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Genetics

Identification of the promoter of the mouse obese gene

FABIENNE CHARLES DE LA BROUSSE, BEI SHAN, AND JIN-LONG CHEN†

Tularik Inc., 270 East Grand Avenue, South San Francisco, CA 94080

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ABSTRACT Primer extension and RACE (rapid amplification of cDNA ends) assays were used to identify and sequence the 5′ terminus of mouse *ob* mRNA. This sequence was used to obtain a recombinant bacteriophage containing the first exon of the encoding gene. DNA sequence analysis of the region immediately upstream of the first exon of the mouse *ob* gene revealed DNA sequences corresponding to presumptive cis-regulatory elements. A canonical TATA box was observed 30–34 base pairs upstream from the start site of transcription and a putative binding site for members of the C/EBP family of transcription factors was identified immediately upstream from the TATA box. Nuclear extracts prepared from primary adipocytes contained a DNA binding activity capable of avid and specific interaction with the putative C/EBP response element; antibodies to C/EBPα neutralized the DNA binding activity present in adipocyte nuclear extracts. When linked to a firefly luciferase reporter and transfected into primary adipocytes, the presumptive promoter of the mouse *ob* gene facilitated luciferase expression. When transfected into HepG2 cells, which lack C/EBPα, the mouse *ob* promoter was only weakly active. Supplementation of C/EBPα by cotransfection with a C/EBPα expression vector markedly stimulated luciferase expression. Finally, an *ob* promoter variant mutated at the C/EBP response element was inactive in both primary adipocytes and HepG2 cells. These observations provide evidence for identification of a functional promoter capable of directing expression of the mouse *ob* gene.

Satiety in vertebrates is controlled by a blood-borne hormone encoded by the obese (*ob*) gene (1). Homozygous recessive mutations of the *ob* gene (*ob/ob*) lead to the gross expansion of adipose tissue. Since animals lacking a functional *ob* gene become phenotypically obese, it has been predicted that the *ob* gene product might play a central role in energy homeostasis and appetite suppression.

The *ob* gene has recently been cloned, facilitating molecular characterization of its encoded protein (2). The *ob* gene product, termed leptin, is a secreted polypeptide produced by adipose tissue. Fat tissue accumulates in response to the intake of excess energy stores, becoming grossly expanded in animals lacking either functional leptin or its putative receptor (3). Under such circumstances, expression of the *ob* gene is markedly elevated (2, 4). These observations give evidence of a feedback loop responsible for controlling vertebrate energy balance. Adipose tissue subsides under conditions of food deprivation, resulting in a reduced level of leptin production and a corresponding increase in appetite. In the well-fed state, excess energy stores accumulate in adipose tissue. Upon maturation and expansion, adipocytes activate expression of the *ob* gene, whose product then serves to quell satiety and stimulate metabolic activity.

Several lines of evidence have indicated that leptin production may be regulated at the level of transcription of its encoding gene. Friedman and colleagues (2) reported that adipose tissue derived from homozygous *ob*-defective animals (*ob/ob*) contains appreciably higher levels of leptin mRNA than that of either heterozygous or wild-type controls. Similar observations have been made using mice bearing homozygous recessive mutations in the diabetes (*db*) gene, which has been predicted to encode the leptin receptor (4). Increased levels of *ob* mRNA have also been observed in obese humans (5, 6). Finally, several recent publications have provided evidence that expression of the *ob* gene is elevated in response to insulin and other blood-borne hormones involved in energy homeostasis (7–9). These observations provide evidence that transcription of the *ob* gene is sensitively balanced with respect to the supply of metabolic energy stores as well as the hormonal factors responsible for controlling energy homeostasis. To initiate studies of the molecular events controlling *ob* gene expression, we have cloned the promoter of the mouse *ob* gene and performed preliminary studies regarding its regulation.*

MATERIALS AND METHODS

Mapping of the *ob* mRNA Transcription Start Site and RACE (Rapid Amplification of cDNA Ends) Sequencing. The transcription start site of the mouse *ob* mRNA was determined by primer extension using two antisense oligonucleotide primers, FCT151 and FCT152, complementary to regions close to the 5′ end of the mouse open reading frame (ORF). Primer extension was carried out using avian myeloblastosis virus reverse transcriptase with products resolved on an 8% polyacrylamide gel. 5′ RACE analysis was performed using a 5′ RACE system kit (GIBCO/BRL) following the manufacturer's recommendations. Fat tissue from mouse C57BL/6J *ob/ob* animals was used as an mRNA source to produce first-strand cDNA. After first-strand synthesis was primed using the ob8 oligonucleotide (see below), the mRNA template was degraded using *Escherichia coli* RNase H. cDNA was then purified and tailed with dCTP using terminal deoxynucleotidyltransferase. PCR synthesis was then carried out using *Taq* DNA polymerase, primed by FCT153 and FCT154. Following amplification the 5′ RACE products were cloned into pSPORT1 (GIBCO/BRL) and sequenced.

Long Distance PCR Amplification of the *ob* Gene First Exon. Long distance PCR amplification of mouse genomic DNA was carried out according to the manufacturer's specifications using an XL PCR kit (Perkin–Elmer) and primers FCT177 and FCT178. Mouse ob PCR products identified by Southern blot analysis were subcloned into pSPORT1 (GIBCO/BRL) and sequenced.

Oligonucleotide Primers. The following primers were used: Ob1, 5′-AATGTGCTGGAGACCCCTGTG-3′; Ob6, 5′-CTTCAGCATTCAGGGCTAACATCCAACTGT-3′; Ob8, 5′-AGGTCATTGGCTATCTGCAGCACA-3′; FTC151, 5′-AGCCACAGGAACCGACACAGGGGTCTC-CAGCACATT-3′; FTC152, 5′-ATAGGCACTGCTTGAAC-

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Abbreviations: RACE, rapid amplification of cDNA ends; ORF, open reading frame.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U52147).

†To whom reprint requests should be addressed.

ATAAGACAGATAGGACCAA-3′; FTC153, 5′-CCGAATTCGCCCAGGAATGAAGTCCAAGCC-3′; FTC 154, 5′-CCGAATTCCGCCAGTGACCCTCTGCTTGGC-3′; FTC177, 5′-CCACGCGTCTGCTCCAGCAGCTGCA-3′; FTC178, 5′-CCGCGGCCGCGCACACTGCTTGCTCTTCCAG-3′; FTC180, 5′-CCGAATTCCTGCAGCTGTGGGAAACCTAACCATCTC-3′.

Isolation of Mouse Genomic Clones. Genomic clones containing the second and third exons of the mouse *ob* gene were obtained by hybridization screening of a bacteriophage λ library using a PCR-amplified probe derived from known *ob*-encoding sequences (2). One clone, designated λ mouse Ob (mOb1), was restricted mapped and sequenced at its insert termini, localizing its 5′ cloning junction roughly 3.5 kb upstream from the second exon of the *ob* gene. A primer (FCT178) derived from the sequence located at the 5′ terminus of λ mOb1 was used in combination with a primer (FCT177) derived from the sequence of the 5′ RACE clone, to obtain a 3.8-kb PCR product (see above). A hybridization probe was prepared from the 5′ end of this long distance PCR product and used to screen a bacteriophage λ library prepared from C57BL/6 genomic DNA. Overlapping clones spanning 24 kb, including the first, second, and third exons of the mouse *ob* gene were isolated and mapped by restriction enzyme digestion and Southern blotting according to standard methods (10).

Construction of *ob* Promoter–Luciferase Fusion Plasmids. A cloned fragment of the mouse *ob* gene encompassing exon 1 was digested with either *Eco*RI alone, *Hin*dIII and *Eco*RI, or *Asp*718 and *Eco*RI to yield fragments of roughly 7, 4, and 0.45 kb. Each fragment contained the same 3′ terminus (an *Eco*RI site located 147 bp downstream from the first exon) with variable amounts of 5′ flanking DNA. These putative promoter fragments were cloned into pGL2-basic (Promega). The 5′ deletions of the putative mouse *ob* promoter were generated by PCR amplification and cloned into pGL2-basic. A site-directed mutation of the putative C/EBP response element was generated by PCR mutagenesis such that the sequence 5′-GTTGCGCAAG-3′ was changed to 5′-GCGAATTCGG-3′.

Preparation of Primary Adipocytes and Electroporation. Epididymal fat tissue was excised from 2-month-old mice (C57BL/6J) and prepared for cell culture by collagenase digestion (11). Isolated adipocytes were transfected by electroporation (12) with recombinant *ob*-luciferase plasmids and then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were harvested 18 hr posttransfection, lysed, and assayed for luciferase enzymatic activity according to the manufacturer's recommendations (Promega). All transfections were carried out in duplicate and repeated at least three times.

Transfection of Cultured HepG2 Cells. Transient transfections were carried out using cultured HepG2 cells by the calcium phosphate precipitation method (13). Five micrograms of promoter-luciferase plasmid DNA was cotransfected with either 1 μg of pMSV expression vector or 1 μg of pMSV-C/EBPα expression vector (14). Samples were coprecipitated with 2 μg of salmon sperm DNA and 0.2 μg of a β-galactosidase internal control expression vector and then applied atop adherent HepG2 cells in six-well tissue culture plates. After 16 hr cells were washed in phosphate-buffered saline and refed with fresh DMEM/F12 culture medium supplemented with 10% fetal bovine serum. After an additional 24 hr cells were harvested, lysed, and assayed for luciferase and β-galactosidase enzymatic activity according to the manufacturer's recommendations (Promega).

Gel Mobility Shift Experiments. Nuclear extracts were prepared from adipocytes isolated from 2-month-old *ob/ob* mice (C57BL/6J) according to published procedures (15). Three double-stranded DNA oligonucleotides were used as binding probes in gel mobility shift assays, one corresponding to the presumed C/EBP response element of the *ob* promoter, one corresponding to the mutated C/EBP binding site (see above), and one corresponding to an optimal C/EBP binding site (16). Binding assays were carried out according to published procedures (17) using 2 μg of adipocyte nuclear extract. Antiserum to C/EBPα was as described (18).

RESULTS

As a first step toward identification of the mouse *ob* promoter, primer extension assays were carried out using two antisense oligonucleotides derived from sequences located close to the 5′ terminus of the *ob* ORF (2). As shown in Fig. 1*A*, both primer extension reactions predicted the *ob* mRNA cap site to be located 56 ribonucleotides 5′ to the initiator (ATG) codon of the *ob* ORF. This measurement did not correspond to the 115 ribonucleotides predicted from the sequence of the cloned, mouse *ob* cDNA (2), consistent with the report that an artifact may have arisen on the generation of the original mouse *ob* cDNA clone (19).

To resolve the nucleotide sequence corresponding to the 5′ terminus of mouse *ob* mRNA, 5′ RACE methods were used to amplify, clone, and sequence the corresponding region. When compared to the sequence of mouse genomic DNA corresponding to the presumed first exon of the *ob* gene (ref. 2; see below), the 5′ RACE sequence diverged from the genomic sequence 31 residues upstream from the ATG codon, leaving ≈25 residues unaccounted for. It was provisionally assumed that these 25 residues corresponded to a small, untranslated exon.

A mouse genomic clone containing the two coding exons of the *ob* gene was obtained and tested by Southern blotting for the presence of the presumptive first exon. Despite containing roughly 3.5 kb of DNA upstream from the first coding exon of the *ob* gene, this genomic clone did not contain sequences corresponding to the 5′ terminal residues of *ob* mRNA (data not shown). The cloning junction of this genomic DNA fragment positioned on the 5′ side of *ob* coding sequences was sequenced. From this sequence a primer was prepared and used together with a primer derived from the presumed first exon in long distance PCRs templated by mouse genomic DNA. The reaction yielded a long distance PCR amplicon of roughly 3.8 kb, which was cloned and sequenced. Such efforts allowed definitive assignment of a small first exon locate roughly 8 kb upstream from the first coding exon of the mouse *ob* gene (Fig. 1*B*).

The DNA sequence of the first exon and upstream DNA of the mouse *ob* gene is shown in Fig. 1*C*. The putative transcription start site is located 25 residues upstream from the first exon/intron boundary and 34 residues downstream from a putative TATA box. A canonical C/EBP response element was identified 15 residues upstream of the TATA box. Given that mRNA coding genes are often preceded by a TATA box (20), and that members of the C/EBP family of transcription factors have been implicated in adipocyte-specific gene expression (21), the presence of these putative regulatory elements was consistent with the tentative identification of the *ob* gene promoter.

To test whether sequences upstream from the small first exon might functionally direct gene expression, various derivatives of the cloned genomic DNA were fused to a luciferase reporter construct and transiently transfected into freshly explanted adipocytes. Two promoter constructs were initially tested. One contained 482 bp spanning from an *Asp*718 restriction site located 458 bp upstream of the transcription start site to the first exon/intron junction (24 bp internal to the *ob* gene). The second construct was prepared from the same DNA fragment but was altered by site-directed mutagenesis to eliminate the putative C/EBP response element. When transfected into epididymal fat cells, the native *ob* promoter fragment directed the synthesis of between 15- and 20-fold higher levels of luciferase enzyme activity than a promoter-free plasmid (Fig. 2). By contrast, the fragment bearing a mutated

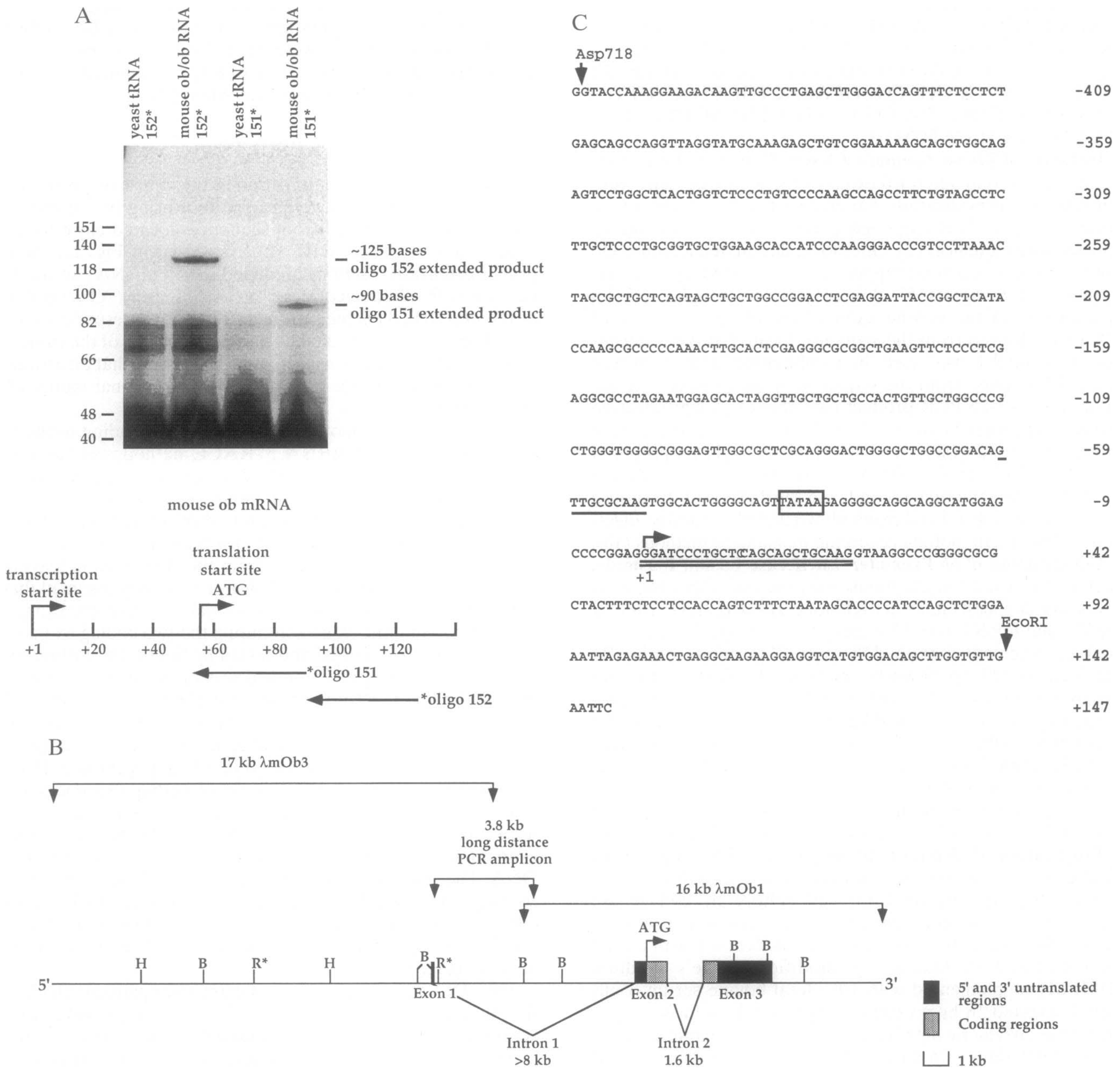


FIG. 1. Molecular organization of the mouse *ob* gene. (*A*) Primer extension mapping of the *ob* gene transcription start site. ^{32}P-labeled FCT151 and FCT152 oligonucleotide primers were annealed to mouse C57BL/6J *ob/ob* fat mRNA and yeast tRNA and extended with reverse transcriptase, and the products were resolved on a denaturing polyacrylamide gel alongside a ^{32}P-labeled size marker. (*B*) Structure of the *ob* gene. Sequencing and restriction mapping analyses of overlapping genomic clones λmOb3, a long distance PCR amplicon, and λmOb1 were used to generate the map shown. *Hin*dIII (H) and *Bam*HI (B) restriction sites are indicated. The −7 kb and +147 *Eco*RI sites (R) used in the promoter deletion analysis are also indicated; * indicates that these sites are not unique within the 24-kb genomic clone. Solid boxes represent noncoding exon sequences. Stippled boxes represent coding regions of the mouse *ob* gene. (*C*) Nucleotide sequence of the region surrounding the first exon of the mouse *ob* gene. The sequenced region is flanked by *Asp*718 and *Eco*RI restriction sites as indicated. Double underline indicates the location of exon 1. Location of the transcription initiation site is marked as position +1. TATA box element is boxed and the putative C/EBP binding site is underlined.

C/EBP site failed to direct luciferase levels any higher than the promoter-free control.

The observations summarized in Fig. 2 provide evidence that DNA sequences located upstream from the first exon of the mouse *ob* gene can promote transcriptional expression in explanted adipocytes. They further indicate that the function of such sequences may be dependent on an intact C/EBP response element. To further test the potential role of C/EBPα in the functional utilization of the mouse *ob* promoter, a variety of promoter constructs were transfected into cultured HepG2 cells, which express little or no C/EBPα protein (21). In this case, duplicate cultures were tested for *ob* promoter function in the presence and absence of an expression vector encoding C/EBPα. Three constructs were initially tested in HepG2 cells. All were linked to the luciferase promoter via an *Eco*RI restriction site located 142 bp downstream from the transcription start site. One extended to an *Asp*718 restriction site located 458 bp upstream from the gene, and the other two extended to *Hin*dIII and *Eco*RI sites located roughly 4 and 7 kb, respectively, upstream of the gene.

As shown in Fig. 3*A*, only the shortest of the three constructs directed significant expression of the luciferase gene in the absence of the C/EBPα expression vector. Upon supplementation of C/EBPα, luciferase expression from this −458-bp

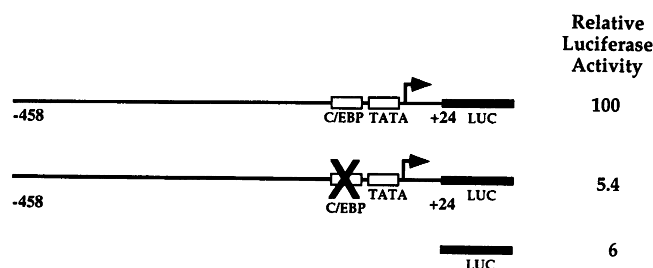
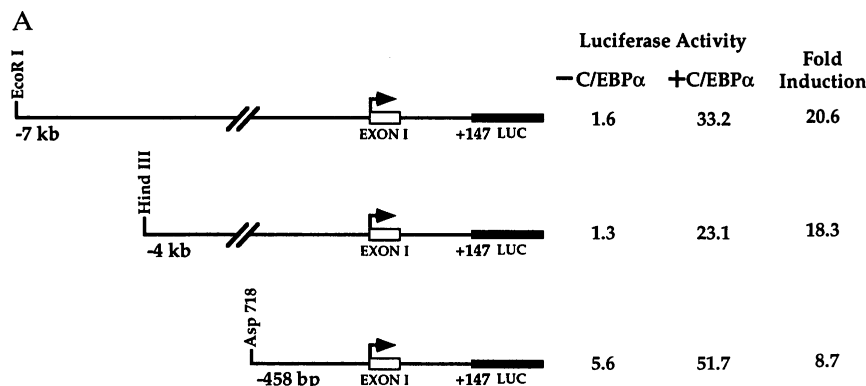


FIG. 2. Expression of luciferase enzymatic activity driven by the mouse *ob* promoter in transfected adipocytes. Isolated adipocytes from mouse epididymal fat pads were transfected by electroporation with (*i*) a construct containing a 482-bp fragment (−458 to +24) of the *ob* gene promoter fused to luciferase reporter; (*ii*) a similar construct bearing a site-directed mutation in the putative C/EBP response element; and (*iii*) a promoter-less reporter plasmid. Cells were harvested 18 hr posttransfection and assayed for luciferase activity.

(*Asp*718) construct was elevated 8.7-fold. C/EBPα supplementation also stimulated expression from the two longer constructs. Luciferase activity specified by the 7-kb (*Eco*RI) fragment was elevated >20-fold when supplemented with the C/EBPα expression vector, whereas expression from the 4-kb (*Hin*dIII) variant was increased 18-fold.

To more closely examine elements of the *ob* promoter that mediate response to C/EBPα, a series of 10 deletion mutants was generated starting with the −458-bp (*Asp*718) construct. As shown in Fig. 3*B*, all deleted variants containing an intact C/EBP response element directed the production of comparable luciferase levels in HepG2 cells cotransfected with the C/EBPα expression vector. A deletion mutant missing the C/EBP site was expressed at levels only slightly higher than the promoter-free luciferase vector. Moreover, a derivative of the −458-bp (*Asp*718) construct that carried a site-directed mutation in the C/EBP response element was similarly defective.

Having obtained provisional evidence for the involvement of C/EBPα in the functional activity of the mouse *ob* promoter, gel mobility shift assays were performed using nuclear extracts prepared from freshly explanted epididymal adipocytes. Double-stranded oligonucleotides were synthesized corresponding to the C/EBP response element of the *ob* promoter, the mutated version tested in transient transfection assays (see Figs. 2 and 3), and a known, optimal C/EBP response element (15). The radiolabeled probes were mixed with adipocyte nuclear extract and subjected to electrophoresis on nondenaturing polyacrylamide gels. Comigrating, shifted complexes were observed for the known C/EBP binding site as well as that derived from the native *ob* promoter yet were not observed for the mutated variant (Fig. 4).



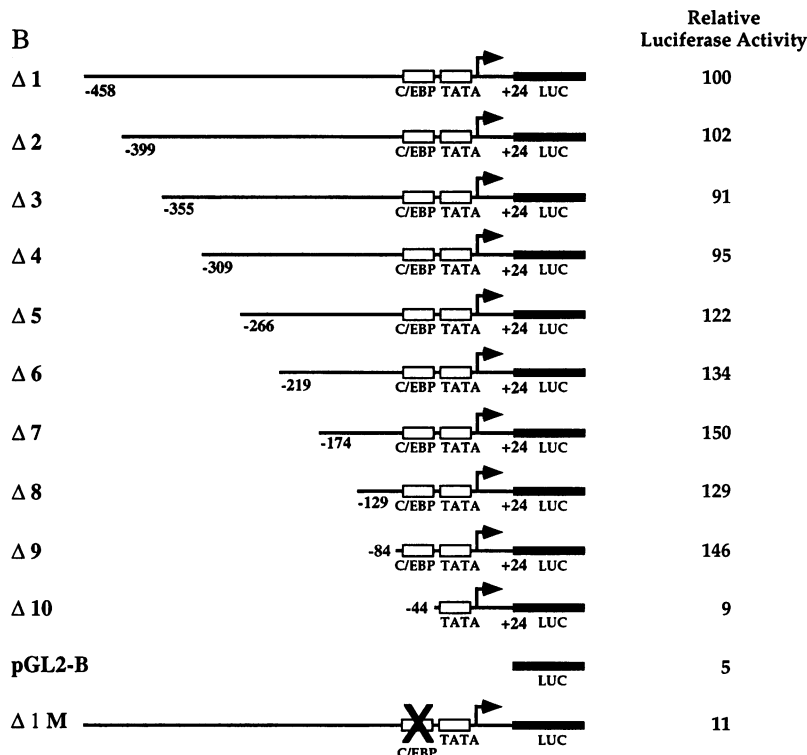


FIG. 3. Activation of the mouse *ob* promoter by C/EBPα. (*A*) Three *ob* promoter luciferase constructs, generated by restriction enzymes *Eco*RI alone (−7 kb/+147 bp); *Hin*dIII/*Eco*RI (−4 kb/+147 bp); and *Asp*718/*Eco*RI (−458 bp/+147 bp) were used in the C/EBPα response assay. Five micrograms of the three different *ob* promoter luciferase constructs were cotransfected into human HepG2 cells with 1 μg of either a murine simian virus (MSV) driven C/EBPα expression plasmid (+C/EBPα) or the MSV control plasmid (−C/EBPα); 0.2 μg of a cytomegalovirus-driven β-galactosidase control plasmid was included in all transfection experiments. Luciferase activity was presented after normalization to β-galactosidase activity. (*B*) A 5′ deletion series of *ob*–luciferase constructs and the site-directed C/EBP mutation construct were cotransfected into HepG2 cells with the MSV-C/EBPα plasmid. Luciferase activity specified by the Δ1 construct was set to 100 and relative luciferase activities to Δ1 are shown.

Antiserum specific to C/EBPα eliminated the predominant complex yet did not generate any artifactual mobility shift when applied in the absence of added nuclear extract. It is notable that treatment of adipocyte nuclear extracts with antiserum to C/EBPα resulted in enhanced binding by the most rapidly migrating band observed in untreated extracts. Since the pattern of gel-shifted complexes was not altered by nonimmune serum, this enhanced band may represent a C/EBP-like protein that was not purged by antibody treatment. It may represent the smaller, 30-kDa translation product of C/EBPα mRNA (22) or one of the other members of the C/EBP family of transcription factors (16). Finally, the sizes of the complexes formed on the *ob* C/EBP response element, as well as the sensitivity of these binding activities to C/EBPα antiserum, were indistinguishable from a known, high-affinity C/EBP binding site (15).

DISCUSSION

The observations outlined in this study provide three lines of evidence for the identification of the transcriptional promoter of the mouse *ob* gene. First, primer extension and RACE assays were used to map and sequence the 5′ terminus of the *ob* mRNA. Such studies revealed the presence of a small, untranslated first exon located roughly 8 kb upstream from the exon encoding the translation initiation codon of the *ob* gene product (2). Second, the DNA sequence immediately upstream from the putative transcription start site was observed to contain a TATA box as well as a potential C/EBP binding site, both hallmarks of mRNA coding genes expressed in mature adipocytes. Third, when linked to a firefly luciferase reporter, the DNA sequences located upstream from the putative transcription start site of the *ob* gene were observed to stimulate gene expression. Evidence of functional promoter activity was observed in primary adipocytes as well as cultured HepG2 cells. Promoter activity was dependent on the integrity of C/EBP binding site in both cell types and was substantially elevated in HepG2 cells upon cotransfection with an expression vector encoding C/EBPα. Finally, gel mobility shift assays provided evidence that the C/EBPα present in adipocyte nuclear extracts is capable of avid and specific interaction with the C/EBP response element of the mouse *ob* promoter. We therefore conclude that these studies firmly identify the promoter of the mouse *ob* gene.

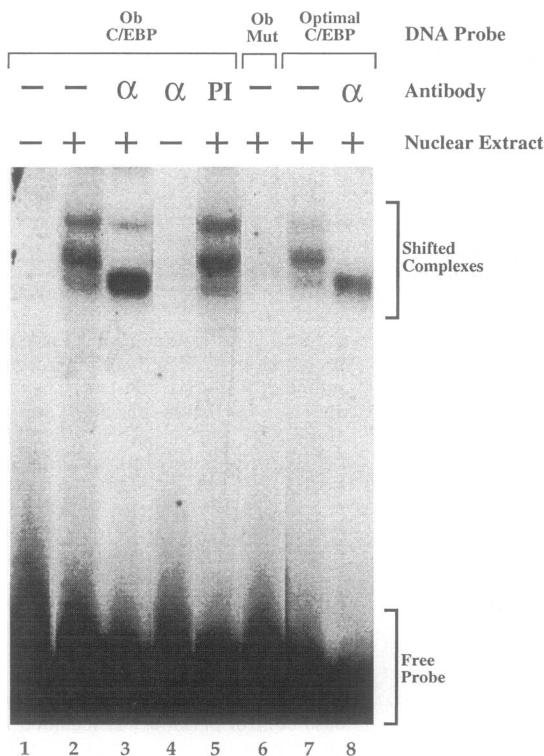


FIG. 4. Gel mobility shift analysis of adipocyte nuclear extracts on the C/EBP binding site of the *ob* promoter. ^{32}P-end-labeled oligonucleotides corresponding to residues −64 to −45 of the *ob* gene promoter (lanes 1–5), a mutated C/EBP binding site used in transfection assays (lane 6), or an optimal C/EBP binding site (lanes 7 and 8) were incubated with 2 μg of nuclear extract from primary adipocytes (lanes 2, 3, and 5–8). In lanes 3, 4, and 8 antiserum directed against C/EBPα was added (α). Preimmune serum (PI) was used in a reaction shown in lane 5.

The observation that *ob* gene expression is positively influenced by C/EBPα is consistent with numerous studies showing the role of this transcription factor in adipogenesis and energy homeostasis. Ectopic expression of C/EBPα is known to specify the adipogenic differentiation of a variety of cell types (23–27), and biochemical experiments have revealed the direct involvement of this transcription factor in the expression of numerous adipocyte-specific genes (28, 29). Gene knockout experiments have recently shown that C/EBPα is required for maturation of adipose tissue (30) and have confirmed the prediction that this transcription factor is a central regulator of mammalian energy homeostasis (31). As such, the involvement of C/EBPα in *ob* gene regulation was easily anticipated (20).

Although C/EBPα appears to represent an important regulator of *ob* gene expression, it is equally clear that other transcription factors will be required to specify its restricted expression in adipose tissue. C/EBPα is expressed in a number of vertebrate tissues, including liver, lung, and intestine (32), that do not express *ob* mRNA (2, 8). As such, it is unlikely that C/EBPα alone is sufficient to activate *ob* gene expression. Further studies will be required to assess the regulatory DNA sequences and transcription factors that restrict *ob* gene expression to mature adipose tissue.

Preliminary evidence of the involvement of regulatory DNA sequences other than the C/EBPα binding site was observed in transient transfection assays included in this study. The two luciferase constructs that contained significant amounts of DNA flanking the state site of *ob* gene transcription (7 and 4 kb) were expressed in cultured HepG2 cells at a significantly reduced level compared with one that contained only 458 bp of upstream DNA. It is possible that negative regulatory DNA sequences present upstream of the *ob* promoter may play a role in restricting *ob* gene expression.

This report, by identifying the *ob* promoter, offers a starting point for comprehensive studies of *ob* gene regulation. Future objectives can now address a number of important questions including how the *ob* gene is induced in response to insulin (8, 9), why its expression is enhanced in animals lacking functional leptin (2) or its putative receptor (4), and how the gene might be regulated in either obese or anorexic human patients.

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