Two 5′-Regions Are Required for Nutritional and Insulin Regulation of the Fatty-acid Synthase Promoter in Transgenic Mice*

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We previously reported that 2.1 kilobase pairs of the 5′-flanking sequence are sufficient for tissue-specific and hormonal/metabolic regulation of the fatty-acid synthase (FAS) gene in transgenic mice. We also demonstrated that the −65 E-box is required for insulin regulation of the FAS promoter using 3T3-L1 adipocytes in culture. To further define sequences required for FAS gene expression, we generated transgenic mice carrying from −644, −444, −278, and −131 to +67 base pairs of the rat FAS 5′-flanking sequence fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Similar to the expression observed with −2100-FAS-CAT transgenic mice, transgenic mice harboring −644-FAS-CAT and −444-FAS-CAT expressed high levels of CAT mRNA only in lipogenic tissues (liver and adipose tissue) in a manner identical to the endogenous FAS mRNA. In contrast, −278-FAS-CAT and −131-FAS-CAT transgenic mice did not show appreciable CAT expression in any of the tissues examined. When previously fasted mice were refed a high carbohydrate, fat-free diet, CAT mRNA expression in transgenic mice harboring −644-FAS-CAT and −444-FAS-CAT was induced dramatically in liver and adipose tissue. The induction was virtually identical to that observed in −2100-FAS-CAT transgenic mice and to the endogenous FAS mRNA. In contrast, −278-FAS-CAT transgenic mice showed induction by feeding, but at a much lower magnitude in both liver and adipose tissue. The −131-FAS-CAT transgenic mice did not show any CAT expression either when fasted or refed a high carbohydrate diet. To study further the effect of insulin, we made these transgenic mice insulin-deficient by streptozotocin treatment. Insulin administration to the streptozotocin-diabetic mice increased CAT mRNA levels driven by the −644 FAS and −444 FAS promoters in liver and adipose tissue, paralleling the endogenous FAS mRNA levels. In the case of −278-FAS-CAT, the induction observed was at a much lower magnitude, and deletion to −131 base pairs did not show any increase in CAT expression by insulin. This study demonstrates that the sequence requirement for FAS gene regulation employing an in vitro culture system does not reflect the in vivo situation and that two 5′-flanking regions are required for proper nutritional and insulin regulation of the FAS gene. Cotransfection of the upstream stimulatory factor and various FAS promoter-luciferase constructs as well as in vitro binding studies suggest a function for the upstream stimulatory factor at both the −65 and −332 E-box sequences.

Fatty-acid synthase (FAS) plays a central role in de novo lipogenesis in mammals (1). By the action of its seven active sites, FAS catalyzes all the reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS activity is not known to be regulated by allosteric effectors or covalent modification. However, the FAS concentration is exquisitely sensitive to nutritional, hormonal, and developmental status in lipogenic tissues (liver and adipose tissue) (1, 2). The concentration or enzyme activity of FAS changes dramatically when animals are subjected to different nutritional manipulations. There is no detectable fatty acid synthesis in animals fasted for 1–2 days, whereas refeeding a high carbohydrate, fat-free diet increases synthesis of FAS dramatically (3). Increased circulating insulin and decreased glucagon levels may participate in the regulation of FAS synthesis. We reported that FAS mRNA is not detectable in the livers of fasted mice and that refeeding a high carbohydrate diet drastically increases the level of FAS mRNA, due to changes in FAS gene transcription (4, 5). The stimulation of FAS gene transcription by feeding was not observed in the livers of streptozotocin-diabetic mice, in which FAS expression was detected at a very low level (5). Administration of insulin to streptozotocin-diabetic mice stimulates the level of FAS mRNA and FAS transcription (5). We mapped an insulin response sequence containing a core E-box to −71 to −50 bp by chimeric construction and transfection into 3T3-L1 adipocytes of serial 5′-deletions of the FAS promoter ligated to the luciferase reporter gene (6, 7). We showed that binding to the −65 E-box by USF1 and USF2, which belong to the basic helix-loop-helix leucine zipper family of transcription factors, is required for the insulin-mediated induction of the FAS gene (8–10). Furthermore, Vaulont and co-workers (11) recently reported that expression of SREBP (sterol regulatory element-binding protein) in the liver and adipose tissue of mice expressing SREBP demonstrates its role in the activation of the FAS promoter (14). Therefore, although studies on transgenic mice over-expressing SREBP demonstrate its role in the activation of the FAS promoter and the involvement of SREBP in FAS regulation by insulin

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are yet to be clarified. In addition to the −65 E-box, other laboratories have reported cis-acting elements present in the FAS gene that may be involved in the hormonal/nutritional regulation. An inverted CCAAT box motif at −99 to −92 bp was shown to be essential for transcriptional down-regulation by cAMP (15). Schweizer and co-workers (16) reported a DNase-hypersensitive site at −568 to −468 bp where nuclear factor Y and Sp1 can cooperatively bind and mediate insulin regulation of the FAS gene. Another DNase-hypersensitive site at +290 to +316 bp, located in the first intron with consensus USF- and CCAAT-binding transcription factor/nuclear factor-1-binding sites, was also implicated as a glucose response element of the FAS gene (17). However, all of the cis-acting sequences identified to date were defined using in vitro culture systems, and the significance of these regulatory elements to in vivo events needs to be established. The only report that addresses the sequence requirement for the FAS gene in vivo is our previous 2.1-kb FAS promoter-CAT transgenic mice studies (18). By examining the transgene expression under various hormonal/nutritional conditions, we demonstrated that the 2.1-kb 5′-flanking sequence is sufficient for tissue-specific and hormonal/nutritional regulation of the FAS gene in the in vivo context.

In this study, we have dissected the FAS promoter region in vivo by generating transgenic mice containing various 5′-deletion FAS promoter-CAT constructs. Unlike the results we reported using transient transfection into 3T3-L1 adipocytes (7, 9), which demonstrated a requirement for the −65 E-box for insulin regulation, we did not detect any CAT expression driven by the −131 FAS promoter region in transgenic mice. FAS promoter sequence to −278 bp directed, although at a very low level, induction of reporter gene expression during feeding and insulin treatment in liver and adipose tissue. However, only when sequence up to −444 bp was present was there a dramatic increase in reporter gene expression by feeding and insulin treatment, which was identical to that of the endogenous FAS gene. In vitro binding and transactivation studies suggest that USF binding at −332 bp may be involved in the maximal induction of the FAS promoter in vivo.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Production of Transgenic Mice**—The −2100-FAS-CAT plasmid containing −2100 to +67 bp of the rat FAS gene in pBlCAT3 has been described previously (18). Transgenic mice harboring −644-FAS-CAT were generated by injection of an agarose gel-purified NotI-KpnI fragment from the −2100-FAS-CAT plasmid containing from −644 to +67 bp of the FAS promoter, CAT, and SV40 polyadenylation sequences into pronuclei of fertilized mouse embryos (Brigham Women’s Hospital Transgenic Mice Facility of the Harvard Medical School). Similarly, transgenic mice harboring −444-FAS-CAT, −278-FAS-CAT, and −131-FAS-CAT were produced using DraIII-KpnI, BsaHI-KpnI, and Ascl-KpnI fragments from the −2100-FAS-CAT plasmid as shown in Fig. 1. Heterozygous transgenic progeny (F1) were obtained by breeding the founders to C57BL/6 wild-type mice. The F1 male and female littermates were bred back to C57BL/6 wild-type mice. Transgene-positive subsequent generation progeny were used for the studies. Transgenic mice carrying the FAS promoter-CAT gene were identified by polymerase chain reaction amplification of tail DNA using the primers 5′-GCCAAAGCTTGCAGCATGGTG-3′ and 5′-AATGGGAAAGC GCACGATTAAA-3′, which amplify a 390-base pair fragment spanning the junction between the FAS promoter and the CAT reporter gene. The reporter gene constructs for −19-FAS-LUC; −67-FAS-LUC, −136-FAS-LUC, and −318-FAS-LUC, which contain the −19/+67, −67/+67, −136/+67, and −318/+67 sequences, respectively, of the rat FAS promoter fused to the luciferase reporter plasmid p0LUC, have been described previously (7). −444-FAS-LUC was constructed by ligating the DraIII-HindIII fragment from the −2100-FAS-LUC plasmid containing sequences from −444 to +67 bp of the rat FAS gene into the promoterless vector p0LUC. −444(−65)-FAS-LUC was constructed following the same procedure as described for the −444-FAS-LUC construct by ligating the DraIII-HindIII fragment obtained from the p2.1KbM-LUC plasmid (9), which carries mutations at the

| Constructs | Restriction fragments |
|------------|-----------------------|
| −2100-FAS-CAT | HindIII / KpnI |
| −644-FAS-CAT | NarI / KpnI |
| −444-FAS-CAT | DraiI / KpnI |
| −278-FAS-CAT | BsaHI / KpnI |
| −131-FAS-CAT | Ascl / KpnI |

**Fig. 1. Constructs used in generating the FAS promoter-CAT transgenic mice.** The upper panel shows the restriction fragments inserted into pBlCAT3 to generate the various FAS promoter-CAT fusion genes indicated in the lower panel.

−65 E-box. The −444/−299-FAS-LUC plasmid contains FAS promoter sequence up to −37 bp fused to the −444/−299 upstream sequence. This FAS promoter construct was generated by deleting the promoter sequence between −298 and −38 bp by site-directed mutagenesis (CLONTECH). Expression vectors for USF1 and USF2 have been described previously (9).

**Animal Treatments**—The animals had access to food pellets containing 58% carbohydrate ad libitum. Diabetes was induced by intraperitoneal injections of streptozotocin (20 mg/100 g of body weight) following a 6-h fasting period as described previously (5). Diabetes was confirmed by a high blood glucose level (>250 mg/dl) by Glucostix (Bayer). Insulin was administered to diabetic mice as a combined dose of regular insulin (3 units/100 g of body weight; Lilly) given intraperitoneally and Lente insulin (10 units/100 g of body weight; Lilly) given subcutaneously as described previously (5). Diabetic mice were studied 85–90 h following streptozotocin treatment. Insulin-treated mice were studied 5 h following insulin injection.

**Cell Culture and Transient Transfection Assays**—3T3-L1 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For transient transfection of 3T3-L1 fibroblasts, 5 µg of the experimental reporter plasmid with 250 ng each of expression vectors for USF1 and USF2 were cotransfected using the calcium phosphate coprecipitation method along with 10 ng of expression vector for cytomegalovirus-β-galactosidase, used as control of transfection efficiency. Control cells were cotransfected with 500 ng of pcDNA3.1 (Invitrogen). Cells were washed twice with phosphate-buffered saline 16–18 h after transfection and changed into Dulbecco’s modified Eagle’s medium containing 1% serum. Forty-eight hours after transfection, cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For transient transfection of 3T3-L1 fibroblasts, 5 µg of the experimental reporter plasmid with 250 ng each of expression vectors for USF1 and USF2 were cotransfected using the calcium phosphate coprecipitation method along with 10 ng of expression vector for cytomegalovirus-β-galactosidase, used as control of transfection efficiency. Control cells were cotransfected with 500 ng of pcDNA3.1 (Invitrogen). Cells were washed twice with phosphate-buffered saline 16–18 h after transfection and changed into Dulbecco’s modified Eagle’s medium containing 1% serum. Forty-eight hours after transfection, cells were harvested, and luciferase and β-galactosidase activities were assayed (Dual-Light kit, Tropix Inc.). Transfections were carried out in quadruplicate, and at least two different preparations were used for each plasmid.

**RNA Isolation and Ribonuclease Protection Assays**—Tissues excised by rapid dissection and frozen in liquid nitrogen were pulverized using a ceramic mortar and pestle. Total RNA was isolated from the frozen tissues using Trizol (Life Technologies, Inc.) following the manufacturer’s procedures. A 150-bp fragment of the mouse FAS cDNA sequence was amplified with the primers FAS-HindiII (5′-TTTTTTAAGCT-TAGGGGTGCAGCTGCTCTCA-3′) and FAS-XhoI (5′-GGCAT
GTCTAGAGGGGTAGTGTAGAAAGAT-3', using Pfu DNA polymerase. The polymerase chain reaction product was digested with HindIII and XhoI and cloned into pCDNA3.0 to generate the plasmid pFAS-RPA. The pCAT-RPA plasmid was generated by inserting into pCDNA3.0 a 180-bp polymerase chain reaction product amplified using CAT-HindIII (5'-AAGCACAAGCTTTACCGCCCTTTATCCAC-3') and CAT-XbaI (5'-ATTACCTCATAGGCGATGAAACGTGTTACGT-3') as primers subsequent to HindIII and XhoI digestion and gel purification. The control plasmid (pTRI-actin-mouse) contained a 250-bp KpnI-XhoI fragment from the mouse β-actin gene (Ambion Inc.). Anti-enzyme RNA probes were generated by in vitro transcription of the HindIII-digested plasmids using SP6 polymerase and α-32PUTP. Ten micrograms of total RNA were subjected to the RNase protection assay using the RPA II kit (Ambion Inc.) following the manufacturer's procedures. The protected probes (120 bp for the FAS probe and 150 bp for the CAT probe) were separated on a 5% denaturing polyacrylamide gel containing urea. The gel was dried and exposed to x-ray film.

In Vitro Transcription / Translation of USF—Plasmid DNA containing USF1 sequence cloned into pCDNA3.0 (9) was digested with XhoI and transcribed in vitro with T7 RNA polymerase. The RNA was then translated in vitro using rabbit reticulocyte lysates (Promega) following the manufacturer's procedures. An in vitro translation reaction with no RNA added was used as a negative control. Parallel experiments were set up to allow one set of reactions to produce [35S]Met-labeled protein to monitor the translation product and the other set of reactions to produce unlabeled protein for gel mobility shift assays.

Gel Mobility Shift Assay—The following single-stranded oligonucleotides were synthesized. -65 E-box, 5'AGTCGTACCAAGTGGGAGG-3' and 3'-ATGCTGGTTACACCGGACGCTGAC-5'; and -332 E-boxes, 5'-GCGACAGTGGCAGACCTGGCCC-3' and 3'-GTTCACGGTGGAGCGGGTGG-5'. Double-stranded oligonucleotides were formed as described previously (9). All probes for gel shift assays were labeled using [α-32P]dCTP in the presence of dATP, dGTP, dTTP, and the Klenow fragment of Escherichia coli DNA polymerase. Unlabeled dCTP was added at the end of the labeling reaction to make full-length probes. Reactions containing the indicated amount of in vitro translated USF and 1 × 10^6 cpm oligonucleotide probes were carried out at room temperature for 1 h and subjected to 6% nondenaturing polyacrylamide gel electrophoresis, and autoradiography was performed. For supershift experiments, anti-USF1 antibody (Santa Cruz Biotechnology) was added to the binding reactions for the last 30 min.

RESULTS

Expression of the FAS Promoter-CAT Fusion Gene in Transgenic Mice—We previously reported that 2.1 kb of the 5′-flanking sequence are sufficient for tissue-specific and hormonally regulated expression of the FAS gene in vivo (18). To examine the DNA regions responsible for tissue-specific and hormonal/nutritional regulation of the rat FAS gene in vivo, we have generated multiple lines of transgenic mice carrying the CAT reporter gene driven by various 5′-deletions of the FAS 5′-flanking sequence. Since expression of reporter genes in transgenic animals can be affected by the location of genomic integration and copy number, we analyzed the following number of independent founder lines, which showed essentially the same results: -2100-FAS-CAT, three lines; -644-FAS-CAT, one line; -444-FAS-CAT, two lines; -278-FAS-CAT, three lines, and -131-FAS-CAT, four lines. As shown in Fig. 2, RNase protection analysis of mice carrying -278-FAS-CAT and -131-FAS-CAT did not show CAT mRNA levels in any of the various tissues examined, including liver, adipose tissue, muscle, kidney, heart, and lung. (A very long exposure of the x-ray film, however, showed a very weak but detectable expression of CAT in all tissues of -278-FAS-CAT transgenic mice, but not in -131-FAS-CAT transgenic mice.) In contrast, transgenic mice carrying CAT driven by longer 5′-flanking sequences (–644-FAS-CAT and –444-FAS-CAT) showed a CAT mRNA expression pattern identical to that of -2100-FAS-CAT transgenic mice and produced high levels of CAT expression in liver and white and brown adipose tissue only. The tissue distribution of the CAT mRNA in transgenic mice carrying -2100-FAS-CAT, -644-FAS-CAT, and -444-FAS-CAT fusion genes paralleled that of the endogenous FAS mRNA, which is highly expressed only in lipogenic tissues (liver and adipose tissue) (18), when determined using the same RNA preparations. Actin mRNA (used as a control) was uniformly expressed in all of the tissues we studied. Overall, these results indicate that sequences present between -278 and -444 bp of the 5′-flanking region are responsible for high expression of the FAS gene in lipogenic tissues.

Expression of the Various 5′-Deletion FAS Promoter-CAT Fusion Genes during Fasting/Refeeding in Transgenic Mice—FAS mRNA, as shown in our previous report (5), decreases to very low to undetectable levels when animals are fasted for 48 h and is superinduced when the fasted animals are refed a high carbohydrate diet. We have also shown that changes in transcription of the FAS gene account for nutritional regulation of the FAS gene and that the first 2.1 kb of the 5′-flanking region of the FAS gene are sufficient for this regulation in transgenic mice (18). Furthermore, we have demonstrated, using 3T3-L1 adipocytes in culture, that the -65 E-box is required for insulin regulation of the FAS promoter (7, 9). To dissect the sequence requirement for nutritional regulation of the FAS gene in the in vivo context, transgenic mice carrying...
Transgenic mice were either fasted for 48 h or fasted for 48 h and then refed a high carbohydrate, fat-free diet for 16 h. The CAT reporter gene expression driven by the various FAS promoter regions in liver and adipose tissue of refed mice. RNase protection assays were carried out as described in the legend to Fig. 3 and exposed to BioMax film.

**Fig. 3. Regulation by fasting/refeeding of CAT mRNA expression driven by the various FAS promoter regions in liver and white adipose tissue.** Transgenic mice were either fasted for 48 h or fasted for 48 h and then refed a high carbohydrate, fat-free diet for 16 h. Total RNA isolated from liver and white adipose tissue was submitted to RNase protection assay to determine CAT, FAS, and actin mRNA levels as described under “Experimental Procedures,” and the urea-polyacrylamide gel was exposed for 1 day using regular x-ray film (Fuji). The asterisk indicates an autoradiogram of BioMax film (Eastman Kodak Co.) exposed for 4 days. F and R represent fasted and refed states, respectively. Essentially the same results were obtained in three independent experiments.

The FAS-CAT transgenic mice were also used for comparison purposes. Since we have shown that nutritional regulation of the FAS promoter occurs only in liver and adipose tissue (18), in this study, we examined only these lipogenic tissues for the regulated expression of the CAT transgene. As we have previously shown (4, 5), fasted mice had markedly reduced, undetectable levels of FAS mRNA in both liver and white adipose tissue, and subsequent feeding of a high carbohydrate, fat-free diet dramatically increased FAS mRNA levels (Fig. 3). As shown in Fig. 3, transgenic mice carrying each of the FAS-CAT constructs showed very low, undetectable levels of CAT expression during fasting. Similar to endogenous FAS mRNA levels, upon refeeding of a high carbohydrate, fat-free diet, CAT expression in both liver and adipose tissue was induced, approaching 2 orders of magnitude in transgenic mice carrying the two longer constructs, -644-FAS-CAT and -444-FAS-CAT. We previously reported a similar magnitude of induction with -2100-FAS-CAT transgenic mice (18). On the other hand, transgenic mice carrying -278-FAS-CAT did not express a high level of CAT upon refeeding. An induced expression of CAT mRNA of ~1% of the expression level observed in the -644-FAS-CAT and -444-FAS-CAT transgenic mice was shown only on a longer exposure of the autoradiogram. Moreover, transgenic mice containing the shortest reporter construct (-131-FAS-CAT) did not show detectable CAT expression. This indicates that deletion of the promoter sequence from -278 to -131 bp caused loss of CAT induction by fasting/refeeding. The actin, FAS, and CAT mRNA levels in the livers and adipose tissue of all the transgenic mice were measured simultaneously by RNase protection assay to compare the expression levels in the transgenic mice (Fig. 4). The results demonstrate that sequence up to -131 bp cannot confer nutritional regulation in vivo. Sequences present between -131 and -278 bp are required for the increase in FAS promoter activity during feeding, although the induction is of a lesser magnitude compared with the endogenous gene. Another regulatory region, present between -278 and -444 bp, is required for the dramatic maximal induction observed for the endogenous FAS gene during feeding.

**Insulin Regulation of the CAT Reporter Gene Driven by the Various 5'-Deletion FAS Promoter Regions in Transgenic Mice**—During feeding of a high carbohydrate, fat-free diet, there is an increase in circulating glucose levels. In addition, increased insulin and decreased glucagon levels contribute to the changes in FAS gene expression during feeding. To determine the DNA sequences involved in the regulation of the FAS gene in an insulin-deficient state, the CAT mRNA levels driven by the various FAS promoter regions were examined in streptozotocin-induced diabetes. Our previous reports showed that insulin increases FAS gene transcription in streptozotocin-induced diabetes and that 2.1 kb of the 5'-flanking sequence are sufficient for insulin regulation in vivo (18). As predicted, all the transgenic mice that were made insulin-deficient by streptozotocin treatment had a marked reduction in FAS mRNA levels in both liver and adipose tissue (Fig. 5). Treatment of the diabetic animals with insulin for 5 h resulted in restoration of the FAS mRNA levels. The control actin mRNA levels remained constant throughout the treatment. As shown in Fig. 5, the CAT reporter gene expression driven by the various 5'-flanking regions of the FAS gene was very low to undetectable in all transgenic mice during streptozotocin-induced diabetes. However, as we have previously observed with the -2100-FAS-CAT transgenic mice (18), transgenic mice carrying the two longer promoter sequences (-644-FAS-CAT and -444-FAS-
CAT) showed a dramatic increase in CAT expression upon insulin injection similar to the levels observed in both liver and adipose tissue in refed mice. When the FAS promoter sequence driving CAT expression was deleted up to −278 bp, the CAT mRNA level was increased upon insulin administration, but to a much lower level, which was detected only with a much longer exposure time of the autoradiogram. When the deletion was up to −131 bp, CAT expression was not detectable in liver or adipose tissue upon insulin treatment. These results indicate that the region up to −131 bp does not contain sequences that confer insulin responsiveness in vivo. Sequence present between −278 and −131 bp contains a response element that confers induced CAT transcription, albeit at a much lower level, upon insulin treatment. However, an additional element present between −278 and −444 bp is required for full induction of FAS transcription by insulin.

**Potential Role of USF Binding to a −332 E-box in Activation of the FAS Promoter**—The −65 E-box is not sufficient to increase FAS promoter activity during fasting/refeeding and upon insulin treatment in diabetic mice. A regulatory region present between −278 and −444 bp is required for maximal induction. Upon sequence examination of the FAS promoter between −278 and −444 bp, we found at position −332 an overlapping inverted repeat of an E-box sequence. We therefore examined if the −332 E-box sequence plays a role in FAS promoter activation. In gel mobility shift assays, USF binding to the −65 E-box could be competed away with excess oligonucleotides corresponding to the −332 E-box (Fig. 6). When oligonucleotides containing the −332 E-box were used in the mobility shift assay, a protein-DNA complex was formed in a sequence-specific manner, and unlabeled oligonucleotides were effective as competitors. In addition, anti-USF antibody could supershift the protein-DNA complex, indicating the presence of USF in the −332 E-box protein complex. Overall, these results indicate that the E-box at −332 bp can bind USF as effectively as the −65 E-box. To examine if USF binding to the −332 E-box activates the FAS promoter, we carried out transactivation assays by cotransfecting 3T3-L1 fibroblasts with USF1 and USF2 along with various 5′-deletion FAS promoter-luciferase constructs. The various 5′-deletion constructs containing sequences from −67 to −444 showed similar (−7-fold) transactivation by USF (Fig. 7A). Only when the promoter sequence was deleted to −19 bp was the activation diminished. Moreover, a mutation of the −65 E-box did not abolish transactivation of the FAS promoter by USF (Fig. 7B). USF could also transactivate a construct containing only the basal FAS promoter, but without the −65 E-box, linked to the region containing the −332 E-box (Fig. 7C). Since there is no additional E-box containing sequence up to −444 bp, we conclude that any one E-box, either at −65 or −332 bp, is required for USF activation in cultured cells. These in vitro studies suggest that USF binding to the −332 E-box sequence may be responsible for the maximal activation of the FAS promoter observed in FAS promoter-CAT transgenic mice by refeeding and insulin treatment.

**DISCUSSION**

Previously, we demonstrated that 2.1 kb of the 5′-flanking sequence of the rat FAS promoter are sufficient for tissue-specific regulation and physiologic regulation in insulin-deficient diabetes and in fasted states (18). The deletion analysis in this report demonstrates the presence of at least two regions responsible for nutritional and insulin regulation of the FAS.
Regulation of the FAS Promoter in Transgenic Mice

Gene in lipogenic tissues (liver and adipose tissue). A proximal region between −131 and −278 bp confers nutritional and insulin regulation of the FAS gene, but at a very low magnitude. However, to obtain the high maximal response of FAS promoter activity that is similar to the induction of the endogenous FAS gene by feeding and insulin treatment, a more distal region between −278 and −444 bp is required. These results are surprising in light of our previous observation of an insulin response element between −67 and −52 bp after transient transfection into 3T3-L1 adipocytes (7). Our present transgenic mice studies indicate that, unlike results obtained in cell culture, two upstream regions are required to confer the nutritional and insulin-regulated expression that is characteristic of the endogenous FAS gene. Our present transgenic mice experiments provide clues as to whether the sequence between −67 and −52 bp that we defined in 3T3-L1 adipocytes is required for the regulated expression in vivo. It is possible that the presently identified proximal region between −131 and −278 bp functions only in the presence of the −67/−52 sequence to confer a low level of regulated expression of the FAS gene. However, it is clear that the more distal sequence between −278 and −444 bp is required for maximal response to nutritional and insulin stimulation in vivo. Previously, two DNase I-hypersensitive sites were reported to be important for glucose and insulin regulation of the FAS gene. Schweizer and co-workers (16) located an element responsible for insulin regulation at a hypersensitive site between −568 and −468 bp, and Ferre and co-workers (17) implicated another DNase I-hypersensitive site at +290 to +316 bp as a glucose response element. However, our present transgenic mice studies reveal that these sites are not critical since deletion of sequences that encompass these sites did not prevent regulated expression of the FAS promoter.

USF has been implicated in the transcriptional activation of L-type pyruvate kinase by glucose when the level of insulin is also high (19, 20). Our previous mutational analysis of the −71/−50 region showed that sequences between −68 and −60 bp, which include a core E-box, are essential for recognition and interaction with a trans-acting factor in vitro (7). We also showed that USF1 and USF2 can bind to the FAS insulin response sequence in vitro (8). Moreover, cotransfection of USF1 and USF2 expression vectors with the FAS promoter-luciferase reporter constructs in 3T3-L1 adipocytes increases insulin-stimulated FAS promoter activity. Furthermore, dominant-negative USF1 and USF2 mutants lacking the DNA-binding domain inhibit insulin stimulation of the FAS promoter in this system (9). These studies clearly demonstrated that USF binding to the E-box at −65 bp is required for insulin regulation of the FAS gene in 3T3-L1 adipocytes. Moreover, Vaulont and co-workers (11) recently reported that in USF1 and USF2 knockout mice fed a carbohydrate diet, FAS induction is severely impaired, demonstrating a requirement for USF in FAS gene induction in vivo by glucose/insulin. Our present transgenic mice experiments suggest that in addition to the −65 E-box, an additional USF-binding site at the −332 E-box may be responsible for maximal induction of the FAS gene by fasting/refeeding and insulin. The impairment of FAS induction by glucose/insulin in USF knockout mice may reflect the function of USF at both of these E-boxes. While studying the regulation of the FAS gene by sterol, Magana and Osborne (12) reported the presence of two tandem sites for SREBP, another basic helix-loop-helix leucine zipper family transcription factor, that overlap the −65 FAS insulin response sequence, each occupying half of the E-box. SREBP binding to these sites was reported to be responsible for suppression of FAS gene transcription by sterol. On the other hand, Spiegelman and co-workers (13) reported that SREBP-1c is induced by the refeeding of a carbohydrate-enriched diet and that a mutated SREBP that can bind only to an E-box and not to a consensus sterol regulatory element site transactivates the FAS promoter by binding to the −65 E-box. These authors concluded that SREBP binding to the −65 E-box is responsible for nutritional and insulin regulation of the FAS gene. However, we reported that site-directed mutagenesis of the sequences surrounding the −65 E-box that impair SREBP binding has no effect on insulin regulation of FAS promoter activity in 3T3-L1 adipocytes (9). Goldstein and co-workers (14) demonstrated that overexpression of the truncated active form of SREBP in liver causes a large accumulation of triacylglycerol and the induction of lipogenic genes including FAS. Along with USF, SREBP probably plays an important role in lipogenic gene induction during refeeding. Although SREBP could bind to the −332 E-box sequence in vitro, mutational analysis showed that SREBP does not transactivate the FAS promoter via the −332 E-box. In addition to the −332 E-box, two regions of GC-rich sequence are also found between −278 and −444 bp. These GC-rich sequences may also contribute to the increased expression of the FAS gene by feeding. Further studies are needed to elucidate the precise function of each of these elements during nutritional and insulin regulation of the FAS promoter and to define the SREBP-binding site that functions in vivo and the possible role of the GC-rich sequence in the regulation of the FAS promoter.

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