Isoform 1c of Sterol Regulatory Element Binding Protein Is Less Active Than Isoform 1a in Livers of Transgenic Mice and in Cultured Cells

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Abstract

We have produced transgenic mice whose livers express a dominant positive NH_2-terminal fragment of sterol regulatory element binding protein-1c (SREBP-1c). Unlike full-length SREBP-1c, the NH_2-terminal fragment enters the nucleus without a requirement for proteolytic release from cell membranes, and hence it is immune to downregulation by sterols. We compared SREBP-1c transgenic mice with a line of transgenic mice that produces an equal amount of the NH_2-terminal fragment of SREBP-1a. SREBP-1a and -1c are alternate transcripts from a single gene that differ in the first exon, which encodes part of an acidic activation domain. The 1a protein contains a long activation domain with 12 negatively charged amino acids, whereas the 1c protein contains a short activation domain with only 6 such amino acids. As previously reported, livers of the SREBP-1a transgenic mice were massively enlarged, owing to accumulation of triglycerides and cholesterol. SREBP-1c transgenic livers were only slightly enlarged with only a moderate increase in triglycerides, but not cholesterol. The mRNAs for the LDL receptor and several cholesterol biosynthetic enzymes were elevated in SREBP-1a transgenic mice, but not in 1c transgenic mice. The mRNAs for fatty acid synthase and acetyl CoA carboxylase were elevated 9- and 16-fold in 1a animals, but only 2- and 4-fold in 1c animals. Experiments with transfected cells confirmed that SREBP-1c is a much weaker activator of transcription than SREBP-1a when both are expressed at levels approximating those found in nontransfected cells. SREBP-1c became a strong activator only when expressed at supraphysiologic levels. We conclude that SREBP-1a is the most active form of SREBP-1 and that SREBP-1c may be produced when cells require a lower rate of transcription of genes regulating cholesterol and fatty acid metabolism. (*J. Clin. Invest.* 1997. 99: 846–854.) Key words: SREBP-1 • alternative splicing • cholesterol • fatty acids • transgenic mice

Introduction

Three sterol regulatory element binding proteins (SREBPs)[1] regulate the synthesis of cholesterol and fatty acids in animal cells (1–5). Two of these proteins, designated SREBP-1a and -1c, are derived from a single gene through the use of alternate transcription start sites that produce alternate forms of exon 1 (6–8). The third protein, SREBP-2, is derived from a separate gene, and no alternate forms are known (9).

The SREBPs are synthesized as long precursors of 1,141–1,147 amino acids that are bound to membranes of the endoplasmic reticulum and nuclear envelope (6, 9–11). Each polypeptide chain has three segments: (1) an NH_2-terminal segment of $\sim$ 480 amino acids that is a transcription factor of the basic helix-loop-helix-leucine zipper family; (2) a middle segment of $\sim$ 80 amino acids encompassing two membrane-spanning domains separated by a short hydrophilic sequence of $\sim$ 30 amino acids; and (3) a long COOH-terminal extension of $\sim$ 590 amino acids. The SREBPs are bound to membranes in a hairpin fashion so that the NH_2- and COOH-terminal segments project into the cytosol, and only the short hydrophilic loop (the lumenal loop) projects into the lumen of the endoplasmic reticulum or nuclear envelope (12).

To influence transcription, the SREBPs must be proteolytically cleaved to release the NH_2-terminal segment so that it can enter the nucleus (1). Cleavage takes place in two steps (13, 14). First, a protease clips the proteins at site 1, which is in the middle of the lumenal loop, thereby breaking the covalent attachment between the two membrane-spanning domains. This cleavage allows a second protease to clip the proteins at site 2 in the middle of the first membrane-spanning segment, releasing the NH_2-terminal segment with half of the membrane-spanning sequence still attached. This segment, designated the mature form of SREBP, enters the nucleus and binds to sterol regulatory elements in the promoters of genes encoding the LDL receptor and several enzymes in the cholesterol biosynthetic pathway, including 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, farnesyl diphosphate synthase, and squalene synthase (1, 2, 4, 15). Binding leads to transcriptional activation through interaction of the SREBPs with other transcription factors, including SP-1 (16, 17) and NF-Y (18). The SREBPs also activate transcription of genes encoding HMG CoA reductase and several enzymes of fatty acid metabolism, including acetyl CoA carboxylase, fatty acid synthase, lipoprotein lipase, and stearoyl CoA desaturase-1 (2, 3, 5, 19, 20). These latter effects are mediated by interaction

with sequences other than the classically defined sterol regulatory element (20, 21).

The proteolytic release of SREBPs from membranes is controlled by the sterol content of cells. When cells are deprived of sterols, cleavage at site 1 is active, and cleavage at site 2 follows rapidly (14). When cells are overloaded with sterols, cleavage at site 1 is abolished, and cleavage at site 2 can no longer occur. The SREBPs remain membrane-bound and transcription of the target genes declines. The net effect is to reduce the synthesis of cholesterol and fatty acids and their uptake from lipoproteins such as LDL. This feedback mechanism is designed to maintain a constant level of cholesterol in cell membranes, and its disruption by mutation in cultured cells leads to either overaccumulation or deficiency of cholesterol (11, 14, 22).

The individual roles of the three SREBPs are beginning to be dissected in cultured cells and in the livers of intact animals. Although the SREBPs can form heterodimers as well as homodimers, there is no current evidence that heterodimerization is necessary for function (9). The NH_2-terminal segments of SREBP-1a, -1c, and -2 were each found to activate transcription independently when overexpressed by transfection in cultured cells (7, 9). These transient transfection experiments were performed with cDNAs expressing SREBPs under control of the strong cytomegalovirus (CMV) promoter which leads to massive overexpression (13). The differential effects of the individual SREBPs have not been compared at lower, more physiologic, levels of expression.

The first suspicion of separate roles for SREBP-1 and -2 came from measurements of SREBP processing in livers of intact hamsters (23). In these experiments, the 1a and 1c forms of SREBP-1 were not distinguished, and so we refer to them generically as SREBP-1. In contrast to cultured cells, in which the processing of SREBP-1 and -2 is regulated coordinately (1, 9, 11), hamster liver exhibited a reciprocal pattern of regulation (23). In the basal state the NH_2-terminal segment of SREBP-1, but not SREBP-2, was found in the nucleus. The reverse occurred when the livers were depleted of cholesterol by treatment of the animals with an HMG CoA reductase inhibitor (lovastatin) and a bile acid sequestrant (Colestipol). Under these conditions the amount of nuclear SREBP-1 declined and the amount of both the precursor and nuclear forms of SREBP-2 increased (23). These findings suggested that SREBP-1 and SREBP-2 were playing separate roles in the liver.

To further explore the role of SREBP-1 in liver, we created transgenic mice that overexpress a truncated NH_2-terminal segment of human SREBP-1a, which is constitutively active and does not require proteolysis (2). These animals exhibited massive hepatic enlargement, owing to engorgement with triglycerides and cholesteryl esters. The amounts of the mRNAs for the cholesterol and fatty acid biosynthetic enzymes were markedly elevated, as were the mRNAs for the LDL receptor, lipoprotein lipase, and stearoyl CoA desaturase-1. The overall rates of synthesis of cholesterol and fatty acids were elevated by 5- and 25-fold, respectively, as measured in intact animals. Despite the accumulation of liver lipids, the plasma lipid levels were not elevated. An unexpected finding was a progressive involution of adipose tissue as the animals aged (2).

In these experiments, we have compared the transcriptional activities of SREBP-1a and -1c in transgenic mice and in cultured cells. SREBP-1c differs from SREBP-1a in its extreme NH_2 terminus, which is encoded by exon 1. In human SREBP-1a, this exon encodes 29 amino acids, which includes 8 negatively charged amino acids (6). This sequence forms part of a 42–amino acid acidic transcriptional activation domain of 12 amino acids, which includes 4 negatively charged amino acids encoded by exon 2 (6). When this activation domain was deleted from human SREBP-1a, the protein retained its ability to bind to sterol regulatory element-1 (SRE-1), but it could no longer activate transcription, and so it had a dominant negative effect (10). Exon 1 of human SREBP-1c contains only five amino acids, one of which is negatively charged (6). This produces a 24–amino acid activation domain with 6 negatively charged amino acids when joined to the acidic segment encoded by the common exon 2. These findings raise the possibility that SREBP-1c might be a weaker transcriptional activator than SREBP-1a.

To test this hypothesis, we produced transgenic mice that express the NH_2-terminal fragment of SREBP-1c, and we have compared them with transgenic mice that produce similar amounts of the NH_2-terminal fragment of SREBP-1a. In addition, we have compared the transcription-stimulating activities of the NH_2-terminal fragments of SREBP-1c and -1a when expressed at low levels by transfection with plasmids using promoters that are weaker than the CMV promoter that was used previously. All of the results indicate that SREBP-1c is a much weaker transcriptional activator than SREBP-1a and that the protein retains a relatively greater potency in stimulating genes involved with fatty acid metabolism as compared with those of cholesterol metabolism.

Methods

Material and general methods. Standard molecular biology techniques were used (24). Sequencing reactions were performed on a DNA sequencer (model 373A; Applied Biosystems, Inc., Foster City, CA) using the dideoxy chain termination method. Luciferase assay systems and β-galactosidase assay kits were purchased from Promega (Madison, WI) and Stratagene (La Jolla, CA), respectively. Plasmid pCMVβ-gal, encoding a CMV promoter-driven β-galactosidase reference gene, was obtained from Stratagene. Plasmid pSRE-Luc, a luciferase reporter plasmid containing, 5′ to 3′, three tandem copies of repeats 2 and 3 of the LDL receptor promoter (17), the adenovirus E1b TATA box, and the luciferase gene, was constructed as previously described (25). The luciferase reporter plasmids, pHMG-CoASyn-Luc and pLDLR-Luc, were constructed by PCR amplification of bp −368 to +1 of the hamster HMG CoA synthase promoter region (26) and bp −308 to −61 of the human LDL receptor promoter region (27), respectively, and insertion of the PCR products into pGL2-Basic (Promega), a plasmid containing the luciferase gene. These luciferase reporter plasmids were kindly provided to us by Tularik, Inc. (South San Francisco, CA). The content of cholesterol and triglycerides in plasma and liver was measured as described previously (2).

Plasmid constructions. An expression plasmid, designated pPEPCK-SREBP-1c436, in which the rat phosphoenolpyruvate carboxykinase (PEPCK) promoter region (2.4 kb) was fused to a cDNA encoding amino acids 1–436 of human SREBP-1c (6) followed by two consecutive stop codons was constructed as previously described for pPEPCK-SREBP-1a460 (2) except that a 1.0-kb SalI-Eco47III fragment encoding the 5′-untranslated region and amino acids 1–308 of the SREBP-1c cDNA was used for ligation into the Eco47III-SalI site of pSREBP-1a(330-460) instead of the EcoRI-Eco47III fragment of human SREBP-1a cDNA (2).

Expression plasmids for truncated versions of human SREBP-1a and -1c driven by the thymidine kinase (TK) promoter and CMV promoter were constructed as follows. A 1.4-kb EcoRI-SalI fragment en-

coding the 5′-untranslated region and amino acids 1–460 of human SREBP-1a cDNA and a 1.4-kb SalI fragment encoding the 5′-untranslated region and amino acids 1–436 of human SREBP-1c cDNA were each excised from the intermediate plasmids produced in the process of construction of pPEPCK-SREBP-1a460 (2) and pPEPCK-SREBP-1c436 (see above), respectively. The DNA fragments were blunted and ligated into the NotI-XbaI sites of pTK-HSV-BP2 (13) and an SmaI site of pCMV7 (28). The resulting plasmids were designated pTK-SREBP-1a460, pTK-SREBP-1c436, pCMV-SREBP-1a460, and pCMV-SREBP-1c436, respectively. A luciferase reporter plasmid driven by the rat fatty acid synthase promoter (5) was produced as follows. The fatty acid synthase promoter fragment (bp −212 to +28) was generated by PCR using rat genomic DNA as template with AmpliTaq (Perkin Elmer, Branchburg, NJ). The following primers were used: 5′ primer: 5′-AAAGGTACCGCGTTCCTTG-TGCTCCAGCGCGCGCCTGTG-3′; and 3′ primer: 5′-AAAAAG-CTTCTGGACGGGACGCTGCTGCCGTCTCTCT-3′ (29), where the KpnI site and HindIII were added, respectively. The PCR product was digested with KpnI and HindIII, ligated into the KpnI-HindIII site of pGL2-Basic, and designated pFAS-Luc.

Production of transgenic mice. Techniques used for generating transgenic mice were described previously (2). For transgenic mice expressing human SREBP-1c436, a 4.0-kb SalI-SphI fragment of pPEPCK-SREBP-1c436 was injected into C57BL/6J × SJL F_2 hybrid eggs. 725 fertilized eggs that were injected with the DNA fragment survived to the two-cell stage and were transferred to pseudopregnant recipients. Among the 99 offspring, 30 had integrated the transgene as determined by dot blot hybridization of DNA from tail homogenates. Of 30 founder mice subjected to partial hepatectomy, 9 produced truncated SREBP-1c as determined by immunoblot analysis (2). Mice with high levels of transgene expression in liver were bred to C57BL/6J × SJL F1 mice, and six lines were established. The line with the highest expression was designated TgSREBP-1c436 and was used for this study. For comparative studies, we used a line of previously described transgenic mice SREBP-1a460 (line A) (2), whose level of protein expression in the liver is similar to that of SREBP-1c436.

Mice were housed in colony cages and maintained on a 14-h light/10-h dark cycle. The mice were fed Teklad 4% (wt/wt) mouse/rat diet (No. 7001; Harlan Teklad Premier Laboratory Diets, Madison, WI) or a synthetic low carbohydrate/high protein diet (No. 5789C-3) from Purina Mills Inc. (St. Louis, MO) containing 71% (wt/wt) casein and 4.25% (wt/wt) sucrose. Before time of killing, transgenic animals and littermate controls were placed on the low carbohydrate/high protein diet for 2 wk to induce maximal expression of the transgene. Mice were killed in the nonfasting state during the early phase of the light cycle.

cDNA transfections and luciferase assays. Human embryonic kidney 293 cells were grown in monolayer at 37°C in an atmosphere of 8–9% CO_2 in medium A (Dulbecco's modified Eagle medium containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (vol/vol) fetal calf serum. Each expression plasmid was transfected into 293 cells simultaneously with one luciferase reporter plasmid (pSRE-Luc, pHMGCoASyn-Luc, pLDLR-Luc, or pFAS-Luc) and a control pCMVβ-gal reference plasmid. Transfection studies were carried out with cells plated on 60-mm dishes or 22-mm replicate wells. All subsequent references to experiments in 22-mm wells are listed in parentheses. On day 0, $4 × 10^5$ (or $6 × 10^4$) cells were plated in medium A with 10% fetal calf serum. On day 2, duplicate dishes (or wells) of cells were transfected by the calcium phosphate method with a Stratagene MBS kit according to the manufacturer's protocol. The following plasmids were simultaneously transfected: 0–8 μg (or 0–4 μg) of an expression plasmid, 0.5 μg (or 0.2 μg) of the luciferase reporter plasmid, and 0.2 μg (or 0.1 μg) of the pCMVβ-gal reference plasmid in a final volume of 500 μl (or 200 μl). The total amount of DNA in each transfection was adjusted to 10 μg (or 5 μg) by adding the appropriate amount of pGEM3Zf(+) (Promega), the parent vector for construction of the PEPCK-driven

SREBP-1a and -1c transgenes. 3 h after transfection, the cells were washed once with phosphate-buffered saline and then fed with 5 ml (or 2 ml) of medium A supplemented with 10% fetal calf serum, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol (added in 0.2% ethanol) unless stated otherwise. After 16 h, the cells in each dish (or well) were washed once with phosphate-buffered saline. Some dishes were used for immunoblot analysis. Others were lysed with 400 μl (or 200 μl) of 1× reporter lysis buffer (Promega), and aliquots of the lysate (20 μl) were used for measurement of luciferase and β-galactosidase activities using the standard assay kits described above. For the luciferase assay, photon production was detected as relative light units in a luminometer. For the β-galactosidase assay, the hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside was detected after incubation for 30 min at 37°C with a spectrophotometer at 420 nm. The amount of luciferase activity in transfectants (relative light units) was normalized to the amount of β-galactosidase activity (OD units).

Immunoblotting. Nuclear extracts were prepared from mouse livers or pools of two 60-mm dishes of transfected cells as previously described (1, 2). Each sample (30 μg protein) was subjected to 8% SDS-PAGE, electrophoretically transferred to Hybond C extra membranes (Amersham, Arlington Heights, IL), and incubated with 5 μg/ml of rabbit anti–human SREBP-1 IgG directed against amino acids 31–175 (1). Visualization of membrane-bound antibodies was carried out with the enhanced chemiluminescence Western blotting detection system kit (Amersham) with a horseradish peroxidase–labeled donkey anti–rabbit IgG antibody (Amersham) (2).

Blot hybridization of RNA. Total RNA was prepared from mouse liver using an RNeasy™ Total RNA kit (QIAGEN Inc., Chatsworth, CA). Equal aliquots of total RNA from mice in each group were pooled (total 15 μg) and subjected to Northern blot analysis with the indicated cDNA probes prepared as previously described (2). The resulting bands were quantified by exposure of the filter to a BioImaging Analyzer with BAS1000 MacBus software (Fuji Medical Systems), and the results were normalized to the signal generated from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

RNase protection assay. An RNase protection assay for lipoprotein lipase and hepatic triglyceride lipase was carried out as previously described for lipoprotein lipase (2). A ^{32}P-labeled probe for hepatic triglyceride lipase was prepared as follows. Mouse liver poly(A)$^+$ RNA was reverse transcribed with a poly(A)$^+$ primer using a Superscript II kit (GIBCO BRL, Gaithersburg, MD). The resulting first-strand cDNA mixture was used as a template in a PCR reaction with the following primers based on the published mouse sequence (30): 5′ primer, 5′-AAAAAGCTTGCTAGAAAACTGGATCTG-GAAGAT-3′, and 3′ primer, 5′-AAAGAATTCTAGAAGTCA-TAGTGGGCAATGGGC-3′, where HindIII and EcoRI sites were added, respectively. The resulting 447-bp PCR product was subcloned into pGEM3Zf(+). After linearization with HindIII, antisense RNA was transcribed using T7 RNA polymerase with [α-^{32}P]UTP (3,000 Ci/mmol). The specific activity of the probe was $1.7–2.6 × 10^9$ cpm/μg.

Results

We constructed a transgene encoding amino acids 1–436 of human SREBP-1c under control of the PEPCK promoter. The terminator codon occurs at the same position as the terminator codon in the SREBP-1a460 construct that was described previously (2). Both of these proteins terminate before the first transmembrane segment and enter the nucleus directly without a requirement for proteolysis, thereby making them immune to the normal process of downregulation (11, 22).

Transgenic mice expressing SREBP-1a460 or SREBP-1c436 were treated for 2 wk with a high protein/low carbohydrate diet to induce expression of the transgene, after which hepatic nuclear extracts were prepared and immunoblotted
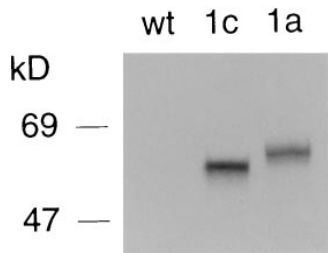
Figure 1. Immunoblot analysis of truncated human SREBP-1 in nuclear extracts from livers of wild-type (*wt*), transgenic SREBP-1c436 (*1c*), and SREBP-1a460 (*1a*) mice. Livers from the groups of mice described in Table I were pooled, and aliquots (30 μg protein) of nuclear extracts were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose filter. Immunoblot analysis was carried out using 5 μg/ml of rabbit anti–human SREBP-1 IgG as the primary antibody. Bound antibodies were visualized as described in Methods. Filters were exposed to film for 10 s at room temperature.

with an antibody against the common region of SREBP-1. For comparative purposes, we chose a line of SREBP-1a460 transgenic mice that express approximately the same amount of the transgene as the SREBP-1c436 transgenic mice (Fig. 1). This line of SREBP-1a transgenic mice (line A) produces approximately two-thirds as much SREBP-1a460 as the B line that was previously studied in detail (see Fig. 5 in reference 2).

Like the previously described line B, the mice from SREBP-1a460 line A exhibited massively enlarged livers that were pale in color, owing to the accumulation of large amounts of triglycerides and cholesteryl esters (Fig. 2, *right*). In contrast, the livers of the SREBP-1c436 mice were only slightly enlarged and only slightly pale when compared with livers of normal mice that were fed the same high protein/low carbohydrate diet (Fig. 2, *left* and *middle*).

Table I compares quantitative parameters of the SREBP-1a460 and SREBP-1c436 transgenic mice after 2 wk of induction with the high protein/low carbohydrate diet. The mean liver weight in the SREBP-1c436 transgenic mice was increased 1.3-fold as compared with normal littermates. This was

much less than the 2.8-fold increase in the SREBP-1a460 transgenic mice. Livers from the SREBP-1c436 mice showed a fourfold increase in triglyceride content and a moderate, but statistically significant, 20% increase in cholesterol content. Both of these values were much less than those observed in the SREBP-1a460 transgenic mice. The level of plasma cholesterol in the SREBP-1c436 transgenic mice was normal, but the level of triglycerides was significantly reduced. Both of these parameters were substantially reduced in the SREBP-1a460 mice.

Fig. 3 shows Northern blots of 14 mRNAs from livers of wild-type and transgenic mice after treatment with the high protein/low carbohydrate diet. The SREBP-1a460 transgenic mice exhibited elevations of 2.5–3.9-fold in mRNAs encoding proteins involved in cholesterol uptake (LDL receptor) and synthesis (HMG CoA synthase, HMG CoA reductase, and squalene synthase); and 9–16-fold elevations in mRNAs encoding enzymes of fatty acid synthesis (acetyl CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1). In sharp contrast, the SREBP-1c436 transgenic mice had no elevations in the cholesterol-related mRNAs, and only 2.1–3.9-fold elevations in the mRNAs involved in fatty acid synthesis. SREBP-1a460 caused modest elevations in the mRNAs encoding endogenous SREBP-1 and SREBP-2, but SREBP-1c436 did not. The Northern blots showed two bands for the SREBP-2 mRNA, which is similar to previous observations and has been attributed to differential polyadenylation (9). Neither transgene caused elevations in mRNAs for cholesterol 7α-hydroxylase or apoproteins AI, B, or E. SREBP-1c436 and SREBP-1a460 both caused elevations in the hepatic mRNA for lipoprotein lipase as measured by a sensitive RNase protection assay, but neither increased the mRNA for hepatic triglyceride lipase (Fig. 4). Although SREBP-1c appeared to be as active as SREBP-1a in increasing lipoprotein lipase mRNA in liver (Fig. 4), it must be borne in mind that the stimulation by both of these transcription factors is relatively weak.

The findings of Fig. 3 suggested that SREBP-1c436, when

Figure 2. Livers from 8-wk-old male wild-type (*left*), transgenic SREBP-1c436 (*middle*), and transgenic SREBP-1a460 (*right*) mice. All mice were fed the low carbohydrate/high protein diet for 2 wk before the time of the photograph.

Table I. Phenotypic Comparison of Wild-Type, Transgenic SREBP-1c436, and Transgenic SREBP-1a460 Mice

Parameter	Wild-Type	TgSREBP-1c436	TgSREBP-1a460
Sex	3 males, 6 females	3 males, 5 females	1 male, 3 females
Body weight (g)	22.4±1.1	25.8±1.5	26.4±1.5
Liver weight (g)	1.2±0.08	1.6±0.1 ($P < 0.05$)*	3.3±0.6 ($P < 0.01$)*
Liver weight/body weight (%)	5.5±0.17	6.2±0.20 ($P < 0.05$)*	12.0±1.3 ($P < 0.001$)*
Epididymal fat pad weight (g)	0.26±0.07	0.24±0.02	0.10
Liver cholesterol content (mg/g)	1.9±0.05	2.3±0.1 ($P < 0.01$)*	10.4±2.0 ($P < 0.001$)*
Liver triglyceride content (mg/g)	9.2±1.6	39±9.1 ($P < 0.01$)*	134±22 ($P < 0.001$)*
Total plasma cholesterol (mg/dl)	72±3.2	74±7.0	46±6.6 ($P < 0.01$)*
Total plasma triglycerides (mg/dl)	200±28	122±11 ($P < 0.05$)*	76±15 ($P < 0.05$)*

Each value represents the mean±SEM of the indicated number of 15–17-wk-old mice of the indicated sex except for the values for epididymal fat pad weight, which were obtained from male mice only. Both sets of transgenic mice were obtained from two litters. The wild-type mice are littermates of the TgSREBP-1c436 mice. The TgSREBP-1a460 mice were derived from line A in Fig. 4 of reference 2. All mice were fed the low carbohydrate/high protein diet for 2 wk before time of killing. Plasma lipids were measured from nonfasted samples. *Values in parentheses denote the level of statistical significance (Student's t test) between the indicated line of transgenic and wild-type mice. For parameters in which the level of significance is $P > 0.05$, no values are shown.

expressed at the levels attained in livers of transgenic animals, was much weaker than SREBP-1a460 in stimulating transcription of genes involved in synthesis of fatty acids and cholesterol. To determine whether this difference also applied to cultured cells, we performed a series of experiments in which human kidney 293 cells produced graded amounts of SREBPs after transfection with cDNAs under control of three different promoters: PEPCK (weak), TK (intermediate), or CMV (strong) (Fig. 5). To measure the transcription-stimulating activities of the SREBPs we cotransfected a cDNA encoding a luciferase reporter gene under control of natural or synthetic

promoters containing one or more sterol regulatory elements. The cells were incubated in the presence of 25-hydroxycholesterol plus cholesterol to suppress sterol-regulated proteolysis and thereby eliminate any contribution from the endogenous SREBPs. To control for transfection efficiency, we also included a cDNA encoding β-galactosidase under control of the CMV promoter. We measured the amount of luciferase activity and normalized it to the β-galactosidase activity observed in the same cells.

Fig. 5 shows a transfection experiment in which the luciferase reporter gene was driven by a synthetic promoter con-
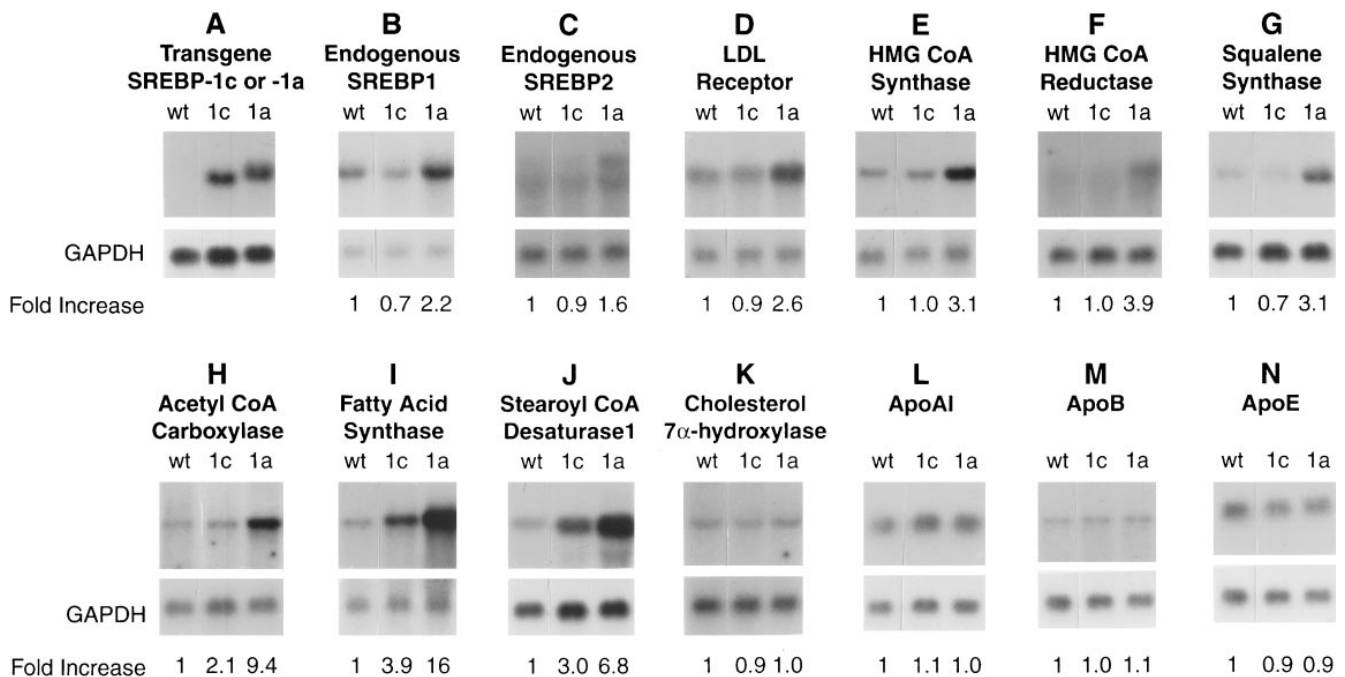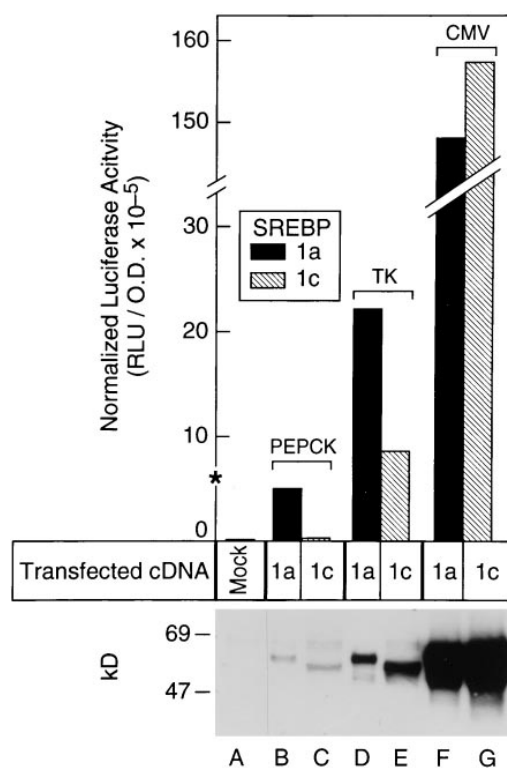

Figure 3. Amounts of various mRNAs in livers of wild-type (*wt*), transgenic SREBP-1c436 (*1c*), and transgenic SREBP-1a460 (*1a*) mice as measured by blot hybridization. Total liver RNA isolated from the mice described in Table I was pooled, and 15-μg aliquots were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probe and with a control ^{32}P-labeled probe directed against GAPDH. The amount of radioactivity in each band was quantified as described in Methods. The fold increase in each mRNA of transgenic mice, relative to that of wild-type control mice, was calculated after correction for loading differences with GAPDH. These values are shown below each blot.
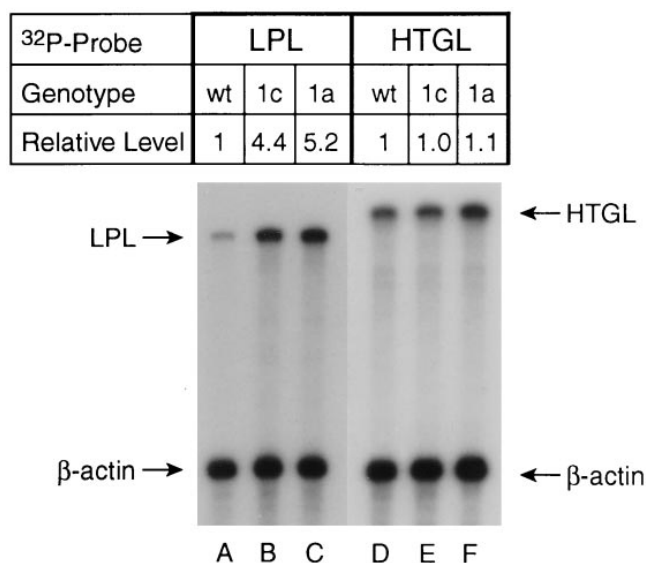
Figure 4. Amount of mRNA for lipoprotein lipase (lanes *A–C*) and hepatic triglyceride lipase (lanes *D–F*) in liver tissue from wild-type (*wt*), transgenic SREBP-1c436 (*1c*), and transgenic SREBP-1a460 (*1a*) mice as measured by RNase protection assay. The animals used for liver RNA are described in Table I. Aliquots of total RNA (10 μg) from the indicated source were hybridized with ^{32}P-labeled ribo-probes, and protected bands were separated by polyacrylamide gel electrophoresis, exposed to film for 12 h at room temperature, quantified on a PhosphorImager, and normalized to β-actin. The calculated data are expressed in relation to the level of wild-type mRNA. *LPL*, lipoprotein lipase; *HTGL*, hepatic triglyceride lipase.

Figure 5. Stimulation of SRE-luciferase reporter gene by transfected SREBP-1a460 and SREBP-1c436 driven by three different promoters. 293 cells (4×10^5 cells per 60-mm dish) were set up for experiments as described in Methods and transfected with 8, 4, or 1 μg of an expression plasmid encoding SREBP-1a460 (*closed bars*) or SREBP-1c436 (*shaded bars*) driven by the PEPCK promoter (bars *B* and *C*), TK promoter (bars *D* and *E*), or CMV promoter (bars *F* and *G*), respectively. pSRE-Luc reporter plasmid (0.5 μg) and pCMVβ-gal reference plasmid (0.2 μg) were simultaneously transfected as described in Methods. In *A*, vector DNA containing no SREBP sequence was transfected to determine the transcriptional activity of endogenous SREBPs. After transfection, all of the cells were cultured in medium A supplemented with 10% fetal calf serum, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol to suppress endogenous SREBP activity. After incubation for 16 h, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of duplicate incubations. Another duplicate set of dishes was harvested for immunoblot analysis. Nuclear extracts (30 μg protein) were prepared and subjected to 8% SDS-PAGE and immunoblotting with 5 μg/ml of rabbit anti–human SREBP-1 IgG as the primary antibody. The filter was exposed to film for 40 s at room temperature. The asterisk on the vertical axis denotes the transcriptional activity of endogenous SREBPs in mock-transfected cells that were incubated in the same experiment under conditions of sterol depletion (medium A supplemented with 5% lipoprotein-deficient serum, 50 μM compactin, and 50 μM sodium mevalonate).

taining three copies of SRE-1 (three copies of repeats 2 and 3 from the LDL receptor promoter). This construct has been shown previously to be extremely sensitive to the level of SREBP in the nucleus (17). In the absence of a cotransfected SREBP cDNA, luciferase activity was low, owing to the 25-hydroxycholesterol in the medium (Fig. 5, lane *A*). The PEPCK promoter produced low, but similar, levels of SREBP-1a460 and SREBP-1c436 as determined by immunoblotting of nuclear extracts (Fig. 5, lanes *B* and *C*). These amounts of SREBP-1a460 increased luciferase activity (Fig. 5, lane *B*), but SREBP-1c did not (Fig. 5, lane *C*). The TK promoter gave higher levels of expression of both SREBP-1a460 and SREBP-1c436 (Fig. 5, lanes *D* and *E*). At these concentrations, both SREBPs stimulated transcription, as monitored by luciferase activity, but the 1a construct had a 2.5-fold greater effect. Expression of both SREBPs was much higher when transcription was driven by the CMV promoter (Fig. 5, lanes *F* and *G*). At these high levels, SREBP-1a460 and SREBP-1c436 both stimulated transcription markedly, and the effectiveness of the two proteins was about equal. For comparative purposes, we also measured the induced level of luciferase activity in cells that were not transfected with any SREBP construct, but instead were incubated in sterol-depletion medium (lipoprotein-deficient serum plus compactin). Under these conditions, the level of luciferase activity reflects the activities of endogenous SREBPs. This value, shown by the asterisk on the vertical axis in Fig. 5, is similar to the level attained in suppressed cells when SREBP-1a460 was produced under the influence of the PEPCK promoter.

Fig. 6 shows curves for the expression of reporter genes driven by the SREx3 element and by SRE-containing fragments of the promoters for HMG CoA synthase and the LDL receptor as a function of the amount of transfected cDNA encoding SREBP-1a460 or SREBP-1c436. When SREBP expression was driven by the PEPCK promoter, SREBP-1a460 was more potent than SREBP-1c436 with all three reporter constructs, and at all concentrations tested (Fig. 6, *A–C*). When
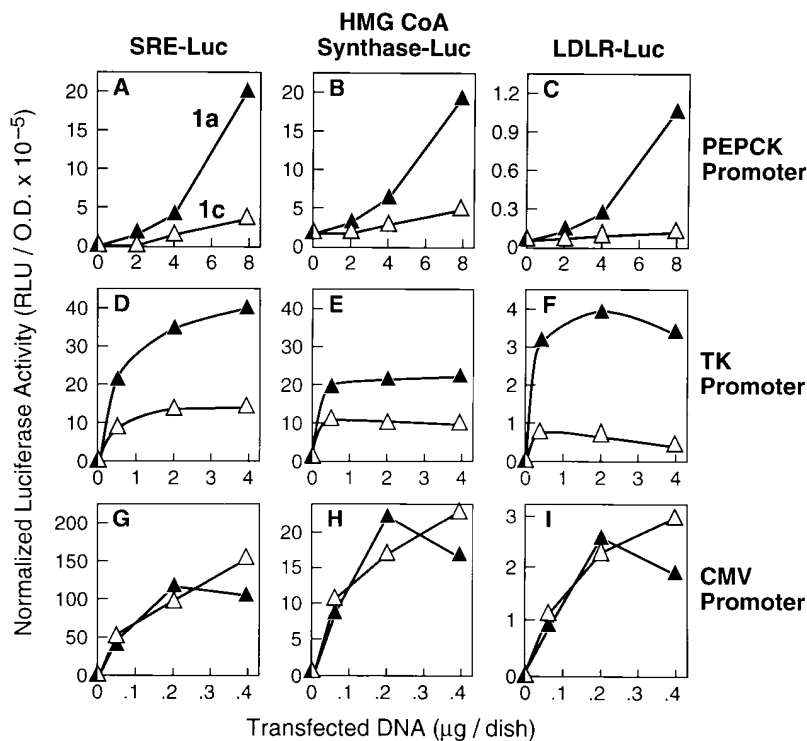
Figure 6. Stimulation of different luciferase reporter genes by transfected SREBP-1a460 or SREBP-1c436 driven by three different promoters. 293 cells (4×10^5 cells per 60-mm dish) were set up for experiments and transfected with the indicated amounts of the SREBP-1a460 (*filled triangles*) or SREBP-1c436 (*open triangles*) cDNA plasmid driven by the PEPCK promoter (*A–C*), TK promoter (*D–F*), or CMV promoter (*G–I*) as described in Methods. A reporter plasmid (0.5 μg) in which the luciferase gene was driven by a synthetic SRE-1 (pSRE-Luc) (*A, D, G*), native HMG CoA synthase promoter (pHMG-CoASynthase-Luc) (*B, E, H*), or native LDL receptor promoter (pLDLR-Luc) (*C, F, I*) was also simultaneously transfected together with the pCMVβ-gal reference plasmid (0.2 μg). After transfection, the cells were cultured in medium A supplemented with 10% fetal calf serum, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol to suppress endogenous SREBP activity. After incubation for 16 h, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of duplicate incubations.
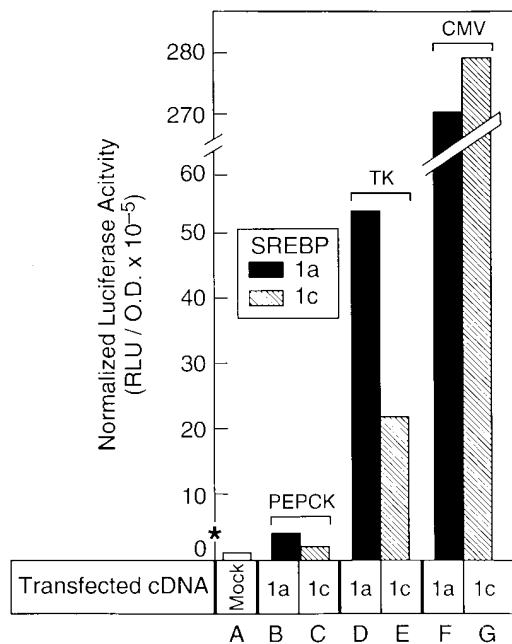
Figure 7. Stimulation of fatty acid synthase–luciferase reporter gene by transfected SREBP-1a460 and SREBP-1c436 driven by three different promoters. 293 cells (4×10^5 cells per 60-mm dish) were set up for experiments as described in Methods and transfected with 8, 4, or 1 μg of an expression plasmid encoding SREBP-1a460 (*closed bars*) or SREBP-1c436 (*shaded bars*) driven by the PEPCK promoter (bars *B* and *C*), TK promoter (bars *D* and *E*), or CMV promoter (bars *F* and *G*), respectively. In *A*, vector DNA containing no SREBP sequence was transfected to determine the transcriptional activity of endogenous SREBPs. After transfection, all cells were cultured in medium A supplemented with 10% fetal calf serum, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol. After incubation for 16 h,

expression was driven by the TK promoter, luciferase activity reached a plateau when the amount of SREBP cDNA exceeded 1 μg/dish (Fig. 6, *D–F*). The level of this plateau was always higher with SREBP-1a460 than with SREBP-1c436. With the CMV promoter, SREBP-1a460 was equal to SREBP-1c436 at all concentrations tested (Fig. 6, *G–I*). The relative effects of each SREBP on the SREx3, HMG CoA synthase, and LDL receptor promoter elements were always similar. However, the absolute levels of luciferase varied. Luciferase activity was always highest for the SREx3 element followed by the HMG CoA synthase element. The LDL receptor promoter was considerably weaker than the other two. Interestingly, the SREx3 element has three copies of the SRE-1; the HMG CoA synthase promoter has two copies (26); and the LDL receptor promoter has one (31).

The fatty acid synthase promoter behaved similarly to the classic SRE-containing promoters in its response to the SREBP-1 constructs (Fig. 7). Using a fragment of the fatty acid synthetase promoter to drive luciferase expression, we found that SREBP-1a460 was more potent than SREBP-1c436 when the two SREBPs were expressed at relatively low levels (PEPCK and TK promoters). When the two SREBPs were expressed at high levels (CMV promoter), their effects on FAS-

the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of duplicate incubations. The asterisk on the vertical axis denotes the transcriptional activity of endogenous SREBPs in mock-transfected cells that were incubated in the same experiment under conditions of sterol depletion (medium A supplemented with 5% lipoprotein-deficient serum, 50 μM compactin, and 50 μM sodium mevalonate).

driven transcription were indistinguishable. Again, the asterisk on the ordinate of Fig. 7 indicates the level of FAS-luciferase expression when endogenous SREBPs were activated by sterol deprivation. This level is approximately the same as the level attained when transcription was stimulated by the PEPCK-driven SREBP-1a construct.

Discussion

The major finding of the current studies is that the NH_2-terminal fragment of SREBP-1c is much less potent than the NH_2-terminal fragment of SREBP-1a in stimulating transcription of all known SREBP-responsive genes. This observation, initially made in livers of transgenic mice, also applies to transfected cells when the level of SREBP expression is restricted to near-physiologic levels. At extremely high levels of expression, such as those attained when SREBP transcription is driven by the CMV promoter, the difference between SREBP-1a and SREBP-1c disappears.

In cultured cells the levels of SREBP-1 expression attained by the weak PEPCK promoter appear to approximate the levels of endogenous SREBPs in cells. This conclusion is based on the observation that the PEPCK-driven constructs produce sufficient SREBP-1a to elicit a level of reporter gene expression that equals the level seen in sterol-deprived cells that have not been transfected with SREBPs (indicated by asterisks in Figs. 5 and 7). Under induced conditions in untransfected cells the amount of active SREBP in the nucleus therefore must be about the same as the amount that is found in the nucleus of suppressed cells after transfection with the PEPCK-driven promoters. At these levels of expression only SREBP-1a is active, and SREBP-1c is relatively silent (see Figs. 5 and 7). Although we cannot make such a direct comparison in the liver, immunoblot analysis suggests that the amount of nuclear SREBP-1c in the transgenic mouse liver is at least as high as the amount of endogenous SREBP-1 in liver nuclei of wild-type mice (data not shown). At these concentrations the NH_2-terminal fragment of SREBP-1a is very active, and the NH_2-terminal fragment of SREBP-1c is much less active.

These observations assume potential significance in light of a recent quantitative analysis of the levels of expression of SREBP-1a and SREBP-1c in cultured cells and various tissues of mice and humans (see companion paper [8]). In multiple lines of cultured cells the predominant form of SREBP-1 was always SREBP-1a. In the most extreme cases, such as mouse 3T3L1 adipocytes, the SREBP-1c transcript was undetectable. In striking contrast, SREBP-1c transcripts outnumbered SREBP-1a transcripts by a ratio of at least 6:1 in livers of mice and humans. SREBP-1c transcripts were also predominant in other organs, including adrenal gland, white and brown fat, brain, and muscle (8).

The predominance of the more active SREBP-1a transcripts in cultured cells is consistent with the relatively large demand of these cells for cholesterol and fatty acids to support cell growth. In such cells the proteolytic cleavage of SREBP-1a and SREBP-2 is accelerated coordinately by sterol depletion (6, 9), and both proteins should activate transcription of genes that supply cholesterol. The predominance of the weak SREBP-1c transcript in liver is harder to rationalize, but it may provide a partial explanation for the previous finding that the proteolytic cleavage of SREBP-1 (now known to be SREBP-1c) and SREBP-2 tend to be regulated in opposite directions

in liver under conditions of sterol deprivation (23). In hamsters on a normal chow diet, an earlier study demonstrated high levels of the NH_2-terminal fragment of SREBP-1, but not SREBP-2, in liver nuclear extracts (23). When the livers were depleted of sterols by treatment of the animals with lovastatin and Colestipol, the amount of nuclear SREBP-1 declined, and the amount of nuclear SREBP-2 increased in parallel with an increase in the mRNAs encoding LDL receptors and enzymes of cholesterol synthesis. Similar observations have been made in studies of C57BL/6J mice (our unpublished observations).

Why does the liver produce a weak activator like SREBP-1c in the basal state? An answer may lie in the observation that SREBP-1c retains a slight but definite ability to stimulate transcription of the genes involved in fatty acid synthesis, but not cholesterol synthesis (Fig. 3). It is possible that the basal level of SREBP-1c supports a basal level of fatty acid synthesis at a time when the demand for cholesterol synthesis is low. When the demand for cholesterol increases, SREBP-1c is replaced by SREBP-2, which may be a stronger activator of SRE-1–dependent promoters. Testing this hypothesis will require production of transgenic mice that produce the dominantly active NH_2-terminal fragment of SREBP-2 in the liver. The prediction is that this fragment of SREBP-2 will be more active than the corresponding fragment of SREBP-1c.

Although these studies show that SREBP-1c is a weaker activator than SREBP-1a, they do not reveal the mechanism for this difference. Fig. 6 reveals that the kinetics of transcriptional activation as a function of SREBP concentration is complex. This is most easily seen when the SREBPs were produced under the influence of the TK promoter. As the amount of the transfected cDNAs increased, the amount of luciferase activity failed to increase, suggesting that the SRE-1–containing promoters had been saturated. The level of this plateau was higher for SREBP-1a than for SREBP-1c. Yet when the amount of SREBPs was increased by an additional order of magnitude through use of the CMV promoter, the amount of luciferase activity increased further, especially with the synthetic promoter containing three copies of SRE-1. With all three SREBP-responsive promoters, the activity of SREBP-1c rose disproportionately so that it became equal to that of SREBP-1a (Fig. 6).

The simplest explanation for the relative inactivity of SREBP-1c at low concentrations lies in the relatively short negatively charged sequence at its NH_2 terminus. Previous studies have shown that complete deletion of this sequence abolishes transcriptional activation and converts SREBP-1 into a pure antagonist (10). A partial reduction in this sequence, as occurs in SREBP-1c, may partially reduce transcriptional activation by reducing the interaction with a required protein that functions as a coactivator (32). This simple explanation is unlikely to provide the whole answer because it does not indicate how the NH_2-terminal fragment of SREBP-1c becomes as active as SREBP-1a when massively overexpressed through the CMV promoter. If SREBP-1c interacts weakly with a coactivator and if the SRE-1 sequence becomes saturated with SREBP-1c, then the rate of transcription should be less than it is when the promoter is saturated with SREBP-1a.

It is likely that the complex kinetics of action of SREBP-1a and -1c reflects different heterodimerizing partners that come into play at different concentrations of these activators. The SREBPs, like other basic-helix-loop-helix-leucine zipper transcription factors (33), are believed to form both homodimers

and heterodimers, although this has not been studied in detail. In addition to the potential for heterodimerization, the SREBPs are known to act in concert with activators such as Sp1 (16, 17) or NF-Y (18). The length of the NH_2-terminal acidic domain may influence these interactions. It may also influence the rate at which complexes of transcription-activating proteins form or dissociate. A full resolution of these questions awaits further studies of SREBP action in cell-free systems.

Acknowledgments

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