Essential Role in Vivo of Upstream Stimulatory Factors for a Normal Dietary Response of the Fatty Acid Synthase Gene in the Liver*

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In the liver, transcription of several genes encoding lipogenic and glycolytic enzymes, in particular the gene for fatty acid synthase (FAS), is known to be stimulated by dietary carbohydrates. The molecular dissection of the FAS promoter pointed out the critical role of an E box motif, located at position −65 with respect to the start site of transcription, in mediating the glucose- and insulin-dependent regulation of the gene. Upstream stimulatory factors (USF1 and USF2) and sterol response element binding protein 1 (SREBP1) were shown to be able to interact in vitro with this E box. However, to date, the relative contributions of USFs and SREBP1 ex vivo remain controversial. To gain insight into the specific roles of these factors in vivo, we have analyzed the glucose responsiveness of hepatic FAS gene expression in USF1 and USF2 knock-out mice. In both types of mouse lines, defective in either USF1 or USF2, induction of the FAS gene by refeeding a carbohydrate-rich diet was severely delayed, whereas expression of SREBP1 was almost normal and insulin response unchanged. Therefore, USF transactivators, and especially USF1/USF2 heterodimers, seem to be essential to sustain the dietary induction of the FAS gene in the liver.

Fatty acid synthase (FAS)¹ plays a central role in de novo lipogenesis in mammals, catalyzing all reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. As for many other lipogenic and glycolytic genes involved in maintenance of energy balance, the expression of the FAS gene is highly dependent on nutritional conditions in liver and adipose tissue. Expression of the gene is barely detectable in starved animals and is stimulated by refeeding a high carbohydrate, fat-free diet (for review see Refs. 1 and 2). This fasting/refeeding transition is accompanied in vivo by an increased circulating insulin level, and it is often difficult to differentiate the effects of insulin from those of carbohydrate metabolism in mediating the regulation of gene expression. To date, the role of insulin, either direct or indirect, in mediating FAS-activated gene expression, is still disputed. It has been proposed that the effects of insulin could be only indirect (3–5), being permissive to allow for effective glucose metabolism (1). In contrast, others have provided evidence for a direct involvement of insulin both in vivo (6) and in vivo (7). In this respect, Sul and co-workers (8) reported, by transfection experiments, that the region between −71/−50 was responsible for mediating the effects of insulin on the rat FAS promoter (see Fig. 1). They further demonstrated that upstream stimulatory factors (USFs), USF1 and USF2, were major components of the complex binding to this region (called IRS for insulin response sequence) (9). USFs are ubiquitous basic helix-loop-helix-leucine zipper (b-HLH-Zip) transcription factors able to interact as homo- and/or heterodimers on E boxes of CANNTG sequence (for review see Ref. 10). The FAS promoter IRS contains such an E box at position −65 (Fig. 1). Mutations impairing binding of USF1 and USF2 to this E box have been demonstrated to abolish the insulin-dependent activation of the FAS promoter (11).

In a recent paper, Kim et al. (12) reported data at variance with the data of Wang and Sul (11), and the authors proposed SREBP1 (sterol response element binding protein 1), instead of USFs, as being the key activator acting through the −65 E box (12). As the USFs, SREBP1s are b-HLH-Zip transcription factors (for review see Ref. 13). Two members of this family, encoded by two separate genes, have been characterized so far, SREBP1 and SREBP2 (14–16). Based on in vivo and ex vivo experiments, it is now assumed that SREBP2 is more specifically devoted to the control of genes involved in cholesterol metabolism and SREBP1 in the control of genes involved in fatty acid metabolism (17–20). Unlike other members of the b-HLH-Zip family, SREBP1s were shown to bind not only to their specific target sites, namely the sterol response elements, but also to canonical E boxes. This unusual ability to bind to two distinct DNA sequences is due to the presence of an atypical tyrosine in the basic DNA-binding domain at a key position that is occupied by an arginine in almost all other b-HLH-Zip proteins (21).

In an effort to characterize the molecular mechanisms underlying the regulation of gene expression by glucose in the liver, we previously reported the generation of USF1 and USF2 knock-out mice (22, 23). To gain insight into the respective roles of USFs and SREBP1s in the regulation of FAS gene expression by glucose and insulin, we have investigated the glucose responsiveness of FAS in USF1 and USF2 knock-out mice. In this paper, we demonstrate that USFs are essential components binding to the −65 E box of the FAS promoter and required for a normal transcriptional response of the FAS gene.

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1 The abbreviations used are: FAS, fatty acid synthase; SREBP, sterol regulatory element-binding protein; b-HLH-Zip, basic helix-loop-helix leucine zipper; USF, upstream stimulatory factor; L-PK, L-type pyruvate kinase; S14, Spot 14; EMSA, electrophoretic mobility shift assay; IRS, insulin response sequence; GIRE, glucose/carbohydrates response element.
to dietary carbohydrates in vivo. In addition, we provide evidence that this response is likely dependent on the presence of USF1/USF2 heterodimers, USF1 and USF2 homodimers being insufficient to promote a rapid response of the FAS gene to dietary glucose.

**EXPERIMENTAL PROCEDURES**

**Animals and Treatments**—USF1- and USF2-deficient mice were generated by gene targeting as described previously (22, 23). To generate the double heterozygous USF1−/− USF2−/− mice, USF1−/+ mice were bred with USF2−/− mice as previously reported (23).

For metabolic studies, animals were fed a high carbohydrate diet for 18 h after a 24-h fast. Mice were sacrificed between 10 and 12 a.m., and tissue samples were stored at −80 °C. Blood samples were collected from the orbital sinus.

Serum insulin levels were determined using an insulin radioimmunoassay kit (Behring Diagnostics) with human insulin as standard.

**RNA Analysis**—Total RNA was purified by a modified guanidium chloride procedure, and Northern blot analysis was conducted as described previously (24). FAS (25) and SREBP1 (26) cDNA probes were radiolabeled with [α-32P]dCTP using the High Prime system (Boehringer Mannheim). Each Northern blot was stripped and reprobed with a ribosomal 18 S cDNA to check for the integrity and the amount of loaded RNAs. The amount of specific mRNA was quantified using a PhosphorImager (Molecular Dynamics).

**Protein Analysis**—Nuclear and whole cell extracts were prepared according to Viollet et al. (27) and Vallot et al. (22), respectively. Western blot analyses were performed with 15 μg of nuclear extracts or 60 μg of whole cell extracts using SREBP1 antibody (K-10, Santa Cruz Biotechnology) or affinity-purified USF2 1–49 antibodies.

For electrophoretic mobility shift assays (EMSA), the DNA-binding reaction was performed as described previously (27) in the presence of either 5 μg of rat liver nuclear extracts or 30 μg of whole cell extracts, 2.5 μg of poly(dI-dC), and 0.1–0.5 ng of end-labeled double-stranded oligonucleotide corresponding to the −65 E box of the FAS promoter (−75 to −52, 5′ GTGTCAACCCATGTGGCCGTAAGGC). For supershift experiments, anti-USF1 and USF2 antibodies were included in the binding reactions (27).

**Data Analysis**—Statistical analysis was performed by the Student’s t test for unpaired data using the StatView software. The significance has been considered at *p < 0.05, **p < 0.01, or ***p < 0.001.

**RESULTS AND DISCUSSION**

**Dietary Carbohydrate-dependent Accumulation of the FAS mRNA Is Altered in USF1- and USF2-deficient Mice**—To determine the impact of USF1 and USF2 deficiency on glucose responsiveness of FAS gene expression, a series of metabolic analyses were performed on either wild type, USF1−/−, or USF2−/− mice. After a 24-h fast, mice were refed a high carbohydrate diet for 18 h. Total liver RNA was purified and analyzed by Northern blot to estimate the content of FAS mRNA (Fig. 1, A and B). Quantitation of this analysis revealed that the amount of FAS mRNA was dramatically reduced, to 23% in the liver of USF1−/− mice and to 21% in the liver of USF2−/− mice, as compared with wild type mice. This reduction in the abundance of FAS mRNA was specific to the fasted/refed transition. Indeed, in ad libitum fed mice or in mice fed a high carbohydrate diet for 5 days, the amount of FAS mRNA was not significantly altered (data not shown). In addition, this reduction was not due to altered circulating insulin level in USF1-deficient mice. Insulin level was indeed found to be similar between USF1−/− and wild type mice (34.6 ± 15.44 (n = 8) and 32.2 ± 13.78 (n = 11) μIU/ml insulin for wild type and USF1−/− groups, respectively), and between USF2−/− and wild type mice (22). Finally, glucose uptake and its utilization by the liver of USF-deficient mice were previously shown to be normal (22, 23).

Taken together, these results indicate that USF1 and USF2 are likely to be specifically and directly involved in the dietary carbohydrate-dependent activation of FAS gene expression.

**USFs Are Major Components Binding to the −65 E box of the FAS Promoter**—To determine the nature of USF complexes binding to the specific −65 E box of the FAS gene promoter, EMSA were performed with rat liver nuclear extracts. USF binding activity on this specific E box was shown to be similar to the USF binding activity previously reported on a canonical E box (27). Indeed, as presented in Fig. 3A, USF1/USF2 heterodimers were largely predominant, whereas the amount of USF1 and USF2 homodimers, as determined after incubation with anti-USF1 (lane 2) and anti-USF2 (lane 3) antibodies, was very low (i.e. <10% of total USF binding activity). The same band shift experiments were performed with liver cellular extracts from USF1- and USF-deficient mice. As shown in Fig. 3B, in USF1-deficient mice, the USF binding activity on the −65 E box of FAS promoter was accounted for by USF2 homodimers; anti-USF1 antibody was without any effect on USF binding activity (lane 2), whereas anti-USF2 antibody fully displaced the complex (lane 3). Exactly the opposite was found in USF2-deficient mice (lanes 4–6), i.e. the USF binding activity was accounted for by USF1 homodimers on the −65 E box of FAS promoter. The incapacity of USF1 homodimers in USF2-deficient mice and USF2 homodimers in USF1-deficient mice to support a normal dietary activation of FAS gene transcription suggests that the heterodimeric species could have a specific role in the regulation of the FAS gene compared with the homodimeric species. This idea is strengthened by the fact that FAS mRNA content was reduced to the same extent in both USF1−/− and USF2−/− mice. Therefore, to address further the question of the specific role of USF1/USF2 heterodimer, we examined FAS gene expression in double heterozygous mice where the USF binding activity, mainly accounted for by the heterodimer, was shown to be reduced to 46% (23). We compared by Northern blot analysis the amount of hepatic FAS mRNA in USF1−/− USF2−/−− mice and wild type mice reared a high carbohydrate diet for 18 h (Fig. 4A). As shown in Fig. 4B, the amount of FAS mRNA in the liver of double heterozygous mice was reduced to 50% of normal, which is to say about 2-fold less than in homozygous USF1−/− and USF2−/− mice. We can speculate that this better response of the FAS gene in double heterozygous mice compared with homozygous mice reflects the higher efficacy of the USF heterodimers compared with both types of homodimers. Indeed, total residual USF binding activity is reduced to the same extent (i.e. to 40–46% of normal) in the three types of knock-out animals (23).

Interestingly, this is the first report enlightening the specific role of USF heterodimer species in nutrient gene regulation. Indeed, for two glucose-regulated genes previously investigated, namely L-PK and S14 genes, the glucose-dependent
gene transcription was reported to be impaired in USF2−/− mice but not in USF1−/− mice, suggesting that, in these latter mice, residual USF2 homodimers are as efficient as the heterodimers to allow for a normal glucose responsiveness of L-PK and S14 genes (22, 23). The differential transactivating properties of USF1 and USF2 dimers in activating different sets of glucose-responsive genes could be functionally achieved through the different nature of USF-binding sites as well as of flanking sequences. Indeed, the L-PK and S14 genes have a regulatory element termed glucose/carbohydrates response element (GIRE) which presents striking similarities (Fig. 1). It consists of two CACGTG type E box motifs whose precise spacing and orientation are critical to create a functional glucose response element (2, 28). This architecture is not found for the −65 FAS E box which is not palindromic and, therefore, does not obey the criteria reported above for the functional glucose/carbohydrates response elements (Fig. 1). Thus, the requirements of these different types of USF-binding sites for USF isoforms can be different. In addition, binding sites for auxiliary factors have been shown to be essential for the S14 and L-PK GIRE (28, 29) and could also be involved in the functional specificity of USF dimers. Finally, it is noteworthy that the role of the −65 FAS E box and of USFs in the dietary response of the FAS gene does not signify that this element is itself the “response element” but that it is required, perhaps with other elements, for this response.

The USF-dependent Defect in Dietary Induction of the FAS Gene Is Not Mediated by SREBP1—Our present study seems to establish the fundamental role of USFs in the response of FAS to dietary glucose. However, following the recent report of Kim et al. (12) on the predominant role of SREBP1 in the nutritional control of FAS, it was important to establish whether SREBP1 level was affected in USF1- and USF2-deficient mice. To this end, we measured by Northern blot analysis the amount of SREBP1 mRNA in the liver of USF1−/− and USF2−/− mice as compared with wild type mice. Fig. 5, A and B, shows that the amount of SREBP1 mRNA was normal in USF1−/− mice and only slightly reduced (30% decreased) in USF2−/− mice as compared with wild type controls. To regulate gene transcription, SREBP must be post-translationally activated by a proteolytic cascade; the mature NH2-terminal domain of the protein is released from the endothelial reticulum membranes into the cytosol and then rapidly translocated into the nucleus (13). We therefore analyzed the concentrations of mature SREBP1 in the liver of USF1- and USF2-deficient mice. As shown in the Western blot of Fig. 6A, the amount of mature SREBP1 seemed to be similar in liver cellular extracts from either wild type, USF1−/−, or USF2-deficient mice, indicating that proteolytic processing of SREBP1 was normal in USF-deficient mice. Furthermore, this mature form of SREBP1 was properly translocated into the nucleus as demonstrated by the similar level of SREBP1 in nuclear extracts from wild type and USF-deficient mice (Fig. 6B). Taken together, these results demonstrate that the absence of USFs is likely the primary event responsible for the dramatic decrease in FAS gene induction upon carbohydrate refeeding and that a normal level of mature SREBP1, in absence of USFs, is unable to support a proper nutritional activation of FAS gene expression.

Therefore, our results are in accordance with the data of Wang and Sul (11) and strongly suggest that USFs are required for the nutrition-dependent activity of the FAS promoter. Of course, these results are not sufficient to rule out the possible involvement of SREBP1. However, Shimano et al. (30) recently reported that in the liver and adipocytes of SREBP1−/− mice, the levels of mRNAs for fatty acid synthesis enzymes, including the FAS mRNA, were normal. Although this could be related to compensatory increase of SREBP2 expression in the liver, this factor was undetectable in adipo-

**Fig. 2.** FAS mRNA content in liver of USF1−/− mice (top panel) and USF2−/− mice (bottom panel) as compared with wild type mice. A, Northern blot analysis in animals fasted for 24 h and refed a carbohydrate-rich diet for 18 h. B, quantitated results are expressed as means ± S.D. of eight mice for both groups. The values obtained in USF1−/− mice and USF2−/− mice are statistically different from those in wild type mice (p = 0.0007 and p = 0.0008, respectively).

**Fig. 3.** USF binding activity in liver extracts from animals fasted 24 h and refed a carbohydrate-rich diet for 18 h using a radiolabeled oligonucleotide corresponding to the specific −65 E box of the FAS promoter. A, EMSA was performed in the presence of 15 μg of rat liver nuclear extracts. Selective depletion of the complexes was realized by adding preimmune serum (−) or specific anti-USF antibodies (Ab) in the binding reactions. B, EMSA was performed as above in presence of 30 μg of liver cellular extracts from USF1- and USF2-deficient mice. The asterisk indicates nonspecific protein complexes.
cytes of SREBP1−/− mice in which the abundance of the FAS mRNA was normal (30). These results are consistent with the idea that, on a physiological point of view, SREBPs play a predominant role in regulating the cholesterol synthesis (through sterol response elements and not E box) and have an auxiliary role only in fatty acid synthesis. In this line, Athanikar and Osborne (31) recently suggested that FAS activation through the E box must occur through more specific regulators of fatty acid metabolism than SREBPs factors to provide the mechanism to regulate independently as well as coordinate the biosynthesis of fatty acids and cholesterol.

It is noteworthy that this interpretation of the respective role of USFs and SREBPs in dietary activation of lipogenic genes is not inconsistent with the observation that overproduction of truncated form of SREBP1 in the liver results in constitutive activation of these genes, especially the FAS gene (19, 32). In this case, indeed, it could be that processed SREBP1 in excess constitutively binds to the E box and activates transcription, bypassing the USF-dependent regulation.

How USFs manage to regulate the transcriptional response to dietary glucose is not yet fully elucidated. Preliminary results in our laboratory suggest that the transactivating poten-
tial of USFs is modulated by a glucose-sensor complex interacting, as USFs, with canonical GIREs. However, whether the FAS gene contains authentic GIREs is not clear. Foufelle et al. (33) reported an element in the FAS gene first intron with similar properties to known glucose-responsive elements. However, this element was demonstrated to be ineffective in supporting the dietary gene regulation in the natural context (29) and to be dispensable for in vivo response of a FAS transgene to carbohydrate refeeding (34). As discussed above, the −65 E box, essential in cell culture for the response to insulin, is not an authentic palindromic GIRE, and its cis-effect has not been verified in vivo. Whether this element is really involved in the response to insulin or rather to insulin-dependent increase of the glycolytic flux is also not yet clear.

As a matter of fact, the sensitivity of the FAS gene to USF deficiencies suggests that this gene belongs to the class of the glucose-responsive genes, such as the L-PK and S14 genes, rather than to that of the strictly insulin-responsive genes, essentially represented by the glucokinase gene (2). Indeed, regulation of the glucokinase gene is normal in USF1 and USF2 knock-out mice (22, 23). Further investigations on knock-out mice are needed to firmly establish that the dependence of dietary-regulated genes on USF really permits us to predict which of them are authentic glucose-responsive genes and which are rather regulated by insulin or other glucose-dependent hormones.

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