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CCAAT/enhancer binding protein α (C/EBPα) undifferentiated protein: A developmentally regulated nuclear protein that binds to the C/EBPα gene promoter

(3T3-L1 adipocyte/preadipocyte differentiation/Sp1/gene transcription)

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ABSTRACT **During differentiation of 3T3-L1 preadipocytes into adipocytes, transcription of the C/EBPα (CCAAT/enhancer binding protein α) gene is activated. The promoter of the C/EBPα gene contains a bipartite cis element with binding sites for C/EBPα undifferentiated protein (CUP) and an Sp1-like GT box binding protein. Binding of CUP to this element is markedly enhanced by its interaction with the Sp1-like protein. CUP, purified ≈100,000-fold from HeLa cell nuclear extracts, appears to be composed of at least two types of subunit. Evidence is presented that a CUP-containing protein complex bridges between the CUP/Sp1-like GT box element and a downstream cis element, which contains a C/EBP binding site. During differentiation of 3T3-L1 preadipocytes into adipocytes, CUP activity or expression decreases as expression of C/EBPα increases. It is suggested that bridging by the CUP-containing protein complex may play a role in transcriptional regulation of the C/EBPα gene.**

During differentiation of preadipocytes into adipocytes, transcription of adipose-specific genes is coordinately activated (1–4). Evidence now indicates that the transcription factor C/EBPα plays an essential role in this process. C/EBPα was identified as a differentiation-induced nuclear factor that binds specifically to the promoters of several genes [the 422(aP2), SCD1, and GLUT4 genes], which are coordinately expressed when 3T3-L1 preadipocytes differentiate into adipocytes (3, 5). Consistent with a role for C/EBPα in coordinating adipocyte gene expression, the C/EBPα gene is transcriptionally activated just prior to expression of these genes (2). It has been demonstrated both in intact 3T3-L1 cells (3, 5) and with a cell-free transcription system (6) that C/EBPα serves as a trans-activator of the promoters of these genes and that mutation of the C/EBP binding site prevents trans-activation (5–7). While compelling, this evidence implicating C/EBPα in differentiation-induced gene expression was indirect. Experiments with antisense C/EBPα RNA (8, 9) have provided unequivocal proof. Expression of antisense C/EBPα RNA prevents differentiation-induced expression of C/EBPα and several adipose-specific genes and blocks cytoplasmic fat accumulation. This and other evidence (10, 11) indicate that C/EBPα plays a key role in the differentiation process.

To investigate the basis for transcriptional activation of the C/EBPα gene during differentiation, Christy *et al.* (2) cloned the mouse gene and identified a DNA sequence within the promoter that binds a protein present in nuclear extracts of preadipocytes but not adipocytes. In the present paper, we describe the purification and characterization of this nuclear protein—i.e., C/EBPα undifferentiated protein (CUP).‡

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EXPERIMENTAL PROCEDURES

Human recombinant Sp1 was from Promega. Mouse 3T3-L1 preadipocytes were propagated and induced to differentiate into adipocytes as described (13). Nuclear extracts from 3T3-L1 cells were prepared by a modification of the protocol of Dignam (14, 15). HeLa cell nuclear extracts were prepared as described by Rosenfeld and Kelly (16).

Gel Retardation and UV Crosslinking Assays. Gel retardation assays were performed essentially as described (17). Reaction mixtures contained 0.5–1 ng of Klenow fragment ^{32}P-radiolabeled oligonucleotide, 1.5 μg of poly[d(IC)], and either 10 μg (with the EF, EF-M1, or EF-M2 probe) or 30 μg (with the CUP site probe) of nuclear extract protein in 24 μl of retardation buffer. After a 15-min incubation on ice, protein–DNA complexes were separated in 5% polyacrylamide gels [30:1, acrylamide/bisacrylamide in 0.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3)] by electrophoresis in 0.25× TBE buffer at 7 V/cm for 6 h for EF (free probe is run off the gels) and 2 h for CUP probes. Affinity chromatographic fractions diluted to 0.1 M KCl were used for gel retardation assays. In addition to the oligonucleotides cited in Fig. 1*A*, the following synthetic oligonucleotides were used: C/EBP422 site from the 422(aP2) gene, GATC/AAGTTGAGAAATTTCT/ (5); C/EBPA site from the C/EBPα gene, GATC/GCGCAGGAGTCAGTGGGCGTTGCGCCACGATCTC/ (2); Sp1 tk from the thymidine kinase gene of herpes simplex virus, GATC/TAAACCCCGCCCAGCG/ (18) (gift of S. Cereghini, Pasteur Institut); and telomere, a non-related sequence, GATC/CCCTAACCCTAACCCTAA/.

After retardation assays, gels were irradiated with a UV lamp at 300 nm for 20 min on ice and subjected to autoradiography. Labeled DNA–protein complexes were excised and gel pieces were incubated in 1× Laemmli buffer containing dithiothreitol (3 mg/ml) for 1 h before loading onto SDS/8% polyacrylamide gels. After electrophoresis, gels were subjected to autoradiography.

DNase I Footprinting. A Bluescript vector containing a fragment of the C/EBPα gene (2) promoter (nucleotides −600 to +5) was cut with *Sty* I (position −138), 5′ end labeled with [γ-^{32}P]ATP and DNA ligase, and then cut with *Sma* I (position −341), generating a labeled noncoding strand. DNase I footprinting was then performed as described by Christy *et al.* (2).

Abbreviations: C/EBPα, CCAAT/enhancer binding protein α; CUP, C/EBPα undifferentiated protein; tk, thymidine kinase.

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‡Nomenclature for the C/EBP family of proteins is that of Cao *et al.* (12).

Purification of CUP. Nuclear extract, prepared from HeLa S-3 cells (16), was dialyzed against buffer D [20 mM Hepes, pH 7.9/17% (vol/vol) glycerol/0.2 mM EDTA/0.2 mM EGTA/12.5 mM $MgCl_2$/2 mM dithiothreitol] containing 0.1 M KCl. To prepare the CUP-site affinity matrix, 0.5 mg of the CUP-site oligonucleotide (Fig. 1*A*) was oligomerized and coupled to 10 ml of CNBr-activated Sepharose 4B (Pharmacia LKB) as described (19). HeLa nuclear extract (16 mg of protein in 4 ml derived from 2 liters of HeLa suspension culture) containing 62.5 μg of poly[d(I-C)] per ml was incubated for 10 min at 4°C and then added to 900 μl (packed volume) of CUP-site affinity matrix equilibrated with buffer D_1 [buffer D containing 0.1% Nonidet P-40 and protease inhibitors (20)] containing 0.1 M KCl. After slow mixing overnight, the matrix was packed into a 1-ml column and washed with 4 column vol of buffer D_1 containing 0.1 M KCl and 150 μg of insulin per ml. To stabilize CUP binding activity, insulin was added to all elution buffers for chromatography. Stepwise elution was carried out with 1 column vol of buffer D_1 (0.2 M KCl) followed by 4 column vol of buffer D_1 (0.3 M KCl). CUP and Sp1-like binding activities were monitored by gel retardation assays with the EF probe. Fractions containing CUP activity were pooled and diluted to 0.1 M KCl with buffer D_1 for a second round of affinity chromatography. Poly[d(I-C)] was added (final concentration, 0.75 μg/ml) and the solution was mixed with 500 μl of fresh CUP binding-site affinity matrix for 5 h at 4°C. The matrix was poured into a 1-ml column and was washed with 4 column vol of buffer D_1 (0.1 M KCl). Elution was accomplished with 2 column vol of buffer D_1 (0.2 M KCl), followed by 4 vol of buffer D_1 (0.3 M KCl). CUP activity, free of Sp1-like binding activity, was eluted with 0.3 M KCl-containing buffer. The pooled CUP-containing fractions, after dilution to 0.1 M KCl with buffer D_1, were stored frozen at −80°C for 2 months without loss of activity.

RESULTS AND DISCUSSION

DNase I footprinting studies (2) showed that a nuclear factor present in 3T3-L1 preadipocytes, but not differentiated adipocytes, binds to the mouse C/EBPα gene promoter between nucleotides −252 and −239. To further characterize this interaction, a synthetic oligonucleotide, EF, corresponding to this region in the promoter (Fig. 1*A*), was subjected to gel retardation analysis with nuclear extracts from preadipocytes and adipocytes (day 13 after induction of differentiation). Three protein–oligonucleotide complexes—i.e., S1, S2, and CUP—were detected with nuclear extracts from preadipocytes, of which only two, S1 and S2, were formed with extracts from adipocytes (Fig. 1*B*). Since the nucleotide sequence of EF is G+C-rich, the possibility was considered that these complexes contained a previously identified DNA binding protein—e.g., Sp1, which binds to similar sequences (21). An excess (40-fold) of an unlabeled high-affinity Sp1 binding site oligonucleotide, Sp1 tk, effectively competed with the EF probe for formation of the S1 and S2 complexes, but not the CUP complex (Fig. 1*C*). However, a 200-fold excess of Sp1 tk reduced CUP complex formation, suggesting the presence of a tightly binding Sp1-like protein in the complex (results not shown). That formation of the CUP complex is specific is indicated by the reduction of complex formation by unlabeled EF (Fig. 1*C*). A 200-fold excess of an unrelated telomeric sequence did not compete for any of the three complexes. Thus, a nuclear factor(s) required for CUP complex formation is lost or becomes inactive during differentiation, while factors required for formation of the S1 and S2 complexes are constitutive.

That Sp1 *per se* is present in preadipocyte nuclear extracts is indicated by the fact that an Sp1 tk probe is gel-shifted by such nuclear extracts (results not shown). Extensive competition studies revealed, however, that Sp1 has a relatively

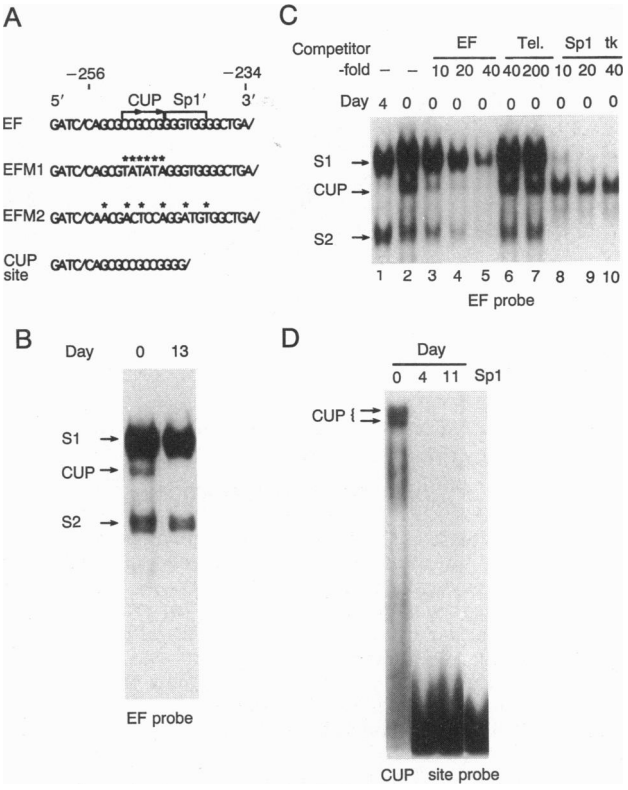

FIG. 1. Effect of state of differentiation and competitor oligonucleotides on gel retardation of EF oligonucleotide by nuclear extracts from 3T3-L1 cells. (*A*) Sequences of synthetic oligonucleotides corresponding to wild-type (EF and CUP) and mutated (EFM1 and EFM2) CUP/Sp1-like GT box element within the C/EBPα gene promoter. Asterisks identify mutated nucleotides. Slashes delimit positions of linkers in both strands. Numbers (−256 and −234) refer to position upstream of transcription start site. (*B–D*) Gel retardation assays using EF or CUP site probes and nuclear extract protein from day 0 3T3-L1 preadipocytes; day 4, 11, or 13 differentiated 3T3-L1 adipocytes; or 60 ng of recombinant Sp1. Levels of unlabeled competitor oligonucleotides are -fold molar excess over labeled EF probe. Tel. and Sp1 tk refer to telomere and Sp1 tk oligonucleotide, respectively. Arrows identify S1, CUP, and S2 complexes as indicated.

low affinity for EF. As Sp1 is a zinc finger DNA binding protein (22), binding to its cognate binding site is prevented by EDTA (22). Similarly, formation of the S1 and S2 complexes was prevented by EDTA, and reversed by zinc, but not by calcium (results not shown). Formation of the CUP–EF complex was unaffected by EDTA. Heat treatment of the nuclear extract at 70°C for 5 min completely blocked formation of the CUP–EF complex, but not the S1–EF and S2–EF complexes. These results suggest that the CUP complex contains a differentially expressed G+C-rich DNA binding protein and an Sp1-like GT box binding protein and that the S1 and S2 complexes contain Sp1 and/or an Sp1-like protein. The possibility that the Sp1-like GT box binding protein is identical to the recently characterized Sp3 GT box binding protein (23) remains to be investigated.

To define the sites of interaction of the nuclear proteins with EF, two mutated EF oligonucleotides were tested in gel retardation assays (results not shown). EF-M1 contains a 6-base mutation between nucleotides −251 and −246, while EF-M2 contains 6 point mutations scattered throughout the EF sequence at positions −254, −251, −249, −246, −243, and −240 (Fig. 1*A*). Competition experiments using EF as probe and preadipocyte nuclear extract showed that a 20-fold excess of unlabeled EF-M1 or Sp1 tk decreased formation of the S1 and S2 complexes but had no effect on formation of the

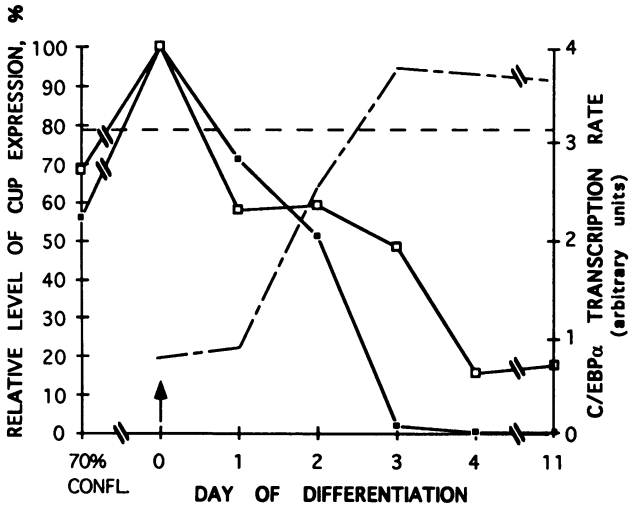

FIG. 2. Decrease in CUP activity during preadipocyte differentiation. 3T3-L1 preadipocytes were propagated and induced to differentiate 2 days after reaching confluence—i.e., on day 0. Gel retardation assays with EF (□) or CUP-site (■) probes were performed with nuclear extracts at different times after induction of differentiation (arrow). Gel retardation activity was normalized to maximal activity on day 0. – – –, Relative activity of a comparable amount of nuclear extract protein from proliferating HeLa cells; – · –, C/EBPα transcription rate [results from Christy *et al.* (2)].

CUP complex. A 20-fold excess of unlabeled EF-M2 had no effect on CUP or S1 and S2 complex formation. In gel retardation shift assays with EF-M1 as probe, the CUP complex was not formed; however, the S1 and S2 complexes were formed. No complexes were detected when EF-M2 was used as probe.

In gel shift experiments with EF as probe, unlabeled CUP site (positions −256 to −243; Fig. 1*A*) or EF prevented formation of the CUP complex (results not shown). Use of a CUP site probe gave rise to a major protein–oligonucleotide complex[§] with nuclear extract from preadipocytes (day 0; Fig. 1*D*). This complex was not formed, however, with nuclear extract from differentiated day 4 or day 11 adipocytes or with purified Sp1 (Fig. 1*D*). The diffuse band below the CUP complex in Fig. 1*D* in day 0 preadipocytes was not consistently observed and was not blocked by competition with unlabeled CUP site. Both unlabeled CUP site and EF were effective competitors of the CUP complex, whereas Sp1 tk and a telomere control oligonucleotide were not (results not shown). Furthermore, competition for CUP site binding was not detected with seven other related consensus binding site sequences for known transcription factors. It can be concluded that two adjoining sites are present in EF (Fig. 1*A*)—a 5′ CUP site to which a protein expressed only by preadipocytes binds and a 3′ site to which a constitutively expressed GT box Sp1-like protein binds.

As nuclear factor binding to the CUP site appeared to be differentiation dependent, the progress of CUP expression was followed by gel retardation analysis during preadipocyte differentiation using both EF and CUP site as probes (Fig. 2).[¶] CUP is expressed by proliferating preadipocytes—i.e., at 70% confluence (Fig. 2) and at 40% confluence (results not shown)—as well as by proliferating HeLa cells (Fig. 2). Maximal CUP activity is achieved as 3T3-L1 cells reach confluence. After induction of differentiation, CUP activity decreases precipitously despite the fact that the cells undergo two additional rounds of mitosis (clonal expansion) during this period (1, 4), reaching a minimum between days 3 and 4 after induction. At this time, there is a concomitant increase in transcription of the C/EBPα gene (2) (Fig. 2). This inverse relationship between CUP activity and expression of C/EBPα extends to other cell and tissue types (results not shown). For example, liver (which expresses genes known to be transactivated by C/EBPα; e.g., the albumin gene) is devoid of CUP activity but expresses a high level of C/EBPα. Conversely, cell lines (i.e., 3T3-C2, HepG2, HeLa, and myoblast/C2C12 cells) and brain tissue that do not express

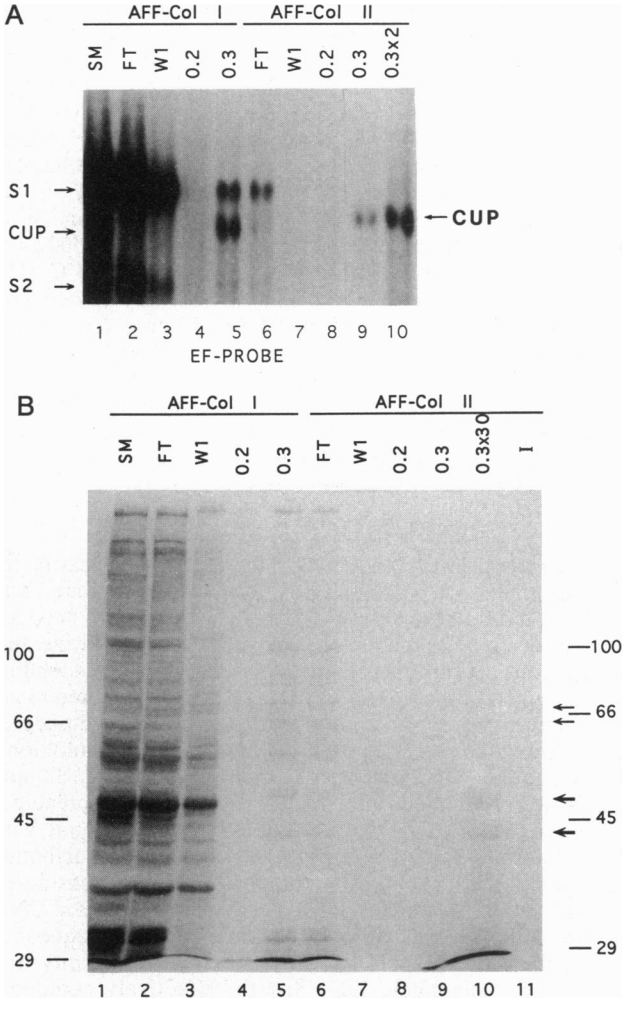

FIG. 3. Analysis of CUP-site binding activity and protein during purification. (*A*) Gel retardation assays using EF as probe and fractions from purification of CUP. Amount loaded in each lane, except lane 10, was equivalent to that of starting material (SM); in lane 10, 2-fold the amount was loaded. FT and W1, flow-through and first wash, respectively, of affinity chromatographic columns (AFF-Col I and AFF-Col II); 0.2 and 0.3 refer to individually pooled 0.2 M and 0.3 M KCl eluates. It should be noted that the CUP complex has a somewhat lower mobility after the second affinity column, which removes Sp1 and/or Sp1-like protein. (*B*) Silver-stained SDS/8% polyacrylamide gels for the fractions shown in *A* except lanes 10 and 11. Amount of each fraction loaded in lanes 1–9 was equivalent to that of the starting material. In lane 10, 30 times the level of 0.3 M KCl eluate (lane 9) was used. In lane 11, only insulin-containing buffer was used. Heavy arrows, major silver-stained bands in lane 10; light arrows, minor silver-stained bands in lane 10. Numbers on left and right refer to molecular masses (kDa) of marker proteins.

[§]Two CUP-containing protein complexes are sometimes observed with nuclear extracts. Results (not shown) suggest that the state of phosphorylation of CUP may be responsible for the double-band pattern.

[¶]The small difference in CUP activity measured with the EF and CUP oligonucleotides between days 3 and 11 is attributable to cross-contamination of ^{32}P activity in the region of the CUP band by the heavily labeled neighboring S1 and S2 bands when the EF probe was used (e.g., see Fig. 1*B*).

Table 1. Purification of CUP from HeLa cell nuclear extract

Stage	Total protein, μg	Total activity,* units	Specific activity, units/mg	Purification, -fold	Yield, %
Nuclear extract	16,000	15,000	940	1	100
CUP affinity chromat. II	≈0.05†	5,000	≈10^8	≈10^5	33

*One unit of binding activity is the amount of CUP that under standard gel retardation conditions retards 0.05 fmol of Klenow fragment-labeled probe.
†Approximation based on intensity of silver staining of SDS/polyacrylamide gels relative to standards.

C/EBPα express CUP. Preadipocyte cell lines, whose capacity to differentiate has been genetically impaired by expression of C/EBPα antisense RNA or by forced expression of c-Myc, express high levels of CUP activity, but not C/EBPα, when subjected to the differentiation protocol. Thus, we suggest that CUP is involved in repression of the C/EBPα gene by inappropriate cell types or at inappropriate stages of development.

As CUP from HeLa cells and 3T3-L1 preadipocytes possess identical properties, HeLa cells, which can be propagated on a larger scale, were used for purification of CUP. Since CUP activity is localized exclusively in the nuclear fraction, the initial step in purification was isolation and extraction of HeLa cell nuclei. Gel retardation assays with the EF were used to monitor the resolution of CUP from proteins in the S1 and S2 complexes. Specificity was verified by competition with both unlabeled EF and CUP site. Purification of CUP was achieved by two rounds of sequence-specific DNA affinity chromatography using Sepharose-4B to which oligomerized CUP binding site (Fig. 1*A*) was covalently linked. As illustrated in Fig. 3*A*, Sp1-like binding activity (S1 and S2 complexes) was resolved from CUP. SDS/PAGE showed that purified CUP contained two major polypeptides of ≈40 and ≈50 kDa (Fig. 3*B*, lane 10). These polypeptides appeared to correspond to two polypeptides from nuclear extract that are UV-crosslinked to EF or the CUP binding site (see below). Two minor polypeptides with molecular masses of ≈65 and ≈70 kDa were also present. As shown in Table 1, the overall yield of CUP binding activity in a typical purification was ≈33%. While protein mass could not be determined accurately at the final stage of purification because of the small amount of protein, we estimate a protein yield of ≈50 ng (based on silver-stained intensity SDS/polyacrylamide gels of CUP relative to standards). The overall purification was ≈100,000-fold. The K_d for binding of purified CUP to EF and CUP site, determined by gel retardation analysis, was ≈10 nM (results not shown).

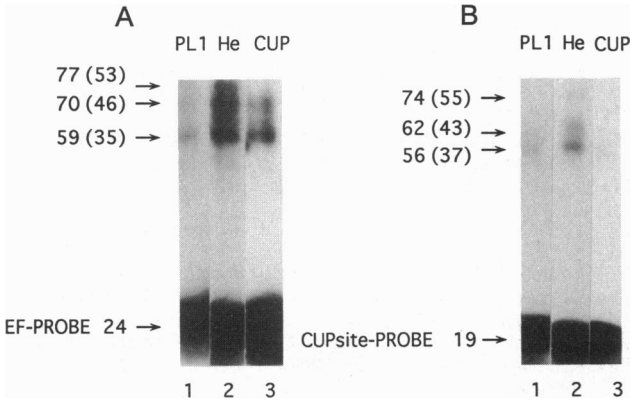

FIG. 4. UV cross-linking of proteins bound to labeled EF and CUP-site oligonucleotides. After gel retardation with labeled EF or CUP site with 3T3-L1 preadipocyte or HeLa cell nuclear extracts or purified CUP, gels were exposed to UV light. Labeled CUP complexes were subjected to SDS/PAGE and autoradiography. Numbers adjacent to arrows refer to apparent molecular masses (kDa) of covalently linked protein-labeled oligonucleotide complexes; numbers in parentheses refer to molecular masses corrected for covalently bound single-strand EF or CUP probes (24 or 19 kDa, respectively).

UV crosslinking experiments with labeled EF and CUP site oligonucleotides and nuclear extracts from either 3T3-L1 preadipocytes or HeLa cells gave rise to the same three labeled UV-crosslinked polypeptides (Fig. 4). Cross-linked polypeptides were not detected by using nuclear extracts from 3T3-L1 adipocytes. Purified CUP gave rise to only two of the three labeled polypeptides. We estimate the molecular masses of the CUP polypeptides (corrected for crosslinked oligonucleotide) to be ≈37 and ≈45 kDa. These approximate the molecular masses of the two major polypeptides in purified CUP—i.e., ≈40 and ≈50 kDa—estimated by SDS/PAGE (Fig. 3*B*, lane 10). Thus, CUP appears to contain at least two different types of subunit. That CUP contains more than a single subunit may account for our inability (*i*) to identify CUP protein by Southwestern blotting procedures; (*ii*) to clone a CUP cDNA by screening several 3T3-L1 preadipocyte expression libraries with an EF probe; or (*iii*) to recover binding activity following elution of purified CUP polypeptides from SDS/polyacrylamide gels, denaturation, and renaturation.

To assess the binding of purified CUP and Sp1 (which served as a homologue of the Sp1-like GT box binding protein that is removed from CUP during purification) in the context of the C/EBPα gene promoter, a 205-bp segment (−343 to −138) containing the CUP and GT box sequences was subjected to DNase I footprinting. It should be noted that this segment of the promoter contains two regions that are differentially footprinted by nuclear extracts from preadipocytes and adipocytes (2). The region between positions −252 and −233 is footprinted by preadipocyte, but not adipocyte, nuclear extract. Another region (between positions −203 and −176) exhibits different, but overlapping, footprints by preadipocyte and adipocyte nuclear extracts. The protected region with preadipocyte nuclear extract extends further 5′ than that protected by adipocyte nuclear extract. The 3′ segment of this region is footprinted by recombinant C/EBPα. As shown in Fig. 5*A*, neither CUP nor Sp1 produced a detectable footprint, although Sp1 generated a hypersensitive site at nucleotide −241 within the GT box (Fig. 5*A*, lane 2). When CUP and Sp1 were added together, however, two strong footprints were generated, one covering the CUP and GT box sites (between −252 and −233) and another between −203 and −187 (Fig. 5*A*, lanes 6–8), while hypersensitivity at position −241 decreased. This pattern is similar to that observed with nuclear extracts from preadipocytes (2) or HeLa cells (results not shown).

The footprint (by CUP and Sp1) at −203 to −187 adjoining the C/EBP binding site was unexpected, since it lies ≈30 bp 3′ to the bipartite CUP site/GT box element (Fig. 5*A*). This region of footprinting is specific for the undifferentiated preadipocyte and is not observed with nuclear extracts from differentiated 3T3-L1 adipocytes (ref. 2; results not shown).‖ The fact that footprinting of this site requires both CUP and Sp1 strongly suggests that a CUP–Sp1 protein complex bridges between the CUP/GT box element and a consensus Sp1 binding site—i.e., GGGCG—between −193 and −189 at the 5′ end of the C/EBP binding site. Evidence that Sp1 (in the bridging complex) binds at this GC box was obtained in gel retardation–competition studies with nuclear extract from

‖Neither adipocyte nuclear extract, which contains Sp1 and/or an Sp1-like protein(s) (Fig. 1 *B* and *C*), nor recombinant Sp1 footprints either the CUP site/GT box element or the site (−203 to −187) adjoining the C/EBP binding site (Fig. 5*A*; ref. 2).

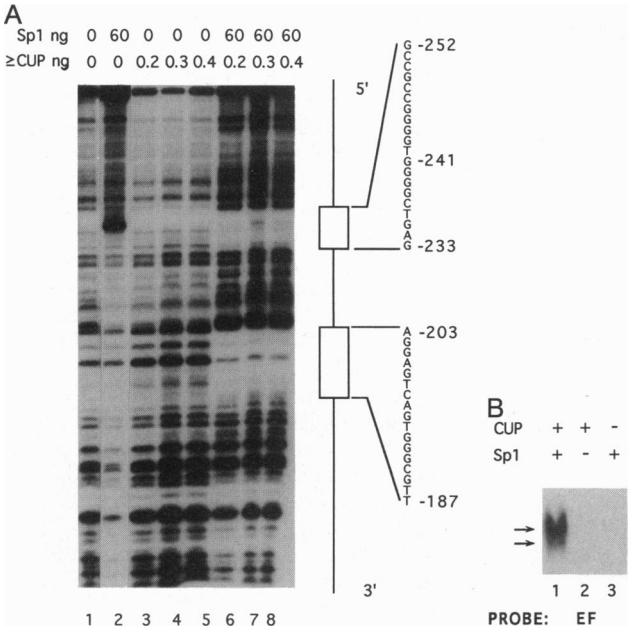

FIG. 5. Synergistic DNase I footprinting by and gel retardation analysis of purified CUP and Sp1. (*A*) A 203-bp fragment of the C/EBPα gene proximal promoter (positions −341 to −138 bp 5′ to transcription start site) was subjected to DNase I protection analysis with purified CUP and/or purified recombinant Sp1 at the indicated levels. Nucleotide sequences of the windows of protection (positions −252 to −233 bp and positions −203 to −187 bp) are shown on the right. (*B*) Gel retardation assays were conducted using EF probe with purified CUP and/or purified recombinant Sp1. Arrows indicate complexes formed.

preadipocytes and EF as probe. Thus, a low level of unlabeled oligonucleotide (C/EBPA), which contains the C/EBP binding site and the adjoining 5′ GC box of the C/EBPα gene promoter, competes with the S1 and S2 Sp1-like complexes but not the CUP complex. In contrast, oligonucleotide containing the C/EBP binding site of the 422(aP2) gene promoter (which lacks a GC box) does not compete with these complexes. Similarly, a CUP site oligonucleotide, which does not compete with the S1 and S2 complexes, competes with the CUP complex. These results were confirmed in gel retardation experiments using C/EBPA as probe and the EF, Sp1 tk, and CUP oligonucleotides as competitors (results not shown). These findings support the view that an Sp1-like protein(s) in the S1 and/or S2 complexes binds to both the GT box (position −245 to −240) adjoining the CUP site and to the GC box (−193 to −189) adjoining the C/EBP binding site.

The apparent synergistic interaction between purified CUP and Sp1 observed in footprinting experiments was verified by gel retardation studies. As shown in Fig. 5*B*, neither CUP nor Sp1 alone at low concentration caused significant gel retardation of EF. However, when both purified proteins were added together, complexes were formed. The strongest synergism between CUP and Sp1 occurred with the EF probe, which contains binding sites for both factors (Fig. 5*B*). Nevertheless, significant synergism was also observed with the CUP probe (results not shown) indicating a protein–protein interaction. Further evidence for a cooperative interaction between these protein factors was obtained in a study of the dependence of gel retardation on nuclear extract protein concentration (results not shown). There are several other examples of interactions between Sp1 and other DNA binding proteins (24, 25).

We suggest that the CUP site/GT box element communicates with the C/EBP binding element in the C/EBPα gene promoter through a CUP/Sp1-like protein/Sp1 bridging complex. Evidence is presented that this complex binds both to the CUP site GT box element and the downstream GC box adjoining the C/EBP binding site. Thus, it would be expected that upon differentiation of preadipocytes into adipocytes the CUP/Sp1-like protein/Sp1 complex, and hence bridging, would be destabilized, since CUP activity or expression decreases during differentiation. We suggest that disruption of the interaction between the CUP site/GT box and C/EBP binding elements might affect transcriptional activation (perhaps derepression) of the C/EBPα gene. Disruption of this interaction due to loss of CUP activity may render the C/EBP binding site accessible to C/EBPα, allowing transcriptional autoactivation of its own gene. Several lines of evidence indicate that C/EBPα is capable of autoactivating transcription of its own gene. A site within the proximal promoter has been shown to bind recombinant C/EBPα (2). Moreover, blocking expression of C/EBPα posttranscriptionally with antisense C/EBPα RNA prevents autoactivation of transcription of the gene (8). Finally, C/EBPα is capable of transactivating transcription mediated through the C/EBP site within the proximal promoter of its own gene (F.-T. Lin and M.D.L., unpublished results). An interpretative model consistent with these findings has been presented (4).

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