Elevated lipogenesis is a key determinant of exaggerated fat deposition in adipose tissue of obese Zucker rats. We previously delineated a region in the fatty-acid synthase promoter, which was responsible for obesity-related overexpression of the fatty-acid synthase (FAS) gene, by negatively regulating the activity of the downstream promoter in lean but not obese rat fat cells. The present study aimed to identify the transcriptional factors acting on this target region. First, functional analysis of mutated FAS promoter constructs in transiently transfected lean and obese rat adipocytes showed that the activity of the obesity-related region relied on the presence of a transcriptionally inactive sterol regulatory element at −150, which counteracted activation through the downstream E-box. Adenovirus-mediated overexpression of a dominant negative form of adipocyte determination and differentiation factor 1 (ADD1) was used to neutralize endogenous ADD1/sterol regulatory element-binding protein (SREBP) transcriptional activity in fat cells, by producing inactive dimers unable to bind target DNA. With this system, we observed that overexpression of FAS in obese rat adipocytes was ADD1/SREBP-dependent. SREBP isoforms expression was assessed in lean and obese rat fat cells and showed no differences in the level of ADD1/SREBP1 mRNA. In addition, equivalent amounts of immunoreactive ADD1/SREBP1 were found in nuclear extracts from lean and obese rat fat cells. In contrast, immunoreactive SREBP2, which was very low in nuclear extracts from lean rats, was induced in obese rat fat cells. Finally, using in vitro binding studies, we showed that SREBP2 was able to displace ADD1/SREBP1 binding from the sterol regulatory element (SRE) site. Thus, we propose a mechanism for obesity-related overexpression of FAS gene in rat adipocyte. ADD1/SREBP1-activated transcription proceeding from the E-box motif is counterbalanced by a negative SRE site acting by limiting the availability of ADD1/SREBP1 in normal fat cells. The negative effect of this site is abolished in obese rat adipocyte nuclei where SREBP2 is induced and can substitute for ADD1/SREBP1 binding to the inactive SRE. These results provide evidence for the implication of SREBPs in the dysregulation of adipocyte metabolism characteristic of the obese state.

Fatty-acid synthase (FAS), a key lipogenic enzyme that catalyzes all steps of the biosynthesis of long chain fatty acids from acetyl-CoA precursors. In conditions of active lipogenesis, it is expressed at high levels in lactating mammary gland, liver, and adipose tissue and is subjected to tight hormonal and nutritional control. In light of a study showing that promoter sequences (contained within a 2.1-kilobase fragment of 5′-untranslated region) were able to confer full hormonal and nutritional regulation to a CAT reporter gene in transgenic mice (1), and in the absence of evidence for post-translational regulation, it is believed that FAS control is exerted primarily at the transcriptional level of gene expression. Key physiological regulators of FAS expression such as glucose (2), insulin and cAMP (3), and thyroid hormone (4) have been identified, but the detailed mechanisms by which FAS gene transcription is ultimately turned on/off remain to be clarified. Recently, cholesterol has been proposed as a new regulator of FAS expression. Its role was first suggested from studies on SREBP(s), the sterol-regulated family of transcription factors (5, 6), and their rat homologue ADD1 (7). SREBPs have been characterized as conditional transacting factors of the bHLH-leucine zipper family, activated upon cholesterol depletion by cleavage of membrane bound precursors (8), and acting on target genes controlling cholesterol uptake and biosynthesis. On one hand, it was found that transgenic mice overexpressing SREBP1 developed fatty livers, in which FAS gene expression was highly stimulated (9). On the other hand, forced expression of ADD1/SREBP1 was shown to induce FAS gene expression in fibroblasts (10). Finally, down-regulation of FAS (11) and also acetyl-CoA carboxylase (12) gene expression by exogenous cholesterol, a major signal inhibiting SREBPs cleavage, was demonstrated in HepG2, pointing out that at least in this cell type, fatty acids and cholesterol biosynthetic pathways might be controlled coordinately.

FAS gene expression is dysregulated in some pathological conditions such as obesity. In rodent, elevated lipogenesis plays a determinant role in the overaccretion of fat, which characterizes the obese state, and it is well known that hypertrophied adipose depots of animals in which obesity results from the disruption of the leptin signaling system (such as ob/ob, db/db mice and fa/fa rats) exhibit dramatically high levels of lipogenic activities, which cannot be normalized by any nutritional or pharmacological treatments (13). As a first step in the elucidation of the mechanism by which the FAS gene is overtrans...
scribed in adipose tissue of obese Zucker fa/fa rats, we used transiently transfected isolated rat fat cells to delineate a region in the FAS promoter, which was responsible for obesity-related overexpression of the gene (14). This region, spanning between −200 and −126 relative to the transcription start site, is footprinted by adipocyte nuclear extracts between −185 and −135 and is able to confer obesity-related overexpression of a CAT reporter gene in transfected rat adipocytes when linked to an heterologous promoter. In the context of the FAS promoter, this region exerts a strong negative effect on the downstream proximal promoter in normal rat adipocytes. This negative effect is abolished in adipocytes from obese rats, leading to constitutive high promoter activity in those hypertrophied fat cells (14).

In the present study, we now demonstrate that the activity of the previously delineated obesity-related region of FAS promoter relies on the presence of a transcriptionally inactive SRE binding site at −150, which limits the availability of nuclear ADD1/SREBPPs. We also show, using adenoviral vectors to express a dominant negative form of ADD1 in isolated adipocytes, that overexpressed FAS activity in obese rat fat cells is ADD1/SREBPPs-dependent. In addition, we observed a specific increase in SREBP2 but not ADD1/SREBP1 in nuclear proteins from obese versus lean rat adipocytes. Finally, we show that SREBP2 is able to displace ADD1/SREBP1 binding from the SRE site, providing a link between SREBP2 activation in obese rat fat cells and loss of negative control exerted by the SRE site on FAS gene transcription.

MATERIALS AND METHODS

Animals and Tissues—All rats used in this study were from the Zucker rat colony established in our laboratory and were bred according to the French guidelines for laboratory animals. Lean (Fa/Fa) and obese (fa/fa) Zucker rats were used. Male and female rats were sacrificed at 4 weeks of age in the fed state, and inguinal subcutaneous adipose tissue was rapidly excised.

Plasmids and Constructs—5'-Deleted FAS-CAT constructs containing various length of rat FAS promoter sequences in front of CAT reporter gene have been described (14). Site-directed mutagenesis of FAS-CAT plasmids was performed in a mixture of transcription factors (Complete™, Roche Molecular Biochemicals) and were separated by SDS-polyacrylamide gel electrophoresis. Protein content was determined as described previously (16). Oligonucleotides were derived from wild type or mutated sequences. Primer pairs were designed so that the reverse primer would contain a HindIII restriction site in order to produce a linear PCR product. Sequences of the primer pairs used for mutagenesis are:

- CGCGCGCGGGCATCGTACCACCGACGGCGGC for mutation of the SRE site at +1,
- GCACCAAAACCCGACGGCGGC for mutation of the E-box at −65,
- GCCGCCGCCCCGATCGTACCACCGACGGCGGC for mutation of the SRE sequence at −150.

The sequences of all mutated constructs were controlled with the T7 sequencing kit (Amersham Pharmacia Biotech). Plasmids pSV-gal or pSV-luciferase (pGL3 from Promega) containing a 0.25-kilobase fragment of the SREBP1 promoter were cotransfected with FAS-CAT and used for normalization of transfection efficiencies. Expression vectors encoding a dominant negative form of ADD1 in isolated adipocytes, the adenoviral vectors were propagated in adenoviral vectors (approximately 5 × 10^6) cells were transfected in 200 μl of DMEM supplemented with 10% fetal calf serum, 20 mM glucose, and antibiotics. In each experiment, a constant number of cells was transfected with increasing amounts of virus (from 10 to 500 pfu/cell). The multiplicity of infection was adjusted to 1.5 ml with DMEM supplemented with 10% fetal calf serum, 20 mM glucose, and antibiotics. In each experiment, a constant number of cells was transfected with increasing amounts of virus (from 10 to 500 pfu/cell). The multiplicity of infection was adjusted to 1.5 ml with DMEM supplemented with 10% fetal calf serum, 20 mM glucose, and antibiotics. In each experiment, a constant number of cells was transfected with increasing amounts of virus (from 10 to 500 pfu/cell).

Northern Blot Analysis—Total RNA were extracted from freshly isolated or cultured mature adipocytes using the single step method (21). RNA were fractionated on formaldehyde/agarose gels, transferred to Hybond N+ membranes (Amersham Pharmacia Biotech) and probed with 32P-labeled cDNAs using the Megaprime random labeling kit (Amersham Pharmacia Biotech). FAS and ADD1 cDNA probes were described previously (7, 22).

Western Blot Analysis of Adipocyte Nuclear Extracts—Nuclear extracts were prepared from freshly isolated lean and obese rat adipocytes as described elsewhere (22). Aliquots of induced primary adipocytes were cotransfected with FAS-CAT and used for normalization of transfection efficiencies. Expression vectors encoding the first 403 amino acids of rat ADD1 or a dominant negative version in which tyrosine 320 was turned to alanine (10) were obtained from J. B. Kim and B. Spiegelman (Dana Farber Cancer Institute, Boston). The plasmid pSREBP2 encoding full-length hamster SREBP2 was obtained from ATCC (Manassas, VA) and pSREBP2del encoding the first 480 amino acids from ATTC (Manassas, VA). pSREBP2del encoding the first 480 amino acids of hamster SREBP2 followed by 30 new amino acids and a stop codon was constructed by deleting an EcoRI fragment in the original pSREBP2.

Rat Mature Fat Cells Isolation and Transfection—Mature fat cells were isolated by collagenase treatment (15) of subcutaneous inguinal adipose tissue. Isolated fat cells were rinsed three times with DMEM and aliquots of fat cell suspension (200 μl) were electroporated in the presence of various plasmid constructs as described previously (14). Immediately after electroporation, cells were incubated in DMEM supplemented with 10% fetal calf serum, 25 mM glucose, and antibiotics. After 48 h, cells were lysed in 250 μl Tris, pH 8, 5 mM dithiothreitol by three freeze-thaw cycles, and a clear cell lysate was obtained after centrifugation at 3000 × g for 15 min at 4 °C. Reporter gene activities were assayed as described elsewhere (16).

Cell Culture and Transient Transfection of 3T3F442A Fibroblasts—3T3F442A fibroblast cell line was obtained from J. Pairault (INSERM, Créteil, France). Cells were maintained in DMEM supplemented with 10% fetal calf serum (ATGC) and antibiotics. Pre confluent cells were trypsinized, washed, and resuspended in DMEM at a concentration of 5 × 10^6/ml. 200-μl aliquots were electroporated at 200 V/960 microfarads in the presence of 10 μg of FAS-CAT, 1 μg of pSV-Luc, and 100 ng of ADD1 expression vector or empty vector. Electroporated cells were divided in three aliquots and plated onto 35-mm dishes in fresh medium. After 2 days, cells were scraped in cold phosphate-buffered saline, pelleted (1200 × g, 5 min, centrifuged in 100 μl of 250 μl Tris, pH 8, 5 mM dithiothreitol, cell lysates were obtained as described above for mature fat cells and assayed for reporter gene activities.

Adenovirus-mediated Overexpression of a Dominant Negative Form of ADD1 in Mature Adipocytes—The dominant negative version of rat ADD1 was subcloned into the EcoRI-linearized pDK6 shuttle vector (17) under the control of the early polyoma virus immediate early promoter. The pDK6 ADD1/DN plasmid was cotransfected in 293 cells together with the CiaI-cut DNA of the E1a adenosine virus vector, Ad gal-nls (18). Recombinant adenosine ADD1/DN plasmids were detected by polymerase chain reaction amplification of viral DNA using ADD1 primers, and one clone was further amplified in 293 cells. The adenovirus vector Adnull, whose expression cassette contains the major late promoter with no exogenous gene, was used as a control (19). Adβgal, which contains the Escherichia coli lacZ gene coding for β-galactosidase driven by the Rous sarcoma virus promoter (19) was also used to determine the efficiency of adenosine-mediated gene transfer in isolated primary adipocytes. The adenosiviral vectors were propagated in 293 cells, purified by cesium chloride density centrifugation, and stored as stocks at −70 °C. Isolated or cultured adipocytes (approximately 5 × 10^6) cells were transduced in 200 μl of DMEM supplemented with viral titers (from 3 to 150 × 10^6 pfu) for 4 h at 37 °C. Then, culture medium was adjusted to 1.5 ml with DMEM supplemented with 10% fetal calf serum, 20 mM glucose, and antibiotics. In each experiment, a constant number of cells was transfected with increasing amounts of virus (from 10 to 500 pfu/cell). The multiplicity of infection was calculated as poste-riori after the exact number of adipocytes had been determined. Pat cell number in 200-μl aliquots was calculated by dividing total lipid content by mean fat cell volume, as described previously (20). Transduced cells were used 24–96 h after viral infection.

In Vitro Protein Binding Studies—pADD1 and pSREBP2-del were used as templates for in vitro transcription/translation in the coupled reticulocyte lysate system, (Promega). In separate reactions, L-[35S]methionine was included, and translated proteins were analyzed on SDS-polyacrylamide gel electrophoresis. Protein content was determined as described by Bradford (23), using ovalbumin as a standard. Proteins were electrophoresed onto HyBond N nitrocellulose membrane (Amersham Pharmacia Biotech). ADD1/SREBP1 was probed with a polyclonal antibody raised against a peptide sequence from amino acids 470–479 of human ADD1/SREBP1 (K10, Biosource) and was detected with IgG7D4 monoclonal antibody raised against amino acids 32–250 of hamster SREBP2. IgG were produced from a hybridoma cell line (ATTC, Manassas, VA) and purified from conditioned medium by protein G-Sepharose affinity chromatography as described by the manufacturer (Amersham Pharmacia Biotech). Primary antibodies against SREBP1 and -2 were used at 1:500 and 0.5 μg/ml dilutions, respectively, and detection of signals was performed using the ECL Western blot detection kit (Amersham Pharmacia Biotech) with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG as secondary antibody.

Determination of Fatty-acid Synthase Activity—FAS activity was measured on cytosolic supernatants obtained by centrifugation (100,000 × g, 1 h, 4 °C) of adipose cell homogenates. FAS activity was measured spectrometrically as described previously (24) and was expressed as nanomoles of NADPH oxidized per minute.
kinase, gel-purified, and 50–100 fmol (typically 30,000 cpm) were used as probes in 20-μl binding reactions containing 20 mM Tris-HCl, pH 7.5, 5% glycerol, 0.02% Nonidet P-40, 100 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl₂, 1 μg of poly(dI-dC). Reticulocyte lysates were added, and the mixture was incubated for 30 min at 37 °C and loaded on a 5% polyacrylamide gel. The gels were dried and autoradiographed.

RESULTS

The SRE Motif at −150 Is Responsible for the Activity of the Obesity-related Region—FAS gene expression is highly stimulated in adipose tissue of obese fa/fa Zucker rats. Using this animal model as a source of mature fat cells in which FAS promoter activity could be studied by transient transfection, we have previously delineated an obesity-related region, which was footprinted by adipocyte nuclear extracts at nucleotide 2185 to 2135. As shown in Fig. 1A, a striking feature of this region was the presence of a SRE at 2150, which perfectly matched that found in the LDL receptor promoter. This SRE sequence has been shown to bind purified SREBP 1 and 2 in footprinting experiments (11) and thus was a good candidate for a role in the activity of the obesity-related region. First, we examined the ability of the SRE sequence to transactivate FAS promoter by ADD1 in 3T3F442A fibroblasts. Growing cells were cotransfected with 20 μg of FAS-CAT constructs, and 1 μg of pSV luciferase, in the presence of 100 ng of an expression vector containing (black bars) or not (white bars) N-terminal 403 amino acids of the ADD1 sequence. Normalized CAT activities were calculated by dividing CAT by luciferase activity measured in the same cell lysate. Values are means ± S.E. obtained from at least three independent experiments in which different plasmid preparations were used. B, activity of FAS-CAT promoter constructs in transfected mature adipocytes isolated from lean (white bars, left panel) and obese (black bars, right panel) rats. Freshly isolated fat cells were cotransfected with 20 μg of FAS-CAT constructs and 5 μg of RSV-β-galactosidase plasmid for normalization. Results were expressed as a percent of the normalized CAT activity measured in cells transfected with the −200FAS-CAT construct. Values are mean ± S.E. obtained from four to seven independent experiments.

![Image of the results](#)

**FIG. 1.** Functional analysis of mutations on SRE and E-box motifs in FAS promoter. A, schematic representation of FAS promoter with the sequences and location of SRE and E-box motifs. Mutations in SRE and E-box were introduced as described under “Materials and Methods,” and the sequence of the mutated motifs (mut) is shown below the wild type sequence; nucleotide changes are in bold letters. B, transactivation of FAS-CAT promoter by ADD1 in 3T3F442A fibroblasts. Growing cells were cotransfected with 20 μg of FAS-CAT constructs, and 1 μg of pSV luciferase, in the presence of 100 ng of an expression vector containing (black bars) or not (white bars) N-terminal 403 amino acids of the ADD1 sequence. Normalized CAT activities were calculated by dividing CAT by luciferase activity measured in the same cell lysate. Values are means ± S.E. obtained from at least three independent experiments in which different plasmid preparations were used. C, activity of FAS-CAT promoter constructs in transfected mature adipocytes isolated from lean (white bars, left panel) and obese (black bars, right panel) rats. Freshly isolated fat cells were cotransfected with 20 μg of FAS-CAT constructs and 5 μg of RSV-β-galactosidase plasmid for normalization. Results were expressed as a percent of the normalized CAT activity measured in cells transfected with the −200FAS-CAT construct. Values are mean ± S.E. obtained from four to seven independent experiments.
of cotransfected ADD1. FAS promoter constructs containing (−1000 and −200), or lacking (−118, −200 SREmut) an intact SRE sequence were all strongly transactivated by ADD1. This suggests that the SRE motif does not functionally contribute to promoter activation by ADD1. In contrast, deletion or mutation of the E-box motif at −65 in the proximal promoter (−35, −118 Eboxmut, −200 Eboxmut) totally abolished the ability of cotransfected ADD1 to stimulate FAS promoter-driven CAT activity. This demonstrates that most if not all transactivation of FAS promoter by ADD1/SREBP1 is exerted through the E-box sequence in the proximal promoter. This is in perfect agreement with the experiments of Kim et al. (26), which showed that a mutant form of ADD1 that can bind E-boxes but not SRE can transactivate the FAS promoter as well as the wild type protein. Furthermore, in both lean and obese rat adipocytes, mutation of the E-box sequence leads to a drastic reduction of FAS promoter activity (Fig. 1C), demonstrating that this motif is a key determinant of promoter activity in mature fat cell. An interesting finding shown in Fig. 1C is the differential effect of SRE mutation in transfected fat cells from lean and obese rats. As shown in the left panel, the obesity-related region located between −200 and −118 exerted negative effects on FAS promoter activity in lean rat adipocytes, since CAT activity measured in cells transfected with −200FAS-CAT is 2−3-fold lower than that expressed in cells transfected with shorter promoter −118FAS-CAT construct. Importantly, mutation of the SRE sequence also induced a 2−3-fold increase in promoter activity, leading to CAT activities as high as those measured in cells transfected with the −118 proximal promoter only. This strongly suggests that SRE motif at −150 is responsible for the negative effect of the obesity-related region on FAS gene transcription. This is further supported by the absence of effect of SRE mutation in transfected obese rat adipocytes (right panel in Fig. 1C), in which we observed constitutive high reporter gene activities due to the inability of the obesity-related region to down-regulate proximal promoter. Taken together, these data point out that SRE motif at −150 in FAS promoter, although unable to activate transcription upon binding of transcriptional activators such as ADD1, is responsible for the activity of the obesity-related region.

Since in normal rat adipocytes this region negatively regulates FAS gene transcription, one explanation would be that the SRE motif might exert its effects by titration of factors, limiting their availability for the functionally active E-box sequence. Because ADD1/SREBP family proteins, which recognize SRE, also bind to E-box DNA sequences as other members of the bHLH family (27), and because the E-box at −65 is crucial for FAS promoter activity in mature adipocytes and in various other cellular contexts (11, 26, 28), we hypothesized that ADD1/SREBP family proteins might be the transacting factors responsible for dysregulated expression of FAS promoter in obese rat adipocytes.

FAS Overexpression in Obese Rat Adipocytes Is Dependent upon ADD1/SREBP1 Transcription Factors—To establish the role of the ADD1/SREBP family members, we sought to abolish their transcriptional activity by overexpressing a dominant negative form of ADD1 protein in isolated adipocytes. We used an ADD1 mutant protein, which had been shown previously to sequester wild type ADD1 as inactive dimers unable to bind DNA (10). This dominant negative form of ADD1 (ADD1-DN) was expressed in mature fat cells with an adenovirus-mediated gene transfer system, which, unlike transient transfection, allows high levels of transgene expression in almost every cell of the transduced population. In addition, this system can be used efficiently in nondividing terminally differentiated cell types. To assess the efficiency of adenoviral infection in mature rat adipocytes, cells were incubated with a recombinant adenovirus vector expressing the bacterial lacZ gene under the control of the Rous sarcoma virus promoter, and β-galactosidase activity was measured 4 days postinfection. Fat cells infected with increasing pfu expressed increasing levels of β-galactosidase activity, reaching a plateau around 100 pfu/cell (Fig. 2A). No evidence of cell lysis was observed even at high infection titers, as judged by lipid release in the culture medium (data not shown). Since adipocytes are to be maintained in culture as nonadherent floating cells, histochemistry techniques could not be used to evaluate the percentage of individual cells expressing β-galactosidase. However, RSV promoter-driven β-galactosidase activities measured in adenovirus transduced versus transiently transfected adipose cells differ by more than two ranges of magnitude (50 and 0.5 milliunits/10⁶ cells, respectively), suggesting that gene transfer occurred in a much greater proportion of adipocytes with the adenoviral system. In agreement, a recent study showed that approximately 90% of
fully differentiated 3T3L1 adipocytes expressed a nuclear localizing variant of the \( \beta \)-galactosidase gene upon adenoviral infection (29). It is noteworthy that maximal \( \beta \)-galactosidase activity was 2-fold higher in lean than in obese rat adipocytes (Fig. 2A). Since we observed previously in transient transfection experiments that adipocytes from lean and obese rats expressed similar levels of RSV promoter-driven \( \beta \)-galactosidase activities (16), it can be concluded that adenosivirus gene transfer is slightly more efficient in adipocytes isolated from lean rather than obese rats. Mature adipocytes were then incubated with the adenosivirus containing the ADD1-DN cDNA or with an empty vector (null). Fig. 2B shows that ADD1-DN mRNA expression was significant 24 h postinfection in transduced adipocytes from both lean and obese rats, whereas no signal could be detected in cells infected with the empty vector. When infection was performed under conditions of maximal efficiency (>100 pfu/cell), ADD1-DN messenger RNA was expressed at high levels relative to endogenous ADD1 (data not shown). Since specific antibody for the dominant negative form of ADD1 was not available, direct comparisons of the contents of ADD1-DN protein in transduced lean and obese rat adipocytes could not be performed.

The effect of adenoviral overexpression of ADD1-DN on the activity of FAS in adipocytes is shown in Fig. 3. As observed previously (22), FAS activity in obese rat fat cells was expressed 10-fold above the level measured in lean rats, and adenoviral incubation of fat cells per se (with the empty vector) did not influence FAS activity whatever the rat phenotype. Strikingly, 4 days after infection with ADD1-DN virus, FAS activity was drastically reduced in lean rat fat cells to nearly undetectable values. This clearly indicates that ADD1-DN expression inactivates a factor(s), which is mandatory for FAS expression in adipocytes. ADD1-DN has been shown to act primarily by suppressing DNA binding activity of dimerization partners. Since it is believed that ADD1/SREBP1s do not need other bHLH partners to activate transcription of target genes (6), it is very likely that FAS suppression in ADD1-DN-infected fat cells arises from the inactivation of endogenous ADD1/SREBP1s transcriptional activity. Having established the implication of ADD1/SREBP in FAS expression in primary rat fat cells, we investigated whether ADD1-DN expression could normalize FAS activity in obese rat fat cells. In adipocytes from obese rats, adenoviral overexpression of ADD1-DN led to a significant reduction of FAS activity (from 10,000 to 2500 nmol/min/10^6 cells), demonstrating that most of the FAS overexpression in obese, as in lean, rat fat cells is ADD1/SREBP1s-dependent. It is noteworthy that even under the conditions of maximal gene transfer efficiency used in this experiment, obese rat adipocytes still express significant levels of FAS activity. We cannot exclude that this residual expression arises from ADD1-DN-independent factors. Alternatively, inability of transduced obese rat fat cells to express sufficient amounts of ADD1-DN to achieve complete neutralization of endogenous ADD1/SREBP1s could also be invoked. However, given the long half-life of FAS protein, around 45 h (30), a 25% residual activity after 4 days is concordant with a complete block in enzyme synthesis by adenoviral ADD1-DN and suggests that the remaining FAS activity is likely the result of high expression levels in obese rat fat cells. To summarize, these data establish that FAS activity in adipose tissue is strongly dependent on ADD1/SREBP1s transcriptional activity. In addition, the observation that ADD1-DN can almost completely normalize FAS activity in obese rat fat cells strongly suggests that overexpression of FAS proceeds from high levels of transcriptionally active ADD1/SREBP1s in nuclei. This would explain why FAS promoter exhibits constitutive high activity in obese rat adipocytes, since increased levels of ADD1/SREBP1s acting on the E-box motif at -65 would escape from negative effects exerted by transcriptionally inactive SRE sequence.

**SREBP2 Is Overexpressed in Adipocytes of Obese Rats**—To definitely assess this possibility, we compared the levels of ADD1/SREBP1s in nuclear extracts from lean and obese rat adipocytes. Fig. 4A shows Western immunoblotting experiments in which nuclear extracts from lean and obese isolated rat adipocytes were probed with a polyclonal antibody against human SREBP1. This antibody detected two major immunoreactive bands in adipocyte nuclear extracts. One of lower molecular weight corresponds to the mature product of proteolytic cleavage translocated to the nucleus and comigrates with a major immunoreactive band in cell extracts from human liver. The other, migrating slower than the 120-kDa molecular mass standard likely represents the uncleaved precursor associated to the nuclear envelope as proposed in studies in which this precursor form of SREBP 1 was also detected in nuclear extracts. The intensity of the mature form did not differ significantly in lean or obese nuclear extracts (Fig. 4A), indicating that ADD1/SREBP1 protein was present at similar levels in nuclei from obese and lean rat adipocytes. In addition, no difference was detected for the high molecular weight precursor form, indicative of normal ADD1/SREBP1 gene expression and maturation in obese rat adipocytes. This is further supported by Northern blot analysis of ADD1/SREBP1 mRNA, using ADD1 cDNA as a probe. As shown in Fig. 4B, no obvious difference in the level of ADD1/SREBP1 mRNA can be detected between lean and obese rats, despite dramatically higher levels of FAS mRNA in obese rat fat cells. Finally, SREBP2 levels were assessed in adipocyte nuclear extracts using a monoclonal antibody against hamster SREBP2. This antibody detected a single immunoreactive band of 68 kDa molecular mass in nuclear extracts from rat adipocytes (Fig. 4C). SREBP2 immunoreactive band was barely detectable in nuclear extracts from lean rats, and its intensity was increased dramatically in nu-
SREBP2 Overexpression in Obese Rat Adipocytes

Fig. 4. Expression of ADD1/SREBP1 and SREBP2 in lean and obese rat adipocytes. A, Western blot analysis of SREBP1 in nuclear extracts. 40 μg of nuclear protein extract prepared from lean (L) or obese (O) rat adipocytes were separated by SDS-polyacrylamide gel electrophoresis and probed with an antibody against human SREBP1 as described under “Materials and Methods.” Human liver protein electrophoresis and probed with an antibody against human SREBP1 (as described under “Materials and Methods.” Human liver protein electrophoresis and probed with an antibody against human SREBP1 respectively. B, Northern blot analysis of ADD1 and FAS mRNA. 40 μg of total RNA from lean (L) or obese (O) isolated adipocytes were separated on formaldehyde/agarose gels, transferred onto nylon membranes, and were probed with 32P-labeled cDNA inserts as described under “Materials and Methods.” C, Western blot analysis of SREBP2 in adipocyte nuclear extracts. 100 μg of nuclear protein prepared from lean (L) and obese (O) rats were analyzed with a monoclonal antibody against hamster SREBP2 (IgG 7D4). A single protein band with molecular mass corresponding to the mature cleaved product (M) is detected.

SREBP2 Can Displace ADD1/SREBP1 Binding from the SRE Site—To establish the link between SREBP2 induction and loss of negative control on FAS gene transcription through the inactive SRE site, the ability of SREBP1 and -2 to occupy the SRE site was studied using proteins obtained in a transcription/translation system. The main finding of the present study is the involvement of ADD1/SREBP1 preferentially binds to SRE rather than FAS E-box and by establishing that SREBP2 can displace SREBP1 from the SRE binding site, data in Fig. 5 provide a link between SREBP2 induction and loss of the negative control by SRE on FAS gene transcription. Alternatively, since SREBP2 also binds FAS E-box, we cannot exclude that it might also act directly through this site in vivo. Thus, raising the levels of intranuclear SREBP2s has the potential to increase transactivation through the E-box active site, whatever the isoform involved.

DISCUSSION

Previous studies on functional regulation of FAS promoter in established cell lines have characterized an E-box at −65 as a crucial motif for promoter activation through ADD1/SREBP1 factors (26). The present data establish that in primary fat cells, an additional SRE motif at −150 also plays an important regulatory role. This SRE motif, despite its perfect homology with the functional SRE-1 of the LDL receptor, is unable to activate transcription from the FAS promoter upon binding of ADD1/SREBP1s and negatively regulates FAS gene expression in mature adipocytes. It is presumed to act by sequestration of ADD1/SREBP1s, limiting their availability for the transcriptionally active E-box motif. This indicates that only rate-limiting amounts of ADD1/SREBP1s are normally present in adipocyte nuclei. This fits well with the key feature of ADD1/SREBP1s regulation, which involves restricted availability of factors in nuclei by means of regulated proteolytic cleavage from membrane bound precursors. Furthermore, the regulatory role of the SRE site of FAS promoter described here in primary adipocytes was not observed in established cell lines, illustrating the importance of a physiologically relevant cellular context for promoter studies.

The main finding of the present study is the involvement of SREBP1s as the primary cause of overexpression of the FAS
SREBP2 Overexpression in Obese Rat Adipocytes

gene in obese rat fat cells. This finding is supported by several lines of evidence. First FAS promoter activity in obese rat adipocytes escaped from the negative effects exerted by the SRE site. Second, using an adenoviral system to overexpress a dominant negative form of SREBPs, we demonstrated that in obese rat fat cells, overexpressed FAS activity depended upon the presence of SREBPs transcriptional activity. The present data, which identify the SRE sequence as the key regulatory motif of the obesity-related region, has to be connected, which our previous report showing that the Sp1 binding site present at –165 also contributed (but was not sufficient alone) to the negative activity of the region. Indeed, SREBP1 and Sp1 have been shown to cooperate for binding to the LDL receptor promoter (31).

ADD1/SREBPs transcriptional activity arise from several individual proteins, all of them exhibiting very similar DNA binding properties. Two separate genes encode SREBP2 and ADD1/SREBP1, the latter being expressed as two different isoforms, SREBP1a and ADD1/SREBP1c as a result of alternative promoter use (32). Although no direct comparisons of SREBP1 and SREBP2 protein relative expression in adipose tissue are available, studies at the level of mRNA showed that adipose tissue expressed ADD1/SREBP1c as the major SREBP1 isoform (25), whereas SREBP2 was present at extremely low levels (33). By Western blot analysis, we observed a differential activation of SREBP2 in obese rat adipocyte nuclei. Furthermore, we showed that SREBP2 is able to displace ADD1/SREBP1 binding from the SRE site, providing a possible link between SREBP2 activation and loss of negative control on FAS gene transcription. This fits well with the recently published observation that overexpressing SREBP2 in transgenic mice resulted in increased FAS gene expression (34).

It is generally believed that ADD1/SREBP1 bind DNA and activate transcription as homodimers. No interaction with other members of the bHLH family as putative dimerization partners has been described so far, and dimer formation within isoforms has been poorly documented. In vitro binding studies to SRE did not provide evidence in favor of the existence of SREBP1/SREBP2 dimers. Whether such a dimer exists in vivo deserves further studies. Our data showing that a dominant negative form of ADD1/SREBP1c is effective in suppressing FAS activity of cells in which SREBP2 is activated might suggest that SREBP1 and -2 can interact. However, since we show that SREBP2 has the potential to increase ADD1/SREBP1 availability for the active E-box by competing for binding to inactive SRE, it is very likely that ADD1-DN acts primarily by neutralizing ADD1/SREBP1.

In intact animals, experiments in which the SREBP1 gene was overexpressed or inactivated (9, 33), and the recent report of SREBP2 overproduction in transgenic mice (34) has established the importance of SREBPs in the regulation of lipid and cholesterol metabolism. Moreover, it is now demonstrated that overexpression of all three SREBPs isoforms, although with different potencies, can increase lipogenesis in transgenic animals (25, 34). However the respective role of the apparently redundant SREBPs isoforms is still not clearly understood. Experiments involving cholesterol depletion in hamster have shown a selective induction of liver SREBP2 (35). Conversely, cholesterol feeding was able to decrease the absolute amount of nuclear SREBPs and specifically SREBP2 gene expression (36), suggesting a predominant role for SREBP2 in cholesterol metabolism. This was further supported by the study of transgenic mice overexpressing SREBP2, which activate their liver cholesterol synthesis in preference to fatty acid synthesis (34). On the other hand, acute changes in ADD1/SREBP1 have been reported during fasting/refeeding in mice adipose tissue (26), identifying this factor as a potential mediator of nutritional and insulin regulation of adipocyte gene expression. In the present work, we describe differential effects of obesity on ADD1/SREBP1 and SREBP2. In the light of the report showing that ADD1/SREBP1 behaves as an insulin-regulated factor sensitive to acute nutritional changes (26), unaltered expression of ADD1/SREBP1 in the isolated adipocytes studied here is not surprising. We demonstrated previously that FAS overexpression in obese rat fat cells could not be normalized in vitro, even when cells were maintained in a controlled nutritional and hormonal environment in which insulin was absent (37). It is very likely, however, that in the intact animal, hyperinsulinemia and altered nutritional conditions characteristic of the obese state might induce ADD1/SREBP1, additionally contributing to altered adipocyte metabolism.

An important still unsolved question relies on why SREBP2 is activated in adipocyte from obese rats. One explanation might be cholesterol depletion in fat cells. Although the obese rats of the Zucker strain studied here do not exhibit major alterations in circulating cholesterol, massive increase in triglyceride stores might influence cholesterol metabolism within the adipocyte. Indeed, adipose tissue has been recognized for a long time as the main site of cholesterol storage in the body (38), with cholesterol accumulating in the lipid droplet. Based on evidence that cholesterol stores in adipose tissues from obese patients were increased as a function of adipose tissue overdevelopment, it is believed that cholesterol and triglyceride accumulate within a relatively constant ratio. Thus, it can be assumed that in hypertrophied adipocytes, high triglyceride deposition might generate cholesterol movement from the plasma membrane to the lipid droplet, leading to relative cholesterol membrane depletion. In agreement, studies from our laboratory have pointed out a decreased cholesterol/phospholipid ratio as the major alteration of adipocyte plasma membrane in obese Zucker rats (39). On the other hand, the cleavage of SREBP2 into mature transcriptionally active form has been shown recently to be activated by the tumor necrosis factor α/sphingomyelinase/ceramide signaling pathway (40). Thus, overproduction of tumor necrosis factor α, a general feature of obese rat fat cells (41), might also contribute to SREBP2 activation through paracrine action.

The family of SREBPs-responsive genes, first limited to the LDL receptor and genes involved in cholesterol synthesis, has been expended to several genes controlling lipid metabolism, such as (in addition to FAS) ACC (12), GPAT (42), and also leptin (26) and lipoprotein lipase (10), which are of considerable importance for adipose tissue metabolism. Especially, ADD1/SREBP1 activation of lipoprotein lipase, the main pathway for fat storage in human adipose tissue in which lipogenesis is low, might be of particular interest. Thus, SREBP 2 activation has the potential to induce profound metabolic changes within the fat cell, in both rodents and humans.

Our observation provides the first evidence for the implication of SREBPs in the enlargement of adipocyte energy stores and obesity. Finally, since SREBP2 induction in obese rat adipocytes might be brought about by factors directly related to the obese status of the cell, such as high triglyceride content or tumor necrosis factor α overproduction, the question of SREBP2 activation as a general feature of fat cell hypertrophy can be raised. Further studies in animal models and also in human obese patients will be needed to establish this point.

Acknowledgments—We are grateful to J. B. Kim and B. M. Spiegelman for providing ADD1 vectors. We thank Jack Gauldie (McMaster University) for the generous gift of PDK6 and Marc Elloit (URA INRA, Ecole Nationale Vétérinaire D’Alfort) for Adgal-nls.
REFERENCES

1. Soncini, M., Yet, S.-F., Moon, Y., Chan, J.-Y. & Sul, H. S. (1995) J. Biol. Chem. 270, 30339–30343
2. Foufelle, F., Gouhot, B., Pégarier, J.-P., Perdereau, D., Girard, J. & Ferre, P. (1992) J. Biol. Chem. 267, 20543–20546
3. Paulauskis, J. D. & Sul, H. S. (1989) J. Biol. Chem. 264, 574–577
4. Stapleton, S. R., Mitchell, D. A., Salati, L. M. & Goodridge, A. G. (1990) J. Biol. Chem. 265, 18442–18446
5. Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X. X., Goldstein, J. & Brown, M. S. (1993) J. Biol. Chem. 268, 30339–30343
6. Hua, X. X., Yokoyama, C., Wu, J., Briggs, M. R., Brown, M. S., Goldstein, J. L. & Wang, X. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11603–11607
7. Tontonoz, P., Kim, J. B., Graves, R. A. & Spiegelman, B. M. (1993) Mol. Cell. Biol. 13, 4753–4759
8. Brown, M. S. & Goldstein, J. L. (1997) Cell 89, 331–340
9. Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S. & Goldstein, J. L. (1996) J. Clin. Invest. 98, 1575–1584
10. Kim, J. B. & Spiegelman, B. M. (1996) Genes Dev. 10, 1096–1107
11. Bennett, M. K., Lopez, J. M., Sanchez, H. B. & Osborne, T. F. (1995) J. Biol. Chem. 270, 25578–25583
12. Lopez, J. M., Bennett, M. K., Sanchez, H. B., Rosenfeld, J. M. & Osborne, T. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1049–1053
13. Bray, G. & York, D. A. (1998) Physiol. Rev. 78, 719–808
14. Rolland, V., Le Liepvre, X., Jump, D. B., Lavau, M. & Dugail, I. (1996) J. Biol. Chem. 271, 21297–21302
15. Rodbell, M. (1964) J. Biol. Chem. 239, 375–380
16. Rolland, V., Dugail, I., Le Liepvre, X. & Lavau, M. (1995) J. Biol. Chem. 270, 1102–1106
17. Emige, P. C. R., Wan, Y., Bramson, J. L., Graham, F. L. & Gaudie, J. (1998) J. Immunol. 160, 2531–2538
18. Ouali, O., Genin, P. & Eliot, M. (1995) J. Virol. 69, 6518–6524
19. Lafont, A., Leitard, G., Pacaud, P., Vilde, P., Lemarchand, P. & Escande, D. (1997) Hum. Gene. Ther. 8, 1033–1040
20. Lavau, M., Susini, C., Knittle, J., Blanchet-Hurt, S. & Greenwood, M. R. C. (1977) Proc. Soc. Exp. Biol. Med. 156, 251–256
21. Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. 162, 156–159
22. Guichard, C., Dugail, I., Le Liepvre, X. & Lavau, M. (1992) J. Lipid Res. 33, 679–687
23. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
24. Bazin, R. & Lavau, M. (1982) J. Lipid Res. 23, 839–849
25. Shimomura, I., Shimono, H., Horton, J. D., Goldstein, J. L. & Brown, M. S. (1997) J. Clin. Invest. 99, 838–845
26. Kim, J. B., Sarraf, P., Wright, M., Yan, K. M., Mueller, E., Solanes, G., Lowell, B. B. & Spiegelman, B. M. (1998) J. Clin. Invest. 101, 1–9
27. Kim, J. B., Spotts, G., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towle, H. C. & Spiegelman, B. M. (1998) Mol. Cell. Biol. 18, 2582–2588
28. Moustaid, N., Beyer, R. S. & Sul, H. S. (1994) J. Biol. Chem. 269, 5629–5634
29. Sharma, P. M., Egawa, K., Gustafson, T. A., Martin, J. L. & Olefsky, J. M. (1997) Mol. Cell. Biol. 17, 7386–7397
30. Weis, G. H., Rusen, O. M. & Rubin, C. S. (1990) J. Biol. Chem. 255, 4751–4757
31. Sanchez, H. B., Yieh, L. & Osborne, T. F. (1995) J. Biol. Chem. 270, 1161–1169
32. Hua, X. X., Wu, J., Goldstein, J. L., Brown, M. S. & Hobbs, H. H. (1995) Genomics 25, 667–673
33. Shimano, H., Shimomura, I., Hammer, R. E., Herz, J., Goldstein, J. L., Brown, M. S. & Horton, J. D. (1997) J. Clin. Invest. 100, 2115–2124
34. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L. & Shimano, H. (1998) J. Clin. Invest. 101, 2331–2339
35. Sheng, Z., Otani, H., Brown, M. S. & Goldstein, J. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 935–938
36. Shimomura, I., Bashmakov, Y., Shimano, H., Horton, J. D., Goldstein, J. L. & Brown, M. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12354–12359
37. Briquet-Laugier, V., Dugail, I., Le Liepvre, X., Lavau, M. & Quignard-Boulanger, A. (1994) Am. J. Physiol. 267, E549–E546
38. Krause, B. R. & Hartman, A. D. (1984) J. Lipid Res. 25, 97–110
39. Guerre-Millo, M., Guesnet, P., Guichard, C., Durand, G. & Lavau, M. (1994) Lipids 29, 205–209
40. Scheek, S., Brown, M. S. & Goldstein, J. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11179–11183
41. Spiegelman, B. M. & Hotamisligil, G. S. (1993) Cell 73, 625–627
42. Ericsson, J., Jackson, S. M., Kim, J. B., Spiegelman, B. M. & Edwards, P. A. (1997) J. Biol. Chem. 272, 7298–7305