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Transcriptional activation of the mouse obese (*ob*) gene by CCAAT/enhancer binding protein α

(3T3-L1/preadipocytes and adipocytes/leptin/differentiation/obesity)

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ABSTRACT **Like other adipocyte genes that are transcriptionally activated by CCAAT/enhancer binding protein α (C/EBPα) during preadipocyte differentiation, expression of the mouse obese (*ob*) gene is immediately preceded by the expression of C/EBPα. While the 5′ flanking region of the mouse *ob* gene contains several consensus C/EBP binding sites, only one of these sites appears to be functional. DNase I cleavage inhibition patterns (footprinting) of the *ob* gene promoter revealed that recombinant C/EBPα, as well as a nuclear factor present in fully differentiated 3T3-L1 adipocytes, but present at a much lower level in preadipocytes, protects the same region between nucleotides −58 and −42 relative to the transcriptional start site. Electrophoretic mobility-shift analysis using nuclear extracts from adipose tissue or 3T3-L1 adipocytes and an oligonucleotide probe corresponding to a consensus C/EBP binding site at nucleotides −55 to −47 generated a specific protein–oligonucleotide complex that was supershifted by antibody against C/EBPα. Probes corresponding to two upstream consensus C/EBP binding sites failed to generate protein–oligonucleotide complexes. Cotransfection of a C/EBPα expression vector into 3T3-L1 cells with a series of 5′ truncated *ob* gene promoter constructs activated reporter gene expression with all constructs containing the proximal C/EBP binding site (nucleotides −55 to −47). Mutation of this site blocked transactivation by C/EBPα. Taken together, these findings implicate C/EBPα as a transcriptional activator of the *ob* gene promoter and identify the functional C/EBP binding site in the promoter.**

Since the discovery of the obese (*ob*) mutation (1), the *ob/ob* mouse has been widely investigated as a model of genetic obesity and type II diabetes. Recessive mutations at the *ob* locus produce a phenotype characterized by obesity, hyperglycemia, hyperinsulinemia, hyperphagia, and lowered basal metabolic rate (reviewed in refs. 2–5). The obese gene, which was recently identified by positional cloning (6), encodes a 16-kDa secretory protein, referred to as leptin (or OB protein), that is expressed only in white and brown adipose tissue (6–9). Mutation of the obese gene in *ob/ob* mice gives rise to premature termination of translation of the obese message, producing a nonfunctional protein (6).

The *db/db* mouse exhibits an obese phenotype similar to that of the *ob/ob* mouse (10). Parabiosis experiments with *db/db*, *ob/ob*, and lean mice showed that a blood-borne factor produced by lean mice and overproduced by *db/db* mice can reverse the effects of the obese mutation in *ob/ob* mice (10, 11). This led to the hypothesis that *ob/ob* mice lacked the factor, while *db/db* mice lacked the ability to respond to this factor. Consistent with this hypothesis, the effects of administration of recombinant leptin to *ob/ob* and *db/db* mice are strikingly different. Administration of recombinant leptin causes a dramatic decrease in food intake and a profound loss of body weight by *ob/ob* mice but is without effect on *db/db* mice (12–15). Thus, *db/db* mice appear to lack either the receptor or another component of the signal transmission system for leptin. As might be expected, treatment of lean mice with leptin also reduces food intake and body weight (12, 13, 16).

Relevant to transmission of the leptin signal is the observation that *ob/ob* mice overexpress the obese message (6, 7), while *db/db* mice overexpress both the obese message and leptin (13, 17, 18). Taken together these results suggest that an interruption of the leptin signaling system prevents negative feedback of obese gene expression. It has been postulated that *db/db* mice lack a leptin receptor located in the brain, presumably the hypothalamus. A large body of evidence indicates that the hypothalamus possesses a satiety center that is responsive to signals indicative of the global energy status of the animal (3, 4). Consistent with the occurrence of a leptin receptor in this area of the brain, injection of leptin into the intracerebroventricular region of the brain reduced food intake and weight gain by *ob/ob* mice (14). Moreover, leptin was recently shown to bind with high affinity to hypothalamic plasma membranes (15).

Recent studies show that expression of the obese gene is markedly altered by changes in the nutritional–hormonal status of the animal. Thus, fasting or diabetes drastically lower the level of obese mRNA in adipose tissue, whereas refeeding or insulin administration, respectively, rapidly reverse these effects (7, 18–20). In addition, it has been shown that glucocorticoid administration increases expression of the obese message in adipose tissue (21) and isolated adipocytes in culture (22).

Consistent with a tissue distribution limited to adipose depots, obese mRNA is expressed in adipocyte cell lines (e.g., the 3T3-L1 line) albeit at a much lower level than in adipose tissue (7, 22). During differentiation of 3T3-L1 preadipocytes into adipocytes, the level of obese mRNA increases with kinetics similar to those of other adipocyte genes that are transcriptionally activated by CCAAT/enhancer binding protein α (C/EBPα) (7). In view of the role of C/EBPα as transcriptional activator of adipocyte genes (reviewed in refs. 23 and 24), the present investigation was undertaken to determine whether C/EBPα regulates transcription of the obese gene.

EXPERIMENTAL PROCEDURES

Isolation and Analysis of *ob* Genomic Clones. Two genomic DNA libraries were used to isolate the *ob* promoter. The λ FixII mouse (BALB/cJ) genomic DNA library (a gift from D. Nathans; Johns Hopkins University School of Medicine) was screened with the leptin cDNA fragment isolated previously

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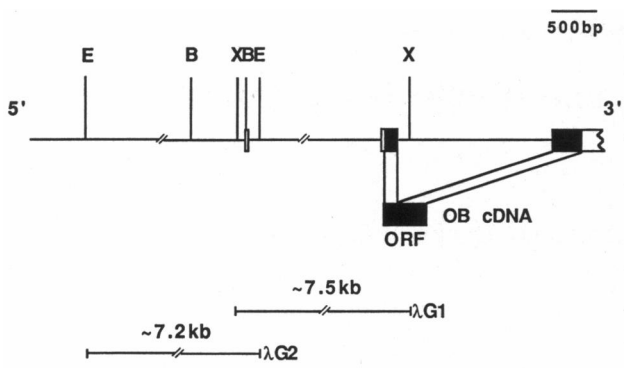
Abbreviations: C/EBPα, CCAAT/enhancer binding protein α; EMSA, electrophoretic mobility-shift analysis.

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ATTCTTGCCT TAATGAAAAA AAAAAAAAAA AAAAACAACA ACCGAAAACA   -612
ACAACAACAA CAAACCAAAC AAACATTGTT TGATCAGGGA CAGGCCCTTG   -562
CCACCTGCCC CTGGGACTGG TCCAGAGCAT CCCTAAACAC ACTTGCGACC   -512
TTCGAAGCAG GTGCATTCTG TGATGTCACA GGATGAAATG AGACGACTGT   -462
TCTTCGGGTA CCAAAGGAAG ACAAGTTGCC CTGAGCTTGG GACCAGTTTC   -412
TCCTCTGAGC AGCCAGGTTA GGTATGCAAA GAGCTGTCGG AAAAAGCAGC   -362
TGGCAGAGTC CTGGCTCACT GGTCTCCCTG TCCCCAAGCC AGCCTTCTGT   -312
AGCCTCTTGC TCCCTGCGGT GCTGGAAGCA CCATCCCAAG GGACCCGTCC   -262
TTAAACTACC GCTGCTCAGT AGCTGCTGGC CGGACCTCGA GGATTACCGG   -212
CTCATACCAA GCGCCCCCAA ACTTGCACTC GAGGGCGCGG CTGAAGTTCT   -162
CCCTCGAGGC GCCTAGAATG GAGCACTAGG TTGCTGCTGC CACTGTTGCT   -112
GGCCCGCTGG GTGGGGCGGG AGTTGGCGCT CGCAGGGACT GGGGCTGGCC    -62
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                +1
ATGGAGCCCC GGAGGGATCC                                    +9
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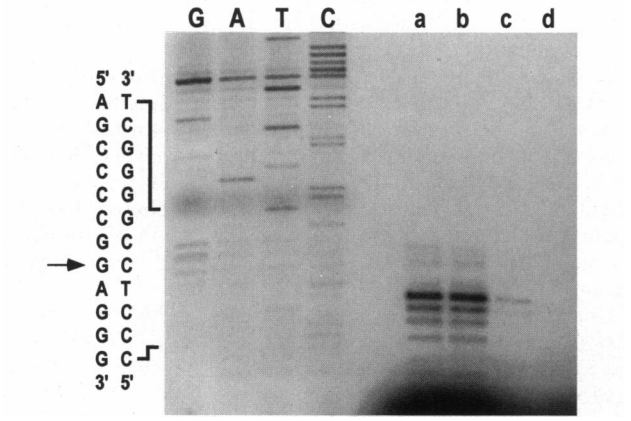

FIG. 1. Nucleotide sequence of the 5′ flanking region of the obese gene and identification of the start site of transcription. (*Top*) Map of the 5′ flanking region. Solid and open boxes indicate the coding region and 5′ and 3′ noncoding regions, respectively. The thin horizontal line identifies the 5′ flanking region and introns. λG1 and λG2 refer to DNA inserts of their respective genomic clones; Ob cDNA refers to obese cDNA. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RI and X, *Xho* I. (*Middle*) Nucleotide sequence of the initial 761 bp of the 5′ flanking region and the untranslated sequence. The transcriptional initiation site is located at +1, a TATA box begins at nucleotide −31 (boldface letters), and three consensus C/EBP binding sites begin at nucleotides −55, −211, and −292 (double underlined). The region footprinted by nuclear extracts from 3T3-L1 adipocytes and recombinant C/EBPα and β (overlined; nucleotides −58 to −42) encompasses the consensus C/EBP binding site between nucleotides −55 and −47 in the proximal promoter. (*Bottom*) Primer extension analysis of obese mRNA. (*Right*) A synthetic oligonucleotide complementary to nucleotides +10 to +28 was end-labeled with ^{32}P and primer extension was carried out with 73 fmol of primer (lanes a, b, and d) or 15 fmol of primer (lane c) and 20 μg of total RNA from adipose tissue of *ob/ob* mice (lanes a, b, and c) or no RNA (lane d). (*Left*) The same unlabeled primer was used to generate the sequencing ladder.

(7). One positive clone with an 18-kb insert was isolated, and the 7.5-kb *Sal* I–*Xho* I fragment derived from this insert was subcloned into pBluescript (KS) (Stratagene) to generate λG1 (Fig. 1 *Top*). The 308-bp *Sal* I–*Eco*RI fragment from the 5′ end of this clone was used to screen a Lambda ZapII mouse (C57BL/6J) genomic library containing 6- to 10-kb *Eco*RI fragments. A clone, λG2, was isolated that encompasses ≈7 kb of the 5′ flanking region as well as exon 1 and 122 bp of intron 1 (Fig. 1 *Top*). Both strands of the proximal promoter segment of λG2 were sequenced (see Fig. 1 *Middle*) by the dideoxynucleotide method (25).

Mapping the Transcriptional Start Site. Primer-extension analysis was conducted as described (26) with two synthetic oligonucleotides (5′ CTTGCAGCTGCTGGAGCAG 3′ and 5′ TTTTCCTCCCTGGGATCC 3′), which are complementary to sequences in exons 1 and 2, respectively, of the mouse obese gene. To determine the length of the primer-extended products, dideoxynucleotide sequencing of the promoter region of the obese gene was performed with an oligonucleotide complementary to exon 1 as primer.

DNase I Footprinting and Electrophoretic Mobility-Shift Analysis (EMSA). DNase footprinting (cleavage inhibition patterns) was carried out essentially as described (27). The −761 to +9 *Bam*HI fragment of the obese gene promoter was subcloned into the *Bam*HI site of pUC18 to generate pUC-Ob770. For DNase I footprinting, the noncoding strand of the segment extending from nucleotide −455 to nucleotide +9 was end-labeled with ^{32}P. DNA probes for EMSA were prepared by labeling the single-stranded oligonucleotides (20–25 nt) with [γ-^{32}P]ATP and T4 polynucleotide kinase and annealing the oligonucleotides. The probes corresponding to the three consensus C/EBP binding sites covered the regions from base pair position −63 to −39, −218 to −199, and −300 to −276 relative to the transcriptional start site, respectively. Incubation with nuclear extracts and electrophoresis were performed essentially as described (28, 29), except that the electrophoresis buffer was composed of 0.38 M glycine, 0.05 M Tris (pH 8.5), and 2 mM EDTA. Recombinant C/EBPα was prepared as described (30), and C/EBPβ was a gift from Steven McKnight (Tularik, San Francisco).

Construction and Transfection of Obese Promoter–Luciferase Gene Constructs. A 768-bp *Bam*HI fragment (nucleotides −760 to +8) from λG2 (Fig. 1 *Top*) was subcloned into the *Bgl* II site of the pGL3-BA luciferase expression vector (Promega), giving rise to pObLuc-760. To construct pObLuc-455 (nucleotides −455 to +8) and pObLuc-159 (nucleotides −159 to +8), pObLuc-760 was cut with *Kpn* I or *Xho* I, respectively, and then religated. To construct pObLuc-580, the *Sau*3AI–*Bam*HI fragment (nucleotides −580 to +8) from λG2 was ligated into the *Bgl* II site of pGL3-BA. Two constructs, pObLuc-m760 and pObLuc-m159, with six mutated nucleotides at positions −52 to −47 (CGCAAG → GAATTC) within the consensus C/EBP binding site located between nucleotides −55 and −47, were constructed by using polymerase chain reactions as described (31), and their authenticity was verified by sequencing.

The *ob* promoter–luciferase constructs were purified for transfection by using Qiagen (Chatsworth, CA) maxiprep columns. 3T3-L1 preadipocytes were maintained and differentiated as described (28, 32). Ten micrograms of the *ob* gene promoter–luciferase constructs were cotransfected by calcium phosphate precipitation along with 10 μg of carrier DNA or with 10 μg of the pMSV-TS[1,3]m42 C/EBPα expression vector (33) into proliferating (≈80% confluent) 3T3-L1 preadipocytes (34). After 48 hr in culture, cell extracts were prepared and assayed for luciferase activity (35).

RESULTS

Isolation and Characterization of the 5′ Flanking Region of the Mouse *ob* Gene. To identify regulatory DNA sequences within the 5′ flanking promoter region of the obese gene, two mouse genomic clones, λG1 and λG2, were isolated (see *Experimental Procedures*). The insert of λG1 contains intron 1 and extends 159 bp into the 5′ flanking region, while the insert of λG2, which overlaps λG1 encompasses ≈7.2 kb of 5′ flanking sequence (Fig. 1 *Top*).

The start-site of transcription was identified by primer extension analysis of total RNA from white adipose tissue of *ob/ob* mice. By using a primer complementary to the 5′ untranslated region encoded by the putative (as identified by cDNA cloning) exon 1, an extension product of 9 nt was obtained (Fig. 1 *Bottom*), thus precluding the occurrence of an exon further upstream. It can be concluded, therefore, that transcription is initiated at the "G" indicated by the arrow in Fig. 1 *Bottom* and that exon 1 is 28 bp in length. Extension analysis using a primer complementary to the 5′ untranslated region encoded by exon 2 gave rise to a 40-nt extension (results not shown) corresponding to the same "G" identified above, thus verifying the size of exon 1. Much longer exposure revealed a minor extension product 90–95 nt longer than the major product. The relevance of this product is unknown, although it does not appear to represent significant alternative splicing of the obese message.

Computer analysis of the 5′ flanking sequence of the obese gene using the SIGNAL SCAN program (36) revealed several consensus sequences for known regulatory elements including a TATA box beginning at position −31 and three potential C/EBP binding sites (37, 38) beginning at positions −55, −211, and −292 from the start-site of transcription (see Fig. 1 *Middle*). As C/EBPα is known to transcriptionally activate many adipocyte genes, it was of interest to ascertain whether the consensus C/EBP binding sites in the obese gene promoter were functional.

Identification of a C/EBP Binding Site in the *ob* Promoter. To identify regulatory elements within the proximal promoter of the obese gene, the region between nucleotides −454 and +9 was subjected to DNase I footprint analysis. Using nuclear extract from 3T3-L1 adipocytes, which is known to contain C/EBPα (27), a strongly protected area was observed between nucleotides −59 and −42 (Fig. 2) that corresponds to the most proximal of the consensus C/EBP binding sites (see Fig. 3*B*). Nuclear extract from undifferentiated confluent preadipocytes, which lack C/EBPα and contain only low levels of C/EBPβ and δ, protected weakly or not at all. Recombinant C/EBPα and C/EBPβ also protected the proximal putative C/EBP binding site. These results are consistent with the previous findings that expression of C/EBPα is activated during differentiation of preadipocytes into adipocytes (30, 39).

Binding was also assessed by EMSA. Thus, oligonucleotides corresponding to the three consensus C/EBP binding sites were compared by using nuclear extracts alone and in combination with antibodies directed against three C/EBP isoforms (C/EBPα, C/EBPβ, and C/EBPδ). Nuclear extracts from adipose tissue and fully differentiated 3T3-L1 adipocytes produced strong gel-shift bands when an oligonucleotide that encompasses the consensus C/EBP binding site between nucleotides −55 and −47 in the *ob* gene promoter were used (Fig. 3 *A* and *B*). The major bands were competitively blocked with both homologous and heterologous C/EBP binding site oligonucleotides. Moreover, the major bands were almost completely supershifted by anti-C/EBPα antibody, but not by anti-C/EBPβ and δ antibodies. In contrast, nuclear extract from 3T3-L1 preadipocytes did not generate the major bands (large arrows in Fig. 3 *A* and *B*) but did produce a weak band that was differentiation-independent (compare bands identified by small arrows in Fig. 3 *A, B,* and *C*). Importantly, the

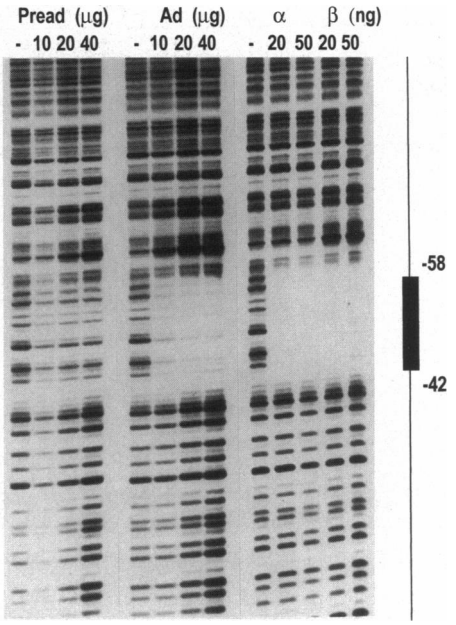

FIG. 2. DNase I footprint analysis of the proximal promoter of the obese gene. The region between nucleotides −455 to +9 of the obese gene was subjected to DNase I footprint analysis of end-labeling the noncoding strand and incubating the DNA with increasing amounts of nuclear extract (in μg) from 3T3-L1 confluent preadipocytes (Pread) (*Left*) or day 5 3T3-L1 adipocytes (Ad) (*Center*) or with increasing amounts of recombinant C/EBPα or β (in ng) (*Right*). The protected region (−58 to −42) is indicated on the right.

major band was supershifted by antibody against C/EBPα, whereas the weak band was supershifted by antibody against C/EBPβ. Antibody against C/EBPδ had no significant effect. It can be concluded that the differentiation-induced bands generated by nuclear extracts from adipose tissue and fully differentiated 3T3-L1 adipocytes contain C/EBPα.

Oligonucleotides corresponding to the two upstream consensus C/EBP sites (beginning at nucleotides −211 and −292) did not produce gel-shift bands with nuclear extracts from adipose tissue or 3T3-L1 adipocytes (results not shown). Therefore, only the proximal site at nucleotide −55 is capable of binding C/EBP.

C/EBPα Transactivates the *ob* Promoter. To ascertain whether C/EBPα can transactivate the *ob* promoter, a series of truncated *ob* promoter–luciferase constructs (i.e., containing from 159 to 760 bp of 5′ flanking sequence) were prepared and cotransfected into 3T3-L1 cells with (or without) a C/EBPα expression vector. All of the constructs containing the C/EBP binding site located between nucleotides −55 and −47 were strongly transactivated by C/EBPα. Consistent with the finding (see above and Fig. 4) that only the proximal C/EBP site is capable of binding C/EBPα, transactivation of all five constructs by C/EBPα was similar regardless of whether they contained either of the two upstream C/EBP sites.

Compelling proof for involvement of the proximal C/EBP site in the transactivation process was provided by mutating this site in two *ob* promoter–luciferase constructs—i.e., the 760-bp (pObLuc-m760) and the 159-bp (pObLuc-m159) constructs. Thus, a six-base mutation in the C/EBP binding site of the proximal promoter completely prevented transactivation by C/EBPα (Fig. 4). It is evident, therefore, that C/EBPα can activate transcription driven by the promoter of the obese gene by interacting with this C/EBP binding site. While other C/EBP binding sites may exist in the distal region of the obese gene promoter, no further transactivation by the C/EBPα expression vector occurred with an ≈7-kb promoter–luciferase construct (results not shown).

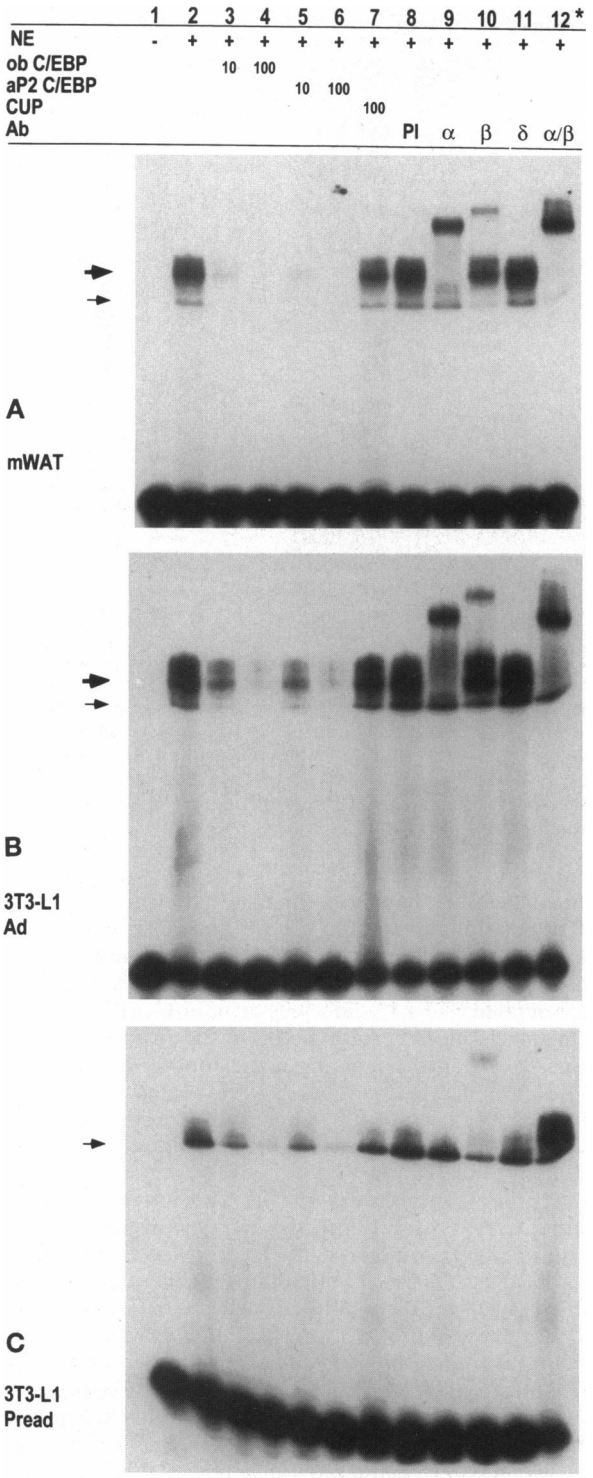

FIG. 3. EMSA with a probe corresponding to the C/EBP binding site in the proximal obese promoter. A ^{32}P-labeled oligonucleotide corresponding to the C/EBP binding site identified by DNase I footprinting (Fig. 2) was incubated with nuclear extract from mouse adipose tissue (*A*); with day 11 differentiated 3T3-L1 adipocytes (*B*); or with confluent 3T3-L1 preadipocytes (*C*). One microgram (*A* and *B*) or 2 μg (*C*) of nuclear protein was used per sample. For competition assays a 10- or 100-fold excess of unlabeled oligonucleotide of a homologous C/EBP-binding site (ob C/EBP) oligonucleotide, a heterologous C/EBP binding site (aP2 C/EBP, 33 bp corresponding to the C/EBP binding site in the 422/aP2 promoter; ref. 27), or a heterologous non-C/EBP binding site (CUP; 24 bp corresponding to the CUP site in the C/EBPα promoter; ref. 46) were added to the preincubation mixture. Supershifting was performed by adding 1 μl of the indicated antiserum to the preincubation mixture. PI refers to preimmune serum, and α, β and δ refer to C/EBPα, β, and δ, respectively. *, Lane 12 in *A* and *B* are as indicated, whereas lane 12 in *C* is identical to lane 2 in *B*—i.e., day 11 3T3-L1 adipocyte nuclear extract.

DISCUSSION

Studies in this (27, 30, 33, 40, 41) and other laboratories (42, 43) have implicated C/EBPα as an indispensable transcriptional activator of adipocyte genes during preadipocyte differentiation (reviewed in refs. 23). In a previous study, we observed that during differentiation of 3T3-L1 preadipocytes, expression of the obese gene and another representative adipocyte gene (stearoyl-CoA desaturase-1) are activated immediately after expression of C/EBPα (7). This finding raised the possibility that transcription of the obese gene may also be regulated by C/EBPα. Examination of the nucleotide sequence of the first 760 bp of 5′ flanking sequence of the gene revealed three consensus C/EBP binding sites located at nucleotides −55, −211 and −292 upstream of the transcriptional start site (Fig. 1 *Middle*). EMSA experiments showed that both C/EBPα and C/EBPβ bind to only one of the proximal consensus sites—i.e,. the site at nucleotide −55 (Fig. 3). DNase I footprinting experiments showed that nuclear extract from 3T3-L1 adipocytes protects this site (but not the sites at −211 and −292), whereas nuclear extract from preadipocytes produces little or no footprint. Consistent with this, EMSA experiments also showed that C/EBPα constitutes both the major C/EBP binding activity in adipocyte nuclear extracts and the major differentiation-dependent change in C/EBP binding activity (see Fig. 3). Transactivation analysis (Fig. 4) with a C/EBPα expression vector and a series of 5′-truncated obese promoter–reporter gene constructs that contain the proximal C/EBP binding site showed that C/EBPα strongly activates reporter gene expression. Moreover, mutation of the proximal C/EBP binding site within the promoter completely blocked transactivation by C/EBPα. Taken together, these findings provide compelling evidence that C/EBPα functions as a transactivator of the obese promoter.

Thus, the obese gene like many other adipocyte genes (reviewed in refs. 23 and 24) is most likely transcriptionally activated by C/EBPα during preadipocyte differentiation. Recent work in Spiegelman's laboratory (44, 45) shows that PPARγ2 (peroxisome proliferator-activated receptorγ2) also plays an important role in initiating transcription of adipocyte genes during differentiation. Although our preliminary attempts to locate a PPARγ2 binding site in or to demonstrate transactivation of the obese promoter by PPARγ2 have been unsuccessful (data not shown), it is possible that a PPARγ2 enhancer lies outside of the promoter sequences we have analyzed thus far. For example, the PPARγ2 binding site in the 422/aP2 gene promoter is located distal (≈5400 bp upstream) to the transcriptional start-site of this gene (44).

In addition to mediating activation of obese gene expression during preadipocyte differentiation (ref. 7 and this paper), the C/EBP site in the promoter may also be involved in conferring hormonal responsiveness to the gene in fully differentiated adipocytes. It has been established that glucocorticoid stimulates the expression of the obese gene message in adipose tissue (21) and isolated adipocytes in culture (22), presumably by inducing transcription of the obese gene. We recently proposed that, while C/EBPα is involved in maintaining expression of adipocyte genes in fully differentiated adipocytes, the rapid induction of C/EBPδ by glucocorticoid in 3T3-L1 adipocytes may function in the transcriptional activation of adipocyte genes by this hormone (28). Consistent with this hypothesis, protein synthesis is required for much of the increase in obese gene mRNA induced by glycocorticoids in isolated adipocytes in culture (22). Further work will be necessary to assess the impor-

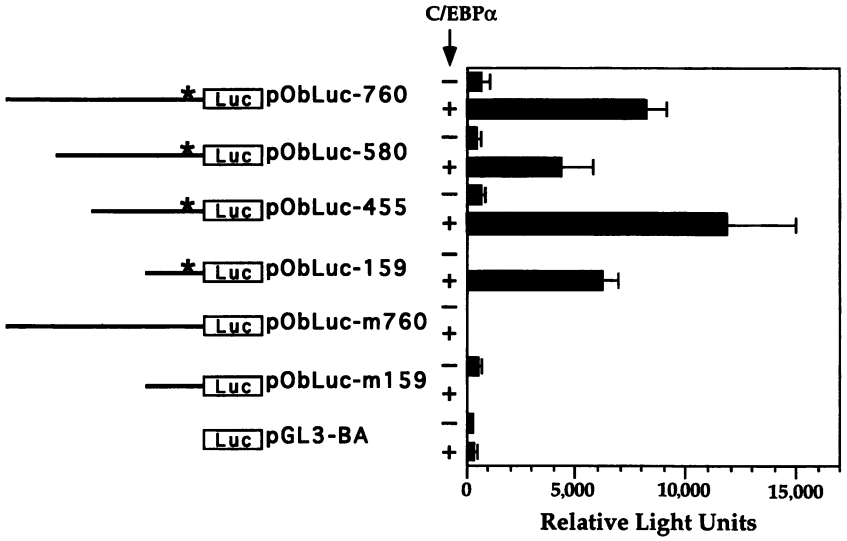

FIG. 4. Transactivation of *ob* promoter–reporter gene constructs by C/EBPα. Various truncated and mutated *ob* promoter sequences were subcloned into a luciferase expression vector (pGL3-BA) as described in *Experimental Procedures*. For pObLuc- or pObLuc-m constructs, 760, 580, 455, and 159 indicate the length in bp of 5′ flanking sequence fused to the luciferase (Luc) gene. pObLuc-m159 and -m760 contain a 6-bp mutation in the proximal C/EBP binding site between nucleotides −55 and −47. Ten micrograms of each construct were cotransfected by calcium phosphate precipitation along with 10 μg of carrier DNA or with 10 μg of the pMSV-TS[1,3] m42 C/EBPα expression vector into 3T3-L1 cells. Cell extracts were prepared and assayed for luciferase activity after 48 hr. Error bars show the standard deviations of triplicate plates. The results are representative of several experiments. The asterisk indicates the approximate position of the consensus C/EBP binding site between nucleotides −55 and −47 in the *ob* promoter. Luciferase activity is given in relative light units.

tance of the C/EBP and glucocorticoid receptor binding sites in the hormonal regulation of expression of the obese gene.

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