Sterol Response Element-binding Protein 1c (SREBP1c) Is Involved in the Polyunsaturated Fatty Acid Suppression of Hepatic S14 Gene Transcription*

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Polyunsaturated fatty acids (PUFA) suppress hepatic lipogenic gene transcription through a peroxisome proliferator activated receptor α (PPARα)- and cyclooxygenase-independent mechanism. Recently, the sterol response element-binding protein 1 (SREBP1) was implicated in the nutrient control of lipogenic gene expression. In this report, we have assessed the role SREBP1 plays in the PUFA control of three hepatic genes, fatty acid synthase, L-pyruvate kinase (LPK), and the S14 protein (S14). PUFA suppressed both the hepatic mRNA_SREBP1 through a PPARα-independent mechanism as well as SREBP1c nuclear content (nSREBP1c, 65 kDa). Overexpression of primary hepatocytes revealed a differential sensitivity of the fatty acid synthase, S14, and LPK promoters to nSREBP1c overexpression. Of the three promoters examined, LPK was the least sensitive to overexpressed nSREBP1c. Promoter deletion and gel shift analyses of the S14 promoter localized a functional SREBP1c cis-regulatory element to an E-box-like sequence (−159 to −131) within the S14 PUFA response region. Although overexpression of nSREBP1c significantly reduced PUFA inhibition of S14CAT, overexpression of other factors that induced S14CAT activity, such as steroid receptor co-activator 1 or retinoid X receptor α, had no effect on S14CAT PUFA sensitivity. These results suggest that PUFA regulates hepatic nSREBP1c, a factor that functionally interacts with the S14 PUFA response region. PUFA regulation of nSREBP1c may account for the PUFA-mediated suppression of hepatic S14 gene transcription.

Polyunsaturated fatty acids (PUFA)§ (both n-3 and n-6) are potent inhibitors of hepatic de novo lipogenesis. PUFA suppression of de novo lipogenesis is due to inhibition of transcription of key genes involved in lipid synthesis, including acetyl-CoA carboxylase, fatty acid synthase, stearyl-CoA desaturase 1, malic enzyme, and L-pyruvate kinase. Long chain n-3 fatty acids like eicosapentaenoic acid (20:5, n-3) also enhance fatty acid oxidation, decrease triglyceride synthesis, and reduce serum triglycerides. By regulating these pathways, PUFA promote a shift from fatty acid synthesis and storage to oxidation (for review see Ref. 1).

The molecular basis for PUFA regulation of hepatic gene expression is complex and involves several mechanisms. For example, PPARα is a ligand-activated nuclear receptor that regulates the expression of mitochondrial, peroxisomal, and microsomal enzymes involved in fatty acid metabolism. Certain hypolipemic drugs like fenofibrate and gemfibrozil as well as fatty acids activate PPARα (2). Studies using the PPARα null mouse indicate that both PPARα-dependent and PPARα-independent pathways are operative in the liver (3). For example, PPARα is required for n-3 PUFA activation of acyl-CoA oxidase (AOX) and cytochrome P450–4A (CYP4A) gene expression, but PPARα is not required for the PUFA-mediated suppression of lipogenic gene expression.

Other studies have shown that hepatic cyclooxygenase expression is restricted to non-parenchymal cells, e.g., Kupffer and endothelial cells (4). Interestingly, hepatic parenchymal cells possess G-protein-linked receptors that respond to prostanooids like prostaglandin E2 and prostaglandin F2α. Prostaglandin E2 treatment of these cells suppresses mRNAs encoding fatty acid synthase (FAS) and the S14 protein (S14). However, n-3 and n-6 PUFA suppression of hepatic lipogenic gene expression is independent of cyclooxygenase (4). The fact that PUFA regulation of lipogenic gene expression is independent of both cyclooxygenase and PPARα implicates other routes for control.

Recent studies by Worgall et al. (5) provide an important clue to another factor involved in PUFA regulation of gene expression. Their studies suggest that unsaturated fatty acids regulate the nuclear abundance of the sterol response element binding protein-1 (SREBP1). Previous studies show that SREBP1 subtypes played a major role in the hormonal/nutrient control of lipogenic gene expression (6–13). SREBP1s are ~130-kDa proteins attached to the endoplasmic reticulum and nuclear membranes through transmembrane-spanning domains. SREBP1 and SREBP2 are encoded by separate genes, and SREBP1 is expressed as two subtypes, 1a and 1c, which arise from differential promoter and exon usage (13). In liver, SREBP1c is the predominant subtype, whereas SREBP1a is the predominant subtype expressed in nonhepatic tissues and most cell lines (7). Cholesterol regulates proteolytic cleavage of SREBP1a and -2 to release a mature (or nuclear) 65-kDa protein, i.e. nSREBP. nSREBP is a basic helix-loop-helix transcription factor that binds sterol response elements (SRE) and some E-boxes in sterol-responsive genes, e.g. hydroxymethyl-
glutaryl-CoA reductase, low density lipoprotein receptor, and fatty acid synthase. Studies in transgenic mice overexpressing different SREBP1c subtypes suggest that SREBP1 may play a major role in the control of lipogenic gene expression, whereas SREBP2 functions in the regulation of cholesterol synthesis (7–10).

Unsaturated fatty acids reduced the nuclear content of SREBP1c in cultured CV1, CHO, and HepG2 cells (5). The linkage of SREBP1 to lipogenic gene expression and the fact that unsaturated fatty acids affect the nSREBP1 levels suggest fatty acid control of lipogenic gene expression might involve the regulation of nSREBP1 abundance. In this report, we have examined the role hepatic SREBP1c plays in the PUFA regulation of rat hepatic lipogenic gene expression. Our studies show that PUFA down-regulates mRNAsREBP1 in liver and cultured primary hepatocytes but not in 3T3-L1 adipocytes. Like other lipogenic genes (5), PUFA suppress SREBP1 gene expression through a PPARα-independent mechanism. PUFA also suppresses the nuclear content of SREBP1 in cultured primary hepatocytes. We have used the S14 gene as a model for lipogenic gene expression because the hormonal/nutrient control of S14 gene transcription parallels that described for several lipogenic genes (1). Moreover, a PUFA-regulatory region has been identified within the S14 proximal promoter (14). With this model we tested the hypothesis that SREBP1c functionally interacts with the PUFA response region (PUFA RR). Our studies have localized a cis-regulatory target for SREBP1c action within the S14 promoter. Moreover, overexpression of SREBP1c significantly affects PUFA regulation of S14 gene expression. These studies provide compelling evidence for SREBP1c as a principal target for PUFA control of S14 gene transcription and possibly other hepatic lipogenic genes.

MATERIALS AND METHODS

Animals—Male Charles River (CD1) rats were meal-fed a high carbohydrate diet (ICN) supplemented with various fats. The composition of the fats used is shown in Table I (14–16). Genfibrozil and WY14,643 were added to some of the high carbohydrate diets at 0.2 and 0.1%, respectively. Feeding of wild type and PPARα-null mice was described previously (3).

Primary Hepatocyte Preparation, Transfection, and Treatments—Primary hepatocytes prepared by collagenase (Liberase, Roche Molecular Biochemicals) perfusion of rat liver were plated at 3 × 10⁶/60-mm Primaria plate for chlamydomenin acetyltransferase (CAT) assay or 10/100-mm Primaria plate for RNA analysis (3, 4). Cells were plated in Williams E medium containing 10 mM glucose, 10% fetal bovine serum, 10 mM lactate, 200 nM insulin, and 10 mM dexamethasone. After a 4–6-h cell attachment period, medium was removed and replaced with Williams E medium containing 10 mM lactate, 200 mM insulin, 10 mM dexamethasone but without serum.

Cells were transfected with CAT reporter vectors (2 µg/plate) and thyroid hormone receptor β1 expression vector (murine leukemia virus TRβ1, 1 µg/plate) and/or SREBP1 expression vectors (see the legend of Figs. 5, 6, and 9 for details) using Lipofectin® (6.6 µg/µl DNA) (Life Technologies, Inc.). Sixteen h later, cells were treated for up to 48 h with Williams E medium containing 25 mM glucose, 1 mM insulin, 1 µM triiodothyronine, and 10 mM dexamethasone and 250 µM fatty acids (Nu-Chek Prep, Elysian, MN) plus 50 µM albumin (3, 4).

RNA Analysis—Total RNA isolated from rat or mouse liver and primary hepatocytes or 3T3-L1 adipocytes was transferred to nitrocellulose for hybridization with 32PitolDNA for S14, FAS, LPK, SREBP1, or CYP4A2 (3, 4). Hybridization was quantified by PhosphorImager analysis (Molecular Dynamics). Our CYP4A2 probe also detected CYP4A3 (93% homologous to CYP4A2). A full-length cDNA for SREBP1/ADD1 (17–19) was obtained from B. Spiegelman (Dana Farber Cancer Center, Boston, MA).

Plasmid Construction—The construction of S14CAT200, S14CAT124, S14CAT155, S14CAT156, S14CAT158, TKCAT222 was described previously (3, 4, 14). SV-Sport (ADD1/SREBP1c) plasmid containing a fragment of 5′-flanking region of CYP4A2 (Walnut 9 23 63 (53% 18:2, n-6; 10% 18:3, n-3)) was further cloned into pBSK+ vector (Stratagene) containing the SV40 early region and the 3′ untranslated region of the viral LTR. The construct was excised as a 5′ HindIII/3′PstI fragment and subcloned into the 5′ HindIII/3′PstI sites of the SV-Sport/ADD1 plasmid that contains the full-length cDNA for SREBP1/ADD1 (93% homologous to CYP4A2). A full-length cDNA for SREBP1/ADD1 was inserted into the 5′ BamHI/3′ HindIII sites of the SREBP1/ADD1 plasmid. The resulting plasmid contains an SV-Sport/ADD1 plasmid and a fragment of 5′ flanking region of CYP4A2 (Walnut 9 23 63 (53% 18:2, n-6; 10% 18:3, n-3)).

RESULTS

PUFA Down-regulates Hepatic SREBP1 Gene Expression in Vivo through a PPARα-independent Mechanism—In CV1, COS, and HepG2 cells, unsaturated fatty acids suppress the nuclear content of SREBP1a (5), a transcription factor involved in the regulation of several lipogenic genes (6–13, 17–20, 25–27). Since PUFA suppress lipogenic gene expression in liver, we examined the effect of different fat diets on hepatic SREBP1 gene expression (Fig. 1).

Rats were meal-fed a high carbohydrate diet containing no fat or supplemented with lard, olive, corn, walnut, or fish oil at 10% w/w for 5 days. Hepatic levels of mRNAs encoding SREBP1, FAS, S14, and LPK (panel A) and serum triglycerides (panel B) were measured. Although our SREBP1 cDNA cannot

| Type of fat | Saturated | Mole% of total | Monounsaturated | Polysaturated |
|------------|-----------|----------------|-----------------|-------------|
| Lard       | 39        | 45             | 11              |             |
| Olive oil  | 14        | 74             | 85              |             |
| Corn       | 13        | 24             | 59 (58% 18:2, n-6) |             |
| Walnut     | 9         | 22             | 63 (53% 18:2, n-6; 10% 18:3, n-3) |             |
| Fish oil   | 26        | 21             | 43 (16.1% 20:5, n-3; 11.2% 22:6, n-3) |             |
PUFA on SREBP1 gene expression are tissue-specific. Since PUFA acts directly on hepatocytes but not 3T3-L1 adipocytes—SREBP1 gene expression were assessed in both primary hepatocytes and adipocytes to suppress lipogenic gene expression (3, 4, 14, 16, 29). The effects of specific fatty acids on SREBP1 gene expression were assessed in both primary hepatocytes (Fig. 3A) and 3T3-L1 adipocytes (Fig. 3B). Fatty acid treatment of primary hepatocytes for 48 h shows that 18:1,n-9 had no effect on the mRNAs encoding FAS, LPK, S14, or SREBP1 when compared with cells receiving no fatty acid. However, the addition of PUFA leads to suppression of mRNA SREBP1 when compared with cells receiving no fatty acid. The extent of inhibition increases as the degree of unsaturation or chain length increases. Thus, cells treated with 20:5,n-3 display the greatest suppression of mRNA, mRNAactin was unaffected by these treatments, whereas mRNAAOX and mRNACYP4A were induced only by 20:5,n-3 (3).

In 3T3-L1 adipocytes, SREBP1a is the predominant subtype (7). PUFA suppress mRNAFAS and mRNAS14 in 3T3-L1 adipocytes through a cyclooxygenase 1-dependent conversion of 20:4,n-6 to prostanoids and subsequent activation of a pertussis toxin-sensitive G-linked receptor-signaling cascade (4, 29). Treatment of 3T3-L1 adipocytes with 18:1,n-9; 20:4,n-6, or 20:5,n-3 had no effect on mRNASREBP1c. Although mRNASREBP1c is suppressed in hepatocytes, mRNASREBP1c is not affected by PUFA in 3T3-L1 adipocytes. Thus, the effects of PUFA on SREBP1 gene expression are tissue-specific. Since
20:4,n-6 inhibition of S14 and FAS gene expression is cyclooxygenase-dependent (29), this result would suggest that prostaglandins may have little effect on SREBP1a gene expression.

**PUFA Suppresses Nuclear Content of SREBP1 in Hepatocytes**—The nuclear content of the mature form of SREBP1a (65 kDa) is suppressed in CV1, CHO, and HepG2 cells following treatment with unsaturated fatty acids (5). As shown above, SREBP1c gene expression in liver and cultured primary hepatocytes is suppressed by PUFA but not by monounsaturated fatty acids (Figs. 1–3). To determine if the decline in SREBP1c gene expression paralleled the decline in the precursor (~130 kDa) or mature form (65 kDa) of SREBP1c protein, Western analysis was performed using crude nuclear extracts from cultured primary hepatocytes treated with either no fat or 20 μM 18:1,n-9 or 20:4,n-6 for 48 h. After fatty acid treatment, nuclear extracts were prepared, and proteins were electrophoretically separated (20). A representative Western blot is shown, and quantitative results from three separate experiments are illustrated (Fig. 4). SREBP1c precursor (~130 kDa) levels were suppressed 26 and 50% by 18:1,n-9 and 20:4,n-6, respectively. The mature (or nuclear, 65 kDa) form was not affected by 18:1,n-9 but was suppressed by 70% in nuclei of 20:4,n-6-treated cells.

The decline in precursor 130-kDa SREBP1c after PUFA treatment is consistent with the decline in mRNA_{SREBP1} levels found both in vivo and in primary hepatocytes (Figs. 1–3). The decline in the nuclear form, however, appears to exceed the change in precursor and might implicate either proteolytic processing of precursor SREBP1c to the nuclear form or the enhanced turnover of nSREBP1c. Others suggest that unsaturated fatty acids affect the conversion of the precursor to the mature form of SREBP1 (5, 30). We find little or no effect of monounsaturated fatty acids on nSREBP1 levels but a profound effect of PUFA on nSREBP1 content. Clearly, additional studies will be required to define how PUFA regulates nSREBP1c levels.

**Effects of nSREBP1c on FAS, LPK, and S14 Gene Transcription**—SREBP1c is a basic helix-loop-helix transcription factor that binds sterol response elements (SRE) and E-boxes (CANNTG) in certain promoters and induces the transcription of several genes involved in cholesterol and fatty acid synthesis (13, 25, 26). The FAS promoter contains both an SRE (at 64 bp) and an E-box (at −64 bp) (25), whereas S14 and LPK promoters contain functional E-boxes at −1440 and −160 bp, respectively (31). The PUFA suppression of mRNA_{SREBP1} and nSREBP1c (Figs. 1–4) coupled with its potential interaction with multiple lipogenic genes makes SREBP1c an attractive candidate to explain the apparent coordinate regulation of lipogenic genes by PUFA.

To assess how nuclear content of SREBP1c affects hepatic lipogenic gene expression, a co-transfection approach was used.
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**Fig. 5.** Differential sensitivity of the FAS, S14, and LPK promoters to overexpressed nSREBP1c. Primary hepatocytes were transiently transfected with either FAS CAT, S14CAT, or LPK CAT at 2 μg/plate each. Cells were co-transfected with either 30 ng or 1 μg of SG5-nSREBP1c (nuclear form, nSREBP1). The total amount of co-transfected plasmid was kept at 1 μg. After 48 h in culture with 1 media change, cells were harvested for CAT activity. Results are expressed as fold induction in CAT activity, mean ± S.D., n ≥ 6. The FAS promoter extends from −265 to +16 bp, a region that contains both the SRE and E-box. The S14CAT promoter extends from −2.8 kilobases to +19 bp. The LPK promoter extends from −4.3 kilobases to +12 bp.

to overexpress nSREBP1c in the presence of three lipogenic gene promoters. Accordingly, primary hepatocytes were co-transfected with SG5-nSREBP1c (see “Materials and Methods”) along with either FAS CAT, S14CAT, or LPK CAT. Results illustrated in Fig. 5 show that as little as 30 ng of co-transfected nSREBP1c expression vector induced both S14 and FAS CAT by −5-fold but had no effect on LPK CAT activity. In fact, as little as 1 ng of co-transfected nSREBP1c was sufficient to induce S14CAT activity −2-fold (not shown). At 1 μg of co-transfected nSREBP1c, FAS, S14, and LPK CAT activity were induced 12-, 10-, and 4-fold, respectively. These results indicate a differential sensitivity of the three promoters for overexpressed nSREBP1c. Of the promoters tested, LPK is the least sensitive to elevated nSREBP1c expression.

**nSREBP1c functionally interacts with SRE elements within the FAS promoter** (25). Although LPK CAT is weakly responsive to overexpressed nSREBP1c (Fig. 5), the target for this action is within the glucose response region, a region containing a direct repeat of E-boxes. Kim et al. (32) report that SREBP1c binds E-boxes in the S14 CHO RR, i.e., between −1.6 and −1.4 kilobases upstream from the transcription start site. Here we use promoter deletion to identify the functional target for nSREBP1c action in the S14 promoter. The full-length S14CAT reporter gene activity (S170) was induced −11-fold by overexpression of nSREBP1c (Fig. 6). Removal of the region extending from −2500 to −220 bp had no effect on this activity. Based on studies by others, this deletion should remove elements associated with the glucose/insulin induction of S14 gene transcription (31). Block deletion of the −1.6/−1.4-kilobase region yielded essentially the same result, i.e., no change in response of S14CAT to overexpressed nSREBP1c (not shown). Thus, the putative S14 CHO RR is not essential for SREBP1c regulation of S14 gene transcription.

**Sequential deletion of the proximal promoter, from −220 to −170 bp and from −170 to −120 bp, led to a progressive decline in the response of S14CAT to overexpressed nSREBP1c.** The S14CAT reporter gene extending to −120 bp (S156) and the reporter gene containing the thymidine kinase (T222) promoter were not induced by overexpressed nSREBP1c. Thus, the effect of nSREBP1c on S14CAT activity is promoter-specific. The functional target for this control is within the S14 PUFA RR, i.e., between −170/−120 bp upstream from the transcription start site.

**nSREBP1c Binds within the S14 PUFA RR—**The PUFA RR within the S14 promoter extends from −220 to −80 bp upstream of the transcription start site (14). nSREBP1c functionally interacts with the PUFA RR between −170 and −120 bp (Fig. 6). EMSA was used to determine if nSREBP1c binds directly to this region. nSREBP1c was prepared by in vitro transcription/translation (see “Materials and Methods”) and extracts from SG5 programmed translations were used as a null control for protein translation. The FAS E-box and SRE were used as positive controls for SREBP1c binding.

**nSREBP1c binds both the FAS-E-box and FAS-SRE (Fig. 7).** nSREBP1c does not bind to the S14 −120/−80 bp element, an element previously reported to bind NFY and CAAT enhancer-binding protein α (21). nSREBP1c bound well to the −158/−116-bp region, a region within the functional target for nSREBP1c action (Fig. 6). No direct binding of nSREBP1c to S14 promoter elements between −220 and −158 was detected (not shown). To confirm this specific association of nSREBP1c with the S14 promoter element (−158/−116 bp), competition gel shift analysis was used (Fig. 7B). [32P]FAS-SRE was used as the labeled probe, and 1 pmol of various DNAs were used to challenge binding. At 1 pmol, both SRE SRE and FAS E-box are effective competitors for nSREBP1c binding. The S14 elements −120/−80, −188/−159, −219/−189, −256/−220 and the LPK E-box failed to compete for binding. Interestingly, the −256/−220 bp element contains an E-box at −234CACGTG, but this DNA fails to compete for SREBP1c binding to the FAS-SRE. Of the S14 DNAs tested, only −158/−116 competed well for binding.

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samples were prepared from translations programmed with SG5 (empty vector). The $^{32}$P-labeled DNAs (FAS SRE, FAS E-box, S14 $\sim$ 120/-80, and S14 $\sim$ 158/-116) used for direct binding are identified below the corresponding lanes. The arrow marks the location of the nSREBP1c-DNA complex, and the asterisk marks the location of a nonspecific band. The nonspecific band generated only in the SG5-programmed extracts and was not competed with any DNA used in this study (not shown). IVTP, in vitro translated protein. Panel B, competition EMSA. The FAS SRE was used as labeled probe. One pmol of competing double-stranded DNA was added to the reaction before $^{32}$P-labeled DNA as described under "Materials and Methods." These results in both panels A and B are representative of three separate studies. The sequence of the competing DNAs is shown under "Materials and Methods."

Further definition of nSREBP1c binding to the $\sim$ 158/-116-bp region involved competition gel shift analysis using mutant versions of the $\sim$ 158/-116-bp element. Fig. 8A illustrates the sequence of the $\sim$ 158/-116-bp element and the location of the 3-bp mutation scan. The results of the competition binding show that mutations between $\sim$ 139 and $\sim$ 131 lead to a loss of competition of SREBP1c for the native $\sim$ 158/-116 element (Fig. 8B). The corresponding sequence for this region is (1$^{\text{39}}$TCGGCTGTA$^{\text{131}}$) (Fig. 8A). In Fig. 8C, we compare the S14 SREBP1c binding sequence with known binding sites found in other promoter (13, 26, 27, 33). SREs are a direct repeat (5$'$-YCCAY-3$'$, where Y represents pyrimidine) separated by a nonessential base. The S14 sequence does not compare favorably with either the consensus SRE or SREs from the low density lipoprotein receptor or FAS promoters. A similar comparison of the S14 sequences with E-box elements reported to bind SREBP1c indicate a better alignment. Based on this comparison, we suggest that nSREBP1c binds an E-box-like element within the S14 proximal promoter at $\sim$ 139/-131 bp.

**Overexpression of nSREBP1c, but Not SRC-1, Abrogates PUFA Suppression of S14CAT Activity—nSREBP1c binds to and functionally interacts with the S14 promoter at $\sim$ 139/-131 bp. This region is within the cis-regulatory target for PUFA control. PUFA suppress hepatocellular mRNAsREBP1c and the nuclear level of nSREBP1c. Taken together, these studies suggest that PUFA controls S14 gene transcription by regulating the nuclear content of a factor binding this region. If this is so, then overexpression of nSREBP1c should eliminate the PUFA suppression of S14CAT activity. To test this hypothesis, primary hepatocytes were co-transfected with nSREBP1c and S14CAT (S170) and treated with specific fatty acids (Fig. 9).

In the absence of co-transfected nSREBP1c, 20:4,n-6 suppressed S14CAT activity by $\sim$ 65%. Transfecting primary hepatocytes with nSREBP1c at 30 ng/plate and 1 µg/plate induced S14CAT activity 5- and 13-fold, respectively (Fig. 9). In these same cells, 20:4,n-6 treatment suppressed S14CAT activity by 30 and 10%, respectively. Thus, increasing the hepatocellular content of nSREBP1c reduces 20:4,n-6-mediated inhibition of S14CAT activity from 65% to $\leq 20\%$ inhibition. In some studies where nSREBP1c overexpression was more stimulatory to S14CAT activity, suppression of S14CAT by PUFA was completely lost.

To establish that the abrogation of PUFA inhibition was not simply due to induction of S14CAT activity, cells were co-transfected with SRC-1, a nuclear receptor co-activator. Unlike nSREBP1c, SRC-1 had no effect on S14CAT activity when co-transfected at $\leq 1$ µg/plate. At 1 µg/plate, SRC-1 induced S14CAT activity $\sim$ 5-fold, a level comparable to that seen with 30 ng/plate nSREBP1c. In contrast to nSREBP1c, elevated SRC-1 expression had no effect on PUFA sensitivity of the S14CAT reporter gene. Similar results were obtained when hepatocytes were co-transfected with RXRα, a co-receptor required for TβR1 binding to the upstream thyroid hormone response elements (not shown). Although overexpression of nSREBP1c, SRC-1, and RXRα can induce S14CAT activity, only nSREBP1c was able to override PUFA suppression of S14CAT activity. These results suggest that the nSREBP1c is a limiting factor required for S14CAT activity in primary hepatocytes. PUFA regulation of mRNA SREBP1c and nSREBP1c may explain the PUFA-mediated suppression of S14 gene transcription.

**DISCUSSION**

The association of SREBP1 with lipogenic gene expression (6–13, 17–20) and the finding that unsaturated fatty acids suppress SREBP1 protein levels in cell lines (5) prompted our analysis of the role this transcription factor played in the PUFA regulation of rat hepatic lipogenic gene expression. Our goals were to 1) to examine the effect of PUFA on hepatic SREBP1 gene expression and protein levels, 2) to determine if nSREBP1c regulates S14 gene transcription and specific lipogenic genes, e.g. FAS and LPK, 3) to determine if nSREBP1c overexpression abrogates PUFA suppression of S14 gene transcription.

The pattern of suppression of mRNA SREBP1 by various fat diets (in vivo) and specific fatty acids (in culture) parallels results found for other lipogenic genes and suggests an apparent coordinate regulatory mechanism. Like other lipogenic genes (1, 3), PPARα is not involved in this regulatory mechanism (Figs. 1–3). The effects of PUFA on SREBP1 gene expression are tissue-specific where PUFA down-regulate mRNA SREBP1c in hepatocytes but does not affect mRNA SREBP1c in adipocytes (Fig. 3). Preliminary transcriptional run-on studies suggest that SREBP1c gene transcription in rat liver is low
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The FAS (25) and SCD1 and SCD2 (33) promoters. and acetyl CoA carboxylase (26) promoters and E-box-like elements from the low density lipoprotein receptor (LDLR) (13), FAS (25), and acetyl CoA carboxylase (26) promoters and E-box-like elements from the FAS (25) and SCD1 and SCD2 (33) promoters.

relative to FAS and S14. In contrast to FAS and S14, SREBP1c run-on transcription was not affected by PUFA, suggesting the mechanism of PUFA control may be post-transcriptional. Thus, the pretranslational regulation of SREBP1c by PUFA may be more like the PUFA-regulated post-transcriptional regulatory mechanism described for glucose-6-phosphate dehydrogenase (34).

The fall in mRNA_{SREBP1c} following PUFA treatment was accompanied by a corresponding fall in the precursor (pSREBP1c) and a more dramatic fall in the mature (nuclear) form of SREBP1c. Recent evidence suggests that SREBP1c proteolytic cleavage is not sensitive to cellular cholesterol levels (35). Others have suggested that fatty acids affected proteolytic cleavage of pSREBP1a in a fashion similar to cholesterol regulation of SREBP1a and -2. An alternative explanation for the changes in nSREBP1c levels might be a rapid turnover. nSREBP1c is phosphorylated through a mitogen-activated protein kinase pathway (36), and this form of covalent modification might be a signal to alter nSREBP1c activity or its turnover. Clearly, additional study will be required to understand how PUFA regulates nSREBP1c levels. Because PUFA also suppresses S14 and FAS gene expression in white adipose tissue and L1 adipocytes (29), PUFA effects on SREBP1c protein levels in these cell types will need to be assessed.

Studies with transgenic mice overexpressing SREBP1c (10) as well as primary hepatocyte studies using dominant negative versions of nSREBP1c (12) support the notion that nSREBP1c is a key factor in lipogenic gene expression. These observations coupled with the fact that several lipogenic genes have SREs and E-boxes that potentially interact with nSREBP1c (25–27) make it attractive to suggest that fatty acid regulation of hepatic SREBP1c can account for the coordinate regulation of multiple lipogenic and related genes. Two lines of evidence suggest this conclusion might be premature. First, although both FAS and S14 bind SREBP1c at either SRE and E-box-like elements and are strongly induced by overexpressed SREBP1c (Fig. 5), only S14CAT is consistently suppressed by PUFA (14). This indicates that the two promoters are sufficiently different and that changes in nSREBP1c levels alone may not be the sole determinant for PUFA regulation of these genes. A second argument against SREBP1c as a coordinator for fatty acid regulation of lipogenic gene expression deals with its role in LPK expression. The E-boxes in the LPK CHO RR (−170/−142 bp) bind SREBP1c weakly (Fig. 6 and Ref. 37), and a full-length LPK CAT reporter gene is weakly sensitive to nSREBP1c when compared with FAS CAT and S14CAT (Fig. 5). Finally, the target for PUFA control in the LPK promoter is located 3′ of the E-boxes, elements that bind HNF4 and NF1 (24, 38). Thus, PUFA regulation of the nSREBP1c levels may account for only part of the PUFA regulation of lipogenic gene expression.

SREBP1c binding to promoters and its function within promoters is augmented by other transcription factors binding nearby elements (25, 26). In the FAS and acetyl CoA carboxylase promoters, Sp1 binds nearby SREs, whereas NFY binds a Y-box near the SRE in the hydroxymethylglutaryl-CoA synthase and farnesyl diphosphate synthase promoters (39, 40). Although a prospective Sp1 site is located at −214 bp in the S14 promoter, its role in S14 gene transcription has not been evaluated. NFY binds the S14 promoter at −104/−99 bp and plays an obligatory role in enhancer-mediated transactivation of S14 gene transcription (21). Any mutation that disrupts NFY binding or substitution of other CCAAT-box-binding proteins,

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3 M. K. Mater, A. P. Thelen, D. A. Pan, and D. B. Jump, unpublished observation.

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**FIG. 8.** SREBP1c binds an E-box-like sequence in the S14-PUFA RR. Competitive gel shift analysis was used to localize the binding site for SREBP1c in the −158/−166-bp region. A, sequence of the −158/−166-bp region. Bracketed sequences were mutated by converting purines to pyrimidines and vice versa. B, 12 oligonucleotide pairs were synthesized, annealed, and used at 1 pmol/reaction (50 nM) in a competitive gel shift binding assay ("Materials and Methods"). The [32P]DNA was the 158/116-bp fragment, and nSREBP1c was generated by in vitro transcription/translation. The lane at the far left represents the binding of SREBP1c with no competing DNA. Lanes labeled Self or FAS-SRE contained unlabeled −158/−116 bp (Self) and FAS SRE as competing DNAs. The numbered lanes represent the binding of SREBP1c in the present of the competing mutant version of the −158/−116 bp. The numbers correspond to the location of the mutations (bracketed in panel A). C, a comparison of the SREBP1c binding site (−138/−131 bp) to known SRE-like elements from the low density lipoprotein receptor (LDLR) (13), FAS (25), and acetyl CoA carboxylase (26) promoters and E-box-like elements from the FAS (25) and SCD1 and SCD2 (33) promoters.

**FIG. 9.** Overexpressed nSREBP1c, but not SRC-1, overrides PUFA suppression of S14CAT activity. Primary hepatocytes transfected with S14CAT (S170) and SG5-nSREBP1c (at 0, 30 ng, or 1 μg/plate, see Fig. 5 for details) or SG5-SRC1 (1 μg/plate). The total amount of co-transfecting plasmid was kept at 1 μg/plate by supplementing with SG5 empty vector. Cells were treated with either 18:1 (n-9) or 20:4 (n-6) for 48 h with one media change. Cells were harvested and assayed for CAT activity, and results are expressed as fold induction of S14CAT activity (open bars) and % inhibition of S14CAT Activity by 20:4,n-6 (solid bars). Mean ± S.D., n ≥ 9.
like CAAT enhancer-binding protein α, essentially inactivates the gene. nSREBP1c functionally interacts with and binds specifically to an E-box-like sequence $^{139}\text{TCGCTG}\text{T}^{131}$ within the S14 PUFA RR. Linker-scanning mutation analysis across the $-146$ to $-129$-bp region leads to a loss of function.² Like NFY (21), nSREBP1c may also play an obligatory role in S14 gene transcription. Additional studies will be required to establish the interaction between nSREBP1 and NFY (at $-104/-99$ bp) as well as other factors upstream of the SREBP1c binding site that facilitate S14 gene transcription.

In conclusion, nSREBP1c binds to and functionally interacts with the S14 PUFA RR at $-139/-131$ bp upstream from the S14 transcription start site. PUFA regulate rat hepatic SREBP1c gene expression and its nuclear content. Together, these observations provide an explanation, at least in part, for the PUFA-mediated suppression of S14 gene expression. PUFA also regulate the activity of PPARα, a nuclear receptor that controls the expression of multiple mitochondrial, peroxisomal, and microsomal enzymes involved in fatty acid oxidation (2). Very long chain n-3 PUFA not only activate PPARα but also directly inhibit triglyceride assembly and secretion (41). The down-regulation of nSREBP1c coupled with the activation of PPARα by n-3 PUFA provides a molecular explanation for the well known shift in hepatic lipid metabolism from lipid synthesis and storage to oxidation associated with ingestion of n-3 PUFA (1). Disruption of this regulatory scheme might contribute to changes in hepatic lipid metabolism associated with obesity and insulin resistance (42, 43).

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