A GC-rich Region Containing Sp1 and Sp1-like Binding Sites Is a Crucial Regulatory Motif for Fatty Acid Synthase Gene Promoter Activity in Adipocytes

IMPLICATION IN THE OVERACTIVITY OF FAS PROMOTER IN OBESE ZUCKER RATS*

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We have previously shown that the proximal 2-kb sequence of the fatty acid synthase (FAS) promoter transfected into rat adipocytes was highly sensitive to the cellular context, displaying an overactivity in obese (fa/fa) versus lean Zucker rat adipocytes. Using deletional analysis, we show here that FAS promoter activity mainly depends on a region from −200 to −126. This sequence exerts a strong negative effect on FAS promoter in adipocytes from lean rats but not in those from obese rats, resulting in a marked overtranscriptional activity in the latter cells. This region, fused to a heterologous promoter, the E1b TATA box, induced differential levels of gene reporter activity in lean and obese rat adipocytes, indicating it harbors fa-responsive elements(s). Whatever the rat genotype, adipocyte nuclear proteins were shown to footprint the same protected sequence within the fa-responsive region, and supershift analysis demonstrated that Sp1 or Sp1-like proteins were bound to this DNA subregion. Compelling evidence that the Sp1 binding site contained in this sequence was implicated in the differential promoter activity in lean versus obese rats, was provided by the observation that mutations at this Sp1 site induced a 2.5-fold increase in FAS promoter activity in adipocytes from lean rats, whereas they had no effect in adipocytes from obese rats.

Fatty acid synthase (FAS) is a multifunctional enzyme that catalyzes all the reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS plays a central role in de novo lipogenesis, and its level of expression is a key determinant of the maximum capacity of a tissue to synthesize fatty acids. In mammals, FAS is expressed at especially high levels in liver and adipose tissue, where it greatly contributes to the regulation of triglyceride-rich lipoprotein production and to appropriate fat storage. In these tissues, FAS enzyme concentrations are under strict nutritional and hormonal control, and many studies using animal models or established cell lines have determined that the regulation of FAS activity is exerted mainly at the transcriptional level of gene expression. In particular, insulin (1), thyroid hormone (2), and glucose (3) act as positive regulators, whereas AMP (1) and polyunsaturated fatty acids (4) are able to suppress FAS gene transcription in an independent manner. In physiological conditions, these signals and presumably others, yet to be defined, are likely to operate in an interactive manner to elicit an integrated control of FAS transcription. It has been known for many years that pathological situations such as obesity are characterized by elevated FAS activity in adipose tissue and liver (5, 6). In particular ob/ob, db/db mice and fa/fa rats in which obesity is due to inherited defects in the newly discovered leptin regulatory pathway (7–9) all share enhanced lipogenic capacity in adipose tissue. Previously, we had observed that adipocyte FAS hyperactivity in genetically obese fa/fa rats was due to proportionate changes in FAS protein and mRNA levels. In addition, run-on analysis using adipocyte nuclei isolated from lean or obese rats has led to the conclusion that FAS gene transcription was the altered step in obese rats (10). Recently the development of a system of transiently transfected rat adipocytes, shown to be well suited for the study of FAS promoter activity, enabled us to demonstrate that the first 2 kb of the 5′-flanking region of the rat FAS gene were able to direct overtranscription in obese rat adipocytes, mimicking the in vivo fatty genotype effect (11). Similarly, using transgenic mice, the same 2-kb promoter region was shown to be sufficient for the hormonal and nutritional regulation of FAS gene transcription (12). The objectives of the present study were 1) to delineate the sequences within the 2-kb promoter region that play a role in FAS gene transcription in the context of the mature unilocular rat adipocyte, and 2) to identify the region(s) involved in the responsiveness of FAS promoter to adipocyte fa-dependent transcription factors. We highlighted a 74-bp (−200 to −126) functional motif as the critically important control region for defining genotypespecific differences in the level of FAS gene transcription. This motif was found to exert a strong negative control on FAS promoter activity in lean but not in obese rat adipocytes. Sp1 was characterized as one of the proteins interacting with this DNA sequence, and we provide evidence of a differential role of the Sp1 binding site in lean and obese rat adipocytes.

MATERIALS AND METHODS

Plasmids and Constructs—The plasmid pl(−2187+65)FAS-CAT was obtained from S. Clarke and contained a XhoI fragment from the rat FAS promoter (13) cloned into the Sail site of promoterless pUC-CAT basic (Promega). From this plasmid, the authentic transcription start site was previously verified by primer extension (11). Deletions with 5′ end points at −1009, −318, −200, −126, −118, and −35 were generated by digestion with appropriate restriction endonucleases. Ends were filled in with Klenow fragment before religation. The −269 to −16 construct was a linker-mediated polymerase chain reaction product of the FAS promoter region ligated in the Sail site of pBlCAT2. The internal deletion of the −318 to −118 region was produced by cutting
Deleterional Analysis of FAS Promoter Activity in Primary Cultured Adipocytes: Evidence of a Strong Negative Regulatory Region between −200 and −126—We have previously shown that the 5′-flanking 2-kb region of the rat FAS gene is able to direct a high level of reporter gene activity in transfected rat adipocytes through correctly initiated transcription (11). In order to identify the sequences essential for transcription of the FAS gene in these cells, progressively shorter fragments of the 5′-flanking region were inserted in front of the coding region of the CAT gene and transfected into rat adipocytes. Co-transfection with RSV-β-galactosidase gene reporter plasmids was routinely carried out in order to correct for transfection efficiency, and CAT activities were normalized to β-galactosidase activities. As seen in Fig. 1, transcription doubled upon deletion of the sequence between −2000 and −1009, suggesting the presence of a weak negative regulatory element in this region. Deletions to −318 and to −269 did not cause any further significant changes in CAT activity. In contrast, the deletion of the sequence between −269 and −118 led to a very significant 4-fold increase in transcriptional activity, pointing to a very strong negative regulatory element localized in this region. This conclusion was further ascertained by the observation that the 2-kb promoter deleted from the region −318 to −118 was four times more active than the intact promoter (Fig. 2A). Finally, upon the truncation to −35, a dramatic loss of CAT activity occurred, indicating the presence of a crucial positive regulatory element within the proximal region of the promoter. Fig. 1 also illustrates FAS promoter activity in transfected adipocytes from obese fa/fa rats. In these cells, CAT expressions driven by −35 and −118 promoter fragments were similar to those measured in adipocytes from lean animals. In contrast, the region from −269 to −118 did not exhibit any negative effect on promoter activity in adipose cells from obese rats. Therefore, a 4-6-fold increase in CAT activity was observed with the −269 to +16 fragment and with longer promoter constructs, in adipocytes from obese as compared with lean rats. The crucial role of the region extending from −269 to −118 on promoter activity is evident from the data presented in Fig. 2A.
In the differential activity of FAS promoter in lean and obese rat adipocytes was further established by two sets of observations. First, the removal of the sequence 2318 to 2118 from the 2-kb promoter abolished the differences between the two genotypes (Fig. 2A). Second, the fusion of the 2269 to 2118 sequence to a heterologous promoter, the TATA box of E1b adenovirus, induced a 3–4-fold increase in CAT activity in obese rat adipocytes, whereas it had no effect in lean rat adipocytes (Fig. 2B). This observation defines the 2269 to 2118 fragment as the fa-responsive region.

To further delineate the functional sequence harboring responsive elements to fa-dependent transcription factors, two additional CAT deletion constructs were created with 5' end points at −200 or −126. Results in Fig. 2C show that the negative regulatory region that acts to suppress FAS transcription in adipocytes from lean rats is restricted to a region spanning from −200 to −126 bp. This localized the fa-responsive element to within this 74-base pair sequence.

FAS Promoter Activity in Unilocular Rat Adipocytes

**Fig. 2.** Functional importance of the −269 to −118 and −200 to −126 FAS promoter fragments on CAT expression in rat adipocytes. 20 μg of FAS-CAT constructs represented as diagrams on the left were cotransfected with 5 μg of RSV-β-galactosidase in adipocytes isolated from lean (white bars) or obese (black bars) rats. The bar graphs on the right show β-galactosidase-normalized promoter activities expressed either as a function of the −2000 CAT value (panel A) or the E1b TATA box-driven CAT value (panel B) in adipocytes from lean rats set arbitrarily to 1 or as absolute values (panel C). Results were obtained from at least four independent experiments performed in triplicate.

**Fig. 3.** DNase I footprinting analysis of FAS promoter with adipocyte nuclear proteins (A and C) or purified human AP-2 and Sp1 (B). 10 fmol of a −318/+65 FAS promoter fragment, labeled on the antisense strand (15,000 cpm/ng) were subjected to DNase I digestion after incubation with increasing (50, 70, and 100 μg) amounts of nuclear proteins from adipocytes or with increasing (0.1, 1, 2, and 5 footprint-forming units) amounts of purified human Sp1 or AP-2 (1, 2, and 5 footprint-forming units). The migration of Maxam-Gilbert sequencing ladders from the same DNA fragment is shown on the right. Boxes protected by adipocyte nuclear extracts are indicated in the middle, and the position of consensus binding sites for Sp1 and AP2 is shown on the left. Panel C represents protection patterns of nuclear extracts from lean or obese rat adipocytes on FPIV using a −318 to +65 FAS promoter fragment labeled on the sense strand as a probe. Increasing amounts (50, 70, and 100 μg) of nuclear proteins from adipocytes of lean or obese rats were used.

FAS Promoter Contains Multiple Nuclear Protein Binding Sites—In an attempt to identify the transcription factors regulating the FAS promoter we next performed DNase I footprinting over the first 300 bp of the FAS promoter using increasing amounts of nuclear extracts from lean rat adipocytes (Fig. 3A). The first region (FP1) protected by adipocyte nuclear...
extracts, from −10 to −40, overlaps with the TATA-box sequence (13) and might therefore correspond to the binding of members of the transcription initiators. The second protected region (FPII), extending from −42 to −64, coincides with the position where liver nuclear extracts were shown to bind to the insulin-responsive sequence (20), suggesting that adipocytes, like hepatocytes, contain insulin-responsive sequence-binding protein(s). The third protected box (FPIII), from −75 to −96, matches with a site recently characterized by Rangan et al. (21) as a CAMP-responsive element. The fourth region (FPIV), ranging from −135 to −180, is located within the fa-responsive region. A fifth protected region (FPV), from −210 to −255, i.e. upstream of the fa-responsive region, was detected.

Since several potential binding sites for Sp1 and AP-2 are present on this promoter fragment (schematically shown on the left in Fig. 3), the probe was footprinted with purified human Sp1 and AP-2 (Fig. 3B). Footprinting with purified AP-2 revealed a true AP-2 binding site, centered at −122, that did not appear to be occupied by adipocyte nuclear proteins. In addition, purified AP-2 also protected the FPIV region and part of the FPV. The Sp1 binding site at −90 was footprinted by human Sp1, in agreement with a recent report of Bennett et al. (22). In addition, two other Sp1 binding sites were detected, in FPIV and FPV.

Identical band protection patterns were observed with adipocyte nuclear proteins from lean and obese rats as shown in Fig. 3C for the fa-responsive region.

Sp1 and Sp1-like Proteins Bind the −180 to −135 Region (FPIV) of the FAS Promoter—The next step in our study was to characterize adipocyte nuclear protein binding to the fa-responsive region of the FAS promoter. For this purpose, gel mobility shift assays were performed using the −180 to −135 footprinted region IV as a probe (Fig. 4). Four specific retarded complexes, C1, C2, C3, and C4, competed away with a 30-fold molar excess of unlabeled FPIV but not with an unrelated oligonucleotide (YY1 consensus binding site), were detected. Since FPIV contains potential binding sites for Sp1, Egr-1, and AP-2 (schematically represented in the lower panel of Fig. 4), competitions with consensus oligonucleotides were performed. Fig. 4A shows that a 100-fold excess of Egr-1 oligonucleotide was unable to disrupt the binding of any complex on the FPIV probe, suggesting that this factor is not involved in complex formation on this region. Moreover, no specific binding of adipocyte nuclear proteins was observed using a labeled Egr-1 consensus oligonucleotide (data not shown). Binding of nuclear extracts on the FPIV probe could not be competed away by a 10- or 30-fold molar excess of AP-2 consensus oligonucleotide, although a 300-fold molar excess produced a weak displacement. This observation together with the inability of anti-AP-2 antibody to produce supershifted bands, suggests that AP-2 is not involved in complex formation of adipocyte nuclear proteins with FPIV. By contrast, C1, C2, and C3 were efficiently competed away by a molar excess of Sp1 consensus oligonucleotide but not by a mutated Sp1 oligonucleotide (data not shown), strongly suggesting that these complexes contain Sp1 or Sp1-related proteins. Moreover, purified Sp1 binding to the FPIV probe leads to a complex that co-migrates with C1 (Fig. 4B). Finally, the identification of C1 and C2 as authentic Sp1 binding complexes was further established by supershifting with anti-Sp1 antibody, C3, not supershifted by anti-Sp1 antibody, might contain other members of the multigene Sp1 family that exhibit the same binding properties as Sp1 (23, 24). Altogether, these experiments demonstrate that Sp1 is present in C1 and C2 binding complexes and that Sp1-like proteins are part of C3. The composition of C4 remains totally unknown.

Sp1 Binding Site in FPIV Is Functional—Gene transfer experiments into the D. melanogaster Schneider cell line (SL2) that lacks endogenous Sp1 and nuclear factor 1 transcription factors were performed in order to further substantiate the possibility that Sp1 or related members of the Sp1 family...
control the level of transcription of the FAS promoter (Fig. 5). In SL2 cells co-transfected with the full-length FAS-CAT construct and the control vector pADH in which the sequence encoding Sp1 has been deleted, we were able to measure significant amounts of FAS-driven CAT activities, indicating that the expression of FAS promoter is not strictly Sp1-dependent. The presence of nuclear factor 1 is also not necessary for FAS expression, in agreement with a previous study using an in vitro transcription assay (25). In SL2 cells transfected with the full-length FAS-CAT construct, forced expression of Sp1 elicited a 3-fold increase in CAT expression, demonstrating the presence of functional Sp1 site(s) in the FAS promoter. A similar effect of Sp1 was observed with the −318 to −65 FAS-CAT construct, suggesting that the functional Sp1 binding sites are present within this region. The removal of the Sp1 site located within the FPV fragment (by deletion of the region from −318 to −200) did not substantially alter the ability of Sp1 to stimulate CAT activity. In contrast, a complete loss of Sp1 stimulation was observed upon transfection of cells with the −118 to −65 FAS-CAT promoter construct or with the full-length promoter in which the −318 to −118 fragment had been deleted. This establishes that Sp1 stimulation of promoter-driven CAT activity arises exclusively from the site located within the −118 to −200 region.

The Mutation of Sp1 Binding Site in FPV Affects FAS Promoter Activity in Adipocytes—The −200/+65 FAS-CAT construct in which the −161 Sp1 binding site had been mutated was transfected in rat adipocytes (Fig. 6). The results show that the Sp1 mutation elicited a 2.5-fold increase in CAT expression in adipocytes from lean rats, suggesting that the Sp1 binding site is partly responsible for the negative effect of the −200 to −126 region on FAS gene transcription. In contrast, the mutation did not modify reporter gene expression in adipocytes from obese animals. The differential effect of the mutated Sp1 binding site in lean and obese rat adipocytes was not explained by the presence of functional Sp1-like proteins demonstrated here on the FAS promoter.

**DISCUSSION**

The cellular context is of critical importance for the study of regulated gene expression. This study provides the first dele-

**Effect of mutation of Sp1 binding site in FPV on promoter activity.** Adipocytes from lean and obese rats were co-transfected with 5 μg of RSV-β-galactosidase and 20 μg of either −200/+65 FAS-CAT construct (wild type) or −200/+65 FAS-CAT in which the Sp1 binding site in FPV had been mutated (Mut Sp1). β-Galactosidase-normalized CAT activities were expressed as a function of the wild type value in adipocytes from lean rat set arbitrarily to 100%. Values are means ± S.E. of three independent experiments.
suggest that the Sp1 binding site is a common trait of the fa-responsive regions. As shown here, mutation of the Sp1 binding site in the fa-responsive region does not alter the capacity of adipocytes from obese rats to direct high transcriptional activity of the FAS promoter. Thus, it is possible that the down-regulating factor(s) acting through the Sp1 binding site might be inactive in obese rat adipocytes. Alternatively, although not detected under the present experimental conditions, adipocytes from obese rats might specifically express a potent activator overriding the negative effect of the Sp family.

The recent discovery that the db mutation, the mouse homolog of fa, invalidates the leptin receptor (8, 9) raises new questions about the molecular pathogenesis of obesity in Zucker rats. In view of the fact that leptin receptor expression in mice is not restricted to the central nervous system but is instead expressed ubiquitously, including in adipose tissue, we cannot rule out the possibility of a direct control of adipocyte gene expression by leptin. The structure of the leptin receptor deduced from the cDNA sequence defines it as a new member of the cytokine receptor superfamily (31). Since the signals transduced via cytokine family receptor complexes are able to modulate the expression of a wide variety of genes, we can speculate that the fa-responsive region of the FAS gene might be an ultimate target of the leptin receptor signaling pathway. Thus, the crucial regulatory region defined here on the FAS promoter could provide a tool for unraveling the leptin signaling pathway.

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