Fatty acid synthase (FAS) plays a central role in de novo lipogenesis in mammals. We have shown that FAS transcription rate is induced dramatically when fasted animals are refed with a high carbohydrate diet or when streptozotocin diabetic mice are given insulin. We also reported that FAS gene transcription was up-regulated by insulin through the proximal promoter region from −71 to −50 and that upstream stimulatory factors (USFs), including USF1 and USF2, interact with this region in vitro. In the present study, by using site-directed mutagenesis of the −71/−50 region and correlating functional assays of the mutated promoter with USF binding activities, we demonstrate that the −65/−60 E-box motif (5′-CATGTG-3′) is functionally required for insulin regulation and that USFs are in vivo components of the insulin response complex. Mutation of the −65/−60 E-box sequence abolished insulin response in both transiently and stably transfected 3T3-L1 adipocytes in the −2.1 kb promoter context, which contains all the necessary regulatory elements of the promoter based on our previous transgenic mice studies, and in the minimal −67 promoter context. Gel mobility shift assays demonstrated that USFs can no longer bind to the −71/−50 promoter region when the E-box is mutated. Cotransfection of USF1 and USF2 expression vectors with the FAS promoter-luciferase reporter constructs increased insulin-stimulated FAS promoter activity. Moreover, cotransfection of dominant negative USF1 and USF2 mutants lacking the DNA binding domain inhibited the insulin stimulation of the FAS promoter activity. On the other hand, site-directed mutagenesis of the −65/−60 E-box surrounding sequences within the overlapped tandem copies of sterol regulatory element-binding protein (SREBP) binding sites prevented SREBP from binding to −71/−50 promoter region in vitro but had no effect on insulin regulation of the FAS promoter in vivo. When rat liver nuclear extracts were used in gel mobility shift assays, only USF-containing protein-DNA complexes that can be supershifted by specific USF antibodies were observed. These results demonstrate that upstream stimulatory factor binding to the E-box at −65 is required for insulin regulation of the fatty acid synthase promoter.

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1 The abbreviations used are: FAS, fatty acid synthase; bHLH, basic helix-loop-helix; IRS, insulin response sequence; L-PK, L-type pyruvate kinase; FCR, polymerase chain reaction; PEPCK, phosphoenolpyruvate carboxykinase; SREBP, sterol response element-binding protein; USF, upstream stimulatory factor.
region is critical for DNA binding and, deletion of this region results in a dominant negative USF, which still dimerizes but lacks DNA binding capacity (18). Introduction of a dominant negative USF into cells can block the function of endogenous wild-type USF proteins (18–20). Although USF proteins were found to be ubiquitously expressed among various tissue and cell types, they can be involved in transcriptional regulation of specific genes. USF binding sites have been found, and the involvement of USF in transcriptional regulation have been studied in a number of cellular and viral genes (19–24). For example, USFs were shown to bind the glucose response element of the L-type pyruvate kinase (L-PK) and S14 gene promoters in vitro (21, 22). However, the role USFs play in mediating glucose response in vivo is yet to be clarified. Evidence supporting (19) as well as against (20) the direct involvement of USF proteins in glucose response of the L-PK and S14 genes was reported.

Based on our previous reports that sequences responsible for insulin regulation of the FAS gene is present in the promoter region at −71/−50 and USFs bind to this region in vitro, this report provides evidence to further define the −65/−60 E-box as a critical requirement for insulin regulation and demonstrate that USF proteins are in vivo components mediating insulin regulation of the FAS promoter activity. Using site-directed mutagenesis, we showed that mutations of the −65/−60 E-box, but not the surrounding sequences within the SREBP binding sites, could effectively abolish the USF binding to FAS −71/−50 region in vitro and disrupt the insulin regulation of the FAS promoter-luciferase reporter constructs in transfection assays. When cotransfected with the FAS promoter constructs, USF1 and USF2 could further activate and dominant negative USF1 and USF2 could inhibit the insulin-stimulated FAS promoter activity. Using gel mobility shift assays, we demonstrated that USF and SREBP proteins independently bind to their exclusive binding sites and that USF interaction with the −65/−60 E-box, but not SREBP, is required for insulin regulation of the FAS transcription.

EXPERIMENTAL PROCEDURES

Plasmid Constructs —The reporter gene constructs of p2.1kb-LUC and p67-LUC, which contain, respectively, the −2.1 kb and −67 bp of wild-type FAS promoter fused with luciferase sequence have been described previously (5). p2.1kbM-LUC and p67M-LUC constructs were made to mutate the FAS −65/−60 E-box sequence 5′-CATGTG-3′ to 5′-TTGG-3′ by site-directed mutagenesis methods (25, 26). In p2.1kbS1-LUC, p2.1kbS2-LUC, and p2.1kbS3-LUC plasmids, which contain the −2.1 kb FAS promoter with mutations disrupting the SREBP binding sites or SREBP and SP1 interaction (7), were also made by site-directed mutagenesis methods (25, 26). In p2.1kbS1-LUC, wild-type FAS promoter sequence 5′-TCA-3′ from position −71 to −69 and 5′-TGG-3′ from position −56 to −54 were changed to 5′-TCA-3′ and 5′-CAA-3′, respectively. In p2.1kbS2-LUC and p2.1kbS3-LUC, a 4-bp (5′-CATG-3′) and a 10-bp (5′-CTAGTCTAGA-3′) sequence were inserted between positions −73 and −72. Expression vectors pFLAG-USF1 and pFLAG-USF2, which contain full-length human USF1 and USF2 cDNA sequences, were made by site-directed mutagenesis (25, 26) of pFLAG-USF1 and pFLAG-USF2 to delete the basic regions of USF1 (amino acids 193–211) and USF2 (amino acids 228–247). SREBP expression vectors, pcDNA-SREBP1 and pcDNA-SREBP2, for active forms of SREBP1a (amino acids 1–490) and SREBP2 (amino acids 1–484) were constructed by inserting the corresponding Fnu DNA polymerase-amplified cDNA sequences into pcDNA3.1 expression vector (Invitrogen). A consensus Kozak sequence was also introduced immediately upstream of the ATG start codon of SREBP1a and SREBP2 by PCR primers.

Cell Culture and Transfection—3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and induced to differentiate to adipocytes by 0.5 ml 1-methyl-3-isobutylxanthine and 0.25 μM dexamethasone as described previously (5). For stable transfection of 3T3-L1 cells, 10 μg of the experimental reporter plasmid DNA and 1 μg of pcDNA3.0 plasmid DNA, which provides the neomycin resistance, were cotransfected into preadipocytes using the calcium-phosphate-DNA coprecipitation method (27) and selected with Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and 300 μg/ml G418 for 2 weeks. G418-resistant colonies were pooled for later differentiation and luciferase activity assay. For transient transfection of 3T3-L1 cells, 10 μg of the experimental reporter plasmid with the indicated amount of expression vectors (when used) were cotransfected into differentiated 3T3-L1 adipocytes using the calcium-phosphate-DNA coprecipitation method. Insulin treatment of the cells and luciferase activity assay were carried out as described previously (5). Triplicate samples were used for each transfection and average luciferase activities were used to calculate -fold changes induced by insulin and -fold stimulation caused by USF cotransfection.

In Vitro Transcription/Translation of USF and SREBP—Plasmid DNA containing USF1, USF2, SREBP1, and SREBP2 cDNA sequences cloned in pcDNA3.0 and pcDNA3.1 (as described above) was first linearized and then transcribed with T7 RNA polymerase using a mRNA capping kit (Stratagene). The mRNAs were then in vitro transcribed to USF and SREBP proteins by programming rabbit reticulocyte lysates (Promega). Manufacturer’s instructions were followed. The in vitro translation reaction with no mRNA added to the rabbit reticulocyte lysate was used as a negative control. Parallel experiments were set up to allow one set of reactions to produce [35S]Met-labeled proteins to monitor the translation products and the other set of reactions to produce unlabeled proteins for gel mobility shift assays.

Gel Mobility Shift Assay—The following single-stranded oligonucleotides were synthesized by Operon, (Alameda, CA).

**IRS-WT**: 5′-AGCTGTCACGCCATGGCCTGCGC-3′

**IRS-M**: 5′-AGCTGTCACGCAATTCGCTGCGCCG-3′

**IRS-S1**: 5′-AGTCGGCTACACCGCAGGCGTCGAC-3′

**IRS-S1**: 5′-AGTCGGCTACACCGCAGGCGTCGAC-3′

**IRS-S1**: 5′-AGTCGGCTACACCGCAGGCGTCGAC-3′

**IRS-S1**: 5′-AGTCGGCTACACCGCAGGCGTCGAC-3′

Underlined sequences are the mutations from the wild-type insulin response sequence (IRS-WT). IRS-M contains the mutation of the 5′-65 to 5′-60 E-box sequence, and IRS-S1 contains the mutation same as described in p2.1kbS1-LUC. Double-stranded oligonucleotides were formed as described previously (5). All probes for gel mobility shift assays were made by labeling with [α-32P]dCTP, in the presence of dATP, dGTP, and dTTP, and Klenow fragment of the Escherichia coli DNA polymerase. Cold dCTP was added at the end of the labeling reaction to make full-length probes. Reactions (20 μl containing the indicated amount of in vitro translated USF and SREBP proteins and 5 × 105 cpm oligonucleotide probes were carried out and applied onto a 6% non-denaturing polyacrylamide gel, and autoradiography was performed as described previously (6). Specific USF1 and SREBP1 antibodies (2 μl each; Santa Cruz Biotech, Inc.) were added to the binding reactions for supershift experiments.

RESULTS

The −65/−60 E-box Sequence Is Critical for Insulin Regulation of the FAS Promoter—Previously, we mapped the insulin response sequence (IRS) at the proximal FAS promoter region from −71 to −50. Based on the results of gel mobility shift assays, we identified both USF1 and USF2 as major components of complexes in rat liver nuclear extracts that bind the FAS IRS in vitro (6). Within this region, there is an E-box sequence (5′-CATGTG-3′) located at −65 to −60. We hypothesized that USF interacts with the E-box in vivo and this interaction is involved in the insulin regulation of the FAS gene transcription. As the first step, we tested the functional importance of the E-box sequence using transfection assays. Based
on the studies in transgenic mice, the −2.1 kb promoter region contains all the necessary regulatory DNA elements and is sufficient to confer insulin responsiveness in vivo (28). Therefore, we mutated the E-box 5′-CATGTTG-3′ sequence to 5′-GAATTC-3′ within the −2.1 kb FAS promoter context and tested the effect of this mutation on the insulin responsiveness of FAS promoter first in transiently transfected 3T3-L1 adipocytes. Insulin response was calculated as the ratio of luciferase activity in the presence of 10 nM insulin versus in the absence of insulin. As shown in Fig. 1A, mutation of the E-box sequence decreased the insulin response from 2.7-fold in p2.1kb-LUC to 1.4-fold in p2.1kbM-LUC in transient transfection assays. Then we tested the insulin response of these two reporter gene constructs using stable transfection assays. Pooled stable 3T3-L1 adipocyte transfectants were assayed by measuring the luciferase activity in the presence of insulin versus in the absence of insulin. Similar to the results of transient transfection experiments, insulin response was decreased from 2.7-fold in p2.1kb-LUC to 1.2-fold in p2.1kbM-LUC (Fig. 1A). These results suggested that the 5′-CATGTTG-3′ E-box sequence within the previously identified −71/-50 region is required for insulin regulation of the FAS promoter, not only when transiently expressed as naked DNA templates but also when stably incorporated into the chromosomes. Next, we examined changes in the insulin response caused by the same mutation in the FAS −67 promoter context, which is the minimal region that we have shown to confer insulin responsiveness in transient transfection assays (5). Mutation of the 5′-CATGTTG-3′ E-box sequence within this minimal promoter context will further demonstrate the functional importance of the −65/-60 E-box. As shown in Fig. 1A, insulin treatment caused a 2-fold response in luciferase activities of p67-LUC, consistent with our previous reports using the same construct (5). However, when the E-box is mutated, adding insulin to the culturing medium did not induce a insulin response of the FAS promoter. Taken together, these results provide evidence that the 5′-CATGTTG-3′ E-box sequence is critical for mediating the insulin response of the FAS promoter. We also found that E-box mutation within the −2.1 kb promoter context decreased the basal promoter activity by 75%, suggesting that, in addition to its involvement in the insulin regulation of the FAS promoter, the E-box sequence is also important for the basal promoter activity. Similar decrease of basal promoter activity was observed by Osborne and co-workers (32), using deletion of −43 to −73 containing the E-box at −65 in the context of −150. Interestingly, we did not observe a decrease of the basal promoter activity by the E-box mutation in the −67 bp promoter context. Since basal promoter activity of p67-LUC is only 5% of the p2.1kb-LUC, it would have been difficult to see the further decrease caused by the mutation. It is also possible that interaction of USF with other transcription factors binding to sequences upstream to −67 may be necessary for the basal promoter activity.

To see if the USF binding to the −65/-60 E-box correlates with the functional data presented above, we carried out gel mobility shift assays using the FAS −71/-50 region as the wild-type probe (IRS-WT) and the same region carrying the 5′-CATGTTG-3′ to 5′-GAATTC-3′ E-box mutation as the mutated probe (IRS-M). In this experiment, in vitro translated USF1 and USF2 proteins were used to bind the 32P-labeled probes. As shown in Fig. 1B, when USF1 and USF2 were added together to form USF heterodimers, they interacted with the IRS-WT probe to form protein-DNA complexes (lane 6) in a migration pattern similar to our previously reported patterns when nuclear extracts were used (6). However, when the IRS-M probes were used, USF proteins could no longer bind (lane 3), and the IRS-M sequence could not compete for the binding of USF to the IRS-WT probe (data not shown). Two additional bands (indicated by the asterisk in Fig. 1B) were also observed and appeared to be nonspecific protein-DNA complexes because they formed with the unprogrammed rabbit reticulocyte lysate, and their patterns did not change when the −65/-60 E-box sequence was mutated (lanes 5 and 2), nor did addition of specific USF antibodies affect these two bands (see Fig. 4A). Combined with the transfection data, in vitro binding analysis demonstrated that when USF cannot bind to the FAS −65/-60 E-box, insulin response is abolished. These studies, therefore, demonstrate that mediation of insulin response is through the −65/-60 E-box and probably through its interaction with USF.

Cotransfection of USF1 and USF2 Further Increases Insulin-stimulated FAS Transcription—In the case of several genes whose transcription regulation involves USF, cotransfection of USF expression vectors with the reporter genes stimulate the reporter gene activity (19, 29, 30). To further investigate the effect of USF on insulin regulation of the FAS promoter activity, we cotransfected USF1 and USF2 expression vectors, i.e., pFLAG-USF1 and pFLAG-USF2 (Fig. 2A), with the FAS-luciferase reporter construct. To directly demonstrate the interaction between USF and the −65/-60 E-box, we used the p67-LUC because this construct represents the minimal insulin-responsive promoter and has the −65/-60 E-box as the only E-box in the promoter region that USF could bind to. Fig. 2B shows the insulin-stimulated luciferase activities from cotransfection of expression vectors relative to the luciferase activity of p67-LUC under minus insulin conditions. Cotransfection of the control expression vector pcDNA3.0 at all concentrations resulted in the insulin-stimulated luciferase activities at about 2-fold level and thus had little effect on the insulin-stimulated p67-LUC activity. However, cotransfection of USF1 and USF2 significantly increased the insulin-stimulated luciferase activities up to 8- (USF1) and 11-fold (USF2) levels. Maximal activation was seen at concentrations of 200 ng of USF expression vector/culture plate, similar to the concentrations previously reported for maximal stimulation of the L-PK gene promoter by USF in transfection assays (19). When the amounts of USF1 and USF2 were further increased to 1 and 2.5 µg/culture plate, the stimulation was reduced, probably due to the interference of the cellular transcription processes by overproduction of the ectopic USF proteins. When equal amounts of USF1 and USF2 were cotransfected together, further increase of insulin-stimulated FAS promoter activity was observed as USF1 and USF2 were cotransfected separately (Fig. 2B). This result is consistent with the idea that USF homodimers can function as well as heterodimers, although in purified nuclear extracts USFs were generally found as heterodimers (31).

Dominant Negative USF1 and USF2 Inhibits Insulin Stimulation of the FAS Promoter Activity—Since USF is expressed ubiquitously in various types of cells and tissues (14), further increase of insulin-stimulated FAS promoter activity by USF could be a general effect of high level expression of transcription factors. To unequivocally establish that USF is involved in insulin regulation of the FAS promoter, we tested the effects of dominant negative forms of USF on the insulin stimulation of the p67-LUC reporter construct. Dominant negative forms of USF1 and USF2, i.e., pFLAG-USF1Δb and pFLAG-USF2Δb, were generated by site-directed mutagenesis from the full-length USF cDNA to delete the basic DNA binding region (Fig. 2A). With their basic regions deleted, mutated USFs still dimerize but cannot bind the target sequence so that the activation of transcription is blocked (18, 19). Similar dominant negative USFs had been used previously and reported by other
investigators to demonstrate the involvement of USF proteins in transcriptional regulation (19, 20). As shown in Fig. 3, when cotransfected with p67-LUC, USF1 and USF2 exerted inhibitory effects on insulin-stimulated FAS promoter activity in a concentration-dependent manner. When the amount of both USF1 and USF2 was increased from 200 ng to 2.5 μg/plate, the insulin-stimulated FAS promoter activities were

**FIG. 2.** Cotransfection of USF expression plasmids further increases insulin-stimulated FAS promoter activity. A, schematic illustration of the USF expression vectors used for cotransfection studies. M indicates the first amino acid methionine of each protein. Δ represents the 8-amino acid FLAG tag (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) inserted after M, b, H, L, and LZ represent the basic, helix, loop, and leucine zipper regions of the USF proteins. PCR-amplified FLAG-USF1 and FLAG-USF2 coding sequences were inserted into the expression vector pcDNA3.0 (Invitrogen) as described under “Experimental Procedures.” Plasmids for FLAG-USF1Δb and FLAG-USF2Δb were constructed by site-directed mutagenesis of pFLAG-USF1 and pFLAG-USF2, respectively. B, cotransfection of pFLAG-USF1 and pFLAG-USF2 expression vectors with p67-LUC into 3T3-L1 adipocytes. p67-LUC (10 μg) was transfected together with the indicated amount of pcDNA3.0, pFLAG-USF1, pFLAG-USF2, and pFLAG-USF1 + pFLAG-USF2 (in equal amounts) expression vectors to triplicate plates. Insulin (10 nM) was added to measure the insulin-stimulated FAS promoter activity. Transfections using only 10 μg of p67-LUC with or without insulin were carried out with the same batch of cells. Insulin-stimulated luciferase activities relative to p67-LUC activity in the absence of insulin are plotted.

**FIG. 1.** E-box sequence is critical for insulin-response of the FAS promoter. A, effect of E-box mutation on insulin response of the FAS promoter transfected into 3T3-L1 adipocytes. Triple plates were transfected with 10 μg of reporter plasmid DNA/plate. Insulin response was calculated as the ratio of luciferase activity in the presence of 10 nM insulin versus in the absence of insulin. For transfection studies presented throughout the paper, independent transfections were repeated for at least three times. 2.1kb and 67 represent reporter constructs p2.1kb-LUC and p67-LUC carrying wild-type FAS promoter up to −2.1 kb and −67, respectively. 2.1kbM and 67M represent reporter constructs p2.1kbM-LUC and p67M-LUC carrying the mutation of the −65/−60 E-box 5′-CATGTG-3′ to 5′-GAATTC-3′ sequence. Transient and stable transfections were performed as described under “Experimental Procedures.” B, gel mobility shift assays of in vitro translated USF proteins using IRS-WT and IRS-M probes. IRS-WT probe carries the wild-type FAS promoter −71/−50 region, and IRS-M probe contains the E-box mutation as described in A. Both probes were labeled with [α-32P] dCTP and in vitro translation reactions of USF1 and USF2 proteins were carried out as described under “Experimental Procedures.” Numbers 1 and 2 indicate the USF-DNA complexes, and the asterisk (*) indicates the positions of two nonspecific protein-DNA complexes formed by control rabbit reticulocyte lysate. Complex 2 is resulted from partial protein degradation caused by freeze/thaw and storage of the in vitro translated USF proteins. This is likely the reason for slight variations of the same band shown in Fig. 4 (A and B).
insulin regulation of the FAS promoter through USFs

**Insulin Regulation of the FAS Promoter through USFs**

Insulin (10 nM) was added to measure the insulin-stimulated FAS promoter activity. Transfections using only 10 μg of p67-LUC reporter plasmid into triplicate plates of 3T3-L1 adipocytes. Insulin (10 nM) was added to measure the insulin-stimulated FAS promoter activity. Transfections using only 10 μg of p67-LUC under conditions with or without insulin were carried out with the same batch of cells. Insulin-stimulated luciferase activity is relative to p67-LUC activity in the absence of insulin.

**USF and SREBP Proteins Bind to the FAS Promoter Activity—**

We performed gel mobility shift assays using in vitro translated USF and SREBP proteins to study their binding to the −71/−50 region, which contains the intact −65/−60 E-box as well as the two tandem copies of SREBP binding sites. Additions of both USF1 and USF2 to the binding reactions containing the IRS-WT probe resulted in specific USF-DNA complexes (Fig. 4A, lane 2). Addition of SREBP1 and SREBP2 resulted in slower migrating SREBP-DNA bands (lane 4), probably because of the larger sizes of SREBP1 and SREBP2. Since SREBP homodimerize and their heterodimerization has not been reported, we also added only SREBP1 or SREBP2 to the binding reactions, which resulted in the same migration of protein-DNA complexes (data not shown). Addition of specific USF and SREBP antibodies to the binding reactions supershifted the protein-DNA complexes (lanes 3, 5, and 6) and thus confirmed the identities of the protein-DNA complexes as USF- and SREBP-containing complexes. When rat liver nuclear extracts were used in the gel mobility shift assay, however, we observed the dominant presence of USF-DNA complexes (lane 7) that comigrated with the USF-DNA complexes formed by in vitro translated USF proteins. No SREBP complexes could be detected on the FAS SRE-containing probe when nuclear extracts were used (lane 9), as other investigators have reported previously (38). Initial addition of USF1 and USF2 to the binding reaction for 30 min, followed by addition of SREBP1 for another 30 min, produced USF-DNA complexes as well as SREBP-DNA complexes (Fig. 4B, lane 4). We did not observe any newly formed protein-DNA complexes migrating between the USF-DNA and SREBP-DNA complexes, suggesting that USF and SREBP do not interact with each other to form USF-SREBP heterodimers and bind the probe. Neither did we observe any newly formed complexes migrating slower than SREBP-DNA bands, suggesting that USF dimers and SREBP dimers do not bind to the same probe molecule at the same time to form USF dimer-SREBP dimer-DNA complexes under these experimental conditions. When we did the reaction in the reciprocal order, i.e. adding SREBP1 first and then USF1 and USF2, we observed the same final banding pattern consisting of only the USF-DNA and SREBP-DNA complexes (Fig. 4B, lane 5). Taken together, the above results suggest that USF and SREBP proteins bind to the FAS −71/−50 promoter region in an independent manner.

**SREBP Proteins Are Not Involved in the Insulin Regulation of the FAS Promoter Activity—**

The presence of exclusive binding sites of USF and SREBP proteins in FAS −71/−50 promoter region makes this region particularly important for two different regulations of FAS transcription, one by insulin and the other by sterol. Although the above results demonstrate the requirement of the −65/−60 E-box sequence in mediating insulin regulation of the FAS promoter, the role of the surrounding nucleotides, especially those within the SREBP binding sites need to be addressed. To examine whether SREBP binding sites in the FAS −71/−50 region are involved in insulin regulation of the FAS promoter, we mutated the SREBP binding sites by altering only the nucleotides surrounding the −65/−60 E-box (p2.1kbS1-LUC in Fig. 4A). In this way, SREBP binding sites are disrupted but the −65/−60 E-box is still intact. Since 4-nucleotide (p2.1kbS2-LUC) and 10-nucleotide (p2.1kbS3-LUC) insertions between the SREBP binding sites and the possible SP1 binding site at position −90 could abolish the SREBP stimulation of the FAS promoter activity (7), we also tested the effects of these two mutations in the −2.1 kb promoter context on the insulin regulation of the FAS promoter. As shown in Fig. 5B, all mutations, along with the wild-type p2.1kb-LUC control plasmid, responded to the addition of 10 nM insulin to the culturing media. The insulin re
Insulin is the principal hormone that controls blood glucose. When circulating insulin is high, there is an increase in lipogenesis in adipose tissue and liver. This process is impaired when insulin is low, and administration of insulin restores the rate to its normal level. FAS is a key lipogenic enzyme, and insulin increases its activity dramatically, not through allosteric effectors or covalent modification but through changes in transcription (3, 4). Rapid and high level induction of the FAS gene by insulin makes FAS an excellent model for studying transcriptional activation by insulin. We had defined the FAS IRS to the proximal promoter region at −71/−50 and had shown that USF proteins bind to the −71/−50 region in vitro. However, the functional requirement of the −65/−60 E-box in the insulin regulation of the FAS promoter activity and involvement of USF proteins in the insulin regulation were not addressed previously. In this paper, we provide evidence that the −65/−60 E-box is required and USFs are in vitro components for insulin regulation by correlating functional assays and USF binding activities to the −65/−60 E-box. When the −65/−60 E-box motif was mutated, FAS −71/−50 region was no longer a target for USF binding, and insulin response of the FAS promoter was abolished (Fig. 1). Cotransfection of USF1 and USF2 expression vectors with the FAS promoter increased insulin-stimulated FAS promoter activity (Fig. 2), and dominant negative USFs lacking the DNA binding activity inhibited the insulin stimulation of the FAS promoter activity (Fig. 3). At a concentration of 200 ng/plate, which exhibited maximal stimulation, USF2 exerts a slightly higher degree of stimulatory effect than USF1 (Fig. 2B). Moreover, when the dominant negative USF were used to block the insulin stimulation of FAS promoter activity, USF1Δb showed a higher degree (down to 30% of the p67-LUC control group) of inhibition than USF2Δb (down to 50% of the p67-LUC control group). This observation suggests that although both USF1 and USF2 are involved in the insulin regulation of the FAS promoter activity, their relative amounts inside the cells are not likely the same. Maybe USF2 is more limited in 3T3-L1 adipocytes so that ectopic USF2 and USF1Δb showed higher degree of stimulatory and inhibitory effect on the insulin regulation of the FAS promoter.

DISCUSSION

Throughout the 2.1-kb rat FAS promoter sequence, there are 13 hexanucleotide sequences that match the 5′-CANNTG-3′ E-box sequence. Besides the −65/−60 E-box, whether or not other E-box sequences within the promoter are also the binding sites of USF proteins remains to be fully investigated. Cotransfection of dominant negative USFs showed inhibitory effect on basal promoter activity of p2.1kbM-LUC but did not of SREBP1 + SREBP2 were added 0.5 h later; lane 5, 1 μl of SREBP1 were added first and 1 μl of USF1 + USF2 were added 0.5 h later. Numbers 1 and 2 indicate the SREBP-DNA complexes. Number 3 indicates the USF-DNA complexes. Asterisk (*) indicates the nonspecific protein-DNA complexes.

FIG. 4. USF and SREBP proteins bind the FAS −71/−50 region independently in vitro. A, gel mobility shift assays of in vitro translated USF, SREBP, and rat liver nuclear extracts. In vitro translated USF and SREBP proteins without [35S]Met labeling and the IRS-WT probe were used. Control lysate (2 μl, lane 1), USF1 + USF2 (lanes 2 and 3), SREBP1 + SREBP2 (lanes 4 and 5), and rat liver nuclear extract (lanes 7–9) were used. USF1 antibody (2 μl, lanes 3 and 8) and SREBP1 antibody (lanes 5 and 9) were added for the supershift reactions. Lane 6 is a longer exposure of lane 3 to clearly show the supershift of USF-DNA complexes by the USF1 antibodies. Numbers 1 and 2 indicate the SREBP-DNA complexes. Asterisk (*) indicates the nonspecific protein-DNA complexes. Complex 2 is resulted from partial protein degradation caused by freeze/thaw and storage of the in vitro translated proteins. This is likely the reason for slight variations of the same band shown in A and B and in Fig. 5C. B, USF and SREBP proteins bind the FAS promoter −71/−50 region independently in vitro. Gel mobility shift assays were carried out as described under “Experimental Procedures.” Lane 1, 2 μl of control lysate; lane 2, total 2 μl of USF1 + USF2; lane 3, 1 μl of SREBP1; lane 4, 1 μl of USF1 + USF2 were added first and 1 μl sponse stayed at the same level (2.0-fold) regardless of the mutation introduced. In the p2.1kbS1-LUC mutant, the −65/−60 E-box sequence is still intact, and USF proteins were thought to still be able to bind to the −71/−50 region. As shown in Fig. 5C, USF proteins indeed formed protein-DNA complexes with the IRS-S1 probe which carries the same mutation as in p2.1kbS1-LUC (lanes 3 and 7). On the other hand, complex formation between SREBP and IRS-S1 probe is impaired (lanes 4 and 8). These results suggest that SREBP proteins and their binding sites are not required to mediate the insulin regulation of the FAS promoter and reinforce the conclusion that the interaction of USF and −65/−60 E-box is required for insulin regulation of the FAS promoter activity.
SREBP binding sites within FAS promoter represents the Boxed structs used in the transfection studies. When p2.1kb-LUC and p2.1kbM-LUC were stably transfected into 3T3-L1 adipocytes (Fig. 1A), the incorporation of the promoter sequence into chromosomes and the maintenance of the chromatin structure showed the same results as in transient transfection assays. These results reinforced our conclusion that the E-box motif at position −65/−60 plays a critical role in insulin regulation of the FAS transcription.

Insulin regulates transcription of numerous genes in a variety of cell types. Although the insulin response elements for some of these genes have been mapped, the physiological relevance of the insulin regulation of many of them has not yet been established. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the rate-limiting step in gluconeogenesis, and suppression of the PEPCK gene by insulin is the most extensively studied case with physiological relevance to insulin action (34–36). At least two cis-acting elements and C/EBP isoforms and HNF-3 as the trans-acting factors may be involved in dominant repression by insulin of cAMP-stimulated PEPCK transcription (35, 37). Although signaling molecules and molecular mechanisms are yet to be fully elucidated, it has been recently demonstrated that P13-kinase but neither Ras mitogen-activated protein kinase nor p70 S6 kinase is required for PEPCK gene repression by insulin (34, 36). In the case of FAS regulation, insulin increases gene transcription, and it might be through different mechanisms than the suppression of the PEPCK gene. From our present study, it is shown that the −65/−60 E-box motif and USF proteins, which are ubiquitously expressed bHLH transcription factors, are utilized as the cis-acting element and trans-acting factors for the insulin regulation of the FAS transcription respectively. It is plausible that distinct transcription factors bind to their cognate response elements and mediate repression or activation of these metabolic genes.

Within the FAS IRS region, two tandem copies of SREBP binding sites also exist. Although SREBP proteins were shown to bind this region by us using in vitro translated protein (Fig. 4) and by others using E. coli expressed recombinant proteins (7), we could not detect the SREBP binding to FAS −71/−50 region using rat liver nuclear extracts. Although gel mobility shift assay is generally considered a very sensitive detection method for protein-DNA interactions, it is possible that the level of SREBP in nuclear extracts is too low to be detected by this method, as in the case of SREBP binding to sterol regulatory element of low density lipoprotein receptor gene promoter (38). It seems that USF and SREBP binding to the FAS promoter −71/−50 region are independent and mutually exclusive sequences are shown in bold. Insertions (4 and 10 bp) were introduced in p2.1kbS2-LUC and p2.1kbS3-LUC upstream of position −72. B, mutations of SREBP binding sites do not affect insulin response of the FAS promoter. p2.1kb-LUC, p2.1kbS1-LUC, p2.1kbS2-LUC, and p2.1kbS3-LUC (10 μg each) were transiently transfected into triplicate plates of 3T3-L1 adipocytes. Luciferase activities relative to the wild-type p2.1kb-LUC under minus insulin conditions (set at 1.0) are plotted. C, gel mobility shift assay of USF and SREBP proteins using wild-type IRS-WT (lanes 1–4) and mutated IRS-S1 (lanes 5–8) probes. Lanes 1 and 5, probe only; Lanes 2 and 6, 2 μl of control lysate; lanes 3 and 7, total 2 μl of in vitro translated USF1 + USF2; lanes 4 and 8, total 2 μl of in vitro translated SREBP1 + SREBP2.
Transcriptional regulation of the FAS promoter through USF1 and USF2.

(Fig. 4), and thus it is possible that distinct signal transduction pathways are involved in recruiting USF and SREBP to the FAS promoter. From the results described in Fig. 5, it seems that the insulin response through USF is separable from the SREBP regulation of FAS. Further investigation of the signal transduction pathways involved is needed to fully understand the molecular mechanisms underlying the activation of the FAS promoter.

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