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Biochemical and Biophysical Research Communications 296 (2002) 111–117

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The E-box like sterol regulatory element mediates the suppression of human Δ-6 desaturase gene by highly unsaturated fatty acids☆

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Received 1 July 2002

Abstract

Δ-6 Desaturase (D6D) catalyzes the first step of the synthesis of highly unsaturated fatty acids (HUFA) that play pivotal roles in many biological functions. The D6D expression is under feedback regulation by dietary HUFA. We co-transfected D6D promoter–reporter constructs to HepG2 cells with an expression vector of nuclear form sterol regulatory element binding protein-1c (SREBP-1c). A 90-bp region of the D6D promoter was required for the activation by SREBP-1c as well as for the suppression of the promoter activity by HUFA. The region contained two candidates of sterol regulatory element (SRE). Mutation analysis identified E-box like SRE (SRE-2) as essential for both SREBP-1c activation and HUFA suppression. SRE-2 has a core sequence of CAGCAG, and is also conserved in stearoyl CoA desatruases. Because HUFA are primarily incorporated into phospholipids (PL), our results suggest that the primary role of SREBP-1c in liver is the regulation of fatty acid supply for PL rather than for triglycerides.

Keywords: Δ-6 Desaturase; Highly unsaturated fatty acids; Sterol regulatory element; Sterol regulatory element binding protein; Arachidonic acid; Transcriptional regulation; Insulin; Phospholipids

Highly unsaturated fatty acids (HUFA) such as arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3) are essential for a variety of physiological functions including brain development, cardiac function, inflammatory response, and hemostasis [1–4]. In addition to being vital components of membrane phospholipids (PL) and functioning in key steps of cell signaling, HUFA govern the expression of a wide array of genes, including those encoding proteins involved with lipid metabolism, thermogenesis, and cell differentiation [5–8].

Δ-6 Desaturase (D6D) is a microsomal enzyme that catalyzes the first and rate-limiting step of the HUFA synthesis from precursor polyunsaturated fatty acids (PUFA) 18:2 n-6 and 18:3 n-3. Cloning human and mouse D6D enabled us to initiate the investigation of the regulatory mechanism of the enzyme [9]. The abundance of D6D mRNA is low in liver when rats are fed diets containing n-6 and n-3 PUFA, whereas the D6D mRNA is increased by a fat-free diet and by a diet containing triolein (18:1 n-9) as the only fat source [9,10]. A nuclear run-on analysis indicates that the transcriptional regulation largely accounts for the change in the D6D mRNA abundance [Tang et al., unpublished data]. Rimoldi et al. [11] reported that the D6D expression is also dependent on insulin. They showed that the D6D mRNA was diminished in diabetic rats, and was restored by insulin administration.

Sterol regulatory element binding proteins (SREBP) are transcription factors that play a pivotal role in the expression of lipogenic genes [12–17]. SREBP are

☆ *Abbreviations:* ACC, acetyl CoA carboxylase; ChREBP, carbohydrate response element binding protein; D6D, Δ-6 desaturase; FAS, fatty acid synthase; FBS, fetal bovine serum; HUFA, highly unsaturated fatty acids; PCR, polymerase chain reaction; PL, phospholipids; PUFA, polyunsaturated fatty acids; SCD, stearoyl CoA desaturase; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein; TG, triglycerides.

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00851-3

synthesized as precursor proteins that bind to the endoplasmic reticulum membrane. The N-terminal domain, a helix-loop-helix leucine zipper transcription factor, is released for nuclear translocation by a sequential two-step cleavage process. This mature protein activates the transcription of genes involved in cholesterol and fatty acid synthesis by binding to sterol regulatory element (SRE) [18]. SRREBP-1 and SREBP-2 are encoded by separate genes. SREBP-1 is expressed as two subtypes, 1a and 1c, which arise from differential promoter and exon usage [18]. SREBP-1c activates the transcription of genes involved in fatty acid synthesis, whereas SREBP-1a activates genes for both fatty acid and cholesterol synthesis [14,16]. Functional SRE is identified in the genes for fatty acid synthesis such as fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and stearoyl CoA desaturase (SCD) [13,19,20]. Accumulating evidence indicates that SREBP-1c mediates the induction of these genes by insulin. Fasting and diabetes reduce the amount of hepatic SREBP-1c mRNA, while carbohydrate-refeeding and insulin administration increases the abundance of SREBP-1c mRNA [15,21,22]. Moreover, SREBP-1 mediates the suppression of several hepatic lipogenic genes by dietary PUFA [20,23–26]. Because the expression of the D6D gene is also dependent on insulin and is suppressed by dietary PUFA, we hypothesized that SREBP-1 regulates the D6D gene by binding its promoter, and tested the hypothesis in this report.

Materials and methods

Cellular abundance of D6D mRNA. HepG2 cells were plated onto 6-well collagen-coated plates and incubated in high-glucose (4.5 g/L) Dullbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). After cells reached confluence, serum was removed from the medium. After 48 h of serum starvation, the medium was changed to one containing 0.4% bovine serum albumin (BSA), 1 μM insulin, 1 μM dexsamethasone, and 20 μg/ml α-tocopherol. Then, cells were treated with 100 μM albumin-bound 18:1 n-9, 20:4 n-6, 20:5 n-3 or 22:6 n-3 (Nu-Check Prep, Elysian, MN) for 24 h. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Real-time quantitative PCR analysis was used to determine the relative abundance of the D6D mRNA. Two μg of total RNA was reverse transcribed with random hexamers using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Each amplification mixture (25 μl) contained 1 ng of cDNA, 100 nM forward primer (5′-CTGCCAACTGGTGGAATCATC), 100 nM reverse primer (5′-ACAAACACGTGCAGCATGTTC), 100 nM dual labeled fluorogenic probe (6-FAM-CAGCACCACGCCAAGCCTAACATCTTC-TAMRA) (Applied Biosystems, Foster City, CA) and Universal PCR master mix (Applied Biosystems, Foster City, CA). The mRNA abundance relative to 18S rRNA was measured using Comparative C_T (Threshold Cycle) Method according to the manufacturer's instruction.

Reporter vector construction. Human D6D cDNA sequence was used to BLAST-search the human genomic database. Clone PAC AC004228 corresponding to the region of human chromosome 11q12.2-13.1 was found to contain all of the exons for D6D and Δ-5 desaturase as well as the entire region spanning the distance between the two genes. The S1 nuclease protection assay revealed that the D6D promoter is TATA-less, and has multiple transcription start sites, −173 and −155 from the translation start site being the main sites [Tang et al., unpublished data]. Subsequently, the D6D promoter was numbered by assigning +1 to the translation start site. A *Kpn*I/*Avi*II fragment corresponding to the sequence of −6431 to +95 was cut from the human clone AC004228. A luciferase reporter construct containing the D6D proximal promoter region of −304/−46 was prepared by cutting the −6431 to +95 fragment with *Sac*I and *Nae*I, and subsequently inserting the −304/−46 sequence into the *Sac*I and *Sma*I sites of pGL3 basic vector (Promega, Madison, WI). Four DNA fragments (sizes from −851 to −46, −466 to −46, −442 to −46, and −425 to −46 bp upstream of the translation start site) were obtained by the polymerase chain reaction (PCR) using one of the following forward primers: 5′-GAGAGGTACCGGCGCGGACAATGTGGGAT, 5′-GAGAGGTACCGAAAGATCCTCCTGGGCCA, 5′-GAGAGG TA CCAGGCGGGGCGACGCGACC, and 5′-GAGAGGTACCGG ATTGGTGCAGGCGCT, for D6D-851, D6D-466, D6D-442 and D6D-425, respectively. A pGL3 basic vector sequence, 5′-CTTTAT GTTTTTGGCGTCTTCCA, was used as the reverse primer for the reaction. The PCR products were ligated into *Kpn*I–*Hind*III sites of the pGL3 basic vector. Two more fragments, D6D-851-426 and D6D-851-377, were also obtained by PCR using the forward primer D6D-851 with reverse primers, 5′-GAGAGAGCTCGCGTCGCCCCGCCT GCC and 5′-GAGAGAGCTCGCCGCCCGAGTTTCCAC, for D6D-851-426 and D6D-851-377, respectively. The products were ligated into the *Kpn*I site in the vector and the *Sac*I site at the D6D-308. Mutated constructs of the putative SRE were created by site-directed mutagenesis using PCR [27]. Briefly, two DNA fragments, upstream and down stream of mutation site, were separately created by PCR using primers that contain a mutated sequence. Then, these two strands were fused and amplified again by PCR. The following primer pairs were used to create mutations (lower case denotes mutations): for mutant1, 5′-CAATGGCAGGgaattCGACGCGACCGGATTGGTG and 5′-GaattcCCTGCCATTGGCCCAGGAG; and for mutant2, 5′-TGCAGGCGCTgaatTcATCGCTGTGGAAACTCG and 5′-gA-attcAGCGCCTGCACCAATCCG. A high-fidelity DNA polymerase Pfx (Invitrogen, Carlsbad, CA) was used for all the above PCR procedures. All of the PCR products were sequenced to confirm the accuracy using the dideoxy-nucleotide-chain-termination method.

Luciferase reporter assay of D6D gene. An expression plasmid of SREBP-1c (pCMV-SREBP1c-436) that contains the mature form of SREBP-1c in pCMV7 vector [28] was obtained from ATCC (Manassas, VA). HepG2 cells were plated onto collagen coated 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. Next day, the cells (about 80% confluent) were co-transfected with one of D6D promoter–luciferase reporter vectors (0.3 μg/well), either pCMV-SREBP1c-436 or an empty control vector (pcDNA 3.1) (10 ng/well), and pRL-SV40 (6 ng/well) using GeneJammer transfection reagent (Stratagene, La Jolla, CA). Then, the cells were incubated in DMEM with 10% FBS, and cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) for 40 h. For HUFA response, the cells were transfected with various D6D promoter–luciferase reporter vectors (0.4 μg/well) and pRL-SV40 reference plasmid (8 ng/well). Following 18 h transfection period, the medium was changed to one containing 1% FBS, 1 μM insulin, 1 μM dexsamethasone, 20 μg/ml α-tocopherol, and 100 μM albumin-bound 18:1 n-9 or 20:4 n-6 (Nu-Check Prep, Elysian, MN), and incubated for 48 h with one change of medium after 24 h. Fatty acid free albumin was purchased from Sigma (St. Louis, MO). The molar ratio of fatty acids and albumin was 4:1. The cells were lysed and luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instruction.

Statistical analysis. Statistical comparison of the groups was made by ANOVA and Scheffe's post hoc test using Statview 5.0.1 (SAS Institute, Cary, NC). Statistical significance is defined as $p < 0.05$.

Results and discussion

First, we tested the response of the D6D promoter to SREBP-1c. We made various D6D–luciferase reporter gene constructs, and co-transfected to HepG2 cells with an expression plasmid of nuclear form of SREBP-1c. As shown in Fig. 1, D6D promoters (D6D-851 and D6D-466) were activated about sixfold by co-transfection of SREBP-1c. Further 5′ deletions (D6D-442, D6D-425, and D6D-308) caused the loss of response to SREBP-1c. The deletion of −376 to −309 region of D6D promoter (D6D-851-377) retained the SREBP-1c response, but the response was lost when the −425 to −309 region was deleted (D6D-851-426 in Fig. 1). This deletion study localized the region required for the SREBP-1c activation within 90 bp (−466 to −377) of the D6D promoter. The presence of SERBP-1c response sequence in the D6D promoter is consistent with studies by others that show an increase in the D6D mRNA in transgenic mice with over-expressed nuclear form SREBP [29] and a dependence of D6D gene expression on insulin [11]. Next, we tested whether HepG2 cells retain the in vivo response of the D6D gene suppression by HUFA. HepG2 cells were cultured and then treated with 18:1 n-9, 20:4 n-6, 20:5 n-3, or 22:6 n-3. The total RNA was isolated and mRNA abundance was measured by real-time quantitative PCR assay. In the presence of 20:4 n-6, 20:5 n-3, and 22:6 n-3, abundance of the D6D mRNA was reduced about 50%, whereas 18:1 n-9 had no effect on D6D mRNA abundance (Fig. 2). These results demonstrate that HepG2 cells retain the in vivo response to HUFA.

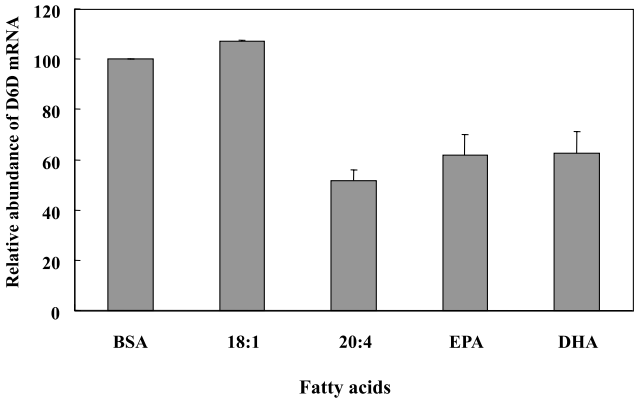

Fig. 2. HUFA suppress the abundance of D6D mRNA in HepG2 cells. Duplicate plates of HepG2 cells were treated with 100 μM of indicated albumin-bound fatty acids. Abundance of the D6D mRNA was measured by quantitative real-time PCR assay. Data are expressed as percentage of the D6D mRNA in cells treated with only BSA.

To localize the site that mediates the suppression of the D6D gene by HUFA, D6D–luciferase reporter constructs were transiently transfected into HepG2 cells and treated with 18:1 n-9 or 20:4 n-6 for 48 h. Then, cells were lysed and luciferase activity was measured. As shown in Fig. 3, the activity of the D6D promoter, D6D-851, was suppressed by 20:4 n-6. The response of truncated D6D promoter constructs revealed that the same D6D promoter region (−466 to −377 bp) as the SREBP response was required for the HUFA suppression.

Sequence analysis of −466 to −377 bp region of D6D promoter by the MatInspector program [30] identified candidate transcription factor binding sites including

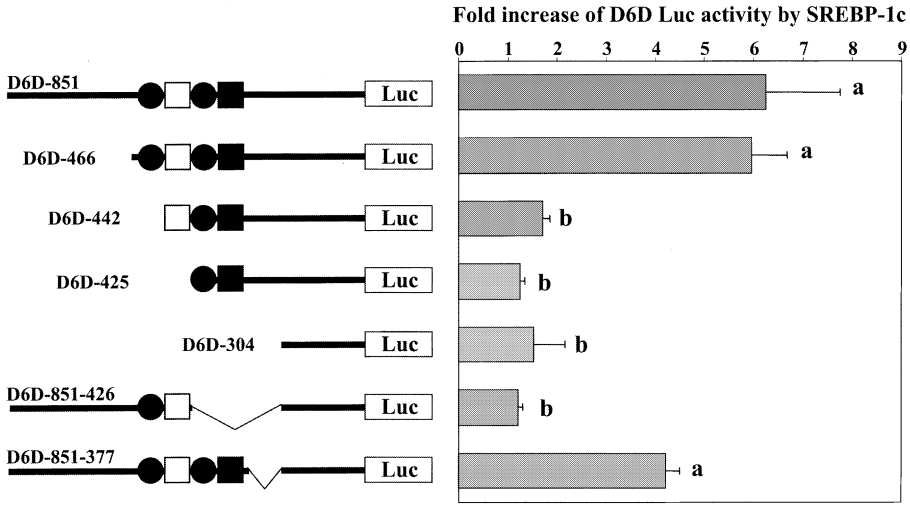

Fig. 1. Activation of the human D6D promoter by SREBP-1c. Various D6D promoter–luciferase constructs (D6D-851, D6D-466, D6D-442, D6D-425, D6D-851-426, and D6D-851-377) were transiently co-transfected to HepG2 cells (three wells per construct) with either a nuclear SREBP-1c expression vector or an empty control vector, and a *Renilla* luciferase reference plasmid. After 24 h incubation, firefly luciferase activity were measured and normalized to *Renilla* luciferase activity. The promoter activity is shown as a fold increase by SREBP-1c expression over the promoter activity of the control vector co-transfection (means ± SD, $n \geqslant 3$ of separate experiments). Groups baring different alphabets differ significantly ($p < 0.05$) by Scheffe's post hoc test. The symbols, SRE-1 (□), E-box like SRE (■), and CCAAT box (●), indicate the presence of candidate elements in the D6D promoter constructs.

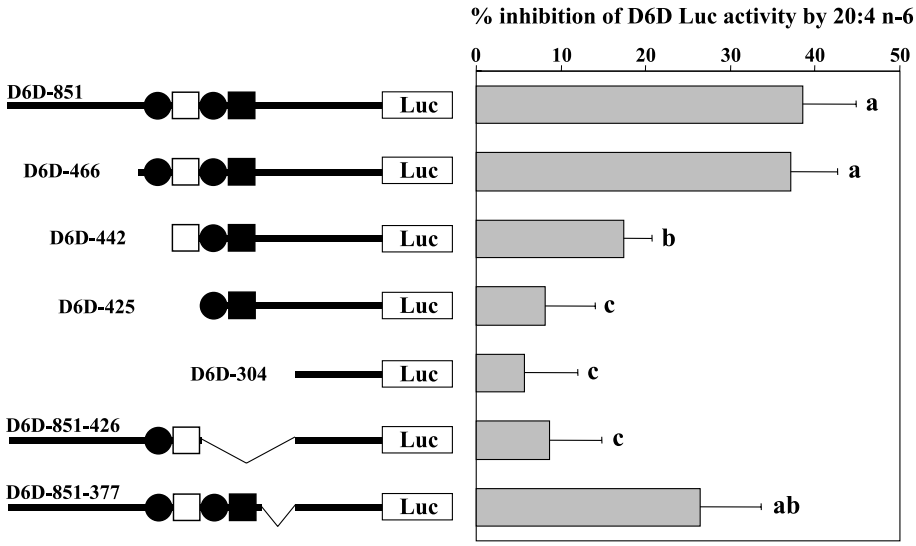

Fig. 3. Suppression of the D6D promoter activity by HUFA. Various D6D promoter–luciferase constructs (D6D-851, D6D-466, D6D-442, D6D-425, D6D-851-426, and D6D-851-377) were transiently co-transfected to HepG2 cells (three wells per construct) with *Renilla* luciferase reference plasmid. After 48 h incubation in medium containing 100 μM of 18:1 n-9 or 20:4 n-6, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. The inhibition by 20:4 n-6 was expressed as percentage decrease in the promoter activity relative to the cells treated with 18:1 n-9 (means ± SD, $n \geqslant 3$ of separate experiments). Groups baring different alphabets differ significantly ($p < 0.05$) by Scheffe's post hoc test. The symbols, SRE-1 (□), E-box like SRE (■), and CCAAT box (●), indicate the presence of candidate elements in the D6D promoter constructs.

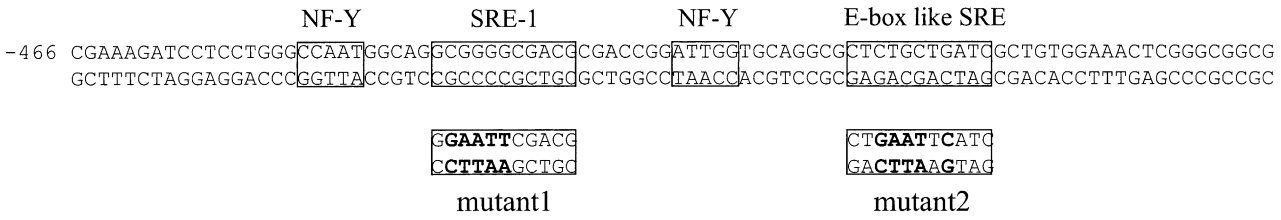

Fig. 4. Location of candidate regulatory sequences and binding sites in the human D6D promoter from −466 to −377. The location and sequence of each mutation are shown below the wild type sequences. SRE, sterol regulatory element; NF-Y, nuclear factor Y binding site.

two SREBP binding sequences and two nuclear factor-Y (NF-Y) binding sequences (Fig. 4). The upstream putative SREBP binding site has 0.75 of core similarity and 0.753 of matrix similarity with the classical SRE (SRE-1), while the other is homologous (0.774 of core similarity and 0.701 of matrix similarity) to the E-box containing SREBP-1 binding element (E-box SRE) described by Kim et al. [31].

To determine which SRE candidate is required for the activation by SREBP and the suppression by HUFA, we mutated each element and tested the response to SREBP-1c and HUFA. As shown in Fig. 5, the constructs that mutated the E-box SRE-like element caused complete loss of both SREBP-1c and HUFA responses. In contrast, mutation of the putative SRE-1 did not change the responses. The functional SRE in the human D6D gene promoter (SRE-2) contains a degenerated E-box sequence, **CAGCAG**, and is conserved in the mouse D6D promoter. Moreover, the SRE-2 sequence is found in the human and mouse SCD SRE (Fig. 6), which were characterized in [20,32,33]. The SRE-2 sequence in SCD1 and 2 also mediates both the activation by SREBP-1 and the suppression by HUFA [20]. A common feature of SRE-2 found in D6D and SCD is a replacement of the core sequence of SRE-1, CACCCC with an E-box like sequence CAGCAG. These results demonstrated that the SRE-2 in the −466 to −377 bp region of D6D promoter is involved in the responses of SREBP and HUFA.

The D6D-442 construct lacks one of two putative NF-Y binding sites (Figs. 1 and 3), but it retains SRE-2. This construct lost responses to both SREBP-1c and HUFA (Figs. 1 and 3), suggesting that NF-Y is required for the activation of D6D promoter by SREBP-1. Concordant with this result, several studies have shown that NF-Y is also required for transactivation of other SREBP-1 regulated genes such as FAS, SCD, SREBP-1c, and S14 [20,34–37]. Taken together, like other lipogenic genes, D6D gene may require NF-Y for the activation by SREBP in liver.

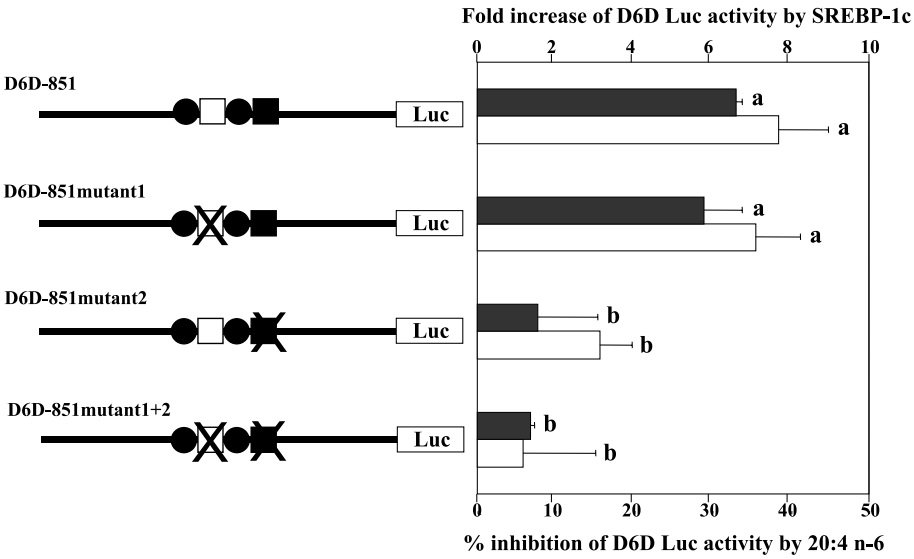

Fig. 5. Mutations in the E-box like SRE impair the SREBP-1c activation (solid bars) and HUFA inhibition (open bars) of the D6D promoter activity. HepG2 cells were transiently transfected with D6D-851, D6D-851mutant1, D6D-851mutant2, or D6D-851mutant1+2 and treated as Figs. 1 and 3. The sequences of SRE (□), E-box like SRE (■), and CCAAT box (●) are indicated. A mutated motif is shown by (×). For SREBP-1c activation, values are shown as a fold activation compared with the activity with control vector. For HUFA suppression, the inhibition by 20:4 n-6 was expressed as percentage decrease in the promoter activity relative to the cells treated with 18:1 n-9 (means ± SD, $n \geqslant 3$ of separate experiments). Groups baring different alphabets differ significantly ($p < 0.05$) by Scheffe's post hoc test.

SRE-1	**KATCACCCCAC**
E-box SRE	**NATCACGTGAY**
SRE-2	
human,mouse D6D	**GATCAGCAGAG**CGCC
mouse SCD1	**GAACAGCAGAT**TGTG
mouse SCD2	**GAACAGCAGAT**TGCG
human SCD	**GAACAGCAGAT**TGCG

Fig. 6. Comparison of sterol regulatory elements (SRE). The sequences of classic SRE (SRE-1) and E-box SRE are described in [31]. SRE in SCD genes are from [20,32,33]. The underline in human SCD shows the footprint by SREBP-1 binding [20].

The principal role of fatty acid synthesis in liver is considered to be the production and secretion of triglycerides (TG) [38]. Because SREBP-1c induces genes for fatty acid synthesis in liver, the conversion of excess glucose to TG for storage has been regarded as the major role of SREBP-1. However, we have shown in this study that SREBP-1c not only activates the D6D gene, but also mediates feedback suppression of the gene by HUFA. Because HUFA are mainly incorporated into PL and are very poor substrates for TG synthesis [39], the primary role of SREBP-1 in liver may be the regulation of fatty acid supply for PL rather than for TG. Indeed, Dobrosotskaya et al. [40] recently reported that phosphatidyl ethanolamine controls maturation of SREBP in *Drosophila* cells, and proposed a hypothesis that the physiological role of SREBP in animals is to regulate the membrane PL synthesis. In addition, HUFA reduce nuclear form SREBP-1 in rat liver [23] and HEK 293 cells [41]. Although the mechanism of this HUFA effect is currently unknown, HUFA esterified into membrane PL may inhibit the maturation of SREBP-1 as a feed back regulation.

Regarding the regulation of fatty acid synthesis for TG storage, acute regulations by dephosphorylation of glycolytic enzymes and ACC have long been known. In addition, carbohydrate response element binding protein (ChREBP) mediates effects of insulin and high glucose, and activates transcription of pyruvate kinase [42] and FAS [43] genes. Thus, ChREBP may be the transcriptional regulator of the conversion of excess glucose to TG storage.

In summary, we showed in this report that SREBP-1c activates the human D6D gene, and that the −466 to −377 bp region was required for the response. The same region was required for the suppression of the D6D gene by HUFA. Mutation analysis showed that E-box like SRE-2, not classical SRE-1, is the functional SREBP-1 binding element in the D6D promoter. SRE-2 replaces the core sequence of SRE-1, CACCCC, with CAGCAG, and is also conserved in mouse and human SCD. Our results suggest that the primary role of SREBP-1c in liver is the regulation of fatty acid synthesis for PL rather than for TG.

Acknowledgments

This study is supported in part by grants from Research Board of University of Illinois at Urbana-Champaign (MTN) and MM Love Chair in Nutritional, Cellular, and Molecular Sciences at University of Texas (SDC).

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