Direct Interaction between USF and SREBP-1c Mediates Synergistic Activation of the Fatty-acid Synthase Promoter*

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To understand the molecular mechanisms underlying transcriptional activation of fatty-acid synthase (FAS), we examined the relationship between upstream stimulatory factor (USF) and SREBP-1c, two transcription factors that we have shown previously to be critical for FAS induction by feeding/insulin. Here, by using a combination of tandem affinity purification and coimmunoprecipitation, we demonstrate, for the first time, that USF and SREBP-1 interact in vitro and in vivo. Glutathione S-transferase pulldown experiments with various USF and sterol regulatory element-binding protein (SREBP) deletion constructs indicate that the basic helix-loop-helix domain of USF interacts directly with the basic helix-loop-helix and an N-terminal region of SREBP-1c. Furthermore, cotransfection of USF and SREBP-1c with an FAS promoter-luciferase reporter construct in Drosophila SL2 cells results in highly synergistic activation of the FAS promoter. We also show similar cooperative activation of the mitochondrial glycerol-3-phosphate acyltransferase promoter by USF and SREBP-1c. Chromatin immunoprecipitation analysis of mouse liver demonstrates that USF binds constitutively to the mitochondrial glycerol-3-phosphate acyltransferase promoter during fasting/refeeding in vivo, whereas binding of SREBP-1 is observed only during refeeding, in a manner identical to that of the FAS promoter. In addition, we show that the synergy we have observed depends on the activation domains of both proteins and that mutated USF or SREBP lacking the N-terminal activation domain could inhibit the transactivation of the other. Closely positioned E-boxes and sterol regulatory elements found in the promoters of several lipogenic genes suggest a common mechanism of induction by feeding/insulin.

Fatty-acid synthase (FAS),² a central enzyme in de novo lipogenesis in mammals, catalyzes all the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS gene transcription is under tight nutritional and hormonal control in lipogenic tissues, namely liver and adipose tissue (1–5). FAS is not known to be regulated by allosteric effectors or covalent modification; rather, its regulation occurs mainly at the transcriptional level. In this regard, other enzymes in the lipid synthesis pathway, such as mitochondrial glycerol-3-phosphate acyltransferase (mGPAT), acetyl-CoA carboxylase (ACC), and ATP-citrate lyase, are also coordinately regulated during fasting/feeding (5–12). Transcription of the FAS and mGPAT genes is not detectable in the lipogenic tissues of fasted mice, whereas feeding a high carbohydrate, fat-free diet increases transcription dramatically, concomitantly with a rise in circulating insulin/glucose and a decrease in glucagon levels (7, 8, 12).

By using 3T3-L1 adipocytes, we originally reported that the −65 E-box present in the FAS promoter, which binds the ubiquitously expressed sterol regulatory factor-1 and -2, mediated insulin activation of the FAS promoter (13, 14). However, by generating mice transgenic for various 5′-deletion and mutant FAS promoter-chloramphenicol acetyltransferase constructs, we found that the −133 FAS promoter construct, containing the −65 E-box, is not sufficient for feeding/insulin-mediated activation of the FAS promoter in vivo. Instead, a region from −278 to −131, containing the −150 sterol regulatory element (SRE), as well as a more upstream region from −444 to −278, containing an additional E-box at −332, are required for high level activation of the FAS promoter by feeding/insulin (15, 16). Furthermore, both the E-box at −65 and the SRE at −150 are required for transcriptional activation of FAS in vivo, as the induction of FAS transcription during fasting/refeeding was abolished in mice transgenic for mutations at these two sites (16).

USFs belong to the class bHLH/LZ transcription factor family and stimulate transcription by binding to E-boxes (5′-CANNTG-3′) as USF-1/USF-2 heterodimers (17, 18). Sterol regulatory element-binding proteins (SREBPs) also belong to the bHLH/LZ family and stimulate transcription by binding to SREs present in the promoters of their target genes (19–23). However, because of an atypical tyrosine residue, which replaces a conserved arginine present in the basic regions of this family of transcription factors, SREBPs can also bind to E-boxes, at least in vitro (24, 25). Nevertheless, we have clearly shown by chromatin immunoprecipitation (ChIP) in transgenic mice that SREBP-1c functions through the −150 SRE and that USFs function through the −65 and −332 E-boxes in vivo (16). Gain-of-function studies using mice doubly transgenic for a truncated active form of SREBP-1a and various FAS-pro-
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moter reporter constructs indicate that SREBP-1a, even when overexpressed during fasting, cannot activate FAS transcription through the −65 E-box in a −131 FAS promoter construct (26). Regardless, loss-of-function studies in SREBP-1c−/− and USF-1−/− knock-out mice clearly demonstrated that these transcription factors are critical in the regulation of FAS transcription (27–29).

So far, two coregulators for SREBP-1a binding and function have been described. These include Sp1 and NF-Y, both of which seem to be important for mediating a transcriptional response to cellular sterol depletion. For example, SREBP-1a was shown to cooperate with Sp1 on adjacent sites to regulate the FAS (30), low density lipoprotein receptor (31), and acetyl-CoA synthetase-1 genes (32), although a direct physical interaction between SREBP-1a and Sp1 in solution has not been reported. Given the apparent requirements for accessory factors in mediating a maximal response of target genes to SREBP-1a during sterol depletion, the identification of an analogous coregulator for SREBP-1c, the isoform specifically induced during feeding/insulin (15, 33), in mediating the lipogenic response would be an important finding, especially because SREBP-1c is an even weaker transcription factor than SREBP-1a on its own (34). The fact that SREBP-1c only modestly activates the FAS promoter in vitro (26), but dramatically activates FAS transcription in vivo (26, 34, 35), implicates the involvement of other factors that may be absent in vitro.

In this study, by using a combination of tandem affinity purification (TAP) and coimmunoprecipitation, we demonstrate that USF and SREBP-1 directly interact in vivo. We have mapped the interaction domains to the bHLH region of each protein as well as an N-terminal region of SREBP-1c. Furthermore, through cotransfection of USF-1 and SREBP-1c in Drosophila SL2 cells, we show that these two factors synergistically activate the FAS as well as the mGPAT promoters. Importantly, we also show that USF and SREBP bind to the proximal mGPAT promoter in vivo in a manner identical to that of FAS. Functional domain mapping using USF and SREBP deletion constructs indicates that the activation domains of both proteins are required for synergy. Furthermore, the presence of closely spaced E-boxes and SREs in several lipogenic genes suggests involvement of USF and SREBP in mediating the transcriptional response to fasting/refeeding in vivo.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Constructs**—USF-1 was cloned into pCTAP-A (encoding an in-frame streptavidin affinity tag followed by a calmodulin affinity tag) (Stratagene) by PCR amplification of human USF-1 in pCDNA 3.0 (14) using primers containing EcoRI and Xhol sites at the 5′- and 3′-ends, respectively, followed by digestion of amplified inserts and vector with the corresponding enzymes. A FLAG tag was also incorporated into the downstream primer. All PCR amplifications for subcloning USF and SREBP were conducted with Pfu Turbo DNA polymerase (Stratagene). Insertion of USF-1 into pCTAP-A was confirmed by sequencing. A Myc tag was added to the C terminus of human SREBP-1c by PCR amplification of SREBP-1a in pCDNA-3.1 (26) using the primers containing an EcoRI site at the 5′-end and an Xhol site and Myc tag at the 3′-end followed by digestion of inserts with EcoRI and Xhol. In all cases where a construct containing the extreme N terminus of SREBP-1c was amplified by PCR from pCDNA3.1-SREBP-1a, the SREBP-1a sequence was converted to SREBP-1c by incorporating nucleotides encoding the first 5 amino acids of SREBP-1c into the 5′-primer. Insertion of the coding region of SREBP-1c-Myc into the correct reading frame was confirmed by sequencing. To construct GST fusions of USF-1 and SREBP-1c, primers were designed to amplify the appropriate region of each transcription factor from pCDNA 3.0-USF-1 and pCDNA3.1-SREBP-1a using EcoRI and Xhol sites at the 5′- and 3′-ends. Inserts were cloned into pGEX-4T-3 vector (Amersham Biosciences) followed by digestion with EcoRI and Xhol. Insertion of the USF and SREBP constructs into the correct reading frame was confirmed by sequencing. To construct Drosophila expression vectors for USF-1 and SREBP-1c, the coding regions of USF-1 and SREBP-1c were amplified from pCDNA 3.0-USF-1 and pCDNA3.1-SREBP-1a using primers containing BamHI and Xhol sites at the 5′- and 3′-ends, respectively. Inserts were ligated into the pPPAC-Sp1 vector (36) after excision of the Sp1 coding sequence by digestion with BamHI and Xhol. Insertion into the correct reading frames was confirmed by sequencing. Reporter constructs for FAS (26) and GPAT (37) have been described previously.

**Tandem Affinity Purification**—293FT cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. The day before transfection, cells were seeded onto 150-mm dishes to achieve a confluency of ~30–40%. Ten 150-mm dishes were each transfected with 10 μg of SREBP-1c-Myc and 5 μg of pCTAP-A-FLAG-USF-1 or 5 μg of pCTAP-A using Lipofectamine (Invitrogen). Cells were maintained in serum free-media for ~16 h following transfection and then replaced with complete media. Approximately 48 h after transfection, cells were harvested by washing twice in PBS followed by scraping. Cells were centrifuged at 1000 × g at 4 °C and resuspended in lysis buffer (Stratagene). The TAP protocol was then performed according to the manufacturer’s instructions, except that the streptavidin-binding reaction was incubated overnight. Eluted proteins were boiled in SDS loading buffer and resolved on 7.5% SDS-polyacrylamide gels. The presence of USF-1 and SREBP-1c-Myc was determined by immunoblotting with M2 anti-FLAG (Sigma) and anti-Myc antibodies (A6, Upstate). For copurification of endogenous SREBP-1 protein with USF-FLAG-TAP, HEK 293F cells were transfected with USF-FLAG-TAP vector or empty TAP vector using 293-Fectin (Invitrogen). Nuclear extracts were prepared according to the method of Andrews and Faller (38) 72 h after transfection and subjected to the TAP protocol. Immunoblotting was performed with anti-FLAG and anti-SREBP-1 antibodies (2A4, Santa Cruz Biotechnology).

**Preparation of Mouse Liver Nuclear Extract**—Mice were fasted for 48 h and then refed a high carbohydrate, fat-free diet for 18 h. Livers were excised and homogenized in cell lysis buffer (2 mM sucrose, 10 mM Tris-Cl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, protease inhibitor mixture). After centrifugation at 75,000 × g for 60
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min, the supernatants were discarded, and nuclei were resuspended in high salt extraction buffer (400 mM NaCl, 10 mM Tris-Cl, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, protease inhibitors) and incubated on ice for 30 min. The extracts were centrifuged for 60 min at 250,000 x g, and supernatants were used as nuclear extracts for subsequent analysis.

Immunoprecipitation—To determine whether SREBP-1 could be coimmunoprecipitated with USF-1, COS-7 cells were cotransfected with 5 μg of pCDNA-FLAG-USF-1 and 10 μg of pCDNA 3.1-SREBP-1a and harvested 48 h after transfection. Cells were washed twice with PBS, scraped, collected by centrifugation at 1000 x g for 10 min, and then lysed by passing through a 21-gauge needle 25 times in Triton X-100 lysis buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM DTT) supplemented with protease inhibitors (Sigma). Lysates were clarified by centrifuging at maximum speed in a tabletop refrigerated centrifuge for 10 min. Antibodies (10 μg/immunoprecipitation) against USF-1 (C-20, Santa Cruz Biotechnology), SREBP-1 (anti-SREBP-1 antibodies (10 μg/immunoprecipitation) against USF-1 (C-20, Santa Cruz Biotechnology)), or control IgG were immobilized on protein A-agarose resin for 2–3 h followed by three washes in the same buffer without Triton X-100. After the last wash, resins were resuspended in 800 μl of Triton X-100 lysis buffer + 200 μl of clarified lysates and incubated overnight with gentle rotation at 4 °C. The following day, immunoprecipitates were washed five times in Triton X-100 lysis buffer followed by two additional washes in the same buffer without Triton X-100. After the last wash, immunoprecipitates were boiled in 50 μl of 1× SDS loading buffer, resolved by 7.5% SDS-PAGE, and transferred to nitrocellulose membranes. Immunoblotting was performed with anti-FLAG and anti-SREBP-1 (2A4, Santa Cruz Biotechnology) antibodies described above except that no anti-USF-1 antibodies (C-20, Santa Cruz Biotechnology) in 1 ml of PBS bead-binding buffer, and immunoprecipitated overnight. Immune complexes were collected with protein A-agarose, washed five times with PBS bead-binding buffer, and eluted with 100 μM glycine, pH 2.5, for 5 min. The eluates were neutralized with 1 M Tris, pH 7.4, and added to GST and GST-SREBP immobilized on glutathione-agarose beads and processed as described.

For copurification of endogenous SREBP-1 with GST-USF-1 FLAG from mouse liver, nuclear extracts were added to immobilized GST or GST-USF-1 FLAG fusion protein and incubated overnight with gentle rotation. Following extensive washing, bound proteins were eluted with glutathione and then subjected to a second round of purification on anti-FLAG resin (Sigma). Eluted complexes were subjected to SDS-PAGE and Western blotting to detect the presence of GST-USF-1 FLAG and endogenous SREBP-1.

Transfection of SL2 Cells for Functional Studies—Drosophila SL2 cells were maintained as described (39). Approximately 16–20 h before transfection, cells were plated at a density of 0.25 × 10⁶ cells/ml into 48-well plates. Cells were transfected using a standard calcium phosphate precipitation protocol. Each well received the indicated amount of pPAC-USF-1 and/or pPAC-SREBP-1c expression vector (generally 1–25 ng) and 500 ng of luciferase reporter construct along with 10 ng of the control plasmid pAC-5.1/V5-His/lacZ for normalization of transfection efficiency. The total amount of DNA transfected per well was brought to 1 μg with the empty expression vector pPAC-0 (470–500 ng/well). Cells were harvested ~48 h after transfection, and luciferase/β-galactosidase activities were assayed using the dual-light system (Applied Biosystems). Each transfection was carried out in triplicate.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation experiments on fasted/refed mice were performed as described previously (16). Primer sequences for amplification...
of the proximal region of the mouse mitochondrial GPAT promoter were 5'-ACAGCACAATCACA-GAGAATGGGGC-3' and 5'-GAGGAGGAGGACTCAGGCTTCCG-GAG-3'. This produces a predicted amplicon size of 182 bp.

RESULTS

Interaction of USF and SREBP-1c in Vivo—Our previous results in transgenic mice have indicated that binding sites in the FAS promoter for both USF and SREBP-1c are required for activation of FAS transcription by feeding/insulin. We also demonstrated that if binding of USF to the E-box at -65 is prevented by mutation, then SREBP does not bind to the -150 SRE in vivo (16, 40). In addition, functional studies in USF/-/- and SREBP-1c/-/- mice showed that FAS transcription is significantly reduced when either factor is missing (27, 28). These highly suggestive data led us to investigate a possible mutual interaction of USF and SREBP-1c. To determine whether USF and SREBP-1c can physically interact in vivo, we employed the tandem affinity purification (TAP) technique (41–44). An expression vector containing full-length SREBP-1c with a Myc tag at the C terminus was cotransfected along with USF-FLAG-TAP vector into 293-FT cells, and the lysates were purified over a streptavidin-binding resin and a calmodulin-binding resin, and the eluates were subjected to Western blotting to detect the presence of USF and SREBP-1c. As expected, USF-FLAG-TAP was efficiently purified over the affinity resins (Fig. 1B, left panel). As shown by a very robust signal in the USF-FLAG-TAP lysates, SREBP-1c-Myc copurified with USF, but no signal was observed in eluates derived from cells transfected with an in-frame fusion of FLAG-tagged human USF-1 with a streptavidin binding domain and a calmodulin binding domain (Fig. 1A). An expression vector containing full-length SREBP-1c with a Myc tag at the C terminus was cotransfected along with USF-FLAG-TAP vector into 293-FT cells, and the lysates were purified over a streptavidin-binding resin and a calmodulin-binding resin, and the eluates were subjected to Western blotting to detect the presence of USF and SREBP-1c. As expected, USF-FLAG-TAP was efficiently purified over the affinity resins (Fig. 1B, left panel). As shown by a very robust signal in the USF-FLAG-TAP lysates, SREBP-1c-Myc copurified with USF, but no signal was observed in eluates derived from cells transfected with an in-frame fusion of FLAG-tagged human USF-1 with a streptavidin binding domain and a calmodulin binding domain (Fig. 1A). An expression vector containing full-length SREBP-1c with a Myc tag at the C terminus was cotransfected along with USF-FLAG-TAP vector into 293-FT cells, and the lysates were purified over a streptavidin-binding resin and a calmodulin-binding resin, and the eluates were subjected to Western blotting to detect the presence of USF and SREBP-1c. As expected, USF-FLAG-TAP was efficiently purified over the affinity resins (Fig. 1B, left panel). As shown by a very robust signal in the USF-FLAG-TAP lysates, SREBP-1c-Myc copurified with USF, but no signal was observed in eluates derived from cells transfected with an in-frame fusion of FLAG-tagged human USF-1 with a streptavidin binding domain and a calmodulin binding domain (Fig. 1A). An expression vector containing full-length SREBP-1c with a Myc tag at the C terminus was cotransfected along with USF-FLAG-TAP vector into 293-FT cells, and the lysates were purified over a streptavidin-binding resin and a calmodulin-binding resin, and the eluates were subjected to Western blotting to detect the presence of USF and SREBP-1c. As expected, USF-FLAG-TAP was efficiently purified over the affinity resins (Fig. 1B, left panel). As shown by a very robust signal in the USF-FLAG-TAP lysates, SREBP-1c-Myc copurified with USF, but no signal was observed in eluates derived from cells transfected with an in-frame fusion of FLAG-tagged human USF-1 with a streptavidin binding domain and a calmodulin binding domain (Fig. 1A). An expression vector containing full-length SREBP-1c with a Myc tag at the C terminus was cotransfected along with USF-FLAG-TAP vector into 293-FT cells, and the lysates were purified over a streptavidin-binding resin and a calmodulin-binding resin, and the eluates were subjected to Western blotting to detect the presence of USF and SREBP-1c. As expected, USF-FLAG-TAP was efficiently purified over the affinity resins (Fig. 1B, left panel). As shown by a very robust signal in the USF-FLAG-TAP lysates, SREBP-1c-Myc copurified with USF, but no signal was observed in eluates derived from cells transfected with an
empty TAP vector. We next transfected 293F cells with USF-FLAG-TAP vector, and nuclear extracts were subjected to purification over the two affinity resins to determine whether endogenous SREBP-1 could be copurified with USF. As shown in Fig. 1B (right panel), a strong signal was observed when the TAP eluates were immunoblotted with an anti-SREBP-1 antibody. These results indicate that USF and SREBP-1 can physically interact in vivo.

To confirm that USF interacts with SREBP-1c in vivo, we also performed coimmunoprecipitation experiments. Cells were cotransfected with FLAG-tagged USF-1 (USF-1-FLAG) and SREBP-1a or Myc-tagged SREBP-1c (Fig. 1A), and then lysates were used for immunoprecipitation with polyclonal antibodies against USF-1 and SREBP-1. After extensive washing, the immunoprecipitates were resolved by 10% SDS-PAGE and analyzed for the presence of USF-1-FLAG and SREBP-1c-Myc by Western blotting. As shown in Fig. 1C, not only USF but also and the eluates were then re-incubated with an anti-FLAG affinity resin. The bound proteins were separated by SDS-PAGE and immunoblotted with anti-FLAG and anti-SREBP-1 antibodies. As shown in Fig. 1D, no signal was observed in either of the purifications with GST alone. However, immunoreactive bands were clearly detected by the SREBP antibody in the GST-USF-FLAG purifications from both fasted and fasted/refed mouse liver. Furthermore, the signal was higher from refed compared with fasted mice, consistent with the known induction of SREBP-1c. These results further confirm the USF-SREBP-1c interaction.

Identification of Domain(s) of USF That Interact with SREBP-1c—To determine whether the interaction between USF and SREBP is direct, we performed a GST pulldown using bacterially expressed USF and in vitro translated SREBP-1c. Full-length USF-1 was expressed as a fusion protein with GST in E. coli (Fig. 2, A and B). Following immobilization of GST-
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A. Structure of Mature Form of SREBP-1c:

B. GST-SREBP (1-436)

C. GST-SREBP (37-436)

D. GST-SREBP (300-436)

E. GST-SREBP (376-436)

D. GST-SREBP (37-153)

E. GST-SREBP (37-299)

F. GST-SREBP (154-299)

G. GST-SREBP (1-349)

H. GST-SREBP (154-436)
USF-1 fusion protein on glutathione-agarose beads, in vitro transcribed and translated and 35S-labeled SREBP-1c was added for overnight incubation. Following extensive washing, eluted complexes were subjected to SDS-PAGE and autoradiography to detect the presence of 35S-SREBP-1c. As shown in Fig. 2C, 35S-SREBP-1c was readily detectable in the purification with GST-USF-1 but not with GST alone. These results demonstrate that the interaction between USF and SREBP-1c is direct and specific.

We next asked which domains of USF are required for interaction with SREBP-1c. As shown in Fig. 2A, USF-1 consists of an N-terminal activation domain (residues 1–196), a bHLH region (residues 197–260), and the C-terminal leucine zipper (residues 261–310). We constructed a series of USF-1 truncations linked to GST and expressed them in E. coli (Fig. 2B). USF-1 fusion proteins were immobilized on glutathione-agarose beads and then incubated with in vitro-translated 35S-SREBP-1c. As shown in Fig. 2C, SREBP-1c efficiently interacted with USF-1-(197–310), even when the entire activation domain has been deleted (lane 4), signifying that the activation domain of USF is not required for interaction with SREBP-1c. However, when the bHLH region of USF was deleted, the interaction with SREBP-1c was abolished (Fig. 2C, lane 6), showing that the bHLH region of USF is required for interaction with SREBP-1c and that the leucine zipper may not be involved in the interaction. In support of this, SREBP-1c did not interact with the leucine zipper region of USF alone. The interaction of SREBP-1c was clearly detected when the bHLH region of USF alone was fused to GST (Fig. 2C, lane 5), indicating that the bHLH region of USF is sufficient for interaction with SREBP. No interaction of SREBP was observed with the activation domain (residues 1–196) of USF alone (Fig. 2C, lane 7), confirming that this region is not involved in the USF-SREBP interaction.

Identification of Domain(s) of SREBP-1c That Interact with USF—The domain structure of the mature form of SREBP-1c is shown in Fig. 3A. The extreme N terminus of SREBP-1c contains the putative activation domain (46, 47), followed by a Pro/Ser-rich region from amino acids 37 to 153 (23). Like USF, the bHLH/LZ region is located at the C terminus. To determine which region(s) in SREBP-1c interact with USF, we generated a series of 5’-deletion constructs linked to GST and expressed them in E. coli (Fig. 3B). Five of these fusion constructs were immobilized on glutathione-agarose beads and incubated with in vitro-transcribed and translated 35S-USF-1. As shown in Fig. 3C, USF efficiently copurified with full-length SREBP-1c-(1–436). USF also copurified with a truncated SREBP-1c-(37–436) that contains all regions except the putative activation domain, indicating that the activation domain of SREBP-1c is not required for interaction with USF. This is consistent with our observation that USF also interacts with SREBP-1a (Fig. 1C, left panel); the only difference between the SREBP-1a and -1c isoforms lies within the first exon that includes four unique amino acids for SREBP-1c. However, when the entire region from 1 to 299 of SREBP-1c was deleted, the interaction with USF was lost (Fig. 3C, lane 5), clearly indicating that the N-terminal region between 37 and 299 is required and that the bHLH region alone is not sufficient for interaction with USF.

To further define the region(s) of SREBP-1c that interact with USF, a second series of truncations was generated and fused to GST (Fig. 3, D and E). Full-length GST-SREBP-1c-(1–436) was included as a positive control in this experiment. As shown in Fig. 3F, in vitro translated 35S-USF-1 efficiently interacted with GST-SREBP-1c-(1–436). None of the first three GST-SREBP fusion constructs depicted schematically in Fig. 3D were capable of interacting with USF (Fig. 3F, lanes 4–6), indicating that these N-terminal regions alone are not sufficient for the interaction. Inclusion of the bHLH region of SREBP in the last two constructs shown in Fig. 3D restored interaction with USF (Fig. 3F, lanes 7 and 8), although deletion of the Pro/Ser-rich region had no effect. This indicates that two regions of SREBP-1c are required for binding to USF as follows: an N-terminal uncharacterized region spanning amino acids 154–299, and the bHLH region spanning amino acids 300–370.

To confirm that the interaction between USF and SREBP is direct, we performed an additional experiment in which in vitro translated 35S-USF-1 was first immunoprecipitated from the reticulocyte lysate reaction prior to incubation with full-length GST-SREBP. As shown in Fig. 3G, purified USF-1 still interacted with SREBP-1c, further confirming our results indicating that the two proteins interact directly.

Synergistic Activation of the FAS and GPAT Promoters by USF and SREBP-1c—Our previous studies have suggested that the effects of USF and SREBP-1c on FAS transcription are not independent and mutually exclusive (15, 16, 26). This is supported by the observation that FAS transcriptional activity in vivo is significantly reduced when either factor is missing (27–29) or when their respective binding sites are mutated (16). This is not the case in 3T3-L1 cells, where transfection of either factor alone results in a relatively potent activation of FAS transcription (15, 26). Regardless, because we found that USF and SREBP-1c directly interact in vitro and in vivo, we hypothesized...
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A

-444-FAS

-248-FAS

SRE

E-BOX

TATA

-332

-150

-65

-150

-65

-248-FAS

-444-FAS

Fold Activation

pPAC-0

pPAC-USF-1

pPAC-SREBP-1c

pPAC-0

pPAC-USF-1

pPAC-SREBP-1c

B

FAS Promoter Activity

C

D

FAS Promoter Activity

pPAC-SREBP-1 (ng)

pPAC-USF-1 (ng)
that introduction of both USF and SREBP-1c would result in a synergistic activation of the FAS promoter. The “synergy index” can be defined here as the ratio of the fold activation of the FAS promoter when USF and SREBP-1c are cotransfected together to the sum of their individual fold activations when each factor is transfected separately. Although reports in the literature vary, a synergy index of 1.5–2.5 seems to be indicative of a high degree of functional cooperativity (48).

To determine whether USF and SREBP-1c can function synergistically in activation of the FAS promoter, we employed Drosophila SL2 cells. Although Drosophila has distantly related orthologs of USF (49) and SREBP (50), these cells lack many mammalian transcription factors and are commonly used as a null background for studying functional transcription factor interactions. Therefore, we reasoned that SL2 cells should provide an ideal minimal system for studying synergy between USF and SREBP-1. To that end, we generated Drosophila expression vectors for human USF-1 and SREBP-1c (pPAC-USF-1 and pPAC-SREBP-1c, respectively). We first asked whether cotransfection of USF and SREBP-1c would result in synergistic activation of the −248 FAS promoter, containing only the E-box at −65 and the SRE at −150 (26). Although introduction of SREBP-1c alone resulted in slight activation of this FAS promoter construct, no significant synergy with USF was observed (Fig. 4A, left panel). We next used the longer −444 FAS-Luc reporter construct that includes, in addition to the E-box at −65, a second E-box at −332 that we have shown previously to be a USF-binding site in vitro and in vivo (15, 26). A high degree of synergy between USF and SREBP-1c was observed with this construct (Fig. 4A, right panel), indicating that binding of USF to the E-box at −65 is not sufficient to mediate maximal synergy with SREBP at the −150 SRE and that the upstream E-box is required for cooperative activation. These results are consistent with our previous in vivo results, where we demonstrated that a −278-FAS promoter fragment has significantly reduced transcriptional activity in refed transgenic mice and that the region from −278 to −444, containing the −332 E-box, is required for high level activation (15). It is also worth noting that cotransfection of USF alone resulted in only a very modest activation of the FAS promoter (−2.5-fold), which is consistent with the lack of FAS transcriptional activation in livers of fasted mice when only USF is bound (16).

In our previous in vivo experiments using transgenic animals, we demonstrated that mutation of the −65 E-box abolishes induction of FAS transcription in response to fasting/refeeding (16). To determine whether the −65 E-box is required for synergistic activation by USF and SREBP, we used a −444 FAS promoter construct containing a mutation at the E-box that prevents binding of either protein (26). As shown in Fig. 4B, mutation of the E-box at −65 not only abolished synergy between USF and SREBP but also reduced the promoter activity to levels similar to that of the promoterless vector pGL2basic (filled bars). Thus, the −65 E-box is crucial for both basal and stimulated activity of the FAS promoter, and these in vitro results are in agreement with our results in vivo.

To determine whether USF can augment FAS promoter activation by SREBP in a dose-dependent manner, and vice versa, we cotransfected increasing amounts of pPAC-SREBP-1c along with constant pPAC-USF-1 (10 ng/well) and the −444 FAS reporter construct. As shown in Fig. 4C, SREBP-1c alone activated the FAS promoter to a maximum of about 20-fold at 10 ng of expression plasmid/well. However, inclusion of 10 ng/well pPAC-USF-1 boosted the activation by SREBP-1c at all concentrations and reached a maximum of ~80-fold at 10 ng of pPAC-SREBP-1c (Fig. 4C). At the highest concentration of SREBP-1c (20 ng/well), we observed a reduction in promoter activity, probably as a result of “squelching.” Importantly, no activation of the promoterless pGL2-basic vector by USF or SREBP was observed in these experiments (Fig. 4C, open squares and circles). Overall, these results indicate that SREBP-1c-mediated activation of the FAS promoter is highly synergistic with USF.

In the inverse experiment, we transfected increasing amounts of pPAC-USF-1 in the presence of 10 ng/well pPAC-SREBP-1c. As shown in Fig. 4D, the cotransfection of SREBP-1c significantly boosted activation by USF-1 to a maximum of about 60-fold. The highest synergy was observed at 10 ng of each transcription factor/well, similar to the results shown in Fig. 4C. At this concentration of each factor in both experiments, the fold activation of the FAS promoter obtained when both USF and SREBP were added together was about three times higher than the sum of their individual fold activations, indicating that USF and SREBP are not acting in an additive and independent manner.

To determine whether the functional collaboration between USF and SREBP-1c might constitute a common mechanism for lipogenic gene induction during feeding/insulin, we next asked whether cotransfection of USF and SREBP-1c would result in synergistic activation of the mGPAT promoter as well. Mitochondrial GPAT catalyzes the condensation of fatty acids with the sn-1 position of glycerol 3-phosphate and, like FAS, is strongly induced by feeding/insulin at the transcriptional level (8, 11). For this purpose we used the −1447 mGPAT promoter,

FIGURE 4. USF and SREBP-1c synergistically activate the FAS promoter in Drosophila SL2 cells. A, SL2 cells were cotransfected with 10 ng of pPAC-USF-1 and/or 10 ng of pPAC-SREBP-1c with the −248 or −444 FAS promoter construct as indicated, and luciferase activity was measured as described under “Experimental Procedures.” Values are expressed as fold activation, where fold activation is calculated as the ratio of normalized luciferase activity observed in the presence of the indicated transcription factor versus in the absence of any expression vector encoding USF or SREBP. All transfections were performed in triplicate, and the total amount of expression vector in each transfection was brought to 500 ng with the empty vector pPAC-O. Results are representative of at least two independent experiments. B, mutation of the E-box at −65 abolishes induction of FAS promoter activity by USF and SREBP. Results are expressed as normalized promoter activity (luciferase/β-galactosidase; ± S.E.) and are representative of at least two independent experiments. C, USF promotes activation of the −444 FAS promoter by SREBP-1c. SL2 cells were cotransfected with increasing pPAC-SREBP-1c and the empty vector pPAC-O or constant pPAC-USF-1 plasmid (10 ng/well) along with the −444 FAS-Luc reporter construct. Normalized FAS promoter activities are plotted as the ratio of luciferase/β-galactosidase (± S.E.). Transfections were performed in triplicate. Activation of the pGL2 basic vector by USF and SREBP-1c is shown as a control. Results are representative of at least two independent experiments. D, SREBP-1c promotes activation of the FAS promoter by USF. SL2 cells were cotransfected with increasing pPAC-USF-1 along with the empty vector pPAC-0 (circles) or constant pPAC-SREBP-1c plasmid (10 ng/well; squares) along with the −444 FAS-Luc reporter construct. Normalized luciferase activities and fold activation are calculated as in C. Results are representative of at least two independent experiments.
which we previously demonstrated to have high activity in 3T3-L1 adipocytes (37) and which has already been shown to be activated by SREBP-1a through an SRE-like element at position 64 (51). There are also several E-boxes in this construct, including one at position 321 which is identical in sequence to that of the E-box in the FAS promoter at position 65. Despite this observation, USF alone was unable to activate the −1447 mGPAT promoter in SL2 cells (Fig. 5A, circles). However, in the presence of 10 ng/well pPAC-SREBP-1c, USF-1 activated the mGPAT promoter in a dose-dependent manner up to a maximum of ~50-fold at a concentration of 10 ng/well (Fig. 5A, squares). Likewise, SREBP-1c alone only weakly activated the mGPAT promoter (Fig. 5B, circles) to a maximum of ~9-fold at a concentration of 10 ng/well. However, in the presence of 10 ng/well USF-1, the activation of mGPAT by SREBP was significantly higher at 5 or 10 ng/well (Fig. 5B, squares). No squelching was observed in the experiment shown in Fig. 5B, probably because we used a lower concentration of SREBP-1c plasmid than in the experiments with the FAS promoter. The fold activation of both transcription factors added together was nearly six times higher than the sum of their individual fold activations (Fig. 5C, left panel), indicating a high degree of synergy between USF and SREBP-1c in activation of the −1447 mGPAT promoter.

The proximal mGPAT promoter contains three potential SREBP-binding sites at −64, −170, and −186 (Fig. 5C). However, only the SRE-like element at −64 was shown to be important for promoter regulation in 3T3-L1 cells (51). Our results are in agreement with this, as removal of the two distal SREs by deletion did not reduce activation by SREBP-1c alone (Fig. 5C, right panel). As shown above, the USF-binding site at −65 in the FAS promoter is not sufficient to mediate synergy with SREBP-1c. Therefore, we next tested whether the single putative USF-binding site present at −321 is sufficient for cooperative activation of the mGPAT promoter with SREBP-1c. For this purpose, we used the shorter −322 mGPAT promoter construct. As shown in Fig. 5C (middle panel), full synergy was still observed with this construct, suggesting that only one E-box for USF is required for synergy with SREBP-1c in activation of the mGPAT promoter. In addition,
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 Cooperative activation was completely abolished in the −86 mGPAT construct that includes the SRE at −64 but lacks the upstream E-box (Fig. 5C). In fact, SREBP-1c-mediated activation of this construct was actually inhibited by coexpression of USF-1 (Fig. 5C, right panel, filled bar). Thus, it is possible that in the absence of a USF-binding site, the interaction of SREBP with USF in solution precludes the binding of SREBP to its target sequence or prevents productive interaction of SREBP with other proteins such as coactivators or general transcription factors.

Occupancy of the FAS and mGPAT Promoters by USF and SREBP in Vivo—We next asked whether USF-1 and SREBP-1 occupy the mGPAT promoter in mouse liver during fasting/refeeding, as we have shown in earlier studies for the FAS promoter. Indeed, by using ChIP analysis, we readily detected in vivo binding of both USF and SREBP-1c to the proximal region of the mGPAT promoter in a manner indistinguishable from what we observed previously for the FAS promoter (16). Specifically, binding of USF-1 to the FAS and mGPAT promoters was detected in both the fasted and refed states, whereas SREBP-1 binding was detectable only during refeeding (Fig. 6). These results suggest a direct role for both USF and SREBP in regulation of the mGPAT promoter during fasting/refeeding in vivo.

The Activation Domains of USF and SREBP Are Required for Synergistic Activation of the FAS Promoter—We hypothesized that synergy between USF and SREBP-1c could potentially arise through two distinct mechanisms. First, because the bHLH regions of both proteins are involved in the physical interaction, synergy between USF and SREBP-1c could arise from recruitment or enhanced binding of SREBP to the promoter through interaction with USF, as suggested by our previous results in vivo. Second, the synergy could also involve recruitment of one or more coactivators by either or both activation domains of USF and SREBP. First, to determine whether the activation domain of USF is required for synergistic activation of the FAS promoter, we made a single deletion construct of USF-1 that has the entire activation domain deleted (amino acids 1–196) but preserves the bHLH/LZ region (amino acids 197–310). We reasoned that if the mechanism of synergy between USF and SREBP-1c results entirely from recruitment of SREBP by USF, removing the activation domain of USF-1 but preserving its bHLH/LZ region would not attenuate the synergy because the 113-amino acid bHLH/LZ domain of USF still both interacts with SREBP-1c (Fig. 2C) and binds E-boxes (52). As shown in Fig. 7A, this truncated USF-1 displayed little transactivation (dotted bar), consistent with others who have reported that the activation domain of USF is indispensable for transcriptional activation (53). We next cotransfected pPAC-USF-1-(197–310) along with full-length SREBP-1c into SL2 cells at the same concentration of each, which gave maximum synergy observed with full-length USF in this experiment. As shown in Fig. 7A, synergy with SREBP-1c was not only abolished with this construct, but cotransfection of the truncated version of USF-1 actually inhibited SREBP-1c-mediated promoter activity by ~70% (Fig. 7A, checkered bar). This suggests that the synergy we have observed cannot be attributed solely to cooperative DNA binding and that recruitment of coactivators may be required for maximal activation.

To determine which domain(s) of SREBP-1c are functionally required for synergy with USF, we next generated a series of N-terminal SREBP-1c deletion constructs (Fig. 7B) and cotransfected them along with full-length USF-1 in SL2 cells. The three deletion constructs employed were pPAC-SREBP-1c-(37–436), pPAC-SREBP-1c-(154–436), and pPAC-SREBP-1c-(300–436), which are deleted for the first 36, 153, and 299 amino acids of SREBP-1c, respectively. As shown in Fig. 7B (middle panel), deletion of the N-terminal region of SREBP-1c abolishes its transcriptional activity, despite the fact that these constructs contain the bHLH/LZ region and should therefore retain the ability to bind DNA. This functionally confirms the results of others who have shown that the putative activation domain of SREBP-1c maps to the N-terminal region (46, 47, 54). When cotransfected along with 10 ng/well pPAC-USF-1, which itself resulted in only a very modest ~5-fold induction (Fig. 7B, middle panel), synergy of the first two SREBP-1c deletion mutants with USF was abolished under conditions in which full-length SREBP-1c was highly synergistic with USF (filled bar). Notably, these two SREBP-1c mutants, SREBP-1c-(37–436) and SREBP-1c-(154–436), appeared to actually inhibit the modest 5-fold activation by USF. To determine whether these deletion mutants can inhibit activation by USF when USF is transfected at a higher concentration, we cotransfected 20 ng/well pPAC-USF-1 along with 10 ng/well of each SREBP-1c deletion construct (Fig. 7B, right panel). Transfection of 20 ng/well pPAC-USF-1 alone resulted in a more robust ~25-fold activation (Fig. 7B, dotted bar), and the first two SREBP-1c deletion constructs displayed a strong inhibition of USF-mediated activation (~74 and ~81%, respectively). However, the shortest construct, containing only the 136-amino acid bHLH/LZ region of SREBP-1c, had no effect on activation by USF (Fig. 7B, checkered bar). Importantly, the first two constructs can clearly
interact physically with USF (as indicated by GST pulldown assay), whereas SREBP-1c-(300–436) was completely unable to interact with USF (Fig. 3C) but still retains the ability to bind DNA (52). These data suggest that the potent inhibition of USF transcriptional activity observed with SREBP-1c-(37–436) and SREBP-1c-(154–436) is not simply because of occupancy of the E-boxes in the FAS promoter by these truncated inactive proteins, but rather that these constructs may interact with USF and function in a dominant negative manner. In addition, these functional deletion studies corroborate the results of our inter-
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FIGURE 8. Schematic of E-box and SRE elements present in the proximal promoter regions of various mouse or rat lipid metabolic genes. For simplicity, only the E-boxes in closest upstream proximity to putative SREs are shown, except in the case of the FAS promoter, which contains an additional E-box at −65. In the mouse LDLR promoter, a putative SRE at −212 and an E-box at −560 were identified by sequence analysis. The putative SRE in the mouse LDLR promoter differs by 1 bp from the well characterized SRE in the human LDLR promoter. In the mouse mGPAT and rat ATP-citrate lyase (ACL) promoters, the E-boxes shown were also identified by sequence analysis.

action domain mapping and strongly imply that physical interactions between USF and SREBP-1c are required for synergistic activation of the FAS and mGPAT promoters.

DISCUSSION

Tissue-specific and hormonal/nutritional regulation of genes depends critically on the activities and protein-protein interactions within and between transcription factors that bind to the cis-acting elements located in their respective promoters. The molecular mechanism for activation of the FAS promoter by feeding/insulin is likely to be complex and probably involves a multitude of cis-acting elements and protein-protein interactions between several different classes of transcriptional regulators. Our previous results and data from this study support a model in which two principal players, USF and SREBP-1c, directly collaborate to regulate FAS transcription. We show, for the first time, that USF and SREBP-1c not only physically interact, but also synergistically activate the FAS promoter as well as the mGPAT promoter. The role of USF in activation of FAS transcription by SREBP-1c in response to feeding/insulin appears to be analogous to the roles of Sp1 and NF-Y in mediating transcriptional activation of several genes, including FAS, by SREBP-1a during cellular sterol depletion. SREBPs may generally require auxiliary factors for maximal transactivation (22, 30–32, 55–59). It is worth noting that we observed a strong activation of the FAS and GPAT promoters by USF and SREBP-1c in Drosophila SL2 cells, which lack Sp1 and NF-Y (31, 36), suggesting that these latter factors are dispensable for high level activation of the FAS promoter, at least in vitro. In contrast, induction of FAS transcription by fasting/refeeding is significantly reduced in USF-1−/− and USF-2−/− knock-out mice (27). Taken together, our results indicate that USF, rather than Sp1 or NF-Y, may be the principal coregulator of SREBP-1c in transcriptional activation of the fatty-acid synthase gene in response to feeding/insulin.

The synergistic activation of the mitochondrial mGPAT promoter by USF and SREBP-1c was an intriguing result, because a role for USF in activation of the mGPAT promoter, to our knowledge, has never been investigated. Previous attempts to show activation of the mGPAT promoter by USF might have failed, as USF alone was unable to activate the mGPAT promoter, as shown in our study. This occurred despite the presence of multiple E-boxes, including one at −321 which is identical in sequence to the −65 E-box in the FAS promoter. In this regard, the ability of USF to activate transcription of target genes independently of other transcription factors may be related to the proximity of the E-box to the TATA-box, as has been shown for activation of the adenovirus major late promoter, where USF and TFIID bind cooperatively (60–62). In fact, the −65 E-box in the FAS promoter is approximately the same distance from the TATA-box as in the adenovirus major late promoter. Thus, the lack of activation of the mGPAT promoter by USF alone may be explained by the absence of any E-box motif in close proximity to the TATA-box. Nevertheless, in the presence of SREBP-1c, USF activates the mGPAT promoter in a dose-dependent manner up to 50-fold (Fig. 5A). Because mGPAT is strongly induced by feeding/insulin in a coordinate manner with FAS (8, 11), it is quite possible that interaction between USF and SREBP-1c mediates activation of mGPAT in vivo. In support of this, the pattern of USF and SREBP-1c binding to the mGPAT promoter during fasting/refeeding by ChIP (Fig. 6) was indistinguishable from what we observed previously for the FAS promoter (16).

In this study we provide clear evidence for cooperative activation of both the FAS and mGPAT promoters by USF and SREBP. In addition to FAS and GPAT, there is suggestive evidence for a role of both proteins in activation of another lipogenic enzyme gene, acetyl-CoA carboxylase-α (ACC-α). Barber et al. (63) used ChIP analysis to show binding of both USF and SREBP-1 to the ovine ACC-α promoter. In this study, USF binding did not change significantly between the nonlactating and lactating states, whereas recruitment of SREBP to the promoter during lactation was associated with a strong induction of ACC-α transcription. In addition, USF was reported to activate the cardiac ACC-β gene through an E-box located in the proximal promoter region (64), and ACC-β has been shown by ChIP to be occupied by SREBP-1 at a nearby site, at least in liver (65). Thus, it is possible that the interactions we have observed here might also be involved in synergistic activation of the ACC
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promoter. Furthermore, the presence of closely spaced E-boxes and SRE-like elements in the promoters of several lipogenic genes (Fig. 8) implicates a possible role for USF and SREBP-1c in mediating the overall transcriptional response to fasting/refeeding in a manner similar to FAS and mGPAT (13–16, 26, 51, 65–74).

The fact that USFs and SREBPs are both highly related bHLH/LZ proteins suggests the obvious possibility that USFs and SREBPs could form a heterodimer on E-boxes, as has already been proposed (63). However, several lines of evidence indicate that this is not the case. First, we did not detect heterocomplex formation between USF and SREBP-1c on the −65 E-box in our earlier gel-shift experiments with either nuclear extracts or recombinant proteins (14). Second, in our ChIP assays, we detected binding of USF, but not SREBP-1, to a truncated −131 FAS promoter containing only the −65 E-box, but lacking the −150 SRE, in transgenic mice (16). Furthermore, in our present domain mapping experiments, we have clearly demonstrated that the isolated bHLH/LZ region of SREBP does not by itself interact with USF (Fig. 3C, lane 5), which would be highly suggestive of heterodimer formation. In addition, others have shown that a truncated SREBP-1 containing only the bHLH/LZ domain efficiently forms homodimers but does not form heterodimers with other Myc family members, including USF and Max (52). It therefore seems likely that if USF and SREBP formed a heterodimer, then the bHLH/LZs of both proteins alone would be sufficient to mediate a stable interaction. Overall, these observations clearly rule out that the mode of interaction between USF and SREBP involves simultaneous binding of each factor to E-boxes.

Together with our previous in vivo data, the present results indicate that the mechanism of synergy may involve both recruitment of SREBP by USF to the FAS promoter followed by interaction of the activation domains of both proteins with yet-to-be identified coactivators. This is supported by our observations that deletion of the N-terminal activation domain of either protein potently inhibits the transactivation ability of its partner. A similar observation has been described for the cooperative interaction between USF and Ets-1 on the human immunodeficiency virus, type 1, enhancer in T cells, where deletion of the activation domain of the latter transcription factor inhibits activation by USF in vitro (75). These authors proposed that recruitment of Ets-1 by USF enables Ets-1 to interact with the basal transcription apparatus. Although the mechanistic details may differ, an analogous situation for the relation between USF and SREBP may explain the results we observe here. Identification of those factors recruited by USF and SREBP-1c would aid in understanding at the molecular level how physical interactions between USF and SREBP lead to synergistic activation of the FAS and mGPAT genes.

In conclusion, we have clearly shown that USF interacts with SREBP-1, and this requires the bHLH domains of both proteins, as well as an additional N-terminal region in SREBP. Cotransfection of USF and SREBP-1c into Drosophila SL2 cells results in a synergistic activation of the FAS as well as the mGPAT promoters, and the activation domains of both proteins are required for synergy. These studies provide a better understanding of the molecular mechanism leading to increased lipogenic gene transcription and lipid storage in feeding/insulin.

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