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Autoregulation of the Human C/EBPα Gene by Stimulation of Upstream Stimulatory Factor Binding

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The human C/EBPα gene promoter shares significant sequence homology with that of the mouse but has a different mechanism of autoregulation. Activation of the murine promoter by direct binding of C/EBPα to a site within 200 bp of the transcriptional start was shown to elevate activity by approximately threefold (R. J. Christy, K. H. Kaestner, D. E. Geiman, and M. D. Lane, Proc. Natl. Acad. Sci. USA 88:2593–2597, 1991; K. Legraverend, P. Antonson, P. Flodby, and K. G. Xanthapoulos, Nucleic Acids Res. 21:1735–1742, 1993). Unlike its murine counterpart, the human C/EBPα gene promoter does not contain a *cis* element that binds the C/EBPα protein. Neither C/EBPα nor C/EBPβ (NF-Il-6) binds the human C/EBPα promoter within 437 bp. However, cotransfection studies show that C/EBPα stimulates transcription of a reporter gene driven by 437 bp of the C/EBPα promoter. Our studies show that the human C/EBPα protein stimulates USF to bind to a USF consensus element within C/EBPα promoter and activates it by two- to threefold. We propose that the human gene employs the ubiquitously expressed DNA-binding protein factor USF to carry out autoregulation. Autoregulation of the human C/EBPα promoter was abolished by deletion of the USF binding site, CACGTG. Expression of human C/EBPβ following transfection did not stimulate USF binding. These studies suggest a mechanism whereby tissue-specific autoregulation can be achieved via a *trans*-acting factor that is expressed in all cell types. Thus, direct binding of the C/EBPα protein to the promoter of the C/EBPα gene is not required for autoregulation.

C/EBPα (CCAAT enhancer-binding protein α) belongs to a family of transcription factors (bZIP proteins) which contain a region rich in basic amino acids and a flanking leucine zipper domain that are necessary for DNA binding and dimer formation (22). A number of bZIP proteins have been cloned and sequenced (2, 8, 21, 29). At least three members of the C/EBP family of proteins show a high level of amino acid homology within the basic region and leucine zipper domains: C/EBPα, C/EBPβ, and C/EBPδ (8). These three nuclear factors are able to form heterodimers that bind DNA (8). C/EBPα consists of four regions which are necessary for its transcriptional activity. Two transactivation domains located in the amino-terminal part of the molecule are important for transcriptional activation, while the basic and leucine zipper regions are necessary for binding to DNA (32). Expression of C/EBPα is regulated during rat liver development (13), adipocyte differentiation (6), and liver regeneration (14, 25). Several lines of evidence suggest a central role of C/EBPα in the maintenance of the quiescent, mature state of adult hepatocytes and adipocytes. A high level of C/EBPα expression is found to be limited to highly differentiated cells. For example, quiescent liver hepatocytes contain a high level of C/EBPα mRNA, but this level is decreased in regenerating hepatocytes (14, 25). C/EBPα regulates several genes expressed in differentiated cells, including the serum albumin (15), stearoyl coenzyme A desaturase (11), α1-antitrypsin (12), insulin-responsive glucose transporter (19), and insulin-like growth factor II (33) genes. Umek et al. show that hormone-regulated increases of C/EBPα binding result in growth arrest of dividing adipocytes (32). Differentiation of mouse preadipocytes into adipocytes is characterized by induction of C/EBPα expression (6, 32).

In the present paper we report the nucleotide sequence of the human C/EBPα 5′-flanking region and the investigation of important *cis* elements within this region. Using transient-cotransfection experiments, we show that the human C/EBPα protein can activate the human C/EBPα promoter. Our attempts to find a binding site for C/EBP proteins were unsuccessful. However, we found that a nuclear factor, upstream stimulatory factor (USF), binds to a specific sequence in the human C/EBPα promoter, and its binding activity increased in response to human C/EBPα but not human C/EBPβ (NF-Il-6) (2). We show that C/EBPα stimulates expression of a reporter gene promoted by the normal, but not a mutant, USF binding site from the C/EBPα promoter.

MATERIALS AND METHODS

Materials. The following oligonucleotides were used in this work: bZIP, 5′-CATGGATGGTATTGAGAAATCTG-3′, containing the C/EBP binding site from the human C3 promoter (18, 35); m-c/ebp1, 5′-AGTCAGTGGGCGTTGC GCCACGATCTCT-3′, corresponding to the sequence of the mouse C/EBPα promoter region from positions −200 to −173 (Fig. 1); h-c/ebp1, 5′-AGGCGG TGGGCGTTGCGCCGCGGCCT-3′, containing the sequence of the human C/EBPα promoter from nucleotides −194 to −165 (Fig. 1); USF/Ad-ml, 5′-AC GGTGTAGGCCACGTGACCGGGTGT-3′, corresponding to the nucleotide sequence of the adenovirus major late promoter which contains a high-affinity binding site for USF (16); USF/cebp, 5′-TACCGACCACGTGGGCGCGGG GGCGAC-3′, having the nucleotide sequence of the human C/EBPα promoter from nucleotides −276 to −249; and Egr-1, 5′-TTGATCTCGCGGGGGCGAG GGGGATCAA-3′, containing the binding site for the Egr-1 protein (9). The above oligonucleotides were synthesized in the Nucleic Acids Core Laboratory of the Department of Molecular and Human Genetics (Baylor College of Medicine). We also used oligonucleotides for Sp1 and AP2 obtained from Promega. Nucleotide sequences of these oligonucleotides are as follows: Sp1, 5′-ATTCG ATCGGGGCGGGGCGAG-3′, and AP2, 5′-GATCGAACTGACCGCCCGCG GCCCGT-3′. For bandshift experiments, double-stranded oligonucleotides were labeled to a high specific activity with [^{32}P]dCTP by filling in with Klenow DNA

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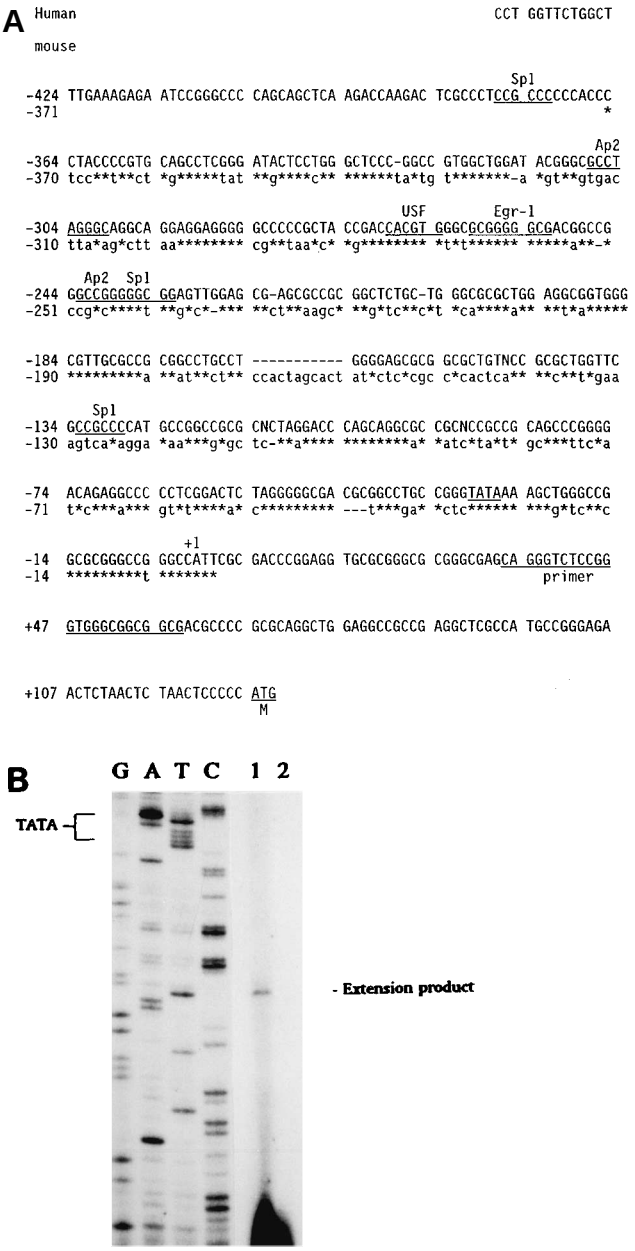

FIG. 1. Human C/EBPα promoter. (A) Nucleotide sequence of the 5′-flanking region of the human C/EBPα gene and its comparison with the mouse C/EBPα promoter. The transcription initiation site (+1), TATA box, and putative binding sites for nuclear proteins (Sp1, AP2, USF, and Egr-1) are indicated. The nucleotide sequence used in primer extension is underlined. Dashed lines indicate the presence of insertion/deletion in one sequence relative to the other. (B) Primer extension analysis documenting the transcription start site of human C/EBPα mRNA. The primer was annealed to 10 μg of poly(A)$^+$ RNA and extended by reverse transcriptase (lane 1). Lane 2, extension of primer by reverse transcription in the absence of RNA. Dideoxy sequencing of the C/EBPα promoter with the same primer. The position of the TATA box homology is shown on the left.

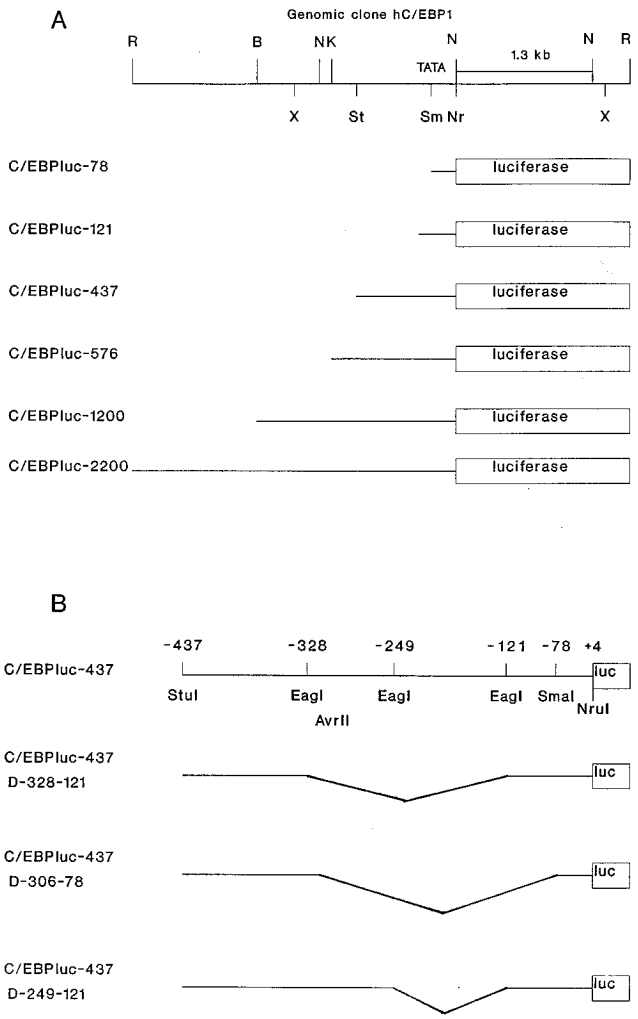

FIG. 2. Restriction map of the human C/EBPα promoter and construction of C/EBP-luciferase plasmids. (A) Restriction map of genomic clone hC/EBP1 and schematic representation of progressive deletion constructs of the C/EBPα promoter. R, *Eco*RI; B, *Bam*HI; N, *Not*I; K, *Kpn*I; X, *Xho*I; St, *Stu*I; Sm, *Sma*I; Nr, *Nru*I. The −2200 bp promoter region was cut by *Eco*RI and *Nru*I and cloned into the *Sma*I site of the pXP1 vector (35). This plasmid was named C/EBPluc-2200. Using *Bam*HI, *Kpn*I, *Stu*I, *Eag*I, and *Sma*I enzymes, progressive deletion plasmids were constructed. (B) Construction of internal deletions within the −437 C/EBPα promoter.

polymerase. Antibodies to C/EBPα, C/EBPβ, and C/EBPδ were prepared in the laboratory of G. Darlington. Anti-C/EBPα serum was raised in rabbits to the C/EBPα peptide KGEPREEDEAKQLA. The anti-C/EBPβ serum was similarly raised to the peptide SKAKKTVDKHSDEYKIRRC. This sequence resides in the leucine zipper domain, and antibodies to this portion of the protein disrupt dimerization and DNA binding. The antiserum to C/EBPδ was raised against the N-terminal amino acids 1 to 206 coupled to histidine. We also used antibodies to C/EBPα and to c-Myc from Santa Cruz Biotechnology. Antisera against human 43-kDa USF and 44-kDa USF were prepared in the laboratory of M. Sawadogo, as was an expression vector for the 43-kDa USF, PN3.

Cell culture and transient-transfection assay. Human hepatoma-derived Hep3B2 cells (1) were maintained as monolayers in M/M (three parts Eagle minimal essential medium, one part Waymouth MAB 87/3) plus 8% horse serum and 2% fetal bovine serum. General tissue culture methods were previously described (35). Coelectroporation of expression vectors was carried out with 5×10^6 cells per sample and 10 μg of the reporter construct and 10 μg of the expression vector. $CaPO_4$ transfection was carried out by a previously published protocol (35) and utilized 5 μg of reporter and 5 μg of the expression construct. The data summarize three to five separate experiments.

Stable clones. Clones of Hep3B2 cells were pooled following DNA-mediated gene transfer of a construct containing wild-type or mutant promoter elements coupled to luciferase as a reporter gene along with a plasmid containing the selectable neomycin resistance (Neo) marker. Analyses of transactivation by C/EBPα or by control DNA (CMV-stop) were carried out by electroporation of the expression vectors into the stable transformants. Luciferase activity was measured 24 h after delivery of the C/EBPα expression vector unless noted otherwise.

Preparation of constructs. The structures of the human C/EBPα gene and its 5′-flanking region are shown in Fig. 2. The coding region of the human C/EBPα gene (1.3 kb) was cut by *Nru*I and *Xho*I. The fragment ends were blunted by

Klenow fragment and cloned into the *Not*I site of a cytomegalovirus (CMV) vector (24) that was restricted by *Not*I and blunted by Klenow fragment. The resulting plasmid was named CMV-C/EBPα. C/EBPα promoter fragments were inserted in front of the luciferase gene of the pXP1 plasmid (26) (Fig. 2). This vector showed low basal activity after transfection into mammalian cells (35). A plasmid, CMV-stop, containing only the CMV promoter was used as a negative control.

Preparation of WCE and NE. For the preparation of whole-cell extracts (WCE), 1×10^6 to 5×10^6 3B2 cells were harvested by scraping and washed twice with phosphate-buffered saline (PBS) without Ca or Mg ions. After pelleting, the cells were suspended in 300 μl of solution E (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 12.5 mM $MgCl_2$, 20% glycerol, 0.1 M KCl, 1% Triton X-100, protease inhibitors). After 5 min of incubation on ice, the lysed cells were centrifuged for 5 min at 10,000 rpm (Sorvall Microspin 12S). Supernatant (WCE) was collected, frozen in liquid nitrogen, and stored at −80°C. Nuclear extracts (NE) were prepared as described previously (18). In some cases the cells were scraped, washed with PBS, and suspended in 150 μl of buffer A (10 mM Tris-HCl [pH 7.6], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT). After 15 min of incubation on ice, cells were homogenized by pulling them through a 23-gauge needle (six to eight strokes), and the sample was centrifuged for 5 min at 10,000 rpm. Nuclei (pellet) were resuspended and salt extracted in 20 to 50 μl of buffer B (20 mM Tris-HCl [pH 7.6], 25% sucrose, 0.420 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) and incubated on ice 30 min. The nuclei were pelleted, and the supernatant (NE) was removed, dialyzed against buffer C (20 mM Tris-HCl, [pH 7.6], 20% glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA), and used immediately.

Electrophoretic mobility shift assay (EMSA). Binding reactions were carried out at room temperature for 30 min. The reaction mixture (20 μl) contained 0.2 to 0.4 ng of probe with or without a 100-fold molar excess of unlabeled oligonucleotides, 10 μg of NE or 30 to 40 μg of WCE, 2 μg of poly(dI-dC) · poly(dI-dC), 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol. Antibodies were added to the reaction mixture before the addition of probe. Protein-DNA complexes were resolved on low-ionic-strength, native, 5% polyacrylamide (acrylamide/bisacrylamide ratio, 30:1.5) gels at room temperature. DNA fragments and oligonucleotides were end labeled by filling in with [^{32}P]dCTP and Klenow polymerase.

Southwestern (DNA-protein) analysis. Nuclear extracts of Hep3B2, HepG2, and HeLa cells were electrophoresed in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (20) and electroblotted onto nitrocellulose membranes. The membranes were blocked by 5% dry milk dissolved in 10 mM Tris-HCl (pH 7.6) with 1 mM DTT. The membranes were incubated at room temperature for 60 min in binding buffer which contained 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 0.25% dry milk, 0.1 mM EDTA, and 10^6 cpm of ^{32}P-end-labeled oligonucleotides per ml. After incubation, the filter was washed with binding buffer without labeled oligonucleotides.

RNA isolation and primer extension. Poly(A)$^+$ RNA was isolated from Hep3B2 cells by using the Fast Track mRNA Isolation Kit (Invitrogen). The synthetic oligonucleotide complementary to residues from +34 to +59 of the human C/EBPα mRNA (Fig. 1, underlined) was annealed to 10 μg of poly(A)$^+$ RNA at 80°C for 10 min and at 42°C overnight. Extension was carried out with 30 U of reverse transcriptase (Promega) under conditions previously described (30). The product was analyzed on an 8% sequencing gel.

Western blotting (immunoblotting). Proteins were electrophoresed in a 10% polyacrylamide gel containing 0.1% SDS and transferred to a nitrocellulose filter. Detection of immunoreactive proteins was performed with the enhanced chemiluminescence system (Amersham).

Site-directed mutagenesis. Deletion of the USF binding site, CACGTG, was carried out with a Transformer Site Directed Mutagenic Kit (Clontech) with the following modifications. The mutagenic primer 5′-CGTCGCCCCCGCGC CGTCGGTAGCGGGGGC-3′ from −249 to −284 (Fig. 1, CACGTG deleted) was annealed to the −437luc and to the pT81-328-249 plasmids. A mutant DNA strand was synthesized with T4 DNA polymerase. After incubation for 1 h, fresh T4 DNA polymerase was added to the reaction mixture, and synthesis was continued for 2 h. Because the sequence CACGTG is a restriction site for *Pml*I, we used this enzyme for primary and secondary selection of mutant plasmids. Deletion of CACGTG was checked by resistance to *Pml*I, by sequencing, and by bandshift assay (see Fig. 10).

RESULTS

Nucleotide sequence of the human C/EBPα promoter. To study the activation of the human C/EBPα promoter, its nucleotide sequence was determined. Figure 1A shows the 5′-flanking region of the human C/EBPα gene from −437 to the first methionine codon. The promoter region of the human C/EBPα gene is GC rich (80%). A sequence with TATA box homology is located in the position identical to that of the mouse C/EBPα TATA box (10). The putative human C/EBPα mRNA cap site would be the adenine residue that is labeled +1. Primer extension experiments demonstrated that the start position of C/EBPα mRNA is precisely as predicted (Fig. 1B). The length of 5′ untranslated mRNA from +1 to the first ATG codon is 126 bp.

Comparison of human and mouse C/EBPα promoters indicated a relatively low level of similarity, although the transcribed regions of both genes are very similar (>80%) (unpublished data). Upstream of the TATA box, the mouse and human C/EBPα promoters possess only 53% sequence identity. The search for known nuclear factor binding sites within the human C/EBPα promoter identified several putative elements, including three GGGCGG boxes (the Sp1 binding site); a perfect binding site for zinc finger proteins, GCGGGGGCG (9); a binding site for helix-loop-helix (HLH)-bZIP (USF) family proteins, CACGTG; and two consensus sequences for AP2 (GCCNNNGGC). These sequences are underlined in Fig. 1A.

Four hundred thirty-seven nucleotides of the human C/EBPα promoter are sufficient for high-level expression of a C/EBPα-luciferase construct. In previous studies, we cloned the human C/EBPα gene and determined its chromosomal localization (17). To identify the region of the human C/EBPα promoter which was important for transcription of this gene, two series of constructs were prepared. In one series, different DNA fragments (with progressive or internal deletions of the C/EBPα promoter) were cloned in front of the luciferase gene of the pXP1 plasmid (Fig. 2). Luciferase activity from the longest promoter fragment (−2200) was consistently less than that of the −1200 and −576 constructs (Fig. 3A). Deletion of nucleotides to −78 resulted in a significant decrease of promoter activity (Fig. 3A). Subsequent analyses of promoter function concentrated on the region within 437 nucleotides of the human C/EBPα promoter. To test for the contributions of various regions to promoter activity, transient-transfection experiments with internal deletions were carried out. Deletion of nucleotides from −328 to −121, as well as from −306 to −78, decreased activity of the human C/EBPα promoter (Fig. 3B). However, a twofold increase of promoter activity was observed after deletion of nucleotides −249 to −121, suggesting the presence of a negative element(s) within this region (Fig. 3B).

Human C/EBPα can activate its own promoter. Autoregulation of mammalian transcription factors by direct binding of the gene product was first described for Jun (3). Mouse C/EBPα can bind to its own promoter (10) and activate expression of a luciferase construct (23). We tested whether or not autoregulation occurs for the human C/EBPα by cotransfection experiments of different C/EBPα-luciferase constructs (Fig. 2) with an expression vector for C/EBPα (CMV-C/EBPα) or control DNA (CMV-stop). Figure 3C shows that C/EBPα expression resulted in two- to threefold induction of activity of the cotransfected 437-bp C/EBPα promoter. Deletion of nucleotides −249 to −121 did not reduce activity substantially, while deletion of nucleotides −306 to −78 resulted in no induction. Autoregulation of the human C/EBPα promoter was observed also with stable clones containing −437 luc C/EBPα promoter integrated into chromosomal DNA (see below). These results suggested that the nucleotide sequence which is necessary for autoregulation is located between nucleotides −306 and −249 of the human C/EBPα promoter.

C/EBPα and C/EBPβ do not bind to the promoter region of human C/EBPα. Because C/EBPα and C/EBPβ bind to the same sequence (18), we have investigated the interaction of both with the promoter region of the human C/EBPα gene. The human C/EBPα promoter was divided into three pieces, −437 to −328, −328 to −249, and −249 to −121, which were subcloned into the pBluescript II KS(−) (Stratagene) vector

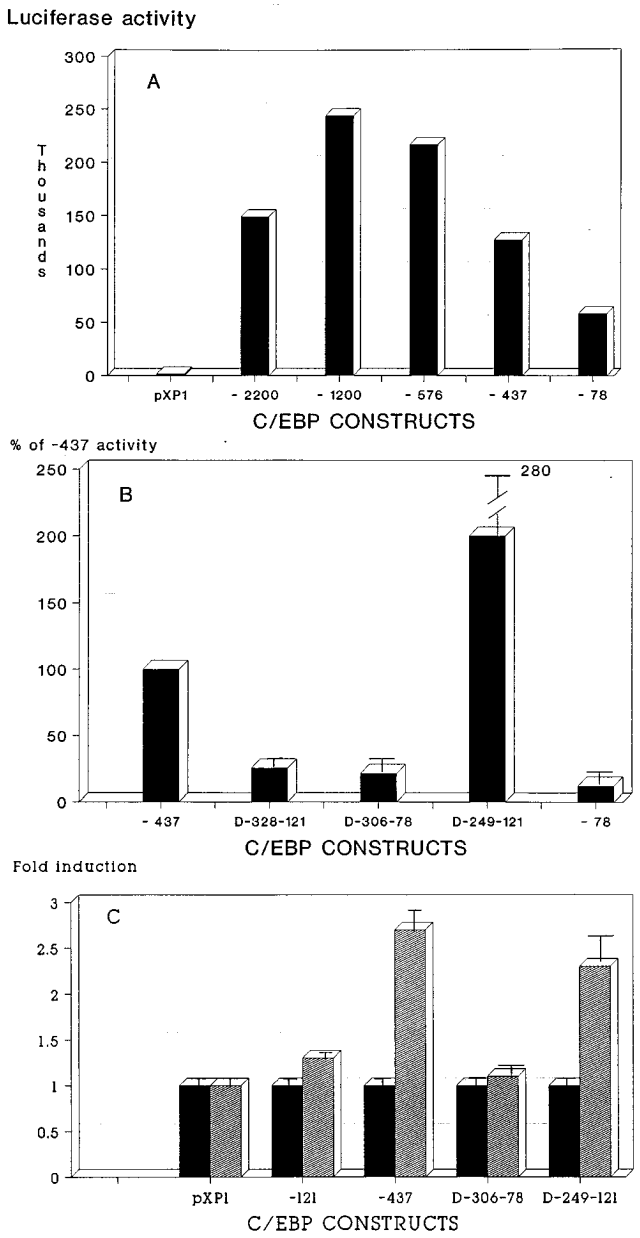

FIG. 3. (A) Four hundred thirty-seven nucleotides of the human C/EBPα promoter direct high-level expression of a C/EBPα-luciferase construct. C/EBPα-luciferase constructs containing different-length pieces of the human C/EBPα promoter were transfected into Hep3B2 cells. Luciferase activity (light units/microgram of cellular protein) was measured 16 h after transfection. (B) Promoter activity of deletion constructs. Luciferase activity is shown as a percentage of the −437 C/EBPα promoter activity. (C) C/EBPα stimulates its own promoter. Different C/EBPα-luciferase constructs were cotransfected into Hep3B2 cells with a C/EBPα expression vector (CMV-C/EBPα [▨]) and with the CMV-stop construct as a control. The fold induction was calculated as the increased luciferase activity in cells cotransfected with C/EBPα above that in cells cotransfected with control DNA (CMV-stop [■]) for each construct.

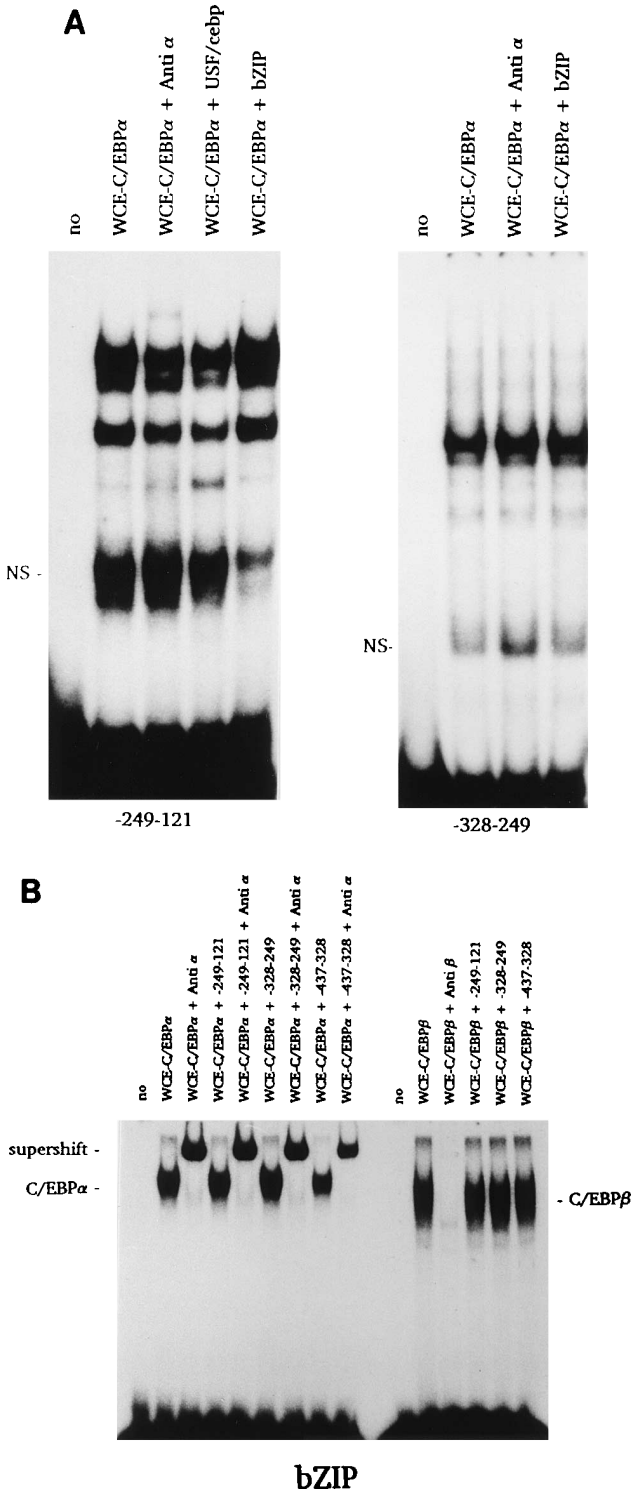

FIG. 4. C/EBP proteins do not bind to the human C/EBPα promoter. (A) EMSA of DNA-protein interactions of the human C/EBPα promoter with Hep3B2 WCE-C/EBPα which contains a high level of C/EBPα. Two promoter fragments (from −249 to −121 and from −328 to −249) were incubated with WCE-C/EBPα. Specific antibodies to C/EBPα and a competitor oligonucleotide, bZIP, were added to binding reaction mixtures before the addition of ^{32}P-labeled DNA probes. NS, nonspecific band (no self-competition was observed). (B) Competition EMSA of DNA-protein interactions. WCEs containing a high level of C/EBPα or -β were incubated first with unlabeled DNA fragments of the C/EBPα promoter and then a ^{32}P-labeled bZIP oligonucleotide (0.4 ng) was added at a molar ratio of 100:1 (unlabeled DNA fragment to ^{32}P-labeled bZIP). Specific antibodies to C/EBPα and to C/EBPβ were added before ^{32}P-labeled bZIP. Lanes no, no proteins.

and used in bandshift assays. For these experiments, WCEs with high levels of C/EBPα (WCE-C/EBPα) and C/EBPβ (WCE-C/EBPβ) binding activities were obtained by transfection of 3B2 cells with CMV-C/EBPα or CMV-C/EBPβ constructs, respectively.

Several DNA-protein complexes were formed after incubation of C/EBPα promoter fragments and WCEs (Fig. 4A). To

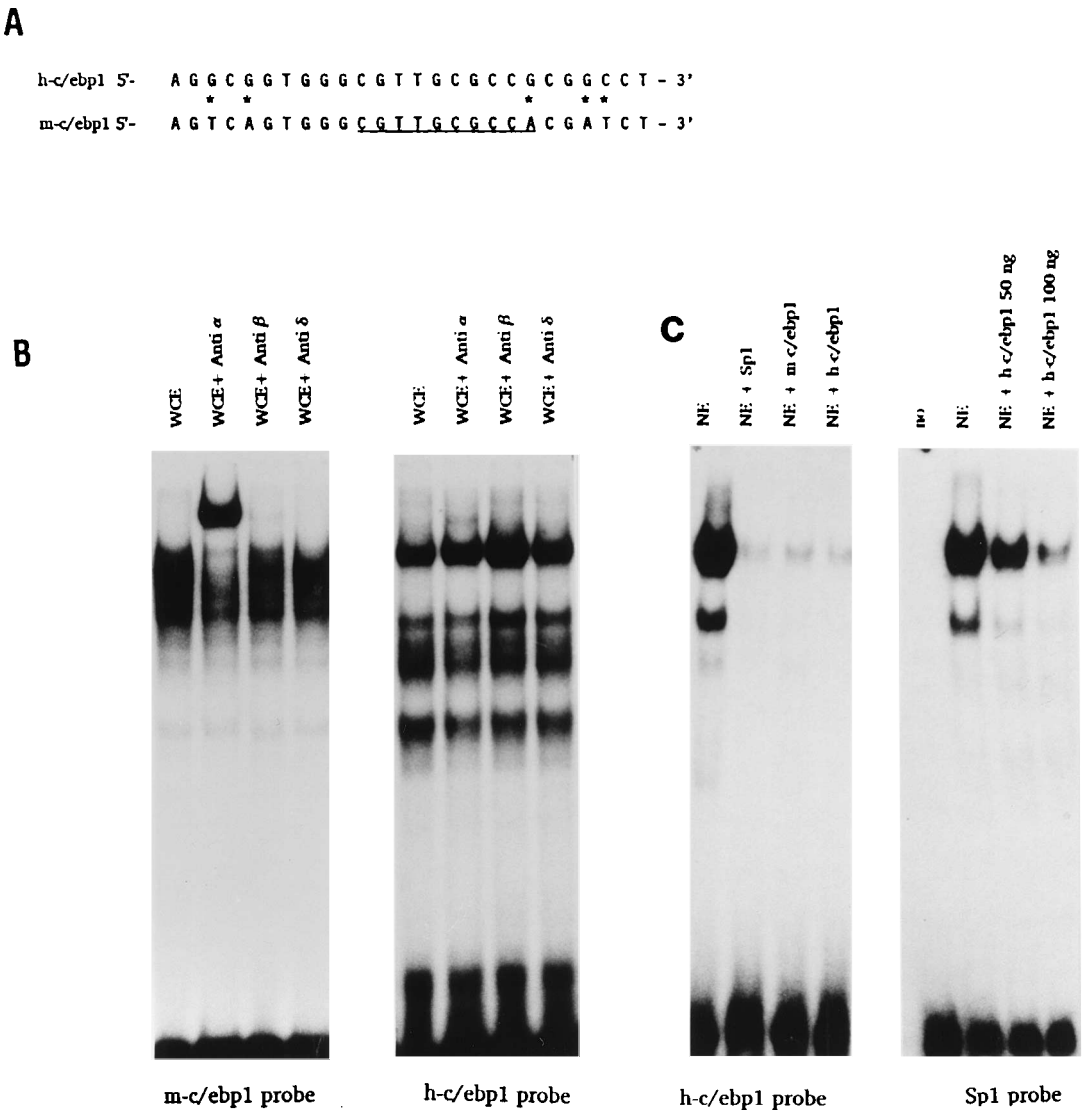

FIG. 5. Sp1-like proteins bind to the human and mouse C/EBPα promoters. (A) Nucleotide sequences of human (h-c/ebp1) and mouse (m-c/ebp1) oligonucleotides. Asterisks show nonhomologous nucleotides. The C/EBPα consensus is underlined. (B) EMSA of protein interactions with ^{32}P-labeled h-c/ebp1 and m-c/ebp1 oligonucleotides. These oligonucleotides were incubated with 20 μg of the same WCE-C/EBPα in the presence and in the absence of antibodies specific to C/EBP proteins. Preimmune serum (1 μl) was added to control mixtures that did not contain antiserum. The gel with m-c/ebp1 was exposed for 12 h, and the gel with the human probe was exposed for 48 h. (C) Competition EMSA of DNA-protein interactions. A total of 5 μg of Hep3B2 NE was incubated with labeled h-c/ebp1 or Sp1 oligonucleotides in the presence of a 100-fold molar excess of different unlabeled oligonucleotides or increasing amounts of h-c/ebp1. Lane no, no proteins.

find possible C/EBP-DNA complexes, three different approaches were pursued: the use of antibodies specific to C/EBPα and -β to supershift protein-DNA complexes, competition of complexes with the fragments by a 100-fold molar excess of unlabeled bZIP oligonucleotides, and EMSA analysis of bacterially expressed protein. Neither antibody changed the patterns of DNA-protein interactions (data for anti-C/EBPβ not shown). Nor were any changes observed after the addition of a bZIP oligonucleotide to the reaction mixtures (Fig. 4A). As shown in Fig. 4B, WCEs prepared from transfected cells contained proteins with C/EBP-specific binding activities. Antibodies to C/EBPα supershifted DNA-protein complexes, while antibodies against C/EBPβ neutralized the binding of the proteins to the bZIP oliogonucleotide. Neither of the complexes was inhibited by fragments of the human C/EBPα promoter, suggesting that no C/EBP binding sites resided within the −437 to −121 region.

In addition, bacterially expressed human C/EBPα did not bind to different pieces of the human C/EBPα promoter, including DNA fragments from −328 to −249 and from −249 to −121, although the recombinant protein did bind to the m-cebp-1 and bZIP oligomers (data not shown). Thus, three lines of experiments failed to identify interaction of C/EBPα and C/EBPβ with the human C/EBPα promoter.

Sp1-like nuclear factors recognized two sequences of the human C/EBPα promoter. The C/EBP binding region of the mouse C/EBPα promoter and the corresponding nucleotide sequence of the human C/EBPα promoter differ by a single nucleotide (Fig. 5A). The binding region of the mouse C/EBPα promoter contains a nucleotide sequence (underlined) almost identical to the C/EBP consensus, TT/GNNGNAAT/G (opposite orientation [Fig. 5A]). Although no C/EBP proteins were found to bind to human h-c/ebp1 sequence by EMSA, several protein-oligonucleotide complexes were formed with this oli-

gonucleotide (Fig. 5B). The addition of antibodies against C/EBPα to the binding reaction mixture resulted in a supershifted band only with the mouse sequence and not with the human sequence. No binding of C/EBPα to h-c/ebp1 was observed after a long exposure of the gel (in Fig. 5B exposure of the bandshift with the mouse probe was 12 h, and exposure with the human probe was 48 h). Supershift of the mouse oligonucleotide was due to a specific interaction with C/EBPα because no change was detected after the addition of antibodies to C/EBPβ or -δ with either oligonucleotide. These results confirmed the data of Christy et al. (10) regarding C/EBP binding to the mouse promoter and indicated that the corresponding region of the human C/EBPα promoter did not contain a binding site for C/EBPα. We have also found that human C/EBPβ bound to the mouse oligonucleotide, m-c/ebp1, but did not bind to human h-c/ebp1 (data not shown).

With the exception of C/EBPα, the electrophoretic mobilities of the DNA-protein complexes are identical with both C/EBP oligonucleotides (human and mouse) (Fig. 5B). Different intensities of the complexes are the result of a longer exposure time. These oligonucleotides contained Sp1 consensus binding sites. Bandshift competition experiments (Fig. 5C) showed that a 100-fold excess of unlabeled Sp1 oligonucleotide inhibited the formation of these complexes with the h-c/ebp1 (Fig. 5C) and oligonucleotides, as did m-c/ebp1. This competition was specific because the addition of a 100-fold excess of other oligonucleotides (bZIP, AP2, USF/Ad-ml, and Egr-1) did not disrupt the complexes (data not shown). To verify the presence of an Sp1 binding site in the human promoter, the opposite competition was performed. A labeled Sp1 oligonucleotide was incubated with Hep3B2 nuclear extract in the presence of increasing amounts of unlabeled h-c/ebp1 oligonucleotide. Two DNA-Sp1 complexes were disrupted by h-c/ebp1 (Fig. 5C). Anti-Sp1 sera shifted only the more slowly migrating complex, suggesting that one complex is truly Sp1 and the faster complex contains an Sp1-like molecule (data not shown). Taken together, the above experiments indicated that the mouse C/EBPα promoter from −203 to −176 possessed binding sites for C/EBPα and -β and for Sp1 or for an Sp1-like protein(s). In contrast, the human C/EBPα promoter did not contain a C/EBPα binding site in this region but contained a sequence recognized by Sp1 and an Sp1-like protein(s).

C/EBPα does not bind to other regions of the human C/EBPα promoter. Analysis of the human C/EBPα promoter region identified a short DNA fragment (−249 to −213) in which three consensus sites for nuclear factors were found: Sp1 (GGGCGG), AP2 (GCCGGGGGC), and a sequence closely resembling C/EBPα (TGGAGCGAG). In addition, the region between −249 and −213 was interesting because the corresponding region of the mouse promoter was bound by a nuclear protein(s) from undifferentiated adipocytes (10). Vasseur-Cognet and Lane have shown that two proteins, C/EBPα undifferentiated protein (CUP) and an Sp1-like protein, interacted with the above region (34). To test for possible interaction of nuclear proteins in this region of the human C/EBPα promoter, a DNA fragment (−249 to −213) was used in EMSA with nuclear extract from HeLa cells transfected with CMV-C/EBPα. Two DNA-protein complexes were observed, and these were both inhibited specifically by the Sp1 oligonucleotide (Fig. 6). Thus, we conclude that the nuclear factor Sp1 and Sp1-like protein(s) can bind in vitro to at least two distinct regions of the human C/EBPα promoter: from −194 to −165 and from −249 to −213.

C/EBPα, but not C/EBPβ, induces binding activity of a nuclear factor which interacts with the CACGTG sequence of the human C/EBPα promoter. Our attempts to identify the

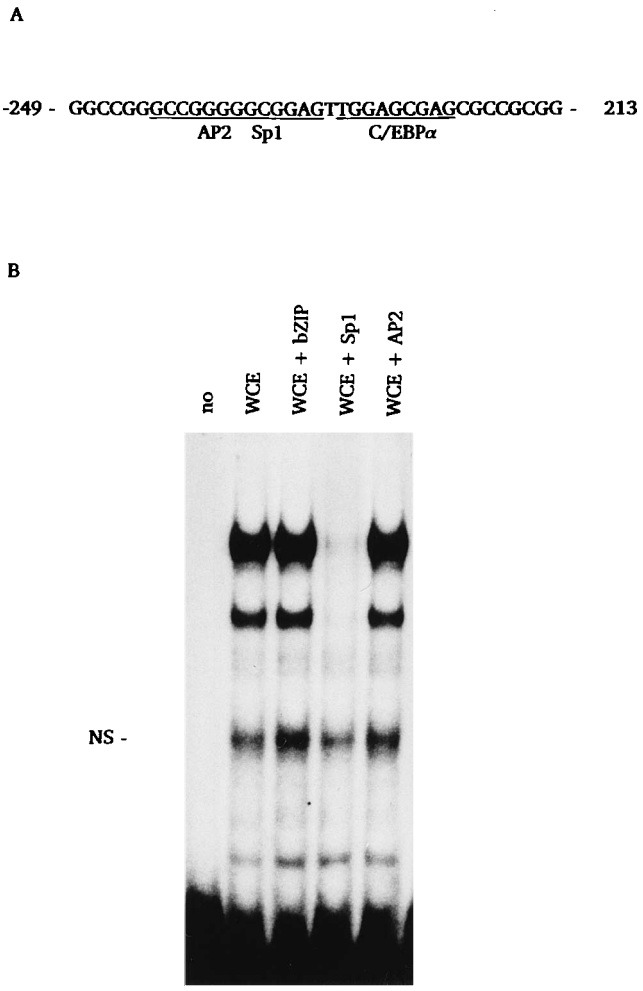

FIG. 6. The promoter fragment from −249 to −213 of the human C/EBPα promoter binds Sp1-like proteins but not C/EBP or AP2. (A) Nucleotide sequence of the −249 to −213 DNA fragment. Putative binding sites for AP2, Sp1, and C/EBPα proteins are underlined. (B) EMSA of DNA-protein interactions on the −249 to −213 DNA fragment. The end-labeled DNA fragment was incubated with WCE isolated from HeLa cells after transfection with the CMV-C/EBPα expression vector. A molar excess (100-fold) of unlabeled oligonucleotides bZIP, Sp1, and AP2 was added. Lane no, no proteins. NS, nonspecific complex (no self-competition was observed).

binding of C/EBPα and -β to the human C/EBPα promoter suggested that neither protein binds within −437 to −121 bp of the proximal sequence. Nevertheless, C/EBPα can stimulate transcription of luciferase constructs (Fig. 3C) containing this same promoter region. We hypothesized that C/EBPα acted indirectly by stimulating activity of another nuclear protein(s) which transactivated the human C/EBPα promoter. To test this possibility, the interactions of WCE-C/EBPα and WCE-C/EBPβ with the promoter region were studied. As a control, we used extracts (WCE-stop) from Hep 3B2 cells after transfection with the CMV-stop construct (Fig. 7). No significant difference in the binding activities of the three WCEs to DNA fragments −437 to −328 or to −249 to −121 was observed (for DNA fragment −437 to −328, data not shown). In contrast, DNA fragment −328 to −249 showed increased binding activity of one nuclear protein in WCE-C/EBPα (Fig. 7A, complex IC). WCE-C/EBPβ contained only a low level (identical to WCE-stop) of this binding activity. This observation was con-

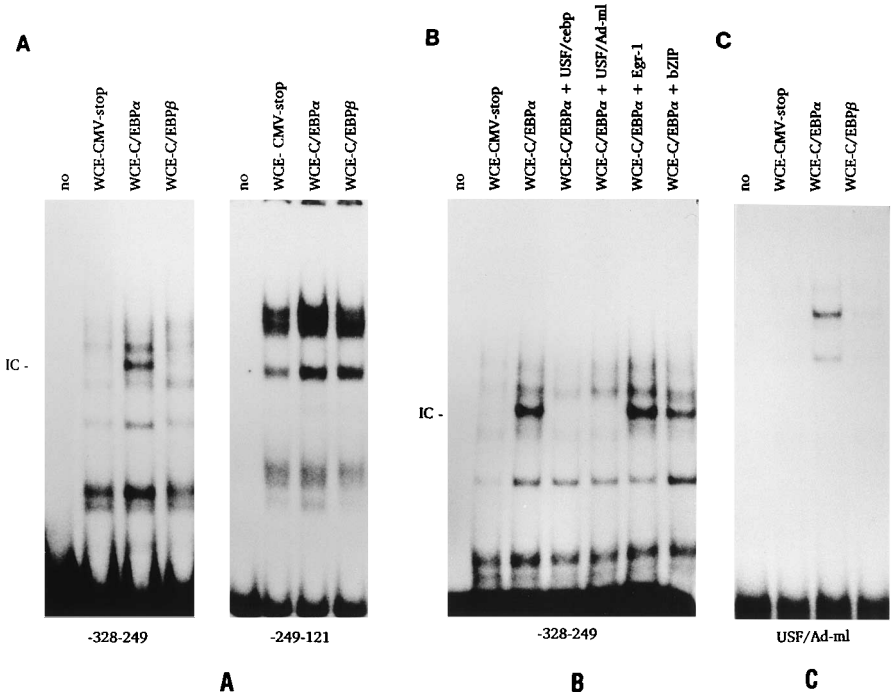

FIG. 7. C/EBPα increased binding activity of a nuclear factor which interacts with the C/EBPα promoter. (A) Interaction of WCE-C/EBPα and WCE-C/EBPβ with the human C/EBPα promoter. End-labeled DNA fragments −328 to −249 and −249 to −121 were incubated with these WCEs and analyzed by EMSA. The position of the inducible complex (IC) is indicated. (B) Competition EMSA of binding of inducible protein to an end-labeled −328 to −249 promoter fragment. (C) USF binding activity is increased in WCE-C/EBPα but not in WCE-C/EBPβ. ^{32}P-labeled USF/Ad-ml oligonucleotide which contained a high-affinity binding site for USF (16) was incubated with 20 μg of WCEs and analyzed by EMSA. Lanes no, no proteins.

sistent with the cotransfection experiments (Fig. 3C) and suggested that C/EBPα increased binding activity of this nuclear factor. To further characterize the interaction of this inducible protein with the C/EBPα promoter, different unlabeled oligonucleotides were added to the binding mixtures. Because the −328 to −249 DNA fragment contained putative binding sites for the HLH-bZIP family proteins (CACGTG) and for zinc finger proteins (GCGGGGGCG) (Fig. 1), we tested oligonucleotides containing well-known high-affinity binding sites for these proteins: USF/Ad-ml and Egr-1 (see Materials and Methods). Oligonucleotides USF/cebp and bZIP were also examined. The results of competition studies are shown in Fig. 7B. Oligonucleotides USF/Ad-ml and USF/cebp, in a 100-fold excess, inhibited the inducible complex (IC), while Egr-1 and bZIP did not. Because oligonucleotides USF/Ad-ml and USF/cebp share only one common element, CACGTG, we concluded that the core binding element for the inducible protein is likely to be CACGTG. Thus, C/EBPα induced binding activity of a nuclear factor which can recognize the CACGTG nucleotides of the human C/EBPα promoter. USF/Ad-ml was used in bandshift experiments (Fig. 7C). After a short exposure, binding activity of this nuclear factor is observed only in WCE-C/EBPα. Upon longer exposure, this binding activity is observed in all lanes. Thus, human C/EBPα can stimulate binding activity of a nuclear factor which recognizes a CACGTG site in the human C/EBPα promoter.

The nuclear factor binding to the CACGTG sequence of the human C/EBPα promoter is identical to USF. The CACGTG sequence is a target for several transcription factors, including USF (16), TFE 3 (5), Myc/Max (7, 28), and Max/Mad and Max/Mxi (4, 36). To determine the nature of the inducible nuclear factor which binds to CACGTG in the human C/EBPα promoter, we initially estimated its molecular weight by using the Southwestern method. Nuclear extracts of 3B2, HepG2, and HeLa cells were electrophoresed under denaturing conditions, blotted onto membrane, and probed with ^{32}P-labeled USF/cebp oligonucleotide. One major protein of different nuclear extracts interacted with the nucleotide sequence of USF/cebp oligonucleotide (Fig. 8A). Its molecular mass was about 43 kDa, corresponding to one of the USF proteins (16, 27). Figure 8B indicates that this nuclear factor was heat stable, as has been reported for USF (16). Two polyclonal antisera, one against 43-kDa USF and the other against 44-kDa USF were used in EMSA to confirm the identity of USF. The specificities of these antibodies have been described (31). The complex was supershifted upon the addition of either of the antibodies to USF proteins, while no effect was detected upon the addition of antibodies to c-Myc or to C/EBPα (Fig. 8C). Thus, molecular weight, heat stability, target sequence specificity to CACGTG, and interaction with specific antibodies to USF revealed that the inducible nuclear factor was identical to USF.

It is known that the nucleotide sequence CACGTG is a target for Myc family transcription factors (7, 28). By using monoclonal antibodies to human c-Myc protein, we tested the possible interaction of Myc/Max heterodimers of different cells with the CACGTG sequence of the human C/EBPα promoter. Nuclear extracts of Hep 3B2, HepG2, and HeLa cells did not contain Myc/Max DNA-binding complexes which were able to be detected by EMSA with the CACGTG oligonucleotide (Fig. 8C and data not shown). Nevertheless, we cannot exclude this interaction in vivo.

C/EBPα induces binding activity of USF. Given that overexpression of human C/EBPα in Hep3B2 cells increased USF binding, we compared the protein levels of USF in cells transfected with the C/EBPα expression vector, CMV-C/EBPα, and in the cells transfected with CMV-stop constructs. NE were

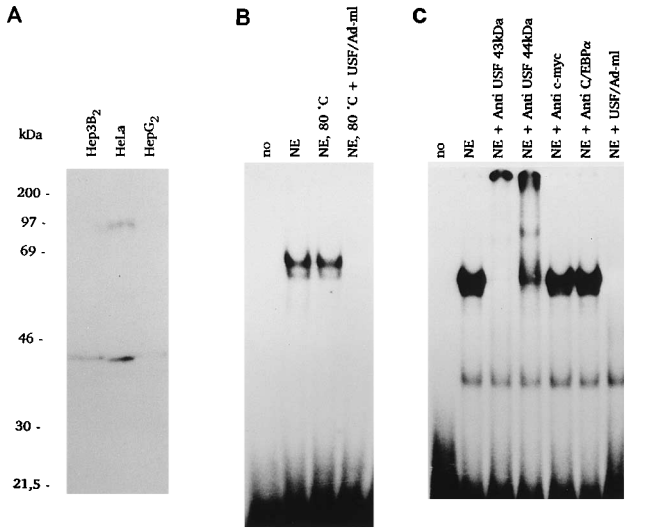

FIG. 8. The inducible nuclear factor is identical to USF. (A) Southwestern gel blotting analysis of interaction of USF/cebp oligonucleotide with NE from Hep3B2, HeLa, and HepG2 cells. A total of 100 μg of each NE was analyzed as described in Materials and Methods. The positions of marker proteins are shown on the left. (B) The inducible nuclear factor is heat stable, as is USF. Hep3B2 NE was heated at 80°C for 10 min, incubated with labeled USF/cebp, and analyzed by EMSA. The addition of specific competitor USF/Ad-ml abolishes binding. (C) Antibodies to USF recognize the inducible nuclear protein. The ^{32}P-labeled USF/cebp probe was incubated with Hep3B2 NE in the presence of antibodies to different nuclear factors and analyzed by EMSA. Lanes no, no proteins.

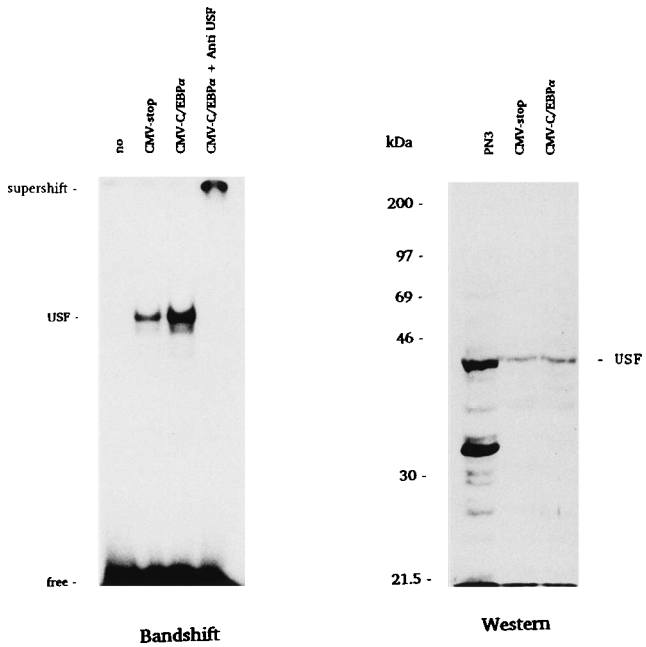

FIG. 9. C/EBPα increases binding activity of USF. NEs isolated from Hep3B2 after transfection with CMV-stop or with CMV-C/EBPα were used in a bandshift experiment (left) and in Western blot analysis (right). (Bandshift) A total of 5 μg of NEs was incubated with 0.1 ng of USF/Ad-ml probe under the conditions described in Materials and Methods and loaded on a 5% polyacrylamide gel. Antibodies to the 43-kDa USF protein were added before the addition of probe. (Western) Proteins were separated in a 10% polyacrylamide gel with 0.1% SDS, blotted onto nitrocellulose filter, and probed with antibodies to 43-kDa USF. The positions of marker proteins are shown on the left. PN3 is a NE of Hep3B2 transfected with the PN3 construct, which codes for human 43-kDa USF. Lane no, no proteins. free, free probe.

isolated 20 h after transfection and used in both bandshift and Western experiments (Fig. 9). Binding activity of USF in NE from cells overexpressing human C/EBPα was three- to four-fold higher than that in NE from control cells. Antibodies to 43-kDa USF supershifted the inducible complex, confirming these results.

Western analysis of NE isolated from Hep3B2 after transfection with CMV-C/EBPα, CMV-stop, and PN3 (a construct which codes for human 43-kDa USF) was performed. The 43-kDa immunoreactive protein corresponds to full-length human USF; the smaller protein presumably was a product of degradation. As shown, the level of immunoreactive 43-kDa polypeptide is almost the same in the extracts from CMV-C/EBPα and CMV-stop transfected cells. These results indicated that overexpression of human C/EBPα in Hep3B2 cells resulted in an increase of USF binding but did not substantially change its protein level.

The USF binding site is crucial for activation of the human C/EBPα promoter by C/EBPα. To investigate the role of the USF binding site in regulation and autoregulation of the C/EBPα gene, two expression constructs of the human C/EBPα promoter which deleted the CACGTG USF binding site were made. One mutant construct, −437 luc mut, contained the entire C/EBPα promoter from +1 to −437 but lacked the CACGTG sequence. We also deleted the USF binding site within pT81-328-249, a plasmid that contained a basal, 81-bp thymidine kinase promoter region coupled to a piece of the C/EBPα promoter from −328 to −249. A bandshift assay with normal and mutant −328 to −249 DNA fragments is shown in Fig. 10A. The mutant DNA fragment did not contain a USF binding site. The same result was observed with the −437 luc mut plasmid (data not shown).

Normal and mutant pT81-328-249 plasmids were tested for activation by USF in transient-cotransfection experiments with the USF expression vector, PN3. As shown in Fig. 9, transfection of PN3 into Hep3B2 resulted in a high level of expression of 43-kDa human USF. Under these conditions, the cotransfected normal pT81-328-249 construct showed a 3.5-fold induction of luciferase activity, whereas the mutant construct did not register changes in activity (Fig. 10B). Therefore, USF activated the reporter construct through binding to the CACGTG nucleotides (USF binding site).

To test the contribution of USF to C/EBPα promoter activity within Hep3B2 cells, normal and mutant (CACGTG-deleted) −437 luc constructs and pT81-328-249 constructs were integrated into chromosomal DNA as described in Materials and Methods. Populations of G418-resistant stable clones were pooled and assayed for luciferase activity. Baseline luciferase activity of normal and mutant, stably transformed populations showed that deletion of the USF binding site resulted in loss of activity (Fig. 11A). Transient-transfection experiments and the identification of nuclear proteins interacting with the human C/EBPα promoter allowed us to suggest that C/EBPα increases the binding activity of USF, which then transactivates the C/EBPα promoter. To test this hypothesis, we used the populations of stable clones containing normal and mutant C/EBPα promoter/luciferase constructs to study their activation by C/EBPα. C/EBPα activated its own wild-type promoter threefold in cells stably transfected with a wild-type promoter, −437 luc normal, but did not change the activity of the mutant construct lacking the CACGTG USF binding site (Fig. 11B). These results indicated that autoactivation of the C/EBPα promoter was abolished by deletion of the USF binding site and led us to propose that C/EBPα activates its own promoter through a USF binding site.

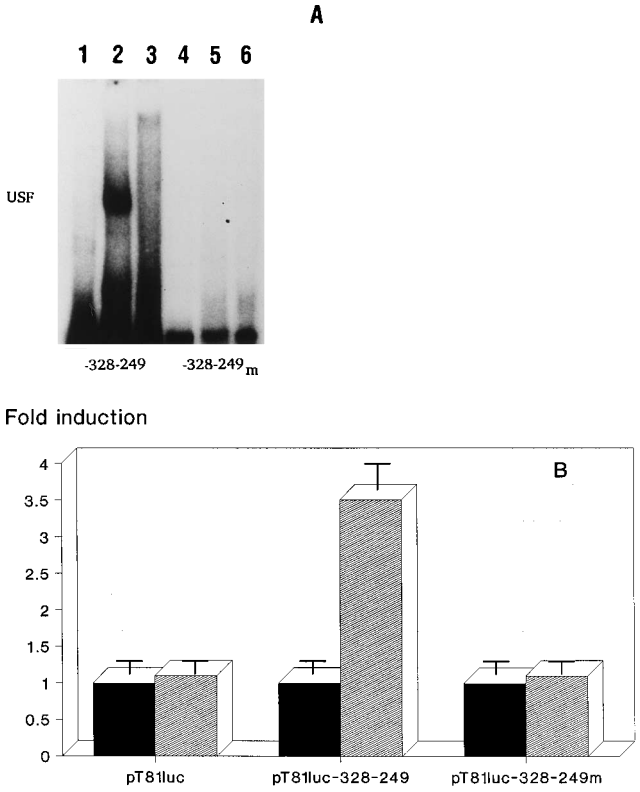

FIG. 10. USF activates the C/EBPα promoter by binding to the CACGTG sequence. (A) Binding of USF to normal and mutant C/EBPα promoter fragment −328 to −249. DNA fragments were end labeled and incubated with PN3 NE. Lanes 1 and 4, mixtures without proteins; lanes 2 and 5, incubation with PN3 NE; lanes 3 and 6, incubation with PN3 NE in the presence of specific competitor USF/Ad-ml. The position of the USF-DNA complex is shown on the left. (B) USF activates the wild-type C/EBPα promoter and does not activate the promoter lacking the CACGTG consensus site. Normal and mutant pT81-328-249 constructs were cotransfected with PN3 (the USF expression vector) into Hep3B2, and luciferase activity was measured 16 h after transfection. The pT81 vector was used as a control. ■, CMV-stop; ▨, PN3.

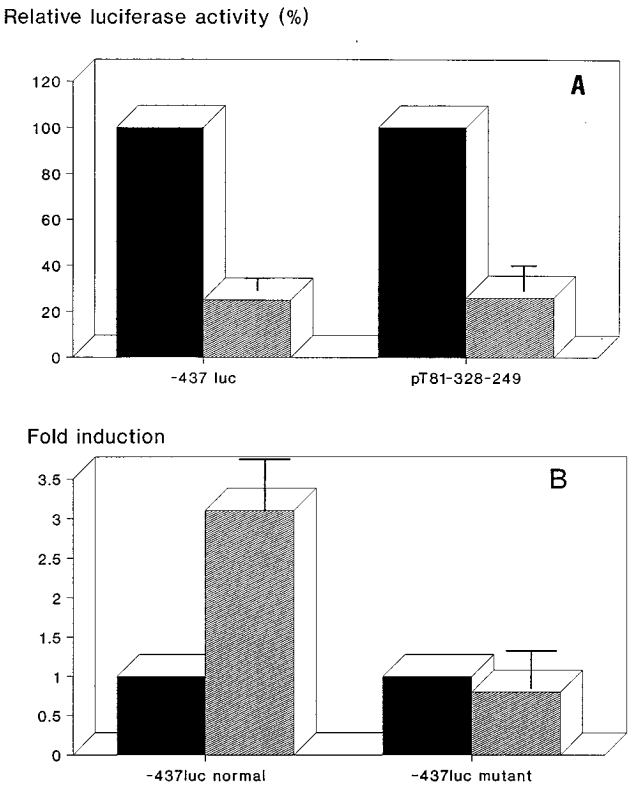

FIG. 11. (A) The USF binding site CACGTG within human C/EBPα promoter is crucial for its activity in Hep3B2 cells. Normal (■) and mutant (▨) plasmids −437luc and pT81-328-249 were transfected into Hep3B2 cells and placed under G418 selection for 2 weeks. G418-resistant colonies were pooled and assayed for baseline luciferase activity (A) and activity induced by electroporation of either CMV-stop (■) or CMV-C/EBPα (▨) (B). Luciferase activity was determined 16 h after delivery of plasmids.

DISCUSSION

In this paper we describe the nucleotide sequence of the human C/EBPα promoter and an investigation of nuclear factors that regulate human C/EBPα gene expression. Sequence comparison of mouse and human C/EBPα promoters indicated a relatively low level of identity (~55%). Transient-transfection experiments indicated that the first 437 nucleotides of the human C/EBPα promoter contained elements necessary for high-level expression of this gene. A search for known target sequences showed the presence of several putative binding sites for nuclear factors. Here we show that a USF site is likely to be an important component in the regulation of human C/EBPα expression.

In the case of Jun, the mechanism of autoregulation included a direct interaction of DNA-binding proteins with their promoter regions. Autoregulation of the mouse C/EBPα protein has been described (10, 23). The human C/EBPα promoter has a potential C/EBP binding site in the same position as does the mouse promoter, and C/EBPα can activate the human 437-bp C/EBPα promoter (Fig. 3). However, no direct binding of C/EBP proteins to the part of the human promoter corresponding to the binding site in the mouse promoter was found. Although several specific complexes were formed as result of protein-DNA interaction, specific antibodies to C/EBP proteins did not recognize any of these proteins (Fig. 4 and 5). An additional loose consensus C/EBPα binding site (−229 to −221) (Fig. 6) was also incapable of interacting with C/EBP proteins. Instead, an Sp1 or Sp1-like protein(s) bound both sequences of interest. An Sp1-like protein(s) also bound to the corresponding region of a mouse C/EBPα promoter (Fig. 5). Christy et al. have shown that interaction of an oligonucleotide identical to m-c/ebp1 with nuclear extracts from adipocytes resulted in the formation of four DNA-proteins complexes, two of which were complexes with C/EBPα (10). It is possible that the other two were complexes with an Sp1-like protein(s). The identification of two distinct recognition sequences for Sp1-like proteins was not surprising, because the human C/EBPα promoter was GC rich and contained several putative Sp1 binding sites (Fig. 1).

Vasseur-Cognet and Lane have identified within the mouse C/EBPα promoter a bipartite *cis* element-containing binding sites for CUP and an Sp1-like GT box binding protein (34). We have studied binding of nuclear proteins from HeLa cells (containing a high level of CUP) to the corresponding region of the human C/EBPα promoter (from −249 to −213). We found specific interaction of Sp1-like proteins but failed to determine any interaction with other nuclear proteins. The differences between the mouse and the human C/EBPα promoters almost certainly result in different mechanisms of regulation and autoregulation.

Our data clearly demonstrate that C/EBPα and C/EBPβ could not bind to the −437 to −121 promoter region of the

human C/EBPα gene. We suggest that the mechanism of activation of the human C/EBPα promoter by C/EBPα includes induction of the DNA-binding activity of the USF nuclear factor. Two different forms of USF, 43-kDa USF1 and 44-kDa USF2, are encoded by two distinct genes and are expressed in virtually all cells (27, 31). We have shown that the DNA-binding activity of USF was stimulated by overexpression of human C/EBPα. Human C/EBPβ did not stimulate USF binding. Transient-transfection experiments indicated a 3.5-fold induction of the C/EBPα promoter by USF (Fig. 10B). Construction of deletion mutants allowed the investigation of the role of the CACGTG USF binding site in regulation and autoregulation of C/EBPα promoter. Expression of a reporter in transient assays and in stable clones showed that the USF binding site is an important element of the C/EBPα promoter. Its deletion decreased C/EBPα promoter activity 70 to 75%. This observation is in good agreement with the data of Legraverend et al., indicating significant contribution of USF to mouse C/EBPα promoter activity (23). Deletion of the CACGTG nucleotides within the human C/EBPα promoter abolished activation of the promoter by C/EBPα and suggested that the human C/EBPα gene activates its own promoter through the USF binding site. It is possible that other sites also activate the C/EBPα promoter. It should also be noted that we have not investigated the role of TFE 3, whose activity is also mediated through a similar binding sequence. As gel shift analysis would not detect TFE 3, it is formally possible that C/EBPα also activates TFE 3 binding.

Our findings provide a paradigm of tissue-specific autoregulation that utilizes a general, ubiquitous factor (USF) which is stimulated by a tissue-restricted nuclear factor (C/EBPα). The mechanism by which C/EBPα enhances USF binding and transactivation is currently under study. It is clear that enhancement by C/EBPα is specific, as we were not able to detect any alteration of USF binding activity in cells after transfection by C/EBPβ, another bZIP protein. This mode of autoregulation is novel. The generality of this mechanism needs to be examined in other genes that show autoregulatory properties. It is possible that autoregulation by direct binding of the gene product may work in concert with activation of more generally expressed factors such as USF.

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REFERENCES

1. **Aden, D. P., A. Foge, S. Plotkin, I. Damjanow, and B. B. Knowles.** 1979. Controlled synthesis of HBsAgin in a differentiated human liver carcinomas-derived cell line. Nature (London) **282:**615–616.
2. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9:**1897–1906.
3. **Angel, P., K. Hattori, T. Smeal, and M. Karin.** 1988. The jun proto-oncogene is positively autoregulated by its product, jun/AP-1. Cell **55:**875–885.
4. **Ayer, D. E., L. Kretzner, and R. N. Eisenman.** 1993. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell **72:**211–222.
5. **Beckmann, H., L. Kuosu, and Kadesch.** 1990. TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. Genes Dev. **4:**167–179.
6. **Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. **3:**1146–1156.
7. **Blackwell, T. K., L. Kretzner, M. Blackwood, R. N. Eisenman, and H. Weintraub.** 1990. Sequence-specific DNA binding by the c-Myc protein. Science **250:**1149–1151.
8. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5:**1538–1552.
9. **Christy, B., and D. Nathans.** 1989. DNA binding site of the growth factor inducible protein Zif 268. Proc. Natl. Acad. Sci. USA **86:**8737–8741.
10. **Christy, R. J., K. H. Kaestner, D. E. Geiman, and M. D. Lane.** 1991. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. USA **88:**2593–2597.
11. **Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Geiman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane.** 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev. **3:**1323–1335.
12. **Costa, R. H., D. R. Grayson, K. G. Xanthopoulos, and J. E. Darnell, Jr.** 1988. A liver-specific DNA-binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, alpha1-antitrypsin, albumin, and simian virus 40 genes. Proc. Natl. Acad. Sci. USA **85:**3840–3844.
13. **Diehl, A. M., P. Michaelson, and S. Q. Yang.** 1994. Selective induction of CCAAT/enhancer binding protein isoforms occurs during rat liver development. Gastroenterology **106:**1625–1637.
14. **Diehl, A. M., and S. Q. Yang.** 1994. Regenerative changes in C/EBPα and C/EBPβ expression modulate binding to the C/EBP site in the c-fos promoter. Hepatology **19:**447–456.
15. **Friedman, A. D., W. H. Landschulz, and S. L. McKnight.** 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev. **3:**1314–1322.
16. **Gregor, P. D., M. Sawadogo, and R. G. Roeder.** 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev. **4:**1730–1740.
17. **Hendricks-Taylor, L. R., L. L. Bachinski, M. J. Siciliano, A. Fertitta, B. Trask, P. J. deJong, D. H. Ledbetter, and G. J. Darlington.** 1992. The CCAAT/enhancer binding protein (C/EBPα) gene maps to human chromosome 19q13.1 and the related nuclear factor NF-IL6 (C/EBPβ) gene maps to human chromosome 20q13.1. Genomics **14:**12–17.
18. **Juan, T. S., D. R. Wilson, M. D. Wilde, and G. J. Darlington.** 1993. Participation of the transcription factor C/EBP delta in the acute phase regulation of the human gene for complement component C3. Proc. Natl. Acad. Sci. USA **90:**2584–2588.
19. **Kaestner, K. H., R. J. Christy, and M. D. Lane.** 1990. Mouse insulin-responsive glucose transporter gene: characterization of the gene and transactivation by the CCAAT/enhancer binding protein. Proc. Natl. Acad. Sci. USA **87:**251–255.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2:**786–800.
22. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
23. **Legraverend, K., P. Antonson, P. Flodby, and K. G. Xanthopoulos.** 1993. High level activity of the mouse CCAAT/enhancer binding protein (C/EBPα) gene promoter involves autoregulation and several ubiquitous transcription factors. Nucleic Acids Res. **21:**1735–1742.
24. **MacGregor, G. R., G. P. Nolan, S. Fiering, M. Roederer, and L. A. Herzenberg.** 1989. Use of E. coli lacZ (B-Galactosidase) as a reporter gene. Methods Mol. Biol. **7:**1–17.
25. **Mischoulon, D., B. Rana, N. L. Bucher, and S. R. Farmer.** 1992. Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBPα) gene expression during hepatocyte proliferation in the regenerating liver and in culture. Mol. Cell. Biol. **12:**2553–2560.
26. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–457.
27. **Pognonec, P., and R. G. Roeder.** 1991. Recombinant 43-kDa USF binds to DNA and activates transcription in a manner indistinguishable from that of natural 43/44-kDa USF. Mol. Cell. Biol. **11:**5125–5136.
28. **Prendergast, G. C., and E. B. Ziff.** 1991. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science **251:**186–189.
29. **Ron, D., and J. F. Habener.** 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev. **6:**439–453.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sirito, M., S. Walker, Q. Lin, M. T. Kozlowski, W. H. Klein, and M. Sawadogo.** 1992. Members of the USF family of helix-loop-helix proteins bind DNA as homo as well as heterodimers. Gene Expr. **2:**231–240.
32. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT-enhancer

binding protein: a component of a differentiation switch. Science **251:**288–292.
33. **van Dijk, M. A., R. J. T. Rodenburg, P. Holtzhuizen, and J. S. Sussenbach.** 1992. The liver specific promoter of the human insuline-like growth factor 11 gene is activated by CCAAT/enhancer binding protein (C/EBP). Nucleic Acids Res. **20:**3099–3104.
34. **Vasseur-Cognet, M., and M. Lane.** 1993. CCAAT/enhancer binding protein α (C/EBPα) undifferentiated protein: a developmentally regulated nuclear protein that binds to the C/EBPα gene promoter. Proc. Natl. Acad. Sci. USA **90:**7312–7316.
35. **Wilson, D. R., T. S. Juan, M. D. Wilde, G. H. Fey, and G. J. Darlington.** 1990. A 58-base-pair region of the human C3 gene confers synergistic inducibility by interleukin-1 and interleukin-6. Mol. Cell. Biol. **10:**6181–6191.
36. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.