Inhibition of CCAAT/Enhancer-binding Protein α and β Translation by Upstream Open Reading Frames*

(Received for publication, December 12, 1997)

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The transcription factor CCAAT/enhancer-binding protein (C/EBP) α is a bZIP transcription factor whose expression is restricted to specific cell types. Analysis of C/EBPα mRNA and protein levels in various mammalian cells indicates that expression of this gene is controlled both transcriptionally and post-translationally. We report here that C/EBPα translation is repressed in several cell lines by an evolutionarily conserved upstream open reading frame (uORF), which acts in cis to inhibit C/EBPα translation. Mutations that disrupt the uORF completely abolished translational repression of C/EBPα. The related C/EBPβ gene also contains an uORF that suppresses translation. The length of the spacer sequence between the uORF terminator and the ORF initiator codon (7 bases in all c/ebpα genes and 4 bases in c/ebpβ homologs) is precisely conserved. The effects of insertions, deletions, and base substitutions in the C/EBPα spacer showed that both the length and nucleotide sequence of the spacer are important for efficient translational repression. Our data indicate that the uORFs regulate translation of full-length C/EBPα and C/EBPβ and do not play a role in generating truncated forms of these proteins, as has been suggested by start site multiplicity models.

The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; ORF, open reading frame; uORF, upstream ORF; PCR, polymerase chain reaction; wt, wild-type.

* This work was supported in part by NCI, Department of Health and Human Services, under contract with Advanced BioScience Laboratories. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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In view of the important role of C/EBPα in cellular differentiation, the mechanisms that regulate the tissue specificity and developmental timing of C/EBPα expression are of considerable interest. Surveys of C/EBPα expression show that whereas C/EBPα transcripts are present at varying levels in many mammalian tissues and cell lines (Refs. 11 and 12; see also Fig. 1), C/EBPα protein occurs in only a subset of these cell types. Cells in which the C/EBPα protein has been detected include differentiated hepatocytes (1, 12), adipocytes (13), intestinal epithelial cells (14), myelomonocytic progenitor cells (15), ovarian follicles (16), and type II cells of the lung (17). The disproportionate levels of C/EBPα mRNA and protein observed in certain cells indicates that post-transcriptional regulation plays a role in restricting C/EBPα expression.

In this study, we have investigated the molecular mechanism underlying post-transcriptional control of C/EBPα expression. We demonstrate that C/EBPα translation is inhibited in transfected cell lines by a short, evolutionarily conserved upstream open reading frame (uORF) located 7 bases upstream of the C/EBPα ORF (18). uORF-mediated repression was also observed for the related C/EBP family member C/EBPβ. The uORF repressed C/EBPα translation by a cis-acting mechanism, and inhibition was overcome by mutations that inhibit translation of the uORF. Certain changes in the length and sequence of the uORF-ORF spacer region also caused translational derepression. Interestingly, we did not detect truncated C/EBPα and C/EBPβ isoforms in these experiments. These findings may necessitate a re-evaluation of models proposing that initiation at internal start sites produces truncated forms of C/EBP proteins (19–22).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa, 3T3-L1, and 293 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker, Inc.) supplemented with 10% fetal bo-
vire serum (Hyclone Laboratories) in the presence of kanamycin, streptomycin, and penicillin. Differentiation of THP-1-L1 cells was performed as described previously (13). HepG2 cells were cultured as described (12). HeLa and 293 cells (30–40% confluent) were transiently transfected in 10-cm plates by standard calcium phosphate coprecipitation procedures using 2.5 μg of test plasmid, 25 μg of internal control plasmid (pMEK) for a total of 20 μg of DNA/plate. The cells were incubated in precipitate for 18–24 h, washed twice with unsupplemented Dulbecco’s modified Eagle’s medium, and then incubated in complete medium for an additional 18–24 h prior to harvesting. 293 cells were transfected with 2.5 μg of test plasmid, 25 μg of internal control plasmid (pMEK), and 5 μg of carrier plasmid (pMEX) plasmid. The cells were incubated in DNA precipitate for 12–15 h, washed twice with unsupplemented Dulbecco’s modified Eagle’s medium, and incubated in complete medium for an additional 12–15 h prior to harvesting. For the experiment of Fig. 7, 10-cm plates of HeLa cells were transfected using 2.5 μg of DNA and 10 μl of DMRIE-C reagent (Life Technologies, Inc.) according to the supplier’s recommendations. Duplicate plates were harvested after 48 h for RNA and mRNA, respectively. For all transfection assays, at least two independent isolates of each recombinant plasmid were tested, and the transfections were repeated independently at least three times.

For stably transfected HeLa cells, transfection conditions were identical to those described above, except that 20 μg of test plasmid were used in addition to 2.5 μg of the selectable marker (pMEX.neo). After ~48 h, cells were split into fresh 10-cm plates and allowed to recover for an additional 48 h. Cells were then fed with complete medium supplemented with G418 (0.3–0.5 units/ml; Life Technologies, Inc.). Pools of ~20–50 independent neo’ transfectants were obtained and analyzed.

**Plasmid Constructions**—Plasmids used for constitutive expression of C/EBPα and C/EBPβ were pMEX-C/EBPα and pMEX-CRP2 (hereafter denoted pMEX-C/EBPβ), respectively (12). C/EBPα expression plasmids containing wild-type and mutant 5’ promoter regions were constructed as follows. PCR was used to introduce both a restriction site immediately upstream of the C/EBPα 5’-leader and mutations within the 5’-leader. PCR (30 cycles of denaturation at 94 °C for 40 s and annealing/extension at 50 °C for 1 min, 20 s) was carried out using the Gene-Amp kit (Perkin-Elmer) under the conditions recommended by the supplier, except that 5% dimethyl sulfoxide was included to aid template denaturation. All PCR-derived fragments were sequenced using Sequenase T7 DNA polymerase and a 7-deaza-dGTP sequencing kit (U. S. Biochemical Corp.) to verify that no extraneous mutations were introduced. Wild-type and mutant 5’-leader constructs of C/EBPα were cloned using SpiI (a site in the pMEX vector polylinker) at the 5’-end and XhoI, which overlaps the c/ebp initiation codon, at the 3’-end. Oligonucleotides used to generate wild-type and mutant C/EBPα constructs were as follows: 5’-oligonucleotide for the wild type and all other mutants, 5’-GATCTCCATGGGAGTTAG; uORF Sp2 5’-GAGCTCCTATGGGGAGTTAG; uORF Sp2 5’- AGCTCCTATGGGGAGTTAG; uORF Sp3 5’-GAGCTCCTATGGGGAGTTAG; uORF Sp3 5’-GATCTCCTATGGGGAGTTAG; and uORF Sp3 5’-GATCTCCTATGGGGAGTTAG. The spacer 5-200 construct was made as follows. The uORF-C/EBPα construct was digested with Stul and, and the linear vector fragment was treated with calf intestinal phosphatase (Boehringer Mannheim) to prevent religation. A 217-base pair fragment was obtained from an MspI digestion of the plasmid pBR322. The ends were made blunt using T4 DNA polymerase (Boehringer Mannheim), and the fragment was ligated to the c/ebp untranslated region vector. The sequence of the fragment double-stranded oligonucleotide was as follows (Sequence 1).

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5’-TC GAC GAG TAG AAG GAC GAC GAT AAG TGA GGC-3’
3’-GTC ATG TTC CTC CTG TTC ACT CGGTTCG-5’
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**RNA Extraction and Northern Blot Analysis**—Total cellular RNA was isolated from transfected cells using RNA STAT-60 (TEL-TEST, Inc.) or Trizol reagent (Life Technologies, Inc.). RNA (15 μg) was separated by electrophoresis using formaldehyde-containing 1.2% agarose gels and then transferred to GeneScreen membrane (DuPont) according to the manufacturer’s instructions. Membranes were hybridized overnight at 42 °C with [α-32P]dCTP-labeled DNA probes. The hybridization solution consisted of 50% formamide, 5° Denhardt’s solution, 5× SSPE (750 mM NaCl, 50 mM NaH2PO4, and 5 mM EDTA), 1% SDS, and 0.1 mg/ml salmon sperm DNA (27). Following incubation, blots were washed with 0.1% SDS and 0.5× SSPE at 65 °C and then exposed to x-ray film (Eastman Kodak Co.). DNA probes for analysis of RNA from transfected cells by Northern blotting were prepared by isolating the following fragments: c/ebpα, 350-base pair PstI-SacI fragment from the 3’-end of the coding region of the murine c/ebpα gene; FLAG, 170-base pair XhoI-HpaI fragment from pMEX-C/EBPα-F (contains the FLAG epitope); C/EBPβ 3’-untranslated from pMEX-C/EBPβ; and C/EBPβ 5’-untranslated from pMEX-C/EBPβ. HindIII fragment excised from the plasmid β2000 (28), and cyclolin, as described by Danielson et al. (29). DNA fragments were labeled to high specific activity with [α-32P]dCTP using the Prime-It II kit (Stratagene).

**Cell Extracts and Western Blotting**—Whole cell extracts were prepared by lysing cells directly in radiomimic precipitation assay buffer (30). Nuclear extracts were prepared as described by Lee et al. (31), except that Buffer A also contained 0.1% Nonidet P-40 (Sigma) and 5 μg/ml leupeptin (Boehringer Mannheim), and nuclear extracts were not dialyzed against Buffer D. Nuclear extracts from tissue samples were prepared according to the method of Gorski et al. (32). Protein concentrations were determined either by the Bio-Rad protein assay or by estimating protein following SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Samples were denatured in sample buffer at 85 °C and then electrophoresed on 10% SDS-polyacrylamide gels (33). Molecular weights of the proteins were determined by comparison to Rainbow molecular weight markers (Amersham Pharmacia Biotech). SDS-polyacrylamide gels were transferred to nitrocellulose (Schleicher & Schuell) by electrophotography. Blots were blocked in 5% nonfat dry milk and Tris-buffered saline, pH 7.6, for 2–6 h at room temperature. Blots were then incubated at room temperature with the antigen-antibody complex was visualized using the Amersham Pharmacia Biotech enhanced chemiluminescence kit. Pan-ERP α-anti-serum was used as described (26). The FLAG epitope was detected using the anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.).
Regulation of C/EBPα and C/EBPβ Translation

**RESULTS**

Post-transcriptional Control of C/EBPα Expression—Fig. 1 shows a survey of C/EBPα mRNA and protein expression in several mammalian cell lines and murine tissues. C/EBPα transcript levels were highest in liver and differentiated 3T3-L1 adipocytes, two cell types in which the C/EBPα protein is abundant. HeLa and HepG2 cells and kidney tissue contained lower but significant levels of C/EBPα mRNA, whereas they lacked detectable C/EBPα protein. Liver contained 5–10-fold more C/EBPα mRNA than kidney (Fig. 1A), but at least 20-fold more C/EBPα protein, as estimated by serial dilution of the protein extract (Fig. 1C). The difference in protein levels is a minimum estimate since the C/EBPα signal in kidney extracts was below the level of detection. This disparity between mRNA and protein levels suggests that a post-transcriptional mechanism contributes to the regulation of C/EBPα expression.

Inhibition of C/EBPα Translation by a Conserved Upstream Open Reading Frame—A comparison of the 5' leaders of rat, mouse, human (34), bovine (35), chicken, and *Xenopus laevis c/ebpa* genes revealed the presence of a short (5-amino acid) conserved ORF (Fig. 2A) (18). This uORF is located precisely 7 nucleotides upstream of the C/EBPα initiation codon in all *c/ebpa* homologs. The evolutionary conservation of the uORF element and its location in the transcript suggest an important function in regulating C/EBPα expression. We suspected that the uORF might suppress translation of the C/EBPα ORF since many eukaryotic mRNAs lack upstream AUG codons, and the insertion of a single functional initiation codon upstream can severely inhibit translation from downstream AUG codons (reviewed in Refs. 36–39).

To investigate the potential role of the uORF in regulating C/EBPα translation, we constructed rat *c/ebpa* genes containing either wild-type or mutant uORF sequences (Fig. 2B) and inserted these into the eukaryotic expression vector pMEX. The mutations included alterations of the uORF initiator (ATG*) and terminator (TAA*) sequences and precise deletion of the uORF (∆uORF). The mutant genes were tested by transient transfection into HeLa cells, a cell line in which expression of the endogenous C/EBPα protein is repressed (Fig. 1). As an internal control, each construct was cotransfected with a constitutive C/EBPβ expression vector (pMEX-C/EBPβ). Transfected cell extracts were analyzed for C/EBPα and C/EBPβ expression by Western blotting using an antibody (pan-CRP) that recognizes both C/EBP proteins (26).

As shown in Fig. 2C (lane 3), a gene containing optimal translation initiation signals in place of the C/EBPα 5'-leader (pMEX-C/EBPα) was expressed at high levels in HeLa cells. In contrast, a construct bearing the wild-type 5'-leader (wt uORF-C/EBPα; lane 4) produced very low amounts of C/EBPα protein. However, the mutants uORFATG* (lane 5) and ∆uORF (lane 7) exhibited high levels of C/EBPα expression, comparable to pMEX-C/EBPα, whereas the uORF* termination codon mutant (uORF*TAA*; lane 6) did not express C/EBPα. The same set of mutations introduced into the murine *c/ebpa* gene gave identical results (data not shown). These findings indicate that translation of the uORF severely inhibits C/EBPα expression in HeLa cells.

To test whether translation initiates at the uORF AUG codon, we generated a construct in which the uORF was fused in frame to the C/EBPα ORF. This vector expressed high levels of a protein that was slightly larger than C/EBPα (Fig. 2C, lane 8). The fact that the fusion protein was strongly expressed from the uORF-ORF construct demonstrates that the uORF initiation codon is utilized efficiently in HeLa cells. Accordingly, the uORF initiation codon is in a favorable context for efficient initiation (40) in all species examined (Fig. 2A). Collectively, the data of Fig. 2C are consistent with a mechanism in which translation of the uORF cistron represses initiation from the downstream C/EBPα AUG codon. Several other eukaryotic genes are also translationally repressed by uORFs (reviewed in Ref. 37), including the gene encoding the Saccharomyces cerevisiae bZIP protein GCN4 (41).

To further establish that the uORF suppresses C/EBPα translation, we examined the expression of wild-type and mutant uORF constructs after stable transfection into HeLa cells. This approach enabled us to readily compare mRNA and protein expression from the engineered constructs. Because forced C/EBPα expression can cause cell growth arrest, we generated a functionally inactive protein by deleting the leucine zipper, thus preventing dimerization and DNA binding. To facilitate detection of the protein, we fused the FLAG epitope (42) to the C terminus, producing a hybrid designated C/EBPα-F. The

**Fig. 1.** C/EBPα expression is regulated at both the transcriptional and post-transcriptional levels. C/EBPα mRNA (A) and protein (B) levels were compared in several mammalian tissues and cell lines. A, total RNA (10 μg) from the indicated adult mouse tissues or cell lines was analyzed by Northern blotting. The blot was probed using a labeled DNA fragment from the mouse *c/ebpa* gene, stripped, and reprobed for β-actin mRNA. B, nuclear extracts prepared from the same cell lines and tissues were analyzed for C/EBPα protein expression. Protein concentrations were estimated by Comassie Blue-stained SDS-polyacrylamide gel electrophoresis analysis, and equivalent amounts of protein were assayed by Western blotting using a peptide antibody that recognizes an internal C/EBPα epitope (1). p42α is the full-length (42-kDa) C/EBPα protein, and p30α is a 30-kDa product proposed to arise from an internal initiation codon within the C/EBPα transcript (20, 21). C, shows the relative C/EBPα protein expression in liver and kidney tissue. Serial dilutions of liver nuclear extract (lanes 1–4) were analyzed by Western blotting and compared with undiluted kidney extract (lane 5). Diff., differentiated; Non-Diff., nondifferentiated.
C/EBPα-F plasmids were cotransfected with a selectable marker, and pools of transfectants were selected and analyzed for C/EBPα-F mRNA and protein expression. Again, the wild-type 5′-leader was found to severely suppress C/EBPα protein expression, and mutation of the uORF AUG codon eliminated this inhibitory effect (Fig. 3). In addition, the uORF-ORF fusion gene was efficiently translated. The differences in protein levels were not due to effects on mRNA expression since similar levels of C/EBPα-F mRNA were detected in each of the transfectant pools. The results further support the conclusion that translational repression of C/EBPα in HeLa cells is controlled by the uORF and confirm that transient transfection is a valid assay for assessing C/EBPα translational control.

To test whether uORF-mediated inhibition occurs in other cell types, we introduced the C/EBPα-F constructs into 293 cells (human embryonic kidney) by transient transfection. As shown in Fig. 4A, the wild-type uORF sequence again strongly reduced C/EBPα expression. Mutations that eliminate uORF translation (uORF(AGT*) and ΔuORF; lanes 5 and 7) reversed this inhibitory effect. The uORF terminator mutant, uORF-(TAA*) (lane 6), was completely repressed, whereas the uORF-ORF fusion construct was efficiently translated (lane 8). Equivalent levels of C/EBPα-F transcripts were produced in each transfection (Fig. 4B). These data corroborate the results obtained using HeLa cells and, together with similar results from HepG2 hepatoma cells (data not shown), demonstrate that uORF-dependent repression operates in a variety of cell types.

**uORF-mediated Translational Repression Is Exerted in**
cis—We next addressed the possibility that the uORF encodes a peptide product that inhibits C/EBPα translation in trans. The uORF(AGT*) mutant was transfected into HeLa cells together with a construct that contains the uORF but lacks the C/EBPα ORF. Transfecting increasing amounts of the uORF expression plasmid did not diminish expression either from uORF(AGT*)-C/EBPα-F or from the control, pMEX-C/EBPα-F (Fig. 5). Although we cannot directly assess synthesis of the uORF-encoded peptide in the cell, the high expression seen from the uORF-ORF fusion construct (Fig. 2C, lane 8) demonstrates that translation initiates efficiently at the uORF in HeLa cells. Thus, the inability of the uORF to repress C/EBPα translation when expressed from a separate transcript suggests that the uORF inhibits C/EBPα expression via a cis-acting mechanism.

The uORF-ORF Spacer Length and Nucleotide Sequence Are Critical for Translational Repression—The spacing between the uORF termination codon and the C/EBPα start site is exactly 7 bases in all c/ebpa homologs characterized (Fig. 2A), suggesting that the spacer length is an important feature for C/EBPα regulation. We examined whether mutations that increase or decrease the spacer length (Fig. 6A) affect translational repression in 293 cells. Insertion of 1–3 cytosines in the spacer did not abolish suppression of C/EBPα translation by the uORF (Fig. 6B, lanes 5–7). In contrast, removing a single cytosine caused a large increase in C/EBPα translation (Fig. 6C, lane 5), and deleting 2 or 3 of the 5 cytosines preceding the ORF also resulted in significant derepression (Fig. 6C, lanes 6 and 7). Thus, decreasing the spacing by 1 nucleotide renders the uORF unable to inhibit C/EBPα translation, demonstrating that the length and/or sequence of the spacer is critical for uORF-mediated repression.

Since small increases in spacer length did not interfere with translational repression (Fig. 6B), we next tested the effects of larger insertions in the spacer element. Spacing was increased by inserting 10 bases (Sp+10) or 10 bases containing a StuI restriction site (Sp+Stu) (Fig. 6A). C/EBPα expression from these constructs remained repressed (Fig. 6C, lanes 8 and 9). In addition, inserting 200 bases into the spacer (in both orientations) did not disrupt the repressive activity of the uORF (Fig. 6C, lanes 10 and 11). The lack of derepression for the larger insertions was unexpected since, in an independent study, reinitiation efficiency at downstream initiation codons was found to improve as the intercistronic distance was increased (43). While the repression observed with the Sp+200 mutant could be due to unforeseen inhibitory sequences within the DNA inserts, it is unlikely that such sequences would occur in both orientations of the insert.

We further investigated whether altering the nucleotide sequence of the spacer would modify the efficiency of repression. We first inspected the C/EBPα spacer for the presence of conserved nucleotides located upstream of the C/EBPα initiation (Kozak) sequence. A cytosine residue at nucleotide −4 relative to the ORF AUG codon was the only conserved base (Fig. 2A). We changed this nucleotide to adenine (Sp C−4→A) (Fig. 6A) as well as constructed a mutant in which nucleotide −4 and its 2 flanking bases were altered (Sp C−4→A) (Fig. 6B). Both of these mutations caused derepression of C/EBPα expression (Fig. 6C, lanes 12 and 13). Collectively, the results of Fig. 6 indicate that the uORF-ORF spacer length and nucleotide sequence are critical for full translational repression.

A Conserved uORF Regulates C/EBPβ Translation—Expression of another C/EBP family member, C/EBPβ, is also regulated post-transcriptionally. Descombes et al. (44) reported that C/EBPβ (or LAP (liver-enriched transcriptional activator protein) transcripts occur in liver, lung, spleen, kidney, brain, and testis, whereas the protein was detected only in liver. These results imply that a translational control mechanism suppresses C/EBPβ protein expression in certain cell types.
Inspection of the 5′-leader of the c/ebpβ gene revealed a conserved uORF located 4 bases upstream of the ORF (Fig. 7A) (22). As depicted in Fig. 7A, the C/EBPβ transcript contains four potential translation start sites (19). The first precedes the uORF and is in frame with the C/EBPβ ORF, the second corresponds to the uORF start site, and the third represents the C/EBPβ initiation codon. A fourth AUG codon forms a potential internal start site that may produce a truncated protein. Mutation of the first, third, and fourth initiation codons indicated that each potential in-frame start site could generate a polypeptide, which were designated FL-LAP (p38), LAP (p34), and LIP (p20), respectively (19). LAP (p34β) is the most common C/EBPβ isoform expressed in vivo (19).

Because of the evidence that C/EBPβ is translationally regulated, we investigated the possibility that the C/EBPβ uORF modulates p34 translation. We mutated the first in-frame AUG codon (ATG1*) and the uORF initiation codon (ATG2*), both individually and in combination (ATG1*/2*). The leucine zipper region was also replaced by the FLAG epitope to facilitate detection of the expressed proteins. Expression was analyzed in transiently transfected HeLa cells and was compared with constructs containing either the wild-type 5′-leader (wt C/EBPβ-F) or a canonical Kozak sequence in place of the 5′-leader region (pMEX-C/EBPβ-F). These experiments showed that the C/EBPβ 5′-leader inhibits expression of p34β (Fig. 7B, lane 3). Mutating the first AUG codon (ATG1*) had only minor effects on p34β expression (lane 4), and p38β was not significantly expressed from any of the constructs. However, altering the uORF initiation codon (ATG2*) caused a large increase in C/EBPβ expression (lane 5), and the double mutant (ATG1*/2*) also expressed high levels of p34β-F (lane 6). These data demonstrate that the C/EBPβ uORF exerts an inhibitory effect on translation of the ORF located immediately downstream.

Surprisingly, in these experiments, we did not detect LIP (p20β), which was predicted to be expressed from constructs containing the wild-type C/EBPβ 5′-leader (19). In addition, p30β, a putative alternative translation product of C/EBPα (20–22), was not produced by any of the C/EBPβ constructs transfected into HeLa or 293 cells (Figs. 3 and 4). These observations, which are discussed in greater detail below, suggest that the 5′-leader regions do not necessarily regulate translation initiation at internal AUG codons.

**DISCUSSION**

In this report, we have examined the molecular basis for post-transcriptional regulation of C/EBPα and C/EBPβ expression. Mutational analysis revealed that the uORF is a potent inhibitor of C/EBPα translation. The uORF initiation codon was readily recognized by the translational machinery, and mutations that abolished uORF translation disrupted repression of the C/EBPβ ORF. The repressive effect of the uORF on C/EBPα translation was not cell-specific since the same results were observed in several cell lines using both transient and stable transfection assays. Although repression was seen in multiple cell lines, there must be a mechanism to overcome repression imposed by the uORF in cells that express the C/EBPα protein abundantly, such as terminally differentiated hepatocytes and adipocytes.

C/EBPβ also contains a conserved uORF that represses translation (Fig. 7). This mechanism could control the induction of p34β translation in response to cell-specific or physiological cues. For example, the human homolog of C/EBPβ, NF-IL6 (45), was shown to be translationally regulated in the pulmonary alveolar epithelial cell line A549 (46). Protein expression from the NF-IL6 transcript was induced upon infection of the cells with respiratory syncytial virus. Enhanced...
Regulation of C/EBPα and C/EBPβ Translation

Fig. 7. The c/ebpβ gene transcript contains a conserved uORF that represses C/EBPβ translation in HeLa cells. A, sequence comparison of 5′-leader regions from several c/ebpβ homologs. A schematic diagram of the C/EBPβ transcript is shown at the top, depicting the relative size and proximity of the uORF to the ORF. The two possible activator proteins resulting from alternative initiation codons (19) are designated p38β and p34β. The putative start site for LIP (p20β) is also indicated. References for the sequences are as follows: rat (12), mouse (62), human (45), chicken (63), and bovine (48). We suggest that induction of C/EBPβ expression in each of these cases involves derepression of uORF-mediated translational inhibition.

The truncated forms of C/EBPα and C/EBPβ (p30α and p20β/LIP, respectively) have been hypothesized to result from alternative translation initiation (19–22). It has been proposed that internal initiation within the C/EBPα or C/EBPβ ORFs occurs by a mechanism that requires the 5′-leader region. The resulting truncated proteins contain a DNA-binding domain, but lack an N-terminal activation domain. LIP, which lacks activating sequences altogether, has been proposed to function as an inhibitory isoform of C/EBPβ. Internal initiation in the C/EBPα and C/EBPβ mRNAs is believed to arise either from “leaky scanning” (19–21) or from termination after uORFs and reinitiation at downstream AUG codons (22, 50).

Our results do not support the hypothesis that C/EBPα and C/EBPβ translation reinitiates at downstream AUG codons within the ORF. The presence of the wild-type C/EBPα 5′-leader diminished translation of p42α (Figs. 3–5), but no truncated proteins such as p30α were produced from any of the C/EBPα constructs. The absence of truncated proteins was not a function of the cell type since these proteins were not observed in any cell line tested, including HeLa, 293, HepG2, and undifferentiated 3T3-L1 cells (data not shown). Similarly, truncated proteins were not expressed from the C/EBPβ constructs (Fig. 7). These results were consistently observed with two different antibodies (pan-CRP and anti-FLAG M2), indicating that antibody specificity does not account for the inability to detect p30α or p20β/LIP. Rather, we have detected only p30α or p20β/LIP in nuclear cell extracts from transfected cells under certain cell lysis conditions. Specifically, the addition of detergents (Nonidet P-40) and the protease inhibitor leupeptin to the lysis buffer prevented the appearance of truncated proteins. The level of endogenous p30α protein observed in differentiated 3T3-L1 cells (Fig. 1B, lane 4) could also be diminished or eliminated by altering the cell lysis protocol. In addition, we have found that a calpain protease cleaves recombinant C/EBPβ to generate a product that is indistinguishable in size from LIP. Collectively, our observations suggest that truncated forms of C/EBPα and C/EBPβ may arise in many cases from proteolysis rather than translation initiation. We propose that the function of C/EBPα and C/EBPβ uORFs is to regulate initiation at the ORFs immediately downstream and not to generate truncated proteins by internal initiation.

Mutations designed to test the mechanism of uORF repres-

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4 M. Baer, A. J. Lincoln, E. Sterneck, and P. F. Johnson, unpublished data.
sion showed that the uORF-ORF spacer is critical for inhibition of C/EBPα translation. Removal of 1–3 cytosine residues significantly decreased translational repression, especially when a single cytosine was deleted. One explanation for this result is that the C/EBPα spacer sequence dictates the efficiency of ribosome release after uORF termination. Support for this notion comes from studies of the yeast gene gnd gene, which encodes a transcriptional activator that regulates the synthesis of several biosynthetic genes in response to amino acid starvation. Gcn4 translation is controlled by four uORFs within the 5′-leader and is regulated primarily by the first and fourth uORFs (41). uORF1 alone inhibits Gcn4 translation by ~50% and is also required for translational derepression in response to amino acid starvation, whereas uORF4 constitutively represses translation of Gcn4. The sequences surrounding the termination codons of both uORFs are critical for regulation of Gcn4 translation (51). Sequences downstream of the uORF1 terminator are required for reinitiation at uORF4 when amino acids are plentiful or at Gcn4 under starvation conditions. A rare proline codon at the 3′-end of uORF4 and sequences just downstream of the terminator appear to be necessary for efficient ribosome release following termination, thereby preventing reinitiation at downstream AUG codons. Thus, sequences 3′ of the uORF1 terminator favor reinitiation downstream, whereas those 3′ of uORF4 favor ribosome release.

C/EBPα translational repression could also involve ribosome release after uORF termination, resulting in weak reinitiation at the C/EBPα AUG codon. Spacer deletions and nucleotide substitution mutants that up-regulate C/EBPα expression may function by decreasing the efficiency of uORF termination/release. An alternative possibility is that these mutations increase initiation efficiency at the C/EBPα start site. However, this explanation seems less likely since the sequence context of the initiation codon should be the predominant factor dictating the efficiency of initiation (40, 52, 53), and the mutations were designed to minimize disruption of the C/EBPα Kozak sequence. In addition, mutants in which uORF translation is disrupted express high levels of C/EBPα, showing that the ORF AUG codon is in a favorable sequence context for initiation. The spacer length may also affect the efficiency of reinitiation, although the derepression seen with spacer deletion mutants could be explained by the removal of nucleotides required for efficient ribosome release. Nevertheless, the stringent conservation of uORF-ORF spacing in c/ebpα and c/ebpβ genes argues that spacer length 

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#### Acknowledgments

We are grateful to E. Sterneck, R. Schwartz, and R. Kirken for critical reading of the manuscript and L. Sewell for expert assistance. We also thank M. Powers for synthesis of oligonucleotides and C. Weinstock and H. Marusiak for help with preparation of the manuscript.

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J. Biol. Chem. 1998, 273:9552-9560.
doi: 10.1074/jbc.273.16.9552

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