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Biochemistry

A 30-kDa alternative translation product of the CCAAT/enhancer binding protein α message: Transcriptional activator lacking antimitotic activity

(3T3-L1 cells/preadipocyte/adipocyte/differentiation/liver)

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ABSTRACT **Full-length (42 kDa) CCAAT/enhancer binding protein α (C/EBPα) (p42) has been implicated in the transcriptional activation of adipocyte genes including the 422(aP2) and C/EBPα genes during differentiation of 3T3-L1 preadipocytes. We have identified a 30-kDa isoform (p30) of C/EBPα that is expressed by 3T3-L1 adipocytes, mouse adipose tissue, and rat liver. *In vitro* translation of wild-type C/EBPα mRNA or transient transfection with a wild-type C/EBPα vector gave rise to similar levels of p42 and p30. Mutational analysis revealed that p30 is an alternative translation product initiated at the third in-frame methionine codon of the C/EBPα message. $p30^{C/EBP\alpha}$ binds to the C/EBP sites within and activates reporter gene expression driven by the 422(aP2) and C/EBPα gene promoters. Although transfection of 3T3-L1 preadipocytes with a strong $p30^{C/EBP\alpha}$ expression vector is insufficient to induce differentiation, this vector advances the differentiation program. Unlike $p42^{C/EBP\alpha}$, which inhibits cell proliferation, $p30^{C/EBP\alpha}$ is not antimitotic. Thus, the N-terminal 12-kDa segment of full-length C/EBPα contains an amino acid sequence necessary for antimitotic activity. During differentiation of 3T3-L1 preadipocytes and during hepatocyte development, the cellular $p42^{C/EBP\alpha}/p30^{C/EBP\alpha}$ ratio changes, raising the possibility of a regulatory role.**

The CCAAT/enhancer binding protein α (C/EBPα) transcriptionally activates a set of genes whose expression gives rise to the adipocyte phenotype (for review, see ref. 1). C/EBPα binds specifically to and transactivates the promoters of several of these genes [the 422(aP2), *SCD1*, and *GLUT4* genes], which are coordinately expressed when preadipocytes differentiate into adipocytes (2–5). Consistent with a role in coordinating adipocyte gene expression, transcription of the C/EBPα gene is activated during differentiation immediately prior to expression of adipose-specific genes (2, 6). Definitive proof that C/EBPα is involved in the differentiation process was provided by studies with C/EBPα antisense RNA (7, 8). Vector-directed expression of C/EBPα antisense RNA during differentiation of 3T3-L1 preadipocytes not only blocked expression of C/EBPα and several adipose-specific mRNAs but also prevented cytoplasmic triglyceride accumulation (7).

Recently, a putative 30-kDa isoform of C/EBPα was detected by Western blot analysis (6, 7). Extracts of differentiated 3T3-L1 adipocytes were found to contain two proteins (42 and 30 kDa or p42 and p32, respectively) recognized by antibody directed against the C-terminal amino acid sequence of C/EBPα. We establish in this report that the 30-kDa species is an alternative translation product of the C/EBPα message. Similar to $p42^{C/EBP\alpha}$, $p30^{C/EBP\alpha}$ transactivates the 422(aP2) and the C/EBPα gene promoters. Unlike $p42^{C/EBP\alpha}$, however, $p30^{C/EBP\alpha}$ is not antimitogenic.

EXPERIMENTAL PROCEDURES

Proliferating 3T3-L1 preadipocytes and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum. Confluent preadipocytes were induced to differentiate into adipocytes as described (9). Nuclei from epididymal adipose tissue of 8-week-old BALB/c mice were lysed for Western blot analysis as described (7). Nuclear extracts from liver tissue of Sprague–Dawley rats were prepared as described (10). Nuclear extract and cytoplasmic proteins for Western blot analysis and gel-retardation assays from 3T3-L1 and 293 cells were prepared by a modification (11) of the procedure of Dignam *et al.* (12).

To construct pBS-m42, pC/EBPα9.7 (13) was digested with *Bam*HI and *Nru* I to remove the 5′ flanking sequence, then filled-in, and religated. This construct was digested with *Hin*dIII to remove most of the 3′ flanking sequence and then religated. The third in-frame ATG in the coding sequence of pBS-m42 was mutated to TTG by site-directed mutagenesis (14, 15) giving rise to pBS-TS^3m42. Truncated pBS-m30 and pBS-TS^3m30 vectors were constructed by removing sequence 5′ of the *Aat* II site (+176 bp) of the C/EBPα coding sequence from pBS-m42 and pBS-TS^3m42 with *Xba* I and *Aat* II, thereby deleting the first two in-frame ATG sites. The rat C/EBPα sequence was removed from pMSV-C/EBPα (16) by digestion with *Bam*HI and *Hin*dIII and was inserted in pBluescript to produce pBS-TS^1r42.

For expression in cells, the C/EBPα cDNA inserts from pBS-m42, pBS-TS^3m42, and pBS-m30 were removed and *Eco*RI linkers were added for insertion into pcDNA1 (Invitrogen) giving rise to pCMV-m42, pCMV-TS^3m42, and pCMV-m30. The C/EBPα cDNA inserts of pBS-m42 and pBS-m30 with *Eco*RI linkers were also inserted into the *Eco*RI site of the pMT2 expression vector (17) giving rise to pAMLP-m42 and pAMLP-m30. Mutants of C/EBPα cDNA, Δ^{AatII}m42 and IN^{MluI}m42, were produced by digesting the C/EBPα gene (pC/EBPα9.7) with *Aat* II followed by deleting the 3′ overhangs and religating or digesting with *Mlu* I followed by filling-in the flanking sites and then religating.

SDS/PAGE and Western blot analysis were performed as described (7). C/EBPα was detected with rabbit antibody against an internal peptide (aa 253–265) or a C-terminal 14-residue peptide of C/EBPα.

Linearized templates (5 μg) from pBluescript were transcribed *in vitro* with T7 RNA polymerase. Each RNA (2 μg) was translated with rabbit reticulocyte lysate. Proteins immu-

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Abbreviations: C/EBPα, CCAAT/enhancer binding protein α; CAT, chloramphenicol acetyltransferase; wt, wild type.

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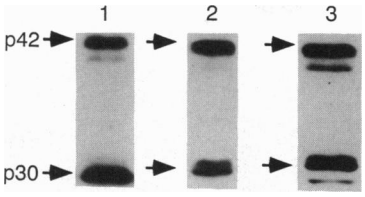

FIG. 1. Detection of a 30-kDa protein in liver, adipose tissue, and 3T3-L1 adipocytes with anti-C/EBPα antibody. After solubilization, 50 μg (liver nuclei) or 100 μg (adipose nuclei) of protein or total cellular protein (200 μg) extracted with SDS from fully differentiated (day 5) 3T3-L1 adipocytes was subjected to SDS/PAGE. Western blot analysis was conducted with antibody against the C-terminal amino acid sequence of C/EBPα. Lanes: 1, liver; 2, adipose; 3, 3T3-L1 cells.

noprecipitated with antibody against preimmune serum or the internal C/EBPα peptide were subjected to SDS/PAGE on 12% gels. *In vitro* transcription and translation of pBS-TS^{1}r42 and pBS-m30 were performed with 5 μg of each transcript for translation and the reaction was for 2 h. Luciferase RNA (1 μg) was translated as negative control. Gel-retardation assays (2) were then carried out.

To generate stable cell lines, 20 μg of pAMLP-m30 and 2 μg of pSV2Neo were transfected into 30% confluent 3T3-L1 preadipocytes by calcium phosphate coprecipitation (18). The 1.5-kb *Bam*HI–*Nru* I and 5.6-kb *Eco*RI–*Nru* I fragments of the C/EBPα gene promoter (from pC/EBPα9.7) were cloned into pBlueCAT (where CAT is chloramphenicol acetyltransferase) and designated p-1.5kb C/EBPαCAT and p-5.6kbC/EBPαCAT (R. J. Christy and M.D.L., unpublished results). The 248-bp *Nsi* I–*Pst* I fragment of the 422/aP2 gene promoter was cloned into pBLCAT3′ and designated p-248CAT as described (19). The linker-scanning mutant of 422(aP2), pΔLS(-122/143)CAT (V. W. Yang and M.D.L., unpublished results), has a *Bam*HI linker sequence inserted in place of the C/EBP binding site. p-248CAT, pΔLS(-122/143)CAT, and promoterless pBLCAT3′, respectively, were transiently transfected into 30% confluent preadipocytes, into a control cell line harboring the pMT2 vector, and into stable cell lines p30-9 and p30-12 that express p30$^{C/EBP\alpha}$. p-1.5kbC/EBPαCAT, p-5.6kbC/EBPαCAT, and promoterless pBlueCAT were also transiently transfected into these stable cell lines. Cells were assayed 2 days later (20).

RESULTS

To determine whether adipose tissue contains immunoreactive p30, nuclear extracts prepared from mouse epididymal fat pads in the presence of protease inhibitors were subjected to SDS/PAGE and then to Western blot analysis. Two major immunoreactive proteins with molecular masses of 42 kDa (full-length C/EBPα) and 30 kDa (relative concentration ratio, 1.8/1.0) were detected in nuclear extract, but not in cytoplasm, with C-terminal (Fig. 1) and internal (data not shown) antibodies. Both the 42- and 30-kDa species were also detected in rat liver and 3T3-L1 adipocytes.

To assess the relative levels of expression of p42 and p30 during differentiation of 3T3-L1 preadipocytes, extracts from cells at different stages of differentiation were analyzed. Neither form was found in confluent preadipocytes; however, expression of both forms began on day 2 of differentiation and reached a maximum on day 4 or 5 (results not shown). The p42/p30 ratio varied between 0.5 and 2.5, achieving a maximum on day 4 or 5, after which the levels of both forms decreased, the decrease of p30 lagging behind that of p42. Since 3T3-L1 cells express a single species of C/EBPα mRNA that reaches a maximum on day 4 and then remains constant (2), changes in the levels and ratio of the two isoforms (after day 4) cannot be due to changes in the level of C/EBPα mRNA.

As the liver undergoes dramatic functional changes during development, it was of interest to ascertain whether changes in the p42/p30 ratio accompany development. Expression of

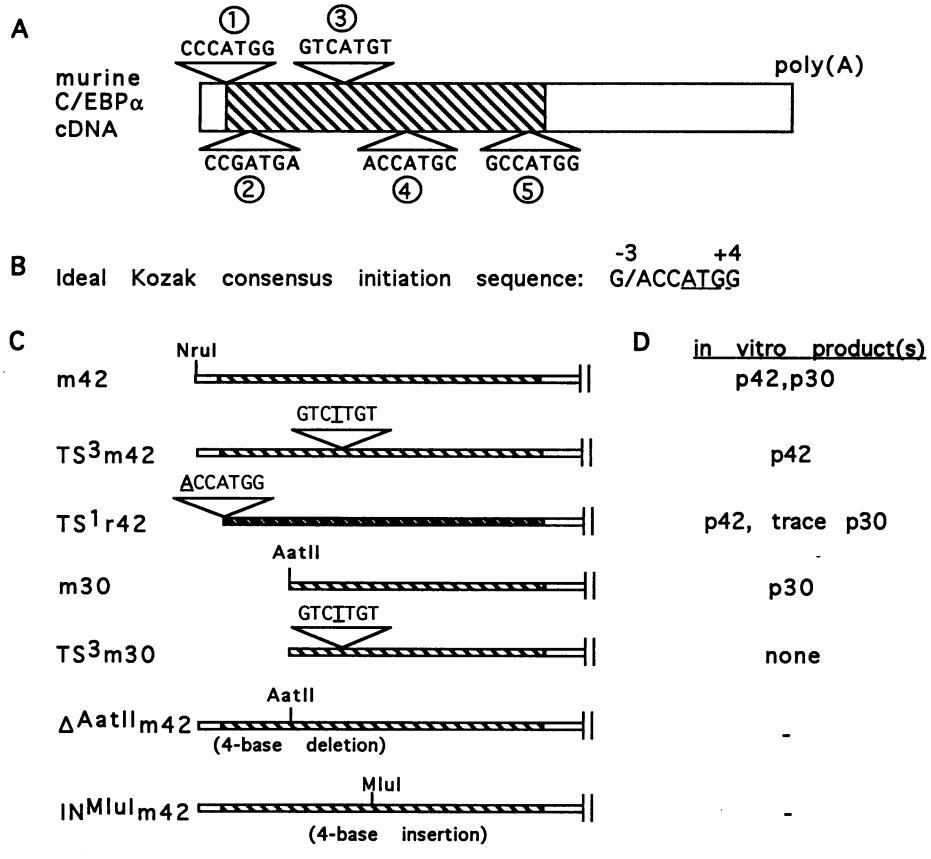

FIG. 2. wt and mutant translational start-site C/EBPα gene constructs and their cell-free translation products. (*A*) Mouse wt C/EBPα cDNA showing potential in-frame translational initiation site sequences. (*B*) An "ideal" Kozak initiation sequence. (*C*) Translational start sites in mouse wt C/EBPα cDNA (m42) and in mouse (m) and rat (r) mutated C/EBPα cDNAs. cDNAs were inserted into vectors either for *in vitro* transcription/translation or for expression in cells. The cDNA inserts encoded full-length 42-kDa (designated 42) or 5′-truncated 30-kDa (designated 30) C/EBPα, mutated (as shown) within the first (TS^1) or third (TS^3) in-frame translation start sites (or adjacent bases) or at specific restriction sites—i.e., the *Aat* II (Δ^{AatII}m42) or the *Mlu* I (IN^{MluI}m42) sites. Δ^{AatII}m42 contains a 4-base deletion between the second and third ATG and IN^{MluI}m42 contains a 4-base insertion (IN) between the third and fourth ATGs. Mutated bases are in boldface type and underlined. (*D*) *In vitro* translation products.

p30 exceeded that of p42 at all stages of development (i.e., from the late fetal stage to day 35 after birth). While the lowest levels of both forms were observed in fetal liver and the highest levels in day 35 liver, the p42/p30 ratio varied from ≈0.67 in fetal liver to a minimum of ≈0.2 on day 14 after which the ratio increased to 0.45 by day 35. The level of C/EBPα mRNA remained virtually unchanged between birth and day 35.

Since both immunoreactive p42 and p30 were present in cell and tissue types that express a single species of C/EBPα mRNA, we considered the possibility that p42 and p30 were alternative translation products of the same message. The sequence of mouse C/EBPα mRNA (13) contains five in-frame AUG codons within the translated region (Fig. 2*A*), the first four of which possess unfavorable contexts ("Kozak" sequences, Fig. 2*B*) for translational initiation (21, 22). Hence, leaky ribosomal scanning (22) of the first two AUG codons in the C/EBPα message could lead to translation initiated at the third in-frame AUG codon to produce the 30-kDa translation product.

To determine whether p30 is an alternative translation product, *in vitro* translation experiments were performed. Messages were generated by *in vitro* transcription of full-length or truncated wild-type (wt) or mutated C/EBPα DNAs (Fig. 2*C*). *In vitro* translation (Fig. 2*D*) of wt C/EBPα message lacking about one-third of the 3′ untranslated sequence generated equal amounts of p42 and p30 (m42, Fig. 2*C*). Mutation of a single base (A → U) in the third in-frame AUG codon abolished formation of p30 but had no effect on formation of p42 (TS^3m42, Fig. 2*C*). By changing the sequence flanking the first AUG codon in the full-length message to an ideal Kozak sequence (i.e., ACCAUGG) and eliminating the G+C-rich 5′ untranslated region, the p42 translation product increased markedly relative to p30 (TS^1r42, Fig. 2*C*), as did translation rate. A 5′ truncation of the wt C/EBPα message to a site (+176 bp) upstream of the third AUG codon, thereby removing the first two AUG codons, gave rise exclusively to the 30-kDa translation product (m30, Fig. 2*C*). Furthermore, a single-base mutation (A → U) in the AUG start codon of the truncated message eliminated the 30-kDa translation product (TS^3m30, Fig. 2*C*).

It was important to verify that translation products of wt and mutated C/EBPα messages detected *in vitro* were the same in the intact cell. Transient transfection of 3T3-L1 preadipocytes with an expression vector encoding the wt message gave rise to similar amounts of p42 and p30 (lane 4, Fig. 3*A*). By changing the sequence adjacent to the first AUG codon of the wt message to an ideal Kozak sequence and deleting the 5′ untranslated region, the relative level of expression of p42 increased markedly (lane 2, Fig. 3*A*). This modification favored initiation at the first AUG codon, at the expense of initiation at the third AUG codon. Deletion of the first two AUG codons led to initiation at the third in-frame AUG giving rise exclusively to the 30-kDa product (lane 3, Fig. 3*A*).

As transient transfection efficiency of 3T3-L1 cells was quite low, further studies were carried out with 293 cells, which exhibit a much higher transfection efficiency and expression level (23). The relative levels of expression of p42 and p30 by 293 cells transfected with expression vectors containing m42, TS^1r42, and m30 were similar to those by 3T3-L1 cells (compare Fig. 3 *A*, lanes 2–4, with *B*, lanes 4, 5, and 2). Additionally, mutation (A → T) of the third in-frame ATG codon in the wt C/EBPα gene eliminated expression of p30 in 293 cells (lane 3, Fig. 3*B*).

Finally, expression of the entire wt C/EBPα gene and two 4-base "out-of-frame" C/EBPα gene mutants (Δ^{AatII}m42 contains a 4-base deletion, and IN^{MluI}m42 contains a 4-base insertion; see Fig. 2*B*) were compared in 293 cells. As the 4-base deletion and the 4-base insertion were in the coding

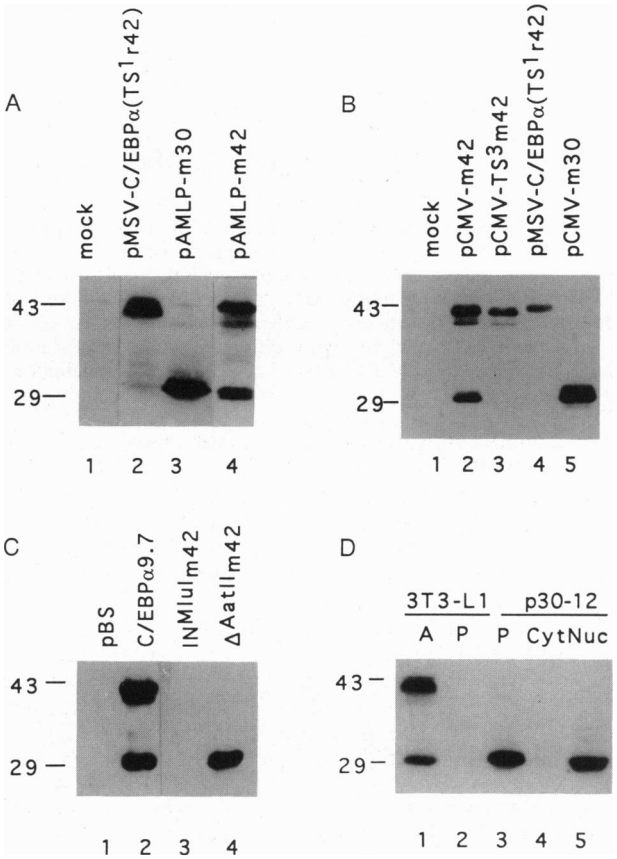

FIG. 3. Translation products of wt and mutated C/EBPα messages by intact cells. wt and mutant C/EBPα expression vectors were transiently (*A–C*) or stably (*D*) transfected either into 3T3-L1 preadipocytes (*A* and *D*) or 293 cells (*B* and *C*). After 48 h proteins (*A*, 200 μg; *B* and *C*, 50 μg; *D*, 200 μg of cell extract and 100 μg of nuclear and cytoplasmic fractions) were resolved by SDS/PAGE and subjected to Western blot analysis with internal anti-C/EBPα peptide antibody. (*A*) pMSV-C/EBPα(TS^1r42) at 20 μg, pAMLP-m30 at 20 μg, or pAMLP-m42 at 40 μg was transfected. (*B*) pCMV-m42, pCMV-TS^3m42, pCMV-m30, or pCMV-TS^3m30 at 20 μg or pMSV-C/EBPα(TS^1r42) at 10 μg was transfected. When pCMV vectors were employed, 2 μg of pRSV-T (a tumor antigen expression vector) was cotransfected to promote plasmid replication. (*C*) pBS (lane 1), pC/EBPα9.7 (lane 2), and C/EBPα mutants IN^{MluI}m42 (lane 3) and Δ^{AatII}m42 (lane 4) at 10 μg was transfected. The pC/EBPα9.7 vector (pBS9.7) contains the entire intronless mouse C/EBPα gene including 5.6 kb of 5′ flanking and ≈1 kb of 3′ flanking sequences. (*D*) A representative stable cell line, p30-12 (pAMLP-m30, transgene copy number = 5), that expresses a high level of $p30^{C/EBP\alpha}$ was subjected to subcellular fractionation into nuclei (lanes Nuc) and cytoplasm (lanes Cyt) prior to differentiation. Unfractionated wt 3T3-L1 preadipocytes (lanes P) and adipocytes (lanes A) served as controls. Molecular masses in kDa are shown.

region of the message, translation 3′ to these sites would be out-of-frame and, if completed, would not produce a protein product recognized by the antibodies whose epitopes reside in the C-terminal one-fourth of the molecule. However, since the deletion in the Δ^{AatII}m42 mutant lies between methionine codons 2 and 3, translation initiated at codon 3 would be expected to give rise to the p30 product recognized by the antibody. wt C/EBPα (C/EBPα9.7) gave rise to both p42 and p30 (lane 2, Fig. 3*C*), whereas the Δ^{AatII}m42 mutant gene gave rise exclusively to p30 (lane 4, Fig. 3*C*) in 293 cells. The IN^{MluI}m42 mutant did not generate a translation product recognized by the antibody (lane 3, Fig. 3*C*). All three transfected constructs gave rise to similar levels of message detected by Northern blot analysis (results not shown). These

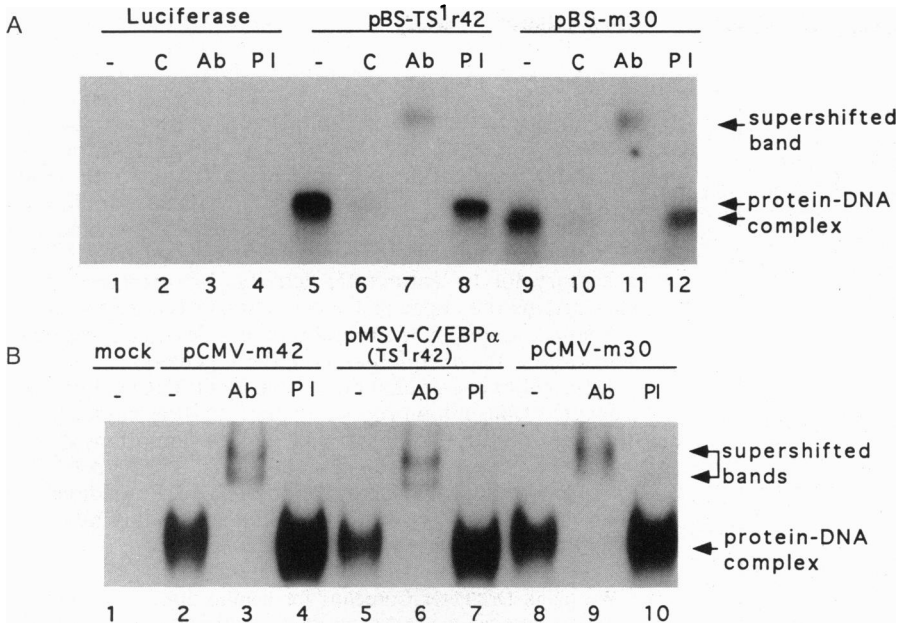

FIG. 4. Gel-retardation analysis of C/EBP binding site oligonucleotides. (*A*) *In vitro* transcription/translation of pBS-TS^{1}r42 and pBS-m30 as in Fig. 3. Gel-retardation analysis was with a synthetic labeled double-stranded oligonucleotide corresponding to the C/EBP binding site (nt −149 to −130) of the mouse 422(aP2) gene promoter (2). Before binding, translated proteins were incubated with labeled probe alone (lanes −), with an excess of unlabeled probe (lanes C), with internal peptide antibody against C/EBPα (lanes Ab), or with preimmune serum (lanes PI). Complexes were resolved by gel electrophoresis. (*B*) 293 cells were transfected with pCMV-m42, pCMV-m30 as in Fig. 3), or pMSV-C/EBPα(TS^{1}r42). After 48 h nuclear extracts were prepared, and gel-retardation assays were performed using oligonucleotides corresponding to the C/EBP binding site (nt −198 to −176) in the C/EBPα gene (13).

results showed that p30 is an alternative translation product of the wt C/EBPα message and, therefore, will subsequently be referred to as p30$^{C/EBP\alpha}$.

Recognition that C/EBPα is antimitotic (24) provided an explanation for the inability of many investigators to establish stable 3T3-L1 cell lines that express p42$^{C/EBP\alpha}$. Numerous attempts in our laboratory to stably transfect 3T3-L1 preadipocytes with pMSV-C/EBPα, an expression vector for p42$^{C/EBP\alpha}$, have been unsuccessful. To determine whether p30$^{C/EBP\alpha}$ also possesses antimitotic activity, a p30$^{C/EBP\alpha}$ expression vector (pAMLP-m30) was cotransfected with pSV2Neo into 3T3-L1 preadipocytes. More than 30 G418-resistant cell lines were obtained of which 12 were propagated for Western blot analysis. Most of these cell lines expressed p30$^{C/EBP\alpha}$ at significant levels. Two of these (i.e., p30-9 and p30-12) that harbored one and five copies of the transgene, respectively, were studied further. Like p42$^{C/EBP\alpha}$, p30$^{C/EBP\alpha}$ is localized exclusively in the nuclear fraction (Fig. 3*D*).

The fact that numerous cell lines that express p30$^{C/EBP\alpha}$ exhibit no evident change in their doubling time indicates that the p30$^{C/EBP\alpha}$ isoform lacks antimitotic activity.§ Cell lines that express p30$^{C/EBP\alpha}$ at a high level exhibit density-dependent growth inhibition and reach the same cell density at confluence as undifferentiated 3T3-L1 preadipocytes. These cell lines failed to undergo significant adipose conversion when maintained at confluence for 7–10 days without exogenous inducers of differentiation (9). Thus, constitutive expression of p30$^{C/EBP\alpha}$ is insufficient to trigger differentiation. It was observed that adipose conversion and the expression of endogenous p42$^{C/EBP\alpha}$ and 422(aP2) protein by 3T3-L1 cells transfected with a p30$^{C/EBP\alpha}$ expression vector occur about 1 day earlier when subjected to the standard differentiation protocol (results not shown). Nevertheless, the maximal extents of adipose conversion and the cellular levels of 42-kDa C/EBPα and 422(aP2) protein were indistinguishable in untransfected and transfected 3T3-L1 adipocytes 5–6 days after the induction of differentiation. Hence, constitutive expression of p30$^{C/EBP\alpha}$ only marginally accelerates differentiation.

§ The possibility was considered the p30$^{C/EBP\alpha}$ might "neutralize" the antimitotic effect of p42$^{C/EBP\alpha}$ through heterodimer formation. Attempts to generate cell lines that constitutively express p42$^{C/EBP\alpha}$ by transfection of the p30-12 cell line (which constitutively expresses p30$^{C/EBP\alpha}$) with pMSV-C/EBPα (TS^{1}r42) were unsuccessful.

The ability of p30$^{C/EBP\alpha}$ to interact with the C/EBP binding site in the 422(aP2) gene promoter was verified in gel-retardation experiments. Both p30$^{C/EBP\alpha}$ and p42$^{C/EBP\alpha}$, generated by *in vitro* translation (Fig. 4*A*) or by transfection of 293 cells with pCMV-m42, pMSV-C/EBPα(TS^{1}r42), or pCMV-m30 (results not shown), form complexes with a C/EBP binding site oligonucleotide. Complex formation was prevented with unlabeled binding site oligonucleotide. Moreover, both complexes were super-shifted by antibody against C/EBPα but not by preimmune serum. Similar results were obtained (Fig. 4*B*) with a labeled oligonucleotide corresponding to the C/EBP binding site in the C/EBPα gene promoter (13). These findings show that p30$^{C/EBP\alpha}$ binds to the C/EBP binding sites within the promoters of two differentially expressed adipocyte genes.

Similar to p42$^{C/EBP\alpha}$, p30$^{C/EBP\alpha}$ transactivates reporter gene expression directed by the promoters of a number of adipocyte genes. As shown in Fig. 5*A*, p30$^{C/EBP\alpha}$ expressed by 3T3-L1 cell lines harboring the pAMLP-m30 vector strongly transactivates CAT expression by the wt 422(aP2) gene promoter but not a 422(aP2) promoter [i.e., ΔLS(-122/-143)], in which the C/EBP binding site is mutated. Similar results were obtained in experiments in which both the pAMLP-m30 vector and p-248[422(aP2)]-CAT were transiently cotransfected into 3T3-L1 preadipocytes (results not shown).

To ascertain whether p30$^{C/EBP\alpha}$ is capable of transactivating expression mediated by the C/EBPα gene promoter, cell lines stably transfected with pAMLP-m30 were transiently transfected with p-1.5kb(C/EBPα)CAT, p-5.6kb(C/EBPα)CAT, or a promoterless CAT reporter gene construct. As shown in Fig. 5*B*, the p30-9 and p30-12 cell lines, which harbor 1 and 5 copies of the p30$^{C/EBP\alpha}$ expression vector, respectively, expressed markedly higher levels of CAT activity driven by the C/EBPα gene promoter than the control cell line. Similar results were obtained with 293 cells.

DISCUSSION

In this report we show that a 30-kDa isoform of C/EBPα, expressed by 3T3-L1 adipocytes and by adipose and liver tissue is an alternative translation product of the C/EBPα message. Expression vectors encoding full-length or 5′-truncated C/EBPα messages lacking the first two in-frame AUG codons led to expression of p42$^{C/EBP\alpha}$ and p30$^{C/EBP\alpha}$ or exclusively p30$^{C/EBP\alpha}$, respectively. Similar findings were

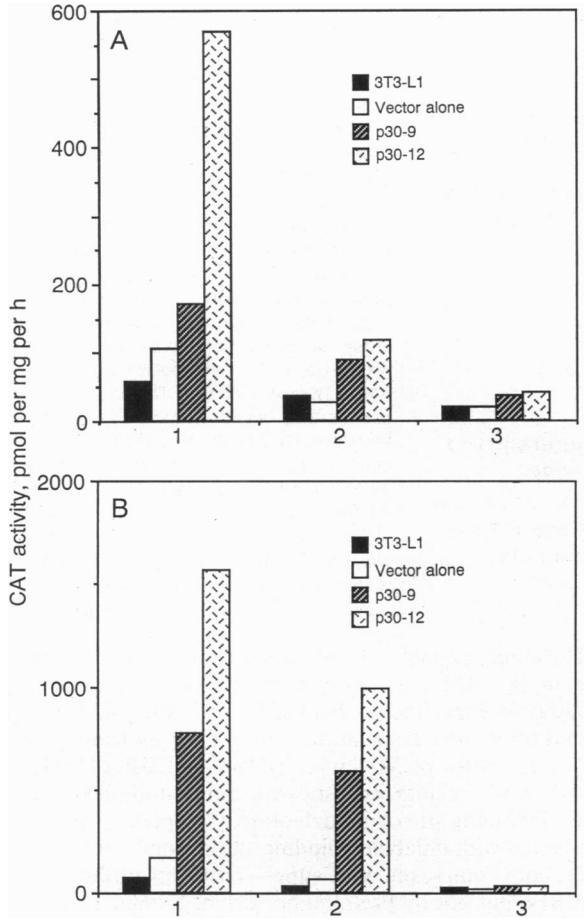

FIG. 5. Transactivation of the 422(aP2) and C/EBPα gene promoters by $p30^{C/EBP\alpha}$. wt 3T3-L1 preadipocytes or cell lines stably transfected with pMT2 or pAMLP-m30 (p30-9 or p30-12) were transiently transfected as follows. (*A*) p-248CAT [containing the 422(aP2) proximal promoter, bars 1], pΔLS(−122/−143)CAT in which a *Bam*HI linker sequence replaces the C/EBP site in p-248CAT (bars 2), or the pBLCAT3′ vector lacking an insert (19) (bars 3), each at 10 μg. (*B*) p-1.5kbC/EBPαCAT (bars 1) or p-5.6kbC/EBPαCAT (bars 2), which contain 1.5 kb or 5.6 kb, respectively, of the 5′ flanking sequence of the mouse C/EBPα gene, or pBlueCAT (bars 3), which lacks an insert.

obtained in cell-free translation experiments. By mutating the third in-frame AUG codon (to UUG) of the message, $p42^{C/EBP\alpha}$ was shown to be the sole product, proving that $p30^{C/EBP\alpha}$ results from initiation at the third in-frame AUG rather than by proteolysis of $p42^{C/EBP\alpha}$. Finally, deleting 4 bases between the second and third AUG codons, thereby changing the reading frame 3′ to the mutation, gave rise only to $p30^{C/EBP\alpha}$. Thus, despite shifting the reading frame 5′ to the p30 translation start site, proper ribosomal initiation occurred (at the third AUG) giving rise to $p30^{C/EBP\alpha}$. Since the nucleotide sequence bracketing the first two AUG codons of the wt C/EBPα message is unfavorable for translational initiation (21), we suggest that these codons are frequently by-passed due to leaky ribosomal scanning (22), allowing alternative initiation at the third AUG. The third AUG need not be in-frame, since the 4-base frame-shift mutation between the second and third AUG codons gives rise to $p30^{C/EBP\alpha}$ (Fig. 3*C*).

In addition to transcriptional activation of adipose genes during preadipocyte differentiation, C/EBPα may act to suppress clonal expansion as adipocytes enter the terminally differentiated state (1). This is suggested by the fact that C/EBPα is antimitotic in 3T3-L1 cells (24) and expression of C/EBPα occurs at the point in the differentiation program when clonal expansion ceases (1). This explains why it has not been possible to constitutively express full-length C/EBPα (i.e., $p42^{C/EBP\alpha}$) in 3T3-L1 cells. In contrast, we found that transfection of 3T3-L1 preadipocytes with a $p30^{C/EBP\alpha}$ expression vector did not interfere with cell proliferation or doubling time. These results suggest that amino acid sequence within the N-terminal 12 kDa of $p42^{C/EBP\alpha}$ is necessary for its antimitotic activity. Nevertheless, $p30^{C/EBP\alpha}$ retains the capacity to transactivate reporter gene expression directed by the promoters of at least two adipocyte genes—i.e., the 422(aP2) and C/EBPα genes.

Descombes and Schibler (25) have identified an alternative truncated translation product of the C/EBPβ message, as a dominant-negative inhibitor that may be important in controlling C/EBP_β activity. Since the $p42^{C/EBP\alpha}/p30^{C/EBP\alpha}$ ratio changes during differentiation of 3T3-L1 preadipocytes and during hepatic development, changes in this ratio may have functional consequences.

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