomes. Together, all of these changes would be expected to lead to a marked increase in the concentration of monounsaturated fatty acids in the transgenic livers, and this was confirmed chromatographically. We conclude that expression of nuclear SREBPs is capable of activating the entire coordinated program of unsaturated fatty acid biosynthesis in mouse liver.**

Sterol regulatory element-binding proteins (SREBPs)[1] are a family of transcription factors that regulate the low density lipoprotein (LDL) receptor and multiple enzymes required for the biosynthesis of cholesterol and fatty acids (see Ref. 1 for review). SREBPs belong to the basic helix-loop-helix leucine zipper family of transcription factors. Unlike other members of the basic helix-loop-helix leucine-Zip family, SREBPs are synthesized as ~1150 amino acid precursors bound to the endoplasmic reticulum and nuclear envelope. The membrane-bound precursor must undergo a sequential two-step cleavage process to release the transcriptionally active $NH_2$-terminal portion of the protein (2). Once cleaved, the ~500 amino acid $NH_2$-terminal nuclear form enters the nucleus and activates transcription by binding to promoter regions of genes containing non-palindromic sterol regulatory elements as well as palindromic sequences called E-boxes (1, 3, 4). Genes involved in cholesterol metabolism that are directly activated by SREBPs include the LDL receptor, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and SREBP-2 (1, 5–8). Genes involved in fatty acid and triglyceride synthesis, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase (GPAT), are also directly activated by SREBPs (9–11).

To date, three isoforms of SREBP have been identified and characterized (1). The human versions of all three isoforms were originally discovered as transcription factors that bound non-palindromic sterol regulatory elements and regulated cholesterol metabolism. Two of these, designated SREBP-1a, and -1c, are derived from the same gene through use of alternative transcription start sites resulting in proteins that produce different first exons (12). The rat homologue of human SREBP-1c (adipocyte determination differentiation-dependent factor 1) was originally cloned as a transcription factor that bound to E-box sequences and promoted adipocyte differentiation (13). The third isoform, SREBP-2, is derived from a second gene (14). All actively growing cultured cells studied to date produce predominantly the SREBP-1a and SREBP-2 isoforms (15). However, in most organs from adult animals, SREBP-1c and SREBP-2 are predominant (15).

When sterols build up in cells, the proteolytic release of SREBPs from membranes is inhibited and transcription of all target genes declines (1). To gain insight into separate roles of each SREBP isoform *in vivo*, we previously produced and characterized transgenic mice that overexpress truncated, transcriptionally active nuclear forms of human SREBP-1a, -1c, or -2 in liver (16–18). The transgenes encode versions of the proteins that terminate prior to the membrane attachment domain. Consequently, these proteins enter the nucleus directly without a requirement for proteolysis. Mice that overexpressed the truncated SREBP-1a isoform (TgSREBP-1a) had dramatically enlarged livers that were engorged with cholesterol as well as triglycerides. The rates of hepatic cholesterol and fatty acid synthesis were increased by 5- and 25-fold, respectively, as measured by the incorporation of [$^3$H]water. Corresponding 5-fold increases were measured in the liver mRNAs for HMG-CoA synthase, squalene synthase, and the

* This work was supported in part by National Institutes of Health Grant HL-20948, the Moss Heart Foundation, and the Perot Family Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Research Fellowship from the Manpei Suzuki Diabetes Foundation of Tokyo, Japan.

§ Supported by the Medical Scientist Training Grant GM08014.

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[1] The abbreviations used are: SREBP, sterol regulatory element-binding protein; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; Glu-6-PD, glucose-6-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; VLDL, very low density lipoprotein; PGDH, 6-phosphogluconate dehydrogenase; SCD, stearoyl-CoA desaturase.

LDL receptor while 20–25-fold increases were measured in ACC and FAS (16). In contrast, mice that overexpressed the nuclear form of human SREBP-1c (TgSREBP-1c) had no increase in hepatic cholesterol synthesis, but had a 4-fold increase in fatty acid synthesis. In agreement with the synthesis studies, no increases were measured in mRNAs for cholesterol synthetic enzymes, but 2- and 4-fold increases were measured in ACC and FAS, respectively (17).

SREBP-2 transgenic mice (TgSREBP-2) had elevated hepatic cholesterol accumulations like those measured in livers from TgSREBP-1a mice, but significantly less triglyceride accumulation. The livers from TgSREBP-2 mice had 28-fold higher rates of cholesterol synthesis, but only 4-fold higher rates of fatty acid synthesis. The mRNAs for the cholesterol synthetic enzymes, HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase were increased by 10–12-fold. The mRNAs for the fatty acid synthetic enzymes ACC and FAS were increased 7- and 15-fold, respectively. Therefore, the SREBP-1 isoforms seem more selective in activating genes involved in fatty acid synthesis while SREBP-2 is more specific for activating genes involved in cholesterol synthesis (18). The mechanism for the relative specificity of each transcription factor is currently not known.

All three lines of transgenic mice had 3–4-fold increases in the hepatic mRNA for the fatty acid modifying enzyme, stearoyl-CoA desaturase (SCD). SCD is a microsomal enzyme that introduces a cis-double bond in the $\Delta^9$ position of saturated fatty acids, resulting in the production of monounsaturated fatty acids (19). The preferred substrates for SCD are palmitoyl (16:0) and stearoyl (18:0) CoA which are converted to palmitoleoyl (16:1) and oleoyl (18:1) CoA, respectively. Two isoforms of SCD, SCD1 and SCD2, are currently known (20, 21). Most organs produce both SCD1 and SCD2. The notable exception is liver, which expresses only the SCD1 isoform (21). The regulation of SCD is of considerable physiologic importance since this enzyme ultimately determines the ratio of monounsaturated to saturated fatty acids in the cell. Alterations in this ratio can change membrane fluidity. Such alterations have been implicated in various disease processes, including cancer, diabetes, obesity, and vascular disease (19).

Several crucial questions regarding the role of SREBPs in fatty acid metabolism remain unanswered: 1) SREBPs markedly enhance the utilization of acetyl-CoA for lipid synthesis, but do they activate transcription of genes encoding enzymes that synthesize acetyl-CoA? 2) Do SREBPs increase the mRNAs encoding enzymes that supply the other substrate for fatty acid synthesis, *i.e.* NADPH? 3) Which isoform of SCD is elevated by SREBP overexpression? 4) Does the contribution of fatty acid overproduction and SCD enhancement lead to a predominance of unsaturated fatty acids in the transgenic livers? 5) Are there relative differences among the SREBP isoforms in eliciting these effects? The current studies were designed to answer these questions.

## EXPERIMENTAL PROCEDURES

*Materials*—All restriction enzymes were obtained from New England Biolabs (Beverly, MA) and DNA manipulations were performed using standard molecular biology techniques (22). *Redivue* [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) was obtained from Amersham Corp. Oleic acid [9,10-$^3$H]- and [1-$^{14}$C]stearoyl-CoA were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Stearic acid, oleic acid, and silica gel plates were purchased from Alltech (Deerfield, IL), stearoyl-CoA from Fluka (Ronkonkoma, NY), and silver nitrate from Calbiochem (La Jolla, CA). All other chemicals used were from Sigma. The content of cholesterol and triglycerides in plasma and liver was measured as described previously (16).

*Transgenic Mice*—Transgene constructs encoding amino acids 1–460 of human SREBP-1a, 1–436 of human SREBP-1c, and 1–468 of human SREBP-2 under the control of the rat phosphoenolpyruvate carboxykinase promoter were described previously (16–18). Two independent experiments were designed to study wild-type and SREBP transgenic mice. In Experiment A, 6 male TgSREBP-1a (line B) (16) and 6 male nontransgenic wild-type littermate mice were studied. Experiment B included 5 male TgSREBP-1c (17), 5 male TgSREBP-2 (line B) (18) mice, and 5 male wild-type control mice. The TgSREBP-2 mice studied were offspring of mice homozygous for the SREBP-2 transgene. Therefore, all of the wild-type mice in Experiment B were derived from TgSREBP-1c nontransgenic littermates.

All mice were housed in colony cages in a 14-h light/10-h dark cycle and were maintained on Teklad 6% (w/w) Mouse/Rat Diet 7002 from Harlan Teklad Premier Laboratory Diets (Madison, WI). Four weeks prior to sacrifice, transgenic and littermate controls were placed on a low carbohydrate/high protein diet (number 5789C-3) from Purina Mills Inc. (St. Louis, MO) containing 71% (w/w) casein and 4.25% (w/w) sucrose. This diet induces the phosphoenolpyruvate carboxykinase promoter resulting in maximal transgene expression. All mice were sacrificed at 34–36 weeks of age, during the early phase of the light cycle, and in the non-fasted state.

*Blot Hybridization of RNA*—Total RNA was prepared from mouse liver using RNeasy™ RNA kit (Qiagen, Chatsworth, CA). For Northern gel analysis, equal aliquots of total RNA were pooled (total, 15 $\mu$g), denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% formaldehyde agarose gel, and transferred to Hybond N$^+$ membranes (Amersham) for hybridization. cDNA probes for ATP citrate lyase, malic enzyme, glucose-6-phosphate dehydrogenase (Glu-6-PD), 6-phosphogluconate dehydrogenase (PGDH), glycerol-3-phosphate acyltransferase (GPAT), and S14 were prepared by reverse transcriptase-polymerase chain reaction using mouse liver poly(A)$^+$ RNA as a template as described previously (16). The polymerase chain reaction primers used to generate these probes are as follows. ATP citrate lyase: 5′,5′-AAGTAGACACTGGTCTGTAACATGGAACAT-3′ and 3′,5′-TCCAGAACACATGAGCATGTAACTGAGCCA-3′ (23, 24). Malic enzyme: 5′,5′-AATTAAGAATTTCGAACGACTGAACTCTGA-3′ and 3′,5′-TCGTTAAATGTGCAATACTTGTTTCGATAC-3′ (25). Glu-6-PD: 5′,5′-CCAGGTGTGTGGGATCCTGAGGGAAGAGTT-3′ and 3′,5′-GGTCCAAAGATCCTGTTGGCAAACCTCAGC-3′ (26). PGDH: 5′,5′-TGGT(G/C)TCCAAGCTGAAGAAGCC-3′ and 3′,5′-TCTTTGGCAGCAGGTGTTTGCC-3′ (27, GenBank accession number U30255), GPAT: 5′,5′-TCCAGAAACCCAGCAGACGA-3′ and 3′,5′-CAGCACCACAAAACTCAGAA-3′ (11). S14: 5′,5′-ATGCAAGTGCTAACGAAACGC-3′ and 3′,5′-AGAAGTGCAGGTGGAACTGGGC-3′ (28). Northern blot analysis was carried out as described previously (16). The bands detected by Northern analysis were quantified by exposing the filter to a BAS1000 Fuji PhosphorImager (Tokyo, Japan), and the results were normalized to the signal generated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

*RNase Protection Assay*—cRNA probes and protection assay conditions for comparison of transgene expression in livers of SREBP-1a, -1c, and -2 mice were prepared as described previously (18). cDNA fragments for mouse stearoyl-CoA desaturase 1 (SCD1) and stearoyl-CoA desaturase 2 (SCD2) were amplified by polymerase chain reaction from first strand cDNA prepared from mouse epididymal fat pad poly(A)+ RNA using the following primers. SCD1: 5′,5′-CCGGCCCACATGCTCCAAGAGATCTCCAGT-3′ and 3′,5′-GTACTCCAGCTTGGGCGGGGGTCCCTCCTC-3′ (20). SCD2: 5′,5′-CTGCAAGAGATCTCTGGCGCTTACTCAGCC-3′ and 3′,5′-CTCATCATCCTGATAGGTGGGGTCATATAG-3′ (21). The RNase protection assay was carried out as described previously except that the amount of cRNA probes for SCD1 and SCD2 was increased to $4 \times 10^5$ cpm (15). The specific activities of the cRNAs were $0.9–1.4 \times 10^9$ cpm/$\mu$g for SCD1 and SCD2 and $1.1–1.6 \times 10^9$ cpm/$\mu$g for $\beta$-actin.

*Stearoyl-CoA Desaturase Activity*—Stearoyl-CoA desaturase activity was measured in liver microsomes that were prepared by homogenizing ~100 mg of liver in 0.5 ml of a 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 $\times$ *g* at 4 °C for 30 min and the resultant supernatant was then centrifuged at 100,000 $\times$ *g* at 4 °C for 30 min. The microsomal pellet was resuspended in a 0.1 M potassium phosphate buffer (pH 7.2) (29). Protein concentrations were determined by the method of Lowry (30). The reaction mixture for the stearoyl-CoA desaturase activity included the following: 60 $\mu$M [1-$^{14}$C]stearoyl-CoA, 2 mM NADH in 10 mM potassium phosphate (pH 7.2), 0.1 M potassium phosphate (pH 7.2), and 100 $\mu$g of microsomal protein in a final volume of 100 $\mu$l (31, 32). The reaction conditions, saponification, and fatty acid extractions were carried out as described (32). [$^3$H]Oleic acid (~2000 dpm/$\mu$l), oleic acid (25 $\mu$g/$\mu$l), and stearic acid (25 $\mu$g/$\mu$l) were added as recovery markers and the extracts were evaporated to dryness under air. Methyl esters were then prepared as

TABLE I

*Phenotypic comparison of wild-type and transgenic mice*

Each value represents the mean ± S.E. In Experiment A, TgSREBP-1a mice were derived from line B in Fig. 4 of Ref. 16, and nontransgenic littermates of 2 litters were used as controls. In Experiment B, TgSREBP-1c mice were derived from mice described in Ref. 17, and TgSREBP-2 mice were derived from line B in Ref. 18. Nontransgenic littermates from 2 litters of TgSREBP-1c were used as controls. All mice in Experiments A and B were 34–36 weeks of age, and were fed the low carbohydrate/high protein diet for 4 weeks before sacrifice. Plasma lipids were measured from nonfasted samples.

| Parameter | Genotype of mice | | |
|---|---|---|---|
| | 6 males | | |
| | Wild-type | TgSREBP-1a | |
| Experiment A | | | |
| Body weight (g) | 29.7 ± 0.8 | 32.7 ± 1.1 | |
| Liver weight (g) | 1.44 ± 0.06 | 5.89 ± 0.33[a] | |
| Liver cholesterol content (mg/g) | 2.44 ± 0.1 | 12.0 ± 1.2[a] | |
| Liver esterified cholesterol content (mg/g) | 0.30 ± 0.04 | 9.9 ± 0.89[a] | |
| Liver triglyceride content (mg/g) | 6.22 ± 0.8 | 183 ± 11[a] | |
| Total plasma cholesterol (mg/dl) | 95 ± 5.4 | 40 ± 4.6[a] | |
| Total plasma triglyceride (mg/dl) | 205 ± 19 | 77 ± 12[a] | |
| | 5 males | | |
| | Wild-type | TgSREBP-1c | TgSREBP-2 |
| Experiment B | | | |
| Body weight (g) | 30.0 ± 0.8 | 31.0 ± 0.9 | 28.4 ± 0.7 |
| Liver weight (g) | 1.52 ± 0.08 | 1.59 ± 0.08 | 1.66 ± 0.09 |
| Liver cholesterol content (mg/g) | 2.28 ± 0.1 | 2.96 ± 0.1[a] | 5.76 ± 0.3[a] |
| Liver esterified cholesterol content (mg/g) | 0.1 ± 0.01 | 0.55 ± 0.09[b] | 3.1 ± 0.06[a] |
| Liver triglyceride content (mg/g) | 5.17 ± 0.1 | 36.1 ± 6.3[b] | 37.9 ± 6.8[b] |
| Total plasma cholesterol (mg/dl) | 100 ± 5.7 | 123 ± 5.7 | 105 ± 4.3 |
| Total plasma triglycerides (mg/dl) | 158 ± 11 | 108 ± 14 | 74.2 ± 7.7[a] |

[a] $p < 0.001$ between wild-type and transgenic mice (Student's t test).
[b] $p < 0.01$ between wild-type and transgenic mice (Student's t test).

described and evaporated to dryness (32).

The products were resuspended in 30 μl of chloroform and spotted on 5% activated argentation plates to achieve separation by thin layer chromatography (TLC) (33). The TLC plates were run in 100% benzene, allowed to dry and sprayed with 0.05% Rhodamine B in methanol. The resulting bands were visualized under UV light and the appropriate fractions were cut into scintillation vials (34). Radioactivity was determined using a liquid scintillation spectrometer. The enzyme activity is expressed as nanomole·min$^{-1}$·mg protein$^{-1}$.

*Tissue Cholesterol and Fatty Acid Composition*—Total lipids were extracted from 300–350-mg aliquots of liver and esterified cholesterol, triglycerides, and total phospholipids were separated on 500-mg silica columns (Varian catalog number 1211-3036) exactly as described (35). After saponification, the fatty acids in each of these samples were methyl esterified and the relative abundance of each fatty acid was quantified by gas-liquid chromatography (36, 37). Liver unesterified and esterified cholesterol also were quantified by GLC (35, 38).

## RESULTS

Table I shows the phenotypic characteristics of the mice used in the current studies. Experiment A included mice overexpressing the nuclear form of human SREBP-1a and their wild-type littermates, while Experiment B included mice that overexpress the nuclear form of human SREBP-1c or SREBP-2. Wild-type littermates from the TgSREBP-1c mice were used as controls in Experiment B. The changes in plasma and liver lipid levels between wild-type and transgenic mice are consistent with previously published results (16–18). Of note, TgSREBP-1a mice had mean liver cholesteryl ester and triglyceride concentrations of 9.9 and 183 mg/g, respectively. Both levels were ~30-fold higher than those measured in control livers. SREBP-1c overexpression resulted in very little change in the cholesteryl ester concentration but it did increase the triglyceride concentration to 36 mg/g. TgSREBP-2 mouse livers had an elevated mean cholesteryl ester concentration of 3.1 mg/g and a triglyceride content of 38 mg/g.

Fig. 1 compares the levels of transgene expression in the livers of the mice described in Table I as measured by the RNase protection assay. In order to estimate the relative expression of each transgene, we designed a cRNA probe that contained the same number of $^{32}$P atoms in the protected fragment. The human SREBP cRNA probes did not produce a protected band in wild-type mouse liver. The level of mRNA encoding the SREBP-1a transgene was arbitrarily set at 1. The SREBP-1c and SREBP-2 transgenes were expressed at levels that were 40 and 60% of the levels of SREBP-1a. The difference in transgene expression precludes any direct comparisons between the three lines of transgenic mice regarding the absolute effects of each SREBP isoform, but they do permit an analysis of the relative ability of each isoform to affect one mRNA *versus* another.

Northern blot experiments were used to further examine the effects of SREBP overexpression on hepatic genes involved in fatty acid metabolism (Fig. 2). ATP citrate lyase generates acetyl-CoA, which is the substrate for cholesterol and fatty acid synthesis. ATP citrate lyase mRNA was increased 4.4-, 3.2-, and 1.5-fold in livers of TgSREBP-1a, -1c, and -2 mice, respectively. The fold changes in the mRNAs for the fatty acid synthetic enzymes, ACC and FAS, were slightly less than previously reported (18), most likely because the mice were sacrificed in the fed state. GPAT, which catalyzes the first committed step in triglyceride synthesis, was significantly increased in livers from SREBP-1a and -1c mice, but not in the SREBP-2 animals. The measured increase was greatest in the TgSREBP-1a mice.

Malic enzyme, Glu-6-PD, and PGDH are enzymes involved in the generation of NADPH required for fatty acid synthesis. The mRNAs for these genes are shown in the second row of Fig. 2. SREBP-1a overexpression increased the hepatic mRNAs for malic enzyme and PGDH by 11- and 6.6-fold, respectively. The most dramatic change was a 31-fold increase in the Glu-6-PD mRNA. SREBP-1c and SREBP-2 overexpression resulted in significant but smaller increases in these mRNAs. S14, a gene known to be induced during lipogenesis (39), was also significantly increased in livers from TgSREBP-1a and -1c mice. However, in livers from TgSREBP-2 mice the mRNA for S14 was slightly less than that measured in control livers. To date, S14 and GPAT are the only genes that have been found to be selectively induced only by the SREBP-1 isoforms in liver. The

fold increase measured in the mRNA for the cholesterol synthetic enzyme, HMG-CoA reductase, was greatest in the Tg-SREBP-2 mice. All fold changes reported in hepatic mRNAs were calculated after correction for loading differences using the signal generated by GAPDH. GAPDH is a glycolytic enzyme, and as such, could be increased in SREBP transgenic mice owing to the apparent increase in lipogenesis. Therefore, the fold increases reported for the various hepatic mRNAs may be slightly underestimated.

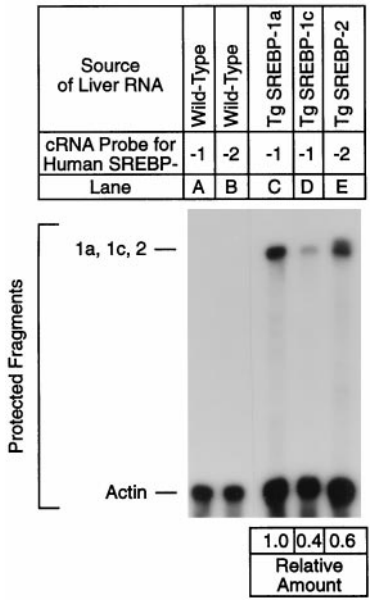


FIG. 1. **Relative amounts of hepatic mRNAs encoding the truncated human transgene as measured by the RNase protection assay.** Total RNA from the livers of wild-type (*lanes A* and *B)*, Tg-SREBP-1a (*lane C*), TgSREBP-1c (*lane D*), and TgSREBP-2 (*lane E*) mice was isolated from the mice described in Table I. Equal aliquots of total RNA (10 $\mu$g) were hybridized at 68 °C to a $^{32}$P-labeled cRNA probe for human SREBP-1 (*lanes A, C,* and *D*) or SREBP-*2* (*lanes B* and *E*) to detect the transgene product. Protected fragments were separated by gel electrophoresis and exposed to film at −80 °C with an intensifying screen. The radioactivity in the gel was quantified by exposure of the filters to a Bio-Imaging Analyzer with BAS1000 MacBas software (Fuji Medical Systems), normalized to the signal generated by $\beta$-actin, and expressed as the fold change relative to the value of the mRNA encoding the SREBP-1a transgene.

Stearoyl-CoA desaturase is the key enzyme responsible for the synthesis of monounsaturated fatty acids (19). We have previously reported that the mRNA for SCD is increased in livers from all three lines of transgenic mice as measured by Northern blotting (16–18). Although SCD1 is reportedly the only SCD isoform present in liver (21), the cDNA probe for SCD used in our previous Northern blots could cross-hybridize with SCD2, if present. To determine whether the measured increase in SCD mRNA in livers from SREBP transgenic mice was due to an increase in SCD1 and/or SCD2 mRNA, we designed cRNA probes for use in a RNase protection assay that would specifically detect SCD1 or SCD2 mRNA.

In order to be certain that the level was within the linear range of the RNase protection assay, we added various amounts of mRNA to the assay tubes. Fig. 3 shows that the intensities of the protected fragments of the SCD1 mRNA increased as increasing amounts of RNA were added to the incubation. Quantifying the protected fragments verified the assay was linear up to 10 $\mu$g of total RNA from all lines of transgenic mice.

Fig. 4 shows the results of the RNase protection assay for SCD1 and SCD2 mRNA transcripts in livers from the wild-type and transgenic mice described in Table I. *Lanes 1* and *2* show the protected bands for SCD1 and SCD2 in 5 $\mu$g of total RNA from the epididymal fat pads of wild-type mice. SCD1 and SCD2 transcripts are both known to be present in white adipose tissue, and therefore, this RNA was used as a positive control (20, 21). *Lanes 3–7* show the protected fragments for SCD1 in livers of the wild-type and transgenic mice described in Table I. The SCD1 mRNA was increased by 2-fold in all three lines of transgenic mice. *Lanes 8–12* show the protected mRNA fragments for SCD2. No SCD2 transcript could be detected in livers from wild-type or TgSREBP-1c mice. However, a protected band for SCD2 was detected in RNA from livers of TgSREBP-1a and TgSREBP-2 mice (*lanes 9* and *12*). The amount of SCD2 transcript in TgSREBP-2 livers is equivalent to the amount found in white fat from wild-type mice, whereas TgSREBP-1a livers had 7-fold more SCD2 transcript than that measured in white fat.

To determine whether the measured increase in SCD mRNA from the transgenic mice resulted in increased enzyme activity,

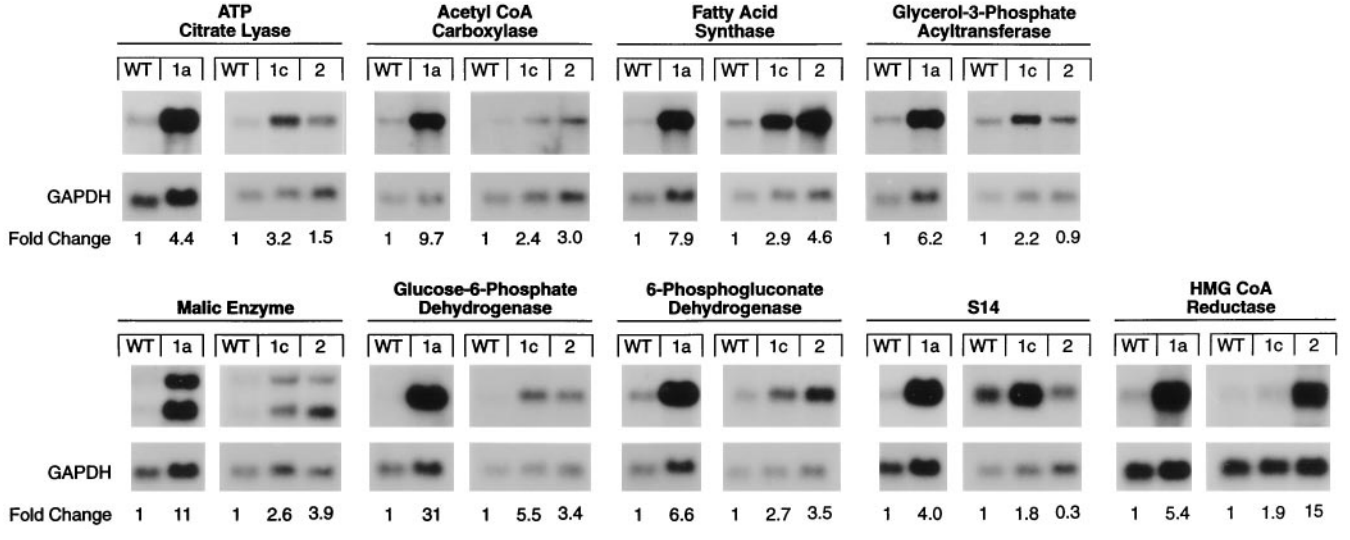


FIG. 2. **Amounts of mRNAs of genes involved in fatty acid metabolism from livers of wild-type and transgenic mice as measured by blot hybridization.** The mice used in this experiment are described in Experiments A and B of Table I. Total RNA isolated from livers of mice was pooled (15 $\mu$g), and subjected to electrophoresis and blot hybridization with the indicated $^{32}$P-labeled probe. The amount of radioactivity in each band was quantified as described in Fig. 1. The fold change in each mRNA, relative to that of the wild-type control, was calculated after correction for loading differences with GAPDH. These values are shown below each blot.

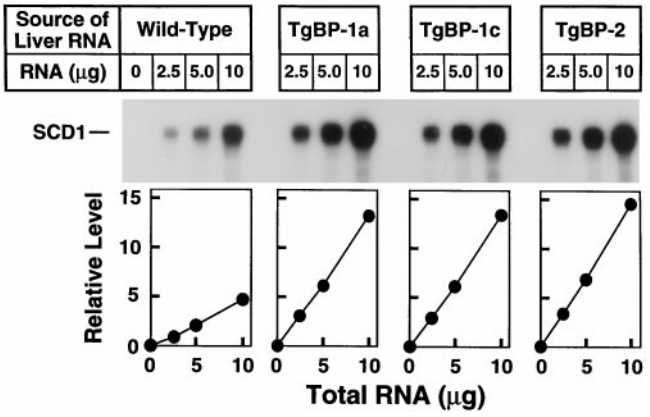


FIG. 3. **Linearity of signals for SCD1 mRNA in wild-type and transgenic mouse livers using the RNase protection assay.** $^{32}$P-Labeled cRNA probe for mouse SCD1 was hybridized to the indicated amount of total RNA from wild-type or transgenic mouse livers for 10 min at 68 °C. Protected fragments were separated by gel electrophoresis and exposed to film for 30 min at −80 °C with an intensifying screen (*top*). The gel was also quantified as described in the legend to Fig. 1, and the results are plotted in the *lower panel*. Levels of SCD1 mRNA are expressed in relation to the signal generated by 2.5 μg of RNA in wild-type liver, which is arbitrarily set at 1.

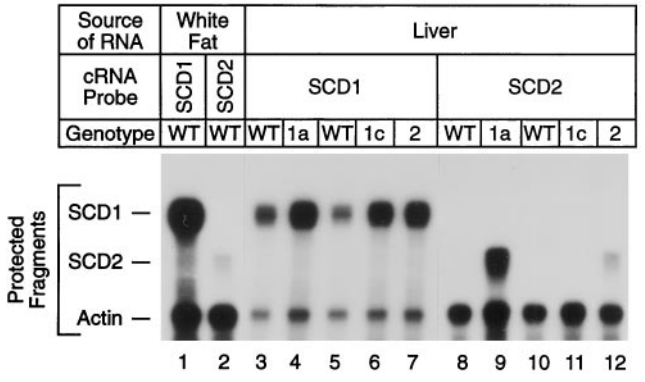


FIG. 4. **Amounts of mRNA encoding SCD1 and SCD2 in livers from wild-type and transgenic mice as measured by the RNase protection assay.** Total RNA was isolated from wild-type epididymal fat and 5 μg was hybridized with a $^{32}$P-labeled cRNA probe for SCD1 (*lane 1*) or SCD2 (*lane 2*) as described under "Experimental Procedures." White fat contains mRNA transcripts for both SCD1 and SCD2, and therefore, was used as a positive control. Total RNA from livers of the mice described in Table I was isolated and equal aliquots (5 μg) were hybridized with a $^{32}$P-labeled cRNA probe for SCD1 (*lanes 3–7*) or SCD2 (*lanes 8–12*). The protected fragments were separated by gel electrophoresis and exposed to film for 30 min at −80 °C with an intensifying screen for SCD1 in liver and for 12 h at room temperature for white fat and SCD2 in liver.

we measured SCD activity in liver microsomes from wild-type and transgenic mice. Table II shows the mean SCD activities as estimated by the rate of conversion of [1-$^{14}$C]stearoyl-CoA to [1-$^{14}$C]oleate by hepatic microsomes. TgSREBP-1a mouse liver microsomes had 5-fold higher SCD enzymatic activities than microsomes from littermate wild-type mice. The activities in livers from TgSREBP-1c and TgSREBP-2 mice were both approximately 2.5-fold higher than wild-type control levels.

The major substrates for SCD are palmitic (16:0) and stearic (18:0) acids, which are converted to palmitoleic (16:1) and oleic (18:1) acids, respectively (19). To determine whether the transgenic mouse livers contained more monounsaturated fatty acids, the various lipid fractions were separated and the relative fatty acid composition in each fraction was measured. Table III shows the relative percentage of 8 major fatty acids measured in the various lipid fractions from wild-type and transgenic mouse livers. In total lipid extracts, TgSREBP-1a livers had a

TABLE II

*Stearoyl-CoA desaturase activities in livers of wild-type and transgenic mice*

Each value represents the mean ± S.E. Liver microsomes were prepared from the indicated mice described in Experiments A and B of Table I. Microsomal protein (100 μg) was incubated with a reaction mixture containing [1-$^{14}$C]stearoyl-CoA for 5 min. The products were saponified, acidified, and the fatty acids were extracted and separated by TLC as described under "Experimental Procedures."

| Genotype of mice | [1-$^{14}$C]Stearoyl-CoA → Oleate |
|---|---|
| | *nmol · min$^{-1}$ · mg protein$^{-1}$* |
| Experiment A | |
| Wild-type | 0.9 ± 0.1 |
| TgSREBP-1a | 5.0 ± 0.3[a] |
| Experiment B | |
| Wild-type | 0.6 ± 0.1 |
| TgSREBP-1c | 1.5 ± 0.1[a] |
| TgSREBP-2 | 1.5 ± 0.1[a] |

[a] $p < 0.001$ between transgenic and wild-type mice (Student's t test).

TABLE III

*Fatty acid compositions of livers in wild-type and transgenic mice*

Liver samples from each mouse were extracted, and the major classes of lipids were separated on a silica column. The lipid fractions were methyl esterified and quantified by GLC as described under "Experimental Procedures." Each value represents the mean from 6 mice in Experiment A and 5 mice in Experiment B. Standard errors for all values were less than 20% of the mean and are omitted from the table for clarity. Bold values denote a level of statistical significance of $p <$ 0.01 between wild-type and transgenic mice (Student's t test).

| Genotype of mice | Fatty acid composition (% of total) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:4 | 22:6 |
| Total fatty acids | | | | | | | | |
| Wild-type | 0.5 | 22 | 2.4 | 12 | 18 | 17 | 16 | 5.5 |
| TgSREBP-1a | 0.4 | **13** | **7.1** | **3.0** | **65** | **3.0** | **2.8** | **0.7** |
| Wild-type | 0.2 | 21 | 1.4 | 14 | 15 | 16 | 18 | 5.8 |
| TgSREBP-1c | 0.5 | 23 | **4.3** | **6.8** | **32** | **13** | **9.8** | **3.4** |
| TgSREBP-2 | 0.5 | **16** | **4.3** | **7.3** | **33** | 17 | **10** | **2.9** |
| Cholesteryl esters | | | | | | | | |
| Wild-type | 0.7 | 53 | 3.9 | 5.9 | 15 | 6.6 | 2.3 | 0 |
| TgSREBP-1a | 0.4 | **13** | **7.6** | **1.1** | **72** | **2.7** | **0.3** | 0 |
| Wild-type | 0.4 | 46 | 2.3 | 6.2 | 19 | 9.0 | 3.0 | 0 |
| TgSREBP-1c | 0.7 | **27** | **5.2** | **1.9** | **46** | 11 | **0.9** | 0 |
| TgSREBP-2 | 0.7 | **17** | **5.9** | **1.8** | **50** | 15 | **1.7** | 0 |
| Triglycerides | | | | | | | | |
| Wild-type | 1.0 | 22 | 4.9 | 2.9 | 37 | 21 | 2.9 | 0 |
| TgSREBP-1a | **0.4** | **11** | **9.8** | **0.8** | **69** | **4.2** | **0.5** | 0 |
| Wild-type | 0.5 | 20 | 2.8 | 3.3 | 35 | 23 | 3.6 | 0 |
| TgSREBP-1c | 0.7 | 22 | **6.7** | **1.2** | **44** | **17** | **1.5** | 0 |
| TgSREBP-2 | 0.8 | **14** | **6.2** | **1.1** | **41** | 22 | **2.9** | 0 |
| Phospholipids | | | | | | | | |
| Wild-type | 0 | 21 | 1.5 | 13 | 12 | 15 | 20 | 6.6 |
| TgSREBP-1a | 0 | **13** | **2.0** | **19** | **24** | **4.3** | **23** | 5.3 |
| Wild-type | 0 | 23 | 0.9 | 18 | 11 | 14 | 18 | 5.1 |
| TgSREBP-1c | 0 | 22 | **1.6** | 15 | **14** | **11** | 20 | 6.5 |
| TgSREBP-2 | 0 | **17** | **1.5** | 16 | **17** | 13 | 19 | 5.4 |

3-fold increase in the relative amount of palmitoleic acid (16:1) and a similar 3.6-fold increase in the relative amount of oleic acid (18:1). On the other hand, the relative amounts of palmitic (16:0) and stearic (18:0) acids were decreased. The total lipid extracts from livers of TgSREBP-1c and TgSREBP-2 mice had similar 3-fold increases in the relative amount of palmitoleic acid and 2-fold increases in the amount of oleic acid.

The hepatic cholesteryl ester fractions from all three lines of transgenic mice contained a 2-fold increase in the relative amount of palmitoleic acid (16:1), while the relative amount of oleic acid (18:1) was 4.8-fold higher in TgSREBP-1a mice and

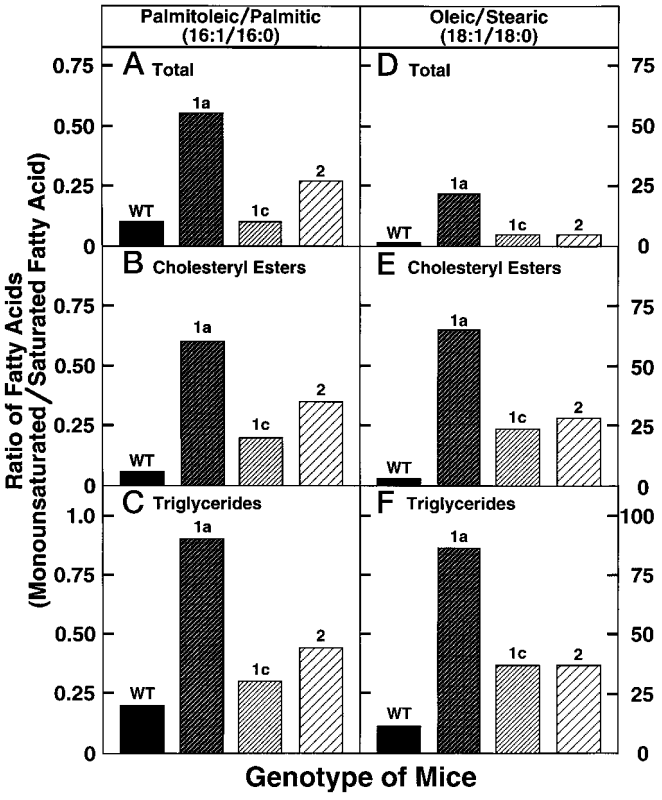


FIG. 5. **The ratio of palmitoleic (16:1) to palmitic (16:0) and oleic (18:1) to stearic (18:0) acids in various lipid fractions extracted from livers of wild-type and transgenic mice.** Total lipids were extracted from livers of wild-type and transgenic mice described in Table I. Lipid fractions were separated on a silica column, methyl esterified, and quantified by GLC as described under "Experimental Procedures." The values used for calculating the ratio of monounsaturated to saturated fatty acids for wild-type mice are the means of the wild-type mice in Experiments A and B presented in Table III. The ratios for the transgenic mice are derived directly from the means presented in Table III. The ratios of palmitoleic (16:1) to palmitic (16:0) acids in total lipid extracts (*panel A*), cholesteryl esters (*panel B*), and triglycerides (*panel C*) from livers of wild-type and TgSREBP-1a, -1c, and -2 mice are shown. The ratio of oleic (18:1) to stearic (18:0) acid for each lipid fraction is presented in *panels D-F*.

2.5-fold higher in TgSREBP-1c and -2 mice (Table III). The changes measured in the relative amounts of monounsaturated and saturated fatty acids in the triglyceride fractions from livers of the TgSREBP-1a, -1c, and -2 mice are similar to the changes measured in the cholesteryl esters. Inasmuch as the cholesteryl ester and triglyceride content in livers of TgSREBP-1a and TgSREBP-2 mice are significantly elevated (see Table I), the absolute monounsaturated fatty acid content in each fraction is dramatically increased. Fatty acid compositional changes in the phospholipid fractions were much less striking, although small increases in the percentage of oleic acid were measured in livers from all lines of transgenic mice (Table III).

The relative increase in the percentage of monounsaturated fatty acids (16:1 and 18:1) measured in livers of transgenic mice was accompanied by significant reductions in the relative percentage of saturated fatty acids (16:0 and 18:0). Fig. 5 shows the ratio of monounsaturated to saturated fatty acids measured in the livers from wild-type and the three lines of transgenic mice. In total lipid extracts (*panels A* and *D*), the 16:1/16:0 ratio was increased in the livers from TgSREBP-1a and TgSREBP-2 mice, but not in livers from TgSREBP-1c mice. The 18:1/18:0 ratio was significantly elevated only in livers from TgSREBP-1a mice. In the cholesteryl ester fractions (*panels B* and *E*) and triglyceride fractions (*panels C* and *F*), the ratio of monounsaturated to saturated fatty acids was significantly increased in all three lines of transgenic mice. The most pronounced changes were again present in livers from TgSREBP-1a mice. The 16:1/16:0 ratios in the cholesteryl ester fractions from TgSREBP-1a, -1c, and -2 livers were increased by 10-, 3-, and 6-fold, respectively (*panel B*), while the 18:1/18:0 ratios were increased by 23-, 9-, and 10-fold, respectively (*panel E*). The hepatic triglyceride fractions also contained increases in the 16:1/16:0 and 18:1/18:0 ratios (*panels C* and *F*), although the absolute fold-change is approximately one-third of that measured in the cholesteryl ester fractions.

## DISCUSSION

The purpose of the current study was to gain insight into the effects of SREBP overexpression on selected aspects of fatty acid metabolism in liver using transgenic mice. In previous studies, we observed increased rates of hepatic fatty acid synthesis in all three lines of transgenic mice as measured by the incorporation of [$^3$H]water into newly synthesized fatty acids (16, 18). The genes required to produce the substrates for fatty acid synthesis as well as the final fatty acid products have not been studied. Inasmuch as TgSREBP-1a mouse livers consistently showed the greatest changes in fatty acid metabolism, the initial discussion will focus on the results obtained in these mice.

Most of the carbon source for fatty acid synthesis comes from pyruvate, whose carbons are incorporated into citrate within mitochondria (40). The citrate then leaves the mitochondria and enters the cytoplasm where ATP citrate lyase cleaves off a two-carbon unit as acetyl-CoA, which is then incorporated into fatty acids and cholesterol. SREBP-1a overexpression resulted in a 4-fold increase in the mRNA for ATP citrate lyase, which would thereby increase the acetyl-CoA substrate required for lipid synthesis.

The synthesis of fatty acids requires a second substrate, NADPH, which supplies hydrogen atoms (40). NADPH is also required for the desaturation reaction catalyzed by SCD (19). Two major pathways exist for the generation of NAPDH, and each is estimated to contribute approximately 50% in rat liver (41). The first reaction is the oxidative decarboxylation of malate to form pyruvate and $CO_2$. This reaction is catalyzed by malic enzyme, which simultaneously generates NADPH from $NADP^-$. The second source of NADPH is from the pentose phosphate shunt. Glu-6-PD and PGDH are the first two enzymes in this pathway and both generate NADPH (40). The livers of SREBP-1a transgenic mice had significantly elevated mRNAs for all three NADPH producing enzymes, indicating both pathways for NAPDH synthesis were activated. Therefore, SREBP-1a not only activates the genes directly responsible for fatty acid synthesis, ACC and FAS, but it also activates the genes responsible for the production of the acetyl-CoA and NADPH required for fatty acid synthesis. Since lipogenesis is globally stimulated in TgSREBP-1a livers, it is possible that transcription of the genes encoding the enzymes responsible for the production of citrate are also increased (*i.e.* glucokinase, phosphofructokinase I, and pyruvate kinase), although these mRNAs were not studied in the current experiments.

To date, SREBP-1a has been shown to directly bind to the promoters and activate the transcription of ACC, FAS, and GPAT (9–11). To our knowledge, the promoters for ATP citrate lyase, malic enzyme, Glu-6-PD, and PGDH have not been studied. Therefore, we cannot conclude SREBP-1a activates the transcription of these genes directly. However, whether the effect is direct or indirect, the end result is an increase in mRNAs encoding multiple enzymes required for the simultaneous production of cholesterol, fatty acids, and triglycerides.

The transcriptional activation of SCD2 by SREBP-1a and SREBP-2 in liver was unexpected. This mRNA was reported to be absent from mouse liver (21) and indeed we failed to detect it in livers of wild-type mice. It did appear in livers of mice expressing SREBP-1a and SREBP-2, indicating that the block in SCD2 expression in liver is not complete and that it might be expressed if the stimulus is strong enough.

The combined increase in SCD1 and SCD2 mRNAs led to an increase in the measured SCD activity in liver microsomes (Table II). This is of possible metabolic importance because the predominant SCD product, oleic acid, is the preferred substrate for acyl-CoA:cholesterol acyltransferase, the enzyme responsible for the esterification of cholesterol (42). The increased esterification of cholesterol prevents the accumulation of potentially toxic-free cholesterol in TgSREBP-1a mouse livers and increases the availability of esterified cholesterol for export in the form of VLDL.[2] As predicted, the increase in SCD activity resulted in a significant increase in the monounsaturated fatty acid content of cholesteryl esters. A very similar fatty acid profile was measured in the triglyceride fractions. Approximately 80% of the fatty acids present in these fractions were monounsaturated with the vast majority being oleic acid. The changes in liver cholesteryl ester and triglyceride fatty acid composition were reflected in the lipids secreted in VLDL from TgSREBP-1a mice.[2] The marked hepatic enrichment of oleic acid can potentially have a wide range of effects on lipid metabolism, such as increased VLDL secretion and accelerated atherosclerosis (43, 44). In cultured cells, the addition of oleic acid to the medium is required for maximal VLDL secretion (44). Therefore, it might be predicted the TgSREBP-1a mice have increased rates of VLDL secretion. This hypothesis is currently under investigation.

The changes measured in the mRNAs for fatty acid synthetic enzymes in livers of TgSREBP-1c and TgSREBP-2 mice were in a similar direction to those observed in the TgSREBP-1a animals, but they were of lower magnitude. In part, this may be attributed to the slightly lower expression of the SREBP-1c and -2 transgenes as compared with TgSREBP-1a (0.4- and 0.6-fold, respectively). The mRNAs for ACC, FAS, and the NAPDH producing enzymes were increased 2–4-fold in TgSREBP-1c and TgSREBP-2 livers. These changes are consistent with the 4-fold increases measured in the rates of fatty acid synthesis using [$^3$H]water in both TgSREBP-1c and TgSREBP-2 mouse livers (18). Interestingly, there was a significant difference between the two SREBP-1 isoforms and SREBP-2 in their ability to activate the lipogenic genes GPAT and S14. Neither mRNA was changed in TgSREBP-2 mouse livers, but both were significantly increased in TgSREBP-1c and -1a livers. In the case of GPAT, this finding is consistent with GPAT promoter-reporter studies in which the relative stimulation of promoter activity was 5.5-fold greater with SREBP-1a than SREBP-2 in HepG2 cells (11). This observation is also consistent with the idea that SREBP-1 is relatively selective as compared with SREBP-2 in activating lipogenic genes (18). The molecular basis of such isoform selectivity is under active study.

The hepatic fatty acid compositions in each lipid fraction were remarkably similar in TgSREBP-1c and -2 mice. Livers from both lines of transgenic mice were enriched with oleic acid in the cholesteryl ester and triglyceride fractions, but to levels that were approximately one-half those measured in TgSREBP-1a livers. The increase in SCD activity was also identical in the TgSREBP-1c and TgSREBP-2 livers. Interestingly, TgSREBP-1c livers did not contain mRNA for SCD2. Whether this is due to lower transgene expression, or to the inability of SREBP-1c to transcriptionally activate SCD2 is not known.

The studies in this paper and the previous papers (16–18) on transgenes encoding truncated SREBPs indicate the potential of high-level expression of nuclear SREBPs to activate the programs for synthesis of unsaturated fatty acids and cholesterol in liver. However, they do not tell us whether the SREBPs carry out this activation under normal physiologic conditions. Answers to this question will come only when methods are found to block the activities of nuclear SREBPs in liver or to modify the target genes so they lose their ability to respond to SREBPs.

*Acknowledgments*—We thank Drs. Michael S. Brown and Joseph L. Goldstein for their continued encouragement and critical reading of the manuscript. We also thank Dr. Stephen Turley and Dr. John Dietschy for access to the GLC and Robin Craddock, Scott Clark, Debra Morgan, and Richard Gibson for excellent technical assistance.

[2] J. D. Horton, H. Shimano, J. L. Goldstein, and M. S. Brown, unpublished observations.

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