Sterol Regulation of Fatty Acid Synthase Promoter

COORDINATE FEEDBACK REGULATION OF TWO MAJOR LIPID PATHWAYS*

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The gene encoding fatty acid synthase, the essential multi-functional enzyme of fatty acid biosynthesis, is shown to be regulated by cellular sterol levels similar to genes that encode important proteins of cholesterol metabolism. We show that expression of the endogenous FAS gene is repressed when regulatory sterols are included in the culture medium of HepG2 cells and that the FAS promoter is subject to similar regulation when fused to the luciferase reporter gene. Mutational studies demonstrate that sterol regulation is mediated by binding sites for the sterol regulatory element-binding protein (SREBP) and transcription factor Sp1, making it mechanistically similar to sterol regulation of the low density lipoprotein receptor gene. It is also demonstrated that SREBP and Sp1 synergistically activate the FAS promoter in Drosophila tissue culture cells, which lack endogenous Sp1. These experiments provide key molecular evidence that directly links the metabolism of fatty acids and cholesterol together.

Coordinate regulation of fatty acid and cholesterol accumulation is essential for balanced membrane biosynthesis and turnover to accommodate metabolic fluctuations that occur during normal cellular growth. Also, these two important lipids are simultaneously required in the liver for regular-ordered assembly of very low density lipoprotein particles, which deliver their lipid load of cholesterol and fatty acids from the liver to other sites in the body to maintain lipid homeostasis (1, 2).

Co-regulation of genes that encode important enzymes of both fatty acid and cholesterol metabolism would be an ideal way to coordinate lipid regulation, and recent work has identified a family of transcriptional regulatory proteins that could link the two pathways together (3–5). Sterol regulatory element-binding proteins 1 and 2 (SREBP 1 and 2)1 are highly related proteins that bind to the same cis-acting elements in the LDL receptor and HMG-CoA synthase promoters and activate expression only when cellular sterol stores are depleted (3, 5, 6). The cDNAs for both were cloned based on amino acid sequence information obtained from the purified proteins (3, 5).

Independently, the rat equivalent of SREBP-1 was cloned from an adipocyte cDNA expression library (4). The rat mRNA was expressed at very high levels in brown fat and was also abundant in white fat and liver. The rat SREBP-1 mRNA was also induced during adipocyte differentiation in cell culture, so it was named the adipocyte determination- and differentiation-dependent factor 1 (ADD1). These observations suggested that SREBP-1/ADD1 is a regulator of genes that are important for lipid accumulation in the adipocyte.

The above studies indicate that the activity of SREBP-1/ADD1 may provide a direct link between the regulation of cholesterol and fatty acid metabolism. In the present paper we demonstrate that expression of the mRNA for fatty acid synthase (FAS), an essential enzyme of fatty acid biosynthesis, is regulated by sterols in a manner similar to genes that encode important proteins of cholesterol metabolism. Moreover, in the region of the FAS promoter that is required for sterol regulation there are two binding sites for SREBP-1. One of them is located close to a binding site for the generic factor Sp1, and we show this site is crucial for sterol regulation. The involvement of SREBP-1 and Sp1 in activation of the FAS promoter is reminiscent of the LDL receptor promoter where both proteins are essential for sterol regulation as well. These experiments provide strong evidence for a direct molecular connection between the regulation of two different classes of cellular lipids that are both required for cellular growth and normal lipoprotein metabolism.

EXPERIMENTAL PROCEDURES

Cells and Media—CV-1 cells were obtained from Dr. K. Cho (University of California, Irvine). HepG2 cells were obtained from the ATCC. All cell culture materials were obtained from Life Technologies Inc. Lipoprotein-deficient serum was prepared by ultracentrifugation as described (7). Cholesterol and 25-OH cholesterol were purchased from Steraloids Inc., and stock solutions were dissolved in absolute ethanol.

Cell Culture—HepG2 cells were plated at 225,000 cells/100-mm dish on day 0. Two days later the dishes were washed three times with phosphate-buffered saline and split into two groups. One set of dishes were refed induced medium (DMEM containing 10% lipoprotein-depleted serum) and one set was fed suppressed medium (same but containing 10 μg/ml cholesterol and 1 μg/ml 25-OH cholesterol) media. Following a 24-h incubation the cells were harvested for RNA by the guanidine lysis procedure, followed by cesium chloride gradient sedimentation (8).

PCR Cloning of FAS cDNA—cDNA was prepared from HepG2 RNA with the aid of a kit from Invitrogen. The cDNA was used as a template with PCR primers designed to two highly conserved regions of the fatty acid synthase mRNA that were chosen by aligning the rat and chicken FAS cDNA sequences. The 5’ primer (5’GACACAGCCTGCTCCTC/ T/AG-3’) was designed to hybridized to a 20-base pair region beginning at nucleotide number 249 of the rat cDNA (where the A of the ATG start...
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Sterol activity in relative light units was divided by the concentration containing 10% heat-inactivated fetal bovine serum. They were seeded at 3 × 10^5 cells/60-mm dish and transfected by a standard calcium phosphate coprecipitation method (13). Plasmid pPACSp1 containing a luciferase reporter construct and the CMV2 early promoter in the pCMV5 vector (a gift of Dr. David Russell of the University of Texas Southwestern Medical Center). The indicated plasmids were excised from PCR II and inserted into the EcoRI site of m13mp18. A clone containing the DNA strand equal to the mRNA was used to generate a probe for S1 nuclease analysis of FAS mRNA.

**S1 Nuclease and Primer Extension Analyses**—S1 nuclease mapping was performed with 10-μg aliquots of total RNA prepared from HepG2 cells that were cultured as described above. The details of the S1 nuclease protocol have been described previously (9, 10). Expression of HMG-CoA synthase and ribosomal protein S17 were analyzed by a primer extension assay with aliquots of the same samples used for FAS RNA analyses. The identity of the primers and the protocol have been described previously (10).

**FAS Promoter Plasmids**—The rat FAS promoter FAS(−1594)CAT (11) was a kind gift of Dr. Steve Clarke (then of Colorado State University; present address University of Texas at Austin). The FAS promoter fragment from −1594 to +65 was excised and inserted upstream of the luciferase gene in pGL2 basic (Promega). To generate −250, −150, and −135 derivatives, PCR primers were designed to hybridize at the corresponding positions and coupled with the common downstream primer at +65. The PCR fragments were re-cloned into the pGL2 basic vector. To generate plasmids D and E (containing the FAS sequence from −150 to −43 or −150 to −73, respectively, linked to a generic TATA sequence), the same PCR primer used to generate −150 was used in combination with a primer designed to hybridize and delete sequences 3′ to position −43 or −73. These fragments were then inserted just upstream of a generic TATA box sequence positioned upstream of the luciferase coding sequence. This minimal “TATA only” plasmid has been described previously (12).

**Transient DNA Transfections—** CV-1 cells were grown in DMEM containing 10% fetal bovine serum and plated on day 0 in 60-mm dishes at 125,000 cells/dish. On day 1 the cells were refed the same medium and transfected by the calcium phosphate co-precipitation method. Precipitates contained 20 μg each of test plasmid and CMV2 β-galactosidase plus 10 μg of salmon sperm DNA in 2 ml of solution. 0.5 ml were added to each of four dishes, which were incubated for 12–16 h at 37 °C and 7% CO_. The dishes were washed three times with phosphate-buffered saline, and duplicate dishes were refed either induced (DMEM containing 10% lipoprotein-depleted serum) or suppressed (same but containing 10 μg/ml cholesterol and 1 μg/ml 25-OH cholesterol) media. Following a 24-h incubation, the cells were harvested by scraping; cells from duplicate dishes were pooled, and extracts were prepared by three freeze-thaw cycles. For activation studies with overexpressed SREBP-1, the cDNA sequence encoding amino acids 1–490 of SREBP-1 was cloned by the polymerase chain reaction from HepG2 cell mRNA as described previously (12) and inserted downstream from the cytolemal virus early promoter in the pCMV5 vector (a gift of Dr. David Russell of the University of Texas Southwestern Medical Center). The indicated amount of this plasmid was included along with the individual FAS-promoter constructs described above. The resulting plasmids have been described previously (12). The FAS mRNA expression was regulated by cellular sterol levels. Total cell RNA was prepared from HepG2 cells cultured in the presence or absence of regulatory sterols as described under “Experimental Procedures.” Triplicate aliquots of RNA from induced (lane 1) or suppressed (lane 2) were analyzed for FAS mRNA by an S1 nuclease assay (9, 10). Probe alone (neg) digested with S1 nuclease is in lane 3. Middle, 20-μg aliquots of the same RNA as above were used to detect transcription of ribosomal protein S17 by a primer extension assay (10). Bottom, 50-μg aliquots of the same RNA as above were used for HMG-CoA synthase mRNA detection by a primer extension assay that generates two 5′ ends (10). This experiment is representative of three separate experiments, and the average -fold regulation value of 3.6-fold (± 0.3) for FAS was determined by analysis with a Bio-Rad PhosphorImager.

**RESULTS**

SREBP-1 was independently identified as a bHLHzip protein involved in activation of sterol-regulated genes and for adipocyte cell differentiation. This suggests that it may link the regulation of sterol and fatty acid metabolism. Therefore, we are interested in characterizing SREBP target genes that are critical proteins of sterol and fatty acid metabolism. The sterol-regulated LDL receptor promoter contains a SRE-1 element, which binds SREBP and is required for sterol regulation. We found an identical match to the LDL receptor 10-base pair SRE-1 in the promoter for rat FAS and we determined that recombinant SREBP-1 binds avidly to this site (see below). The mammalian FAS is a multi-functional enzyme, which contains all of the enzymatic activities required for each cycle of fatty acid chain elongation in one polypeptide chain (16, 17). The FAS gene and its promoter have been isolated and partially characterized previously (18, 19). In addition to the SRE homology, there are three “E-box” sites in the FAS promoter. The E-box is a consensus recognition site for the bHLHzip proteins such as SREBP, and the one at −500 was identified previously as a potential target of SREBP-1/ADD1 activation (4).

If SREBP binding to the FAS promoter were physiologically relevant, then the FAS mRNA should be regulated by sterols in a manner similar to other SREBP activated promoters such as those for the LDL receptor and HMG-CoA synthase. Therefore, we prepared mRNA from human HepG2 cells that were cultured in the presence or absence of regulatory sterols and analyzed FAS mRNA expression by a S1 nuclease protection assay (9, 10). The results of these experiments are summarized in Figs. 1 and 2. Lane 1 represents HMG-CoA synthase, lane 2 represents FAS. The ratio of luciferase activity (activity/h) for each extract was determined by analysis with a Bio-Rad PhosphorImager.

**Enzyme Assays**—Luciferase activities were measured in a luminesimeter with a luciferin reagent from Promega Biotech. β-Galactosidase assays were performed by a standard colorimetric procedure with 2-nitrophenyl-β-D-galactopyranoside as substrate (14). The ratio of luciferase activity in relative light units was divided by the β-galactosidase activity (activity/h) for each extract. The data presented here represent the data from several independent transfections performed in duplicate for each plasmid in Figs. 2 and 4 (see figure legends for the number of individual experiments).

**Protein Purification**—S1 and the recombinant SREBP-1 protein were purified as described (12). The purity and concentration of the purified proteins were assessed by SDS-PAGE analysis performed with marker proteins followed by staining with Coomassie Blue.

**DNAase I Footprinting**—[32P]DNA probes were prepared from the indicated plasmids, incubated with Sp1, SREBP-1, or both proteins together and digested with DNase I as described (9, 15).

Sterols | + | −
--- | --- | ---
Lane 1 | 1 | 2 | 3
FAS
S17
HMG CoA synthase

**Fig. 1. FAS mRNA expression is regulated by cellular sterol levels.** Total cell RNA was prepared from HepG2 cells cultured in the presence or absence of regulatory sterols as described under “Experimental Procedures.” Triplicate aliquots of RNA from induced (lane 1) or suppressed (lane 2) were analyzed for FAS mRNA by an S1 nuclease assay (9, 10). Probe alone (neg) digested with S1 nuclease is in lane 3. Middle, 20-μg aliquots of the same RNA as above were used to detect transcription of ribosomal protein S17 by a primer extension assay (10). Bottom, 50-μg aliquots of the same RNA as above were used for HMG-CoA synthase mRNA detection by a primer extension assay that generates two 5′ ends (10). This experiment is representative of three separate experiments, and the average -fold regulation value of 3.6-fold (± 0.3) for FAS was determined by analysis with a Bio-Rad PhosphorImager.
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assay (Fig. 1, top). The results demonstrated FAS mRNA expression was higher when cells were cultured in the absence of regulatory sterols compared to when cells are fed sterols. The expression of HMG-CoA synthase, an important regulated enzyme of cholesterol biosynthesis, was similarly regulated (Fig. 1, bottom); however, expression of the mRNA for the non-sterol-regulated ribosomal protein S17 was present at equal levels in both RNA samples (Fig. 1, middle). Therefore, FAS mRNA expression is regulated by cellular sterol levels similar to that observed for important genes of cholesterol homeostasis.

Next, the sequence of the rat FAS promoter was fused to the luciferase reporter gene and analyzed by a standard transient expression assay for sterol regulation (Fig. 2a). The ratio of luciferase activity obtained for the wild-type promoter from transfected cells cultured in the absence and presence of regulatory sterols provided a measure of sterol regulation. The full-length promoter exhibited 2.1-fold higher activity in cells that were cultured in the absence of lipoproteins. A deletion of all of the sequence down to −150 resulted in an increase in the degree of regulation to 4.8-fold. This magnitude of regulation is similar to that observed for the endogenous FAS mRNA (Fig. 1) and for the wild-type HMG-CoA synthase and LDL receptor promoters (see the legend to Fig. 2). These results indicated the sequence required for sterol regulation of the FAS promoter is located within the proximal 150 base pairs of the promoter. Thus, the E-box consensus sites upstream of −150 are dispensable for sterol regulation. In addition, the normalized data (Fig. 2b) show that deletion of all sequence upstream of −150 does not drastically affect the absolute level of expression. Similar results were obtained by Moustaïd et al. (19). The sequence remaining in the sterol-regulated −150 construct included the SRE-1 element consensus, an Sp1 binding site, and the remaining proximal E-box motif (Fig. 2a).

Next, we made several more promoter constructs to further define the sequence elements responsible for sterol regulation. The most significant ones are shown in Fig. 2. When we deleted 15 bases from the 5′ side of the −150 construct sterol regulation was reduced from 4.8- to 3-fold (plasmid C). This suggests the SRE-1 site is important for maximal sterol regulation. This plasmid also exhibited a significant drop in basal promoter activity (Fig. 2b), suggesting the SRE homology is required for maximal expression. To evaluate the role of the remaining E-box site, we constructed two additional plasmids. First, the sequence from −150 to −43 was fused to a generic TATA element to provide a basal promoter. This removed the normal FAS TATA box region but retained both the proximal E-box and the SRE-1 element. This plasmid was regulated efficiently by sterols (Fig. 2, plasmid D). However, when we deleted the sequence between −75 and −43 from the 3′ end of this construct, sterol regulation was totally lost (plasmid E).

To determine if the region of the FAS promoter that was required for sterol regulation was directly responsive to SREBP-1, we performed co-transfection studies with plasmid D or E along with a plasmid that encoded amino acids 1–490 of the SREBP-1 protein. This portion of SREBP-1 contains all of the information required for transcriptional activation (6, 12). The results showed the activity of plasmid D was significantly stimulated by co-transfected SREBP-1, however, plasmid E was not stimulated (Fig. 2c). Thus, deletion of the proximal E-box abolished both sterol regulation and activation by SREBP-1. Importantly, when the activation of the FAS promoter (plasmid D) by varying amounts of co-transfected SREBP-1 plasmid was directly compared to activation of the LDL receptor, a very similar dose response was obtained (Fig. 2d). Taken together, all of the above data indicate that the E-box region at −60 is critical for sterol regulation and activation by SREBP-1, the magnitude of sterol regulation and activation by SREBP-1 are similar to that of the LDL receptor, and that the SRE-1 element at −150 is required for maximal regulation and maximal expression.

A DNA probe end-labeled at −250 and extending past the mRNA cap site was used to evaluate SREBP-1 binding in this region by a DNase I footprinting technique. Recombinant SREBP-1 protein bound to both the SRE-1 site at −150 and the proximal E-box motif (Fig. 3a, lane 2), confirming that SREBP-1 binds to both sites that are important for sterol regulation of the FAS promoter. SREBP cannot activate expression efficiently by itself even when its binding site is present in multiple copies (12, 20). In the LDL receptor promoter, there is an Sp1 site close to the SREBP binding site that is also important for sterol regulation. Interestingly, we noted a potential Sp1 binding site at −90 of the FAS promoter, which is close to the proximal SREBP-1 binding site E-box (Figs. 2a and 3b), and we determined that purified Sp1 bound to this site (Fig. 3a, lane 4). When both SREBP-1 and Sp1 were incubated with the DNA probe, both binding patterns were observed (Fig. 3a, lane 3). The recognition sites for SREBP-1 and Sp1 that are included within the footprint regions are indicated on the FAS promoter sequence in Fig. 3b. Based on these studies, it is likely that SREBP and Sp1 function together to mediate sterol-dependent activation of the FAS promoter similar to the LDL receptor promoter.

To specifically evaluate whether the FAS promoter was directly activated by SREBP-1 and Sp1 together, we performed co-transfection studies with SREBP-1 and Sp1 expression plasmids in Drosophila SL2 cells. These cells lack endogenous Sp1, yet they retain all of the accessory regulatory proteins required to respond to exogenously expressed Sp1 (13). We have used this system previously to demonstrate that SREBP-1 and Sp1 synergistically activate the LDL receptor promoter (12, 21). When plasmid D was transfected into SL2 cells alone, a basal level of promoter activity was observed (Fig. 4, lane 1). The addition of plasmids that express either Sp1 or SREBP alone slightly stimulated promoter activity (Fig. 4, lanes 2 and 3). When both expression constructs were included, the activity of the FAS promoter was stimulated 35-fold (lane 4). However, plasmid E, which removes the proximal SREBP binding E-box site, was not activated (lanes 5–8). These experiments confirm that SREBP and Sp1 activate the FAS promoter synergistically and that SREBP-1 and Sp1 are both required for activation.

DISCUSSION

These experiments demonstrate that the gene encoding the important multi-functional enzyme of fatty acid biosynthesis, fatty acid synthase, is regulated by cellular sterols in a manner similar to important genes of cholesterol homeostasis (Fig. 1). The promoter for the FAS gene contains two recognition sites for SREBP that are essential for maximal activity and sterol regulation (Figs. 2–4). One of these important SREBP sites is close to a binding site for the ubiquitous transcription factor Sp1. This is similar to the LDL receptor promoter, where it was previously shown that Sp1 and SREBP are both essential for sterol regulation and promoter activation (12, 22, 23). The present studies demonstrate that SREBP and Sp1 synergistically activate the FAS promoter (Fig. 4) similar to our previous studies for the LDL receptor promoter (12). One of the SREBP-1 sites is a classic SRE-1 site, which is identical to the SREBP-1 site of the human LDL receptor promoter. The other important SREBP-1 site contains an E-box motif, which is the typical recognition site for basic helix-loop-helix proteins like SREBP-1. Kim et al. (24) have shown that SREBP-1 is a unique bHLHZip protein that can bind to both SRE sites and a subset of E-box sites. This dual specificity is
FIG. 2. Regulation of the FAS promoter by sterols and identification of sterol regulatory elements that are activated by SREBP-1. Panel a, promoter deletion series. A scale for the DNA sequence of the rat FAS promoter is shown at the top (18). Notable sequence features are diagrammed on the line corresponding to the wild-type promoter (construct A). There are three E-box consensus sequence elements (CANNNTG), a 10-base pair exact match to the LDL receptor SRE-1, and an Sp1 site that is discussed in the text. Mutant derivatives were made by PCR-assisted mutagenesis as described under “Experimental Procedures.” The sequence retained in each is indicated by the thin line. All of the plasmids were analyzed for sterol regulation by a transient DNA transfection assay (12). -Fold regulation is the ratio of normalized luciferase activity expressed when transfected cells were cultured in the absence or presence of regulatory sterols. The non-sterol-regulated CMV promoter b-galactosidase expression construct was included as an internal control for normalization. The wild-type HMG-CoA synthase and LDL receptor promoters were analyzed similarly, and -fold regulation values for these were 7.9 and 3.7, respectively. The mean -fold regulation value for each plasmid and the standard error for the indicated number of experiments are presented. Panel b, relative activity of FAS promoter constructs. The normalized luciferase activity from cells cultured in the absence of regulatory sterols and transfected by the longest FAS-Luc construct analyzed in panel a was set at 100% and all of the values are plotted relative to this value. Plasmids D and E were compared directly to each other because they are hybrid promoters containing the FAS sequence fused to a generic TATA box. I and S refer to whether the transfected cells were cultured in the absence (I) or presence (S) of regulatory sterols prior to harvest as described under “Experimental Procedures.” Panel c, activation of FAS promoter by co-expressed SREBP-1. CV-1 cells were cultured in DMEM and 10% (v/v) fetal bovine serum and analyzed by a standard transient transfection assay for luciferase expression from plasmid D or E, except that 50 ng of a CMV promoter-based construct expressing amino acids 1–490 of the human SREBP-1 protein was included. -Fold activation corresponds to the ratio of normalized activity obtained with SREBP-1 co-transfection divided by the activity in the absence of co-transfection. In panels b and c, the mean -fold regulation value for each plasmid and the standard error for four independent experiments is presented. Panel d, activation of FAS promoter by over expression of SREBP-1. HepG2 cells were cultured in DMEM and 10% (v/v) fetal bovine serum and analyzed by a standard transient transfection assay for luciferase expression from FAS-150 or the wild-type LDL receptor promoter, except that the SREBP-1 expression plasmid described above was included in increasing concentrations as indicated on the abscissa. -Fold activation is calculated as above.
mediated in part by the substitution of a tyrosine residue in a position that in most other bHLH proteins is occupied by an arginine.

The experiments reported here provide the first example of a classic E-box imparting sterol regulation onto a specific promoter. In fact, in the sterol regulatory region of the FAS promoter there is an archetypal SRE-1 element and the E-box, which both bind SREBP-1. An important question, therefore, is why the E-box is more important for sterol regulation in the FAS promoter than the SRE-1 element? The answer to this question is probably due to some basic properties of the SREBP-1 protein. SREBP-1 apparently cannot activate expression by itself (see Fig. 4), even when synthetic promoters are constructed that contain multiple tandem SRE-1 elements (12, 20). A binding site for a neighboring ubiquitous transcription factor is required. As mentioned above, this ubiquitous factor is Sp1 for the LDL receptor and ACC promoters where the two proteins activate expression synergistically. Since the Sp1 site is closer to the E-box in the FAS promoter, it would probably be easier for Sp1 and SREBP-1 to communicate together when bound at these sites.

Interestingly, Moustaid et al. (19) have identified an insulin-responsive cis-acting site in the FAS promoter, which is in the same region as the E-box recognition sequence identified here as being essential for sterol regulation by SREBP-1. It is possible that SREBP-1 or another bHLHZip protein is responsible for the insulin response, and further studies should clarify this issue.

The involvement of SREBP-1 in the expression of key fatty acid synthetic enzymes as well as for important proteins of cholesterol homeostasis would ensure coordinated levels of cholesterol ester and triglyceride for proper assembly of very low density lipoprotein particles in the liver (1). The fact that SREBP-1 was independently identified as a protein expressed in differentiating adipocytes and involved in sterol regulation suggested it might perform this bridging function, and the
present studies have identified a key target gene to link the two important lipid pathways together. The co-regulation would also be important in growth regulation of all mammalian cells to provide proper amounts of fatty acids and cholesterol for membrane synthesis and assembly.

Studies in both experimental animals and humans have demonstrated that the quantity and quality of fatty acids in the diet have profound effects on very low density lipoprotein secretion, LDL receptor mRNA expression, and plasma cholesterol concentrations that are seemingly independent of the amount of cholesterol in the diet (25–27). It will be interesting to determine if these effects are mediated through alterations in the activity of SREBP-1 and/or -2.

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