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Evidence for Posttranscriptional Regulation of C/EBPα and C/EBPβ Isoform Expression during the Lipopolysaccharide-Mediated Acute-Phase Response†

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The mRNAs of the CCAAT/enhancer-binding *trans*-activator proteins (C/EBPα and C/EBPβ) serve as templates for the differential translation of several isoforms which have specific transcriptional regulatory functions. By using an oligonucleotide corresponding to the C/EBP binding site of the mouse α_1-acid glycoprotein promoter, we detected multiple forms of C/EBPα and C/EBPβ proteins in the mouse liver that have DNA-binding activity. By using specific antisera, we detected C/EBPαs with molecular masses of 42, 38, 30, and 20 kDa that have DNA-binding activity. The pool levels of the 42- and 30-kDa isoforms were high in control nuclear extracts and decreased significantly after lipopolysaccharide (LPS) treatment. The binding activity and protein levels of the 20-kDa isoform are low in controls and increase dramatically after LPS treatment. C/EBPβ isoforms with molecular masses of 35, 20, and 16 kDa were also detected. The 35-kDa pool level did not change whereas the 20-kDa isoform was strongly induced in response to LPS. Western (immunoblot) and Southwestern (DNA-protein) analyses show that $p42^{C/EBP\alpha}$ forms specific complexes with the α_1-acid glycoprotein oligonucleotide in control nuclear extract and that $p20^{C/EBP\beta}$ forms complexes in LPS-treated liver. Our studies suggest that synthesis of specific C/EBPα and C/EBPβ isoforms occurred in the normal liver in vivo and that LPS mediated a differential initiation and inhibition of translation at specific AUG sites within each mRNA. The qualitative and quantitative changes in C/EBPα and C/EBPβ isoform pool levels suggest that LPS or an LPS-stimulated factor can regulate the selection of AUG start sites for both activation and repression of translation. This regulation appears to involve an LPS-mediated down-regulation of initiation at the first AUG codon of the 42-kDa C/EBPα and dramatic translational up-regulation at the fifth AUG codon of the 20-kDa C/EBPα and the third AUG codon of the 20-kDa C/EBPβ. These regulatory events suggest the existence of proteins that may act as translational *trans*-acting factors.

The intronless genes of the CCAAT/enhancer-binding *trans*-activator proteins (C/EBPα and C/EBPβ) each code for a single mRNA (15). These mRNAs code for several proteins that have specific transcriptional regulatory functions (41, 44). This is accomplished by alternative use of different AUG initiation codons within the same open reading frame (ORF). It has been shown that a 5′ ORF, 18 nucleotides in length, positioned 7 nucleotides 5′ of the first C/EBPα AUG codon is essential for the leaky ribosome-scanning mechanism that causes some ribosomes to ignore the first C/EBPα AUG codon and to start translation at internal AUGs (14). The presence of a similar small ORF 5′ to the second AUG initiation codon of C/EBPβ mRNA suggests that start site multiplicity from this mRNA may be governed by the same mechanism (14). The major C/EBPα isoforms produced by alternative initiation of translation are $p42^{C/EBP\alpha}$ (42 kDa) and $p30^{C/EBP\alpha}$ (30 kDa). These isoforms differ significantly in their regulatory functions. For example, $p42^{C/EBP\alpha}$ is a powerful transactivator of several genes coordinately regulated when preadipocytes differentiate into adipocytes and has antimitotic activity associated with adipocyte differentiation (28, 40, 41, 56). $p30^{C/EBP\alpha}$ is initiated from an AUG codon 352 nucleotides downstream of the first AUG (44). This isoform fails to interfere with adipocyte cell proliferation and to induce complete 3T3-L1 differentiation. $p30^{C/EBP\alpha}$ also attenuates transcriptional activation by $p42^{C/EBP\alpha}$. Similar analyses have shown that the C/EBPβ mRNA produces two proteins, LAP (reported to be a 32- to 35-kDa protein) and LIP (20 kDa). The 35-kDa protein ($p35^{C/EBP\beta}$), also known as C/EBPβ or nuclear factor for interleukin-6 expression (NF-IL6), binds to region D of the albumin promoter and acts as a powerful transactivator (44). On the other hand, LIP ($p20^{C/EBP\beta}$) has DNA-binding and dimerization domains and a truncated transcription activation domain, and it exhibits activities that antagonize $p35^{C/EBP\beta}$. Alam et al. (2, 4) have shown that C/EBPα and C/EBPβ bind to the region C binding site of the α_1-acid glycoprotein (AGP-1) promoter, i.e., the acute-phase response element (APRE), and that C/EBPβ is the isoform that binds to this site during the lipopolysaccharide (LPS)-mediated acute-phase response (APR). These studies have raised the possibility that the multiple isoforms of the C/EBPα and C/EBPβ families play specific roles in regulating genes that respond to LPS and that LPS or LPS-induced products, such as cytokines, may regulate the specific initiation of the translation of these C/EBPs.

Inflammatory agents such as bacterial LPS, turpentine, and heavy metals (3, 5–9, 17, 23, 59, 60) stimulate dramatic changes in the binding activities of members of the C/EBP family of transcription factors, i.e., C/EBPα, C/EBPβ, and C/EBPδ (3). By using the oligonucleotide corresponding to the C/EBP-binding site of the AGP-1 promoter (APRE), we have demonstrated a major shift in the DNA-binding activities from C/EBPα to C/EBPβ during the response to LPS (2, 3, 23).

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Furthermore, by assessing DNA-binding activity, we have shown that C/EBPα is the predominant C/EBP isoform in nuclear extracts of untreated mice and that C/EBPβ becomes the major isoform with LPS treatment (2). We showed that concomitant with the increase in C/EBPβ binding activity, the binding activity of C/EBPα is decreased significantly in response to LPS. These studies suggested that the C/EBPα and C/EBPβ levels may be regulated during the APR and that the LPS-mediated regulation of C/EBPα and C/EBPβ binding activities and/or pool levels may be an integral part of the APR.

In this paper we present data from studies on the LPS-mediated regulation of C/EBPα and C/EBPβ isoform synthesis. Our results suggest that the induction as well as repression of C/EBPα and C/EBPβ isoform synthesis is mediated by LPS treatment and is due to differential initiation of translation at multiple AUG sites in the C/EBPα and C/EBPβ mRNAs. We also analyzed the effects of LPS on the rates of transcription of C/EBPα and C/EBPβ genes and on their mRNA and protein pool levels.

MATERIALS AND METHODS

Animals. Male BALB/c mice were obtained from the Charles River Laboratories (Wilmington, Mass.). All animals were maintained on a light-dark (12-h–12-h) cycle and fed a standard chow diet. Animals were injected intraperitoneally (i.p.) with 50 μg of LPS (Sigma, St. Louis, Mo.) in pyrogen-free saline and sacrificed by cervical dislocation at various time points.

Isolation and analysis of RNA. Total cellular RNA was isolated from livers by homogenizing tissues in a solution containing 7.5 M guanidine-HCl, 1% sarcosyl, and 25 mM sodium citrate and separating the RNA by ultracentrifugation through 5.7 M CsCl (20, 24). For Northern (RNA) blotting, RNA samples were resolved by electrophoresis through a 1.4% formaldehyde-agarose denaturing gel buffered with 0.02 M morpholinepropanesulfonic acid and 1 mM EDTA (pH 7.4). The RNAs were then transferred overnight from gels to Nitroplus membranes (Schleicher and Schuell, Keene, N.H.) in the presence of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked for 2 h at 80°C under a vacuum (43).

For the determination of C/EBPα and C/EBPβ mRNA levels, cDNA inserts were isolated from each plasmid by digesting pMSV-C/EBPα (37) with *Hin*dIII and pMSV-C/EBPβ (15) with *Bam*HI-*Eco*RI, and the cDNAs were radiolabeled with a Megaprime DNA labeling kit (Amersham, Arlington Heights, Ill.) before being used to probe Northern blots. The filters were prehybridized for 4 h at 60°C in a solution containing 50% formamide, 4× SSC, 100 μg of sheared and denatured salmon sperm DNA per ml, 3× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 50 mM sodium phosphate, pH 6.5. The filters were then hybridized for 16 to 20 h at 65°C or 60°C in the above solution containing approximately 5×10^6 cpm of ^{32}P-labeled RNA or DNA probe per ml. After hybridization, the filters were washed four times for 15 min at room temperature (RT) in washing solution I (2× SSC, 0.1% SDS) and then three times for 45 min at 55°C in washing solution II (0.1× SSC, 0.1% SDS) (17). The filters were then dried under a heat lamp for 30 min and exposed to X-ray film. The relative amounts of mRNAs for the C/EBP isoforms were determined by densitometric analysis of Northern blot autoradiographs with an Applied Imaging densitometer with Lynx 5.1 densitometry software.

Nuclear run-on transcription analysis. Male BALB/c mice (4 months old) were sacrificed at various times after LPS injection, and nuclei were isolated from fresh tissue homogenates by sucrose gradient centrifugation (36). The nuclear pellet was resuspended in cold nuclear storage buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, and 40% glycerol. Nuclear run-on transcription was performed immediately with 5×10^7 nuclei in the presence of [α-^{32}P]UTP at 30°C for 45 min. The reaction buffer contained 0.5 M Tris-HCl (pH 7.8); 0.25 M NaCl; 1.75 M ammonium sulfate; 0.01 M EDTA; 1 mM each ATP, CTP, and GTP; 1 mg of heparin per ml; and 20 mM $MgCl_2$. Transcription was stopped by adding 10 μg of tRNA, 0.02 M Tris-HCl (pH 7.5), 0.01 M $CaCl_2$, and 25 μg each of DNase I and proteinase K. After incubation for 30 min at 37°C, 0.2 M EDTA and 10% SDS were added and the solution was then incubated at 37°C for 15 min. Nascent RNA was then purified by phenol-chloroform extraction and ethanol precipitation. A total of 10^8 cpm of labeled RNA per assay was hybridized to 5 μg of pMSV-C/EBPα and pMSV-C/EBPβ cDNAs immobilized on nitrocellulose filters (43). The filters were then incubated in a solution containing 0.01 M Tris-HCl (pH 7.4), 0.2% SDS, 0.01 M EDTA, 0.3 M NaCl, 1× Denhardt's solution, 100 μg of yeast tRNA per ml, and 100 μg of polyadenylic acid per ml and then washed once in 2× SSC for 1 h at 65°C, once in 2× SSC containing 10 μg of boiled RNase A per ml for 15 min at 37°C, and once in 2× SSC for 1 h at 37°C. The filters were then dried for 30 min and exposed to X-ray film. The signals on the autoradiographs were quantitated with a scanning densitometer.

Preparation of nuclear protein extracts. Preparation of liver nuclear protein extracts from control and LPS-treated 4-month-old male BALB/c mice has been described (2, 61). Protein concentrations were determined by the method of Bradford (12). Aliquots of the nuclear protein solutions were frozen in liquid nitrogen and stored at −90°C.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were carried out as described by Alam et al. (2). The oligonucleotide 5′-(−127)AGAACATTTTGCGCAAGACATTTCCCAAG(−99)-3′, corresponding to the C/EBP binding site of the AGP-1 promoter, and its complementary strand were used as probes for electrophoretic mobility shift assays or Southwestern (DNA-protein) analysis after being labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (29, 30).

Equal amounts of the complementary strands of the oligonucleotides were heated at 95°C for 10 min in STE buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, and 1 mM EDTA) and allowed to anneal by slowly cooling the solution down to RT.

Southwestern analyses of nuclear proteins. Proteins were electrophoretically separated by heating each sample in a boiling water bath for 5 min in an equal volume of 2× SDS loading buffer and loading the samples onto an SDS–12% polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (35). After electrophoresis, the proteins were then transferred to 0.45-μm-pore-size Westran polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell) (55). Southwestern analysis was performed by a modification of a previously described procedure (57). Briefly, the filters were blocked with 5% (wt/vol) nonfat dry milk in binding buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 60 mM KCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 10% glycerol) for 1 h at 4°C. The filters were then subjected to a binding reaction with ^{32}P-labeled double-stranded oligonucleotides of AGP-1 APRE (10^6 cpm/ml) at 4°C in the presence of 1 μg of salmon sperm DNA per ml and 0.25% nonfat dry milk. The next day they were washed three times for 10 min in binding buffer containing 0.25% nonfat dry milk. After being washed, the filters were blotted on Whatman 3MM paper and the damp blots were placed between two sheets of plastic wrap and exposed to X-ray film.

Western blot (immunoblot) analysis of nuclear extracts. Twenty micrograms of liver nuclear extract was boiled for 5 min at 100°C, subjected to SDS-PAGE on a 12% polyacrylamide gel (35), and transferred to Westran PVDF membranes. The filters were then immunoassayed according to the procedure recommended by the supplier of the ECL Western analysis kit (Amersham). The Western blots were incubated at RT for 1 h in TBS (25 mM Tris, 137 mM NaCl, 5 mM KCl) containing 5% nonfat dry milk (Bio-Rad, Hercules, Calif.) and 0.05% Tween 20 (Sigma). The blots were then incubated in blocking buffer containing appropriate antiserum preparations for 2 h at RT. Antisera specific to C/EBPα and C/EBPβ (15, 37, 38) were diluted 1:10,000. To remove unbound primary antibody, the filters were washed three times in TBS containing 0.05% Tween 20 for 10 min at RT. Then the blots were incubated in washing buffer containing ^{125}I-labeled protein A (Amersham; specific activity, 30 μCi/μg) for 2 h at RT. After four 15-min washes in washing buffer at RT, each damp blot was placed between two sheets of plastic wrap; then the wrapped blots were placed over sheets of X-ray film in photographic cassettes with intensifying screens. After autoradiography (−80°C for 2 days), the signals were quantified with a scanning densitometer.

Antisera. Antisera to C/EBPα and C/EBPβ were prepared against specific oligopeptides (14, 38, 39). The oligopeptide used to prepare the antiC/EBPα is AGPHPDLRTGGGGGGGAGA, which is adjacent to the DNA-binding domain; the C/EBPβ antiserum was prepared by using the oligopeptide LRN LFKQLPEPLLASAG, which is at the carboxy terminus.

Polysome isolation and analysis of binding of nascent peptide chains to APRE oligonucleotide. Polysomes were isolated from control and LPS-treated mouse livers as described previously (13). Minced livers from control mice and from mice 4.5 h after i.p. injection of 50 μg of LPS were homogenized in polysome buffer (50 mM Tris-HCl [pH 7.1], 25 mM NaCl, 5 mM $MgCl_2$, 100 μg of heparin) containing 0.25 M sucrose with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 14,000 × *g* for 10 min at 4°C. The supernate was recovered, and Triton X-100 and deoxycholate were added to final concentrations of 1.0%. Polysomes were then isolated by pelleting through a cushion of 1.0 M sucrose in polysome buffer at 39,000 rpm for 3 h with a Beckman SW40 rotor. The isolated polysomes were resuspended in polysome buffer, dissociated with 25 mM EDTA, and concentrated with a Centricon 10 centrifugal concentrator (Amicon Division, W. R. Grace & Co.). The protein concentration was determined by the method of Bradford (12). These polysomal proteins were then used in a Southwestern analysis as described above.

Isolation of proteins binding to APRE oligonucleotide. DNA-protein complexes (C1 to C4) were isolated by excision from electrophoretic mobility shift gels. Prior to excision, the wet gels were exposed to X-ray film at 4°C overnight. The individual bands were then cut out and electroeluted with gel shift running buffer in an electroeluter (Amicon) for 4 h at 200 V. The eluted fractions were then concentrated with a Centricon 10 centrifugal concentrator (Amicon). The concentrated protein fractions were resolved by SDS-PAGE. The proteins were transferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, Mass.) and were identified according to their molecular weights by ECL-Western blotting (Amersham) and Southwestern analyses. ECL-Western blotting was carried out according to the instructions of the distributor.

Construction of amplification vectors. The amplification vector pMSV-C/EBPβ-SVori was constructed by cloning the *Pvu*II-*Hin*dIII fragments from

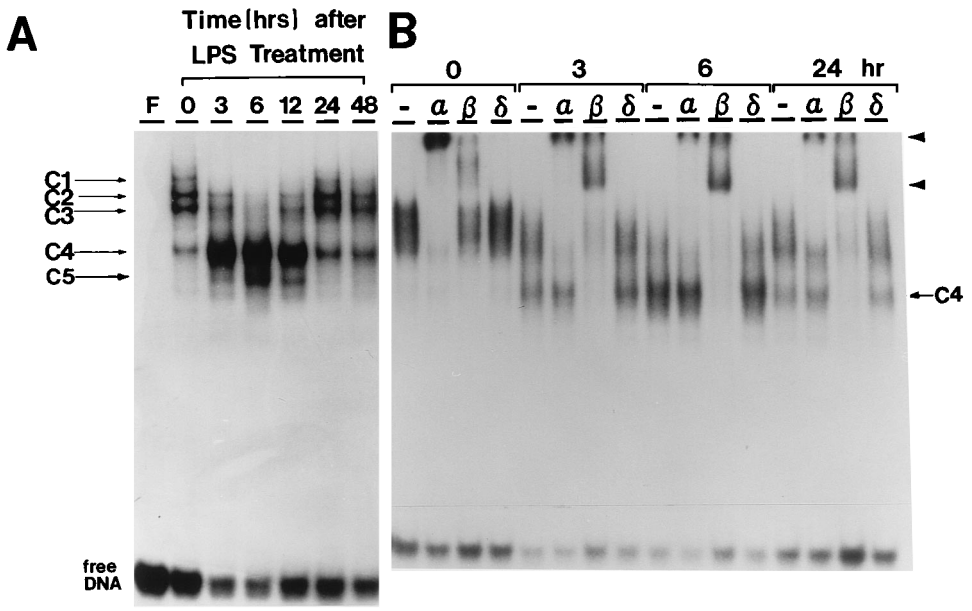

FIG. 1. (A) A time course of DNA-binding activity of the C/EBP binding site (APRE) of the mouse AGP-1 promoter with nuclear proteins from livers of LPS-treated mice. Electrophoretic mobility shift assays were performed with 3 μg of nuclear proteins isolated from fresh livers of control or LPS-injected BALB/c mice. LPS (50 μg) was injected at the times indicated above the lanes. The double-stranded oligonucleotide corresponding to the APRE of the AGP-1 promoter was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The positions of the DNA-protein complexes are indicated with arrows. Lane F contains the free-DNA probe. (B) Super mobility shift analyses of the C1, C2, C3, and C4 DNA-protein complexes formed with the APRE of the AGP-1 promoter. Supershift assays were done to demonstrate that the nuclear proteins involved in the formation of DNA-protein complexes with the AGP-1 APRE are members of the C/EBP family. Monospecific antibodies against synthetic peptides unique to the C/EBPα, C/EBPβ, and C/EBPδ isoforms were used, as indicated by α, β, and δ, respectively, above the lanes. In the lanes labeled −, no antibody was added to the reaction mixture. Nuclear extracts from control and LPS-treated mouse livers were made at the time points indicated. The arrowheads indicate the positions of the bands formed by supershifting with C/EBPα or C/EBPβ antibody.

pSVori (31, 49) into *Eco*RI-digested pMSV-C/EBPβ (15). The identity of each clone was confirmed by restriction enzyme mapping and Southern analysis with ^{32}P-labeled SVori probe.

Cell culture, transfection, and preparation of nuclear extracts. COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (49) and seeded at a density of 10^6 cells per dish (100 mm) 1 day before transfection. Transfections with 5 μg of each plasmid DNA were done by the DNA-calcium phosphate coprecipitation method (32), followed by a glycerol shock procedure. After 48 h, the cells were harvested and nuclear extracts containing each C/EBP isoform were prepared as described by Schreiber et al. (51). The nuclear extracts were centrifuged at 4°C for 5 min in a microcentrifuge, and the supernatants were frozen in aliquots at −90°C. The protein concentrations of the nuclear extracts were determined according to the method of Bradford (12).

RESULTS

Loss of C/EBPα binding activity and increase of C/EBPβ binding activity in mouse liver nuclear proteins during the LPS-mediated APR. The binding activities of C/EBPα and C/EBPβ toward the oligonucleotide of the C/EBP binding site of the AGP-1 promoter, i.e., the APRE, are altered during the LPS-mediated activation of the APR (2, 4). By using the APRE oligonucleotide (−99 to −127 bp) in gel shift and supershift assays, we previously demonstrated that C/EBPα is the predominant C/EBP isoform of control nuclear proteins that binds to the APRE and that C/EBPβ, which had virtually no binding activity in controls, becomes the predominant isoform binding to the APRE in LPS-treated mice (2, 4). These data suggested dramatic changes in the binding activities of C/EBPα and C/EBPβ during the APR; these differences may be due to a loss of protein binding activity or changes in their pool levels. In the present study we conducted a time course analysis of the binding of nuclear proteins to the APRE in order to demonstrate the pattern of changes in C/EBPα- and C/EBPβ-containing DNA-protein complexes formed during the response to and recovery from one injection of LPS (50 μg). Liver nuclear proteins from untreated mice formed three major DNA-protein complexes, C1, C2, and C3, and a C4 complex whose appearance is variable but whose band is consistently lower than those of C1 to C3 (Fig. 1A). The relative proportion of the C4 complex increased significantly by 3 h after LPS treatment, reached a peak by 6 h, and then declined gradually. A band that appears below that of the C4 complex, i.e., C5, follows the temporal pattern of appearance and disappearance of the C4 complex but is usually seen in variable amounts in both Southwestern and Western analyses. In contrast, the C1 and C2 complexes disappeared completely by 6 h after LPS treatment, and this was followed by a gradual reappearance between 12 and 24 h. At the same time there is a significant reduction but not a complete loss of the C3 complex. At 24 h the C1 complex is still relatively weak, suggesting that its reformation is not yet complete, whereas the intensities of the C2 and C3 complexes are indicative of their recoveries to control levels. To show that the four DNA-protein complexes included members of the C/EBP family of transcription factors, we performed supershift assays (Fig. 1B) with monospecific antibodies against synthetic peptides unique to the C/EBPα and C/EBPβ isoforms (15, 37). The C1 and C2 complexes were supershifted by antibody specific to C/EBPα, the C3 complex was supershifted by antibody specific to C/EBPα and C/EBPβ, and the C4 complex was supershifted by antibody specific to C/EBPβ. The C4 complex, which is present only at very low levels in control nuclear extracts, is clearly the predominant isoform in the nuclear extracts of LPS-treated animals (Fig. 1B). These results demonstrate that the C/EBP-protein complexes formed with the APRE contain the C/EBPα isoform(s) in control nuclear extracts and C/EBPβ in the nuclear extracts from treated animals (2, 4). There was no detectable supershift

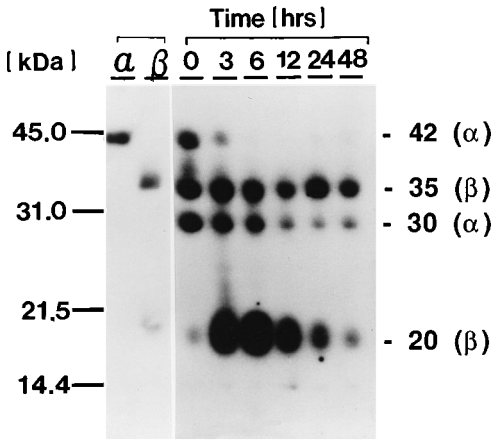

FIG. 2. Southwestern blot analysis of liver nuclear proteins that bind to the APRE of the AGP-1 gene after LPS treatment. BALB/c mice were injected with 50 μg of LPS and then sacrificed at the time points indicated. Nuclear proteins (30 μg) prepared from fresh livers were subjected to SDS-PAGE, blotted onto Westran PVDF membranes, and probed with 10^6 cpm of ^{32}P-APRE oligonucleotide per ml as described in Materials and Methods. The locations of the molecular size standards are shown on the left. The band between the 42- and 35-kDa bands represents the 38-kDa isoform. Lanes α and β contain nuclear extracts from COS-1 cells transfected with expression plasmids for C/EBPα and C/EBPβ, respectively, as described in Materials and Methods.

with C/EBPδ, indicating that this protein is not a major factor in the APR in the liver in vivo.

Identification of C/EBP proteins that bind to the APRE. Previous studies have shown that C/EBPα (42- and 30-kDa) and C/EBPβ (35- and 20-kDa) isoforms are expressed by 3T3-L1 adipocytes, adipose tissues, and liver (11, 39, 41, 44). In these studies we used the APRE oligonucleotide for Southwestern blotting analysis to estimate the apparent molecular weights and binding activities of C/EBP homodimers in liver nuclear proteins of control and LPS-treated animals (Fig. 2). Nuclear extracts isolated from fresh livers of control mice as well as of LPS-treated mice (at various times after LPS treatment) were subjected to SDS-PAGE, and the proteins were transferred to Westran PVDF membranes and probed with the ^{32}P-labeled double-stranded APRE oligonucleotide. The intensity of the 42-kDa band, which is the molecular mass reported for C/EBPα, was relatively high in control nuclear extracts but was 60% lower 3 h after LPS induction and 95% lower at 6 to 12 h. In some experiments there is a slight increase at 48 h, which is suggestive of the recovery of this protein to its control level (data not shown). A second band, at ~38 kDa, showed the same kinetic pattern as the 42-kDa protein. Although this protein exhibits weak binding activity (low-intensity band, Fig. 2), the high intensity of the 38-kDa band on Western analysis indicates a high pool level (see Fig. 3). The band at 35 kDa, which is in the molecular mass range reported for C/EBPβ (15), showed very strong binding activity in the control nuclear extracts and throughout the induction period, although there appears to be a slight decrease in binding activity at 12 and 48 h. The 30-kDa band has a delayed response to LPS, exhibiting a very strong binding activity with control nuclear extract during the first 6 h of LPS treatment, after which there is a significant decrease in activity that lasts from 12 to 48 h. Finally, the band at 20 kDa showed a very low level of binding activity in the control nuclear extract, but within 3 h after LPS treatment a 9.5-fold increase in its binding activity was detected, with the activity peaking at 6 h and then gradually declining. Thus, four major protein bands, the 42-, 35-, 30-, and 20-kDa homodimers, and a minor band of 38 kDa were detected by Southwestern analyses; the 42-, 38-, and 20-kDa isoforms followed the time course of DNA-binding activity (Fig. 1A). The 42-kDa protein corresponds in molecular mass to full-length C/EBPα (37, 38), whereas the 20-kDa isoform paralleled the pattern of appearance of the C4 complex and could play a role in the formation of that complex.

Analysis of the pool levels of C/EBP isoforms during the APR. The Southwestern analyses clearly show significant qualitative and quantitative changes in binding of C/EBPα and C/EBPβ isoforms to the APRE oligonucleotide during the APR. These changes may be due to either altered protein pool levels or modifications of protein structure that affect binding activity. To determine the C/EBP pool levels and to identify the proteins that bind to the APRE in response to LPS, we assayed C/EBPα and C/EBPβ protein levels by Western immunoblotting with specific anti-C/EBP antibodies.

Western immunoblotting with anti-C/EBPα antibody revealed seven protein bands of 61, 42, 38, 35, 30, 27, and 20 kDa (Fig. 3A). After treatment with LPS, the levels of five bands (61, 42, 38, 35, and 30 kDa) decreased. At the same time, a major band at 20 kDa increased dramatically, reached a peak at 3 to 6 h, and then gradually declined. The formation of this protein(s) appears to be induced in response to LPS. Another minor band, at 27 kDa, also showed this induction pattern.

Anti-C/EBPβ detected three isoforms of 35, 27, and 20 kDa in control nuclear extracts and five isoforms in nuclear extracts from livers of LPS-treated mice (Fig. 3B). The latter include the proteins detected in the control nuclear extract plus the 24- and 16-kDa isoforms induced by LPS. The pool level of the 35-kDa C/EBPβ, which has been implicated as the functional *trans*-acting factor that responds to IL-6, does not appear to change after LPS treatment. The levels of four bands migrating at 27, 24, 20, and 16 kDa increased 1.5-, 7.1-, 4.8-, and 9-fold, respectively, in response to LPS, which is consistent with the induction pattern of the C4 complex (Fig. 1). However, only the 20-kDa form has DNA-binding activity; the others are only detectable immunochemically (compare Fig. 2 and 3). These experiments show that the binding activity and pool levels of the 20-kDa C/EBPβ isoform parallel the kinetic properties associated with the LPS-mediated stimulation of C/EBPβ-DNA (C4) protein complex formation.

Comparison of the C/EBP isoforms detected by DNA-binding activity analyses and specific antibody interaction has revealed several members of both the C/EBPα and C/EBPβ families that fail to bind to the APRE. For example, the 61- and 27-kDa forms detected by anti-C/EBPα (Fig. 3A) are not detected by DNA-binding activity analyses, and the 38-kDa isoform shows very weak binding activity (Fig. 2). Similarly, the 27- and 24-kDa isoforms detected by anti-C/EBPβ (Fig. 3B) are not detected by the DNA-binding activity assay (Fig. 2). Since these forms lack detectable DNA-binding activity, they may represent negative regulators similar to GADD153, CHOP, and Id (10, 16, 27, 50). Alternatively, they may represent products of degradation of the higher-molecular-weight forms which have weaker or reduced binding activities. In summary, the immunoblot and DNA-binding activity data suggest that LPS stimulates the formation of the $p20^{C/EBP\alpha}$ and $p20^{C/EBP\beta}$ isoforms and inhibits the formation of $p42^{C/EBP\alpha}$ and that the kinetics of their appearance or disappearance parallels the kinetics of the DNA-binding activity.

Identification of APRE-binding C/EBPα and C/EBPβ isoforms in control and LPS-treated nuclear extracts. In order to identify the specific C/EBP isoforms involved in the formation of C1 to C4, these complexes were isolated from band shift gels and the proteins were analyzed by Southwestern and Western

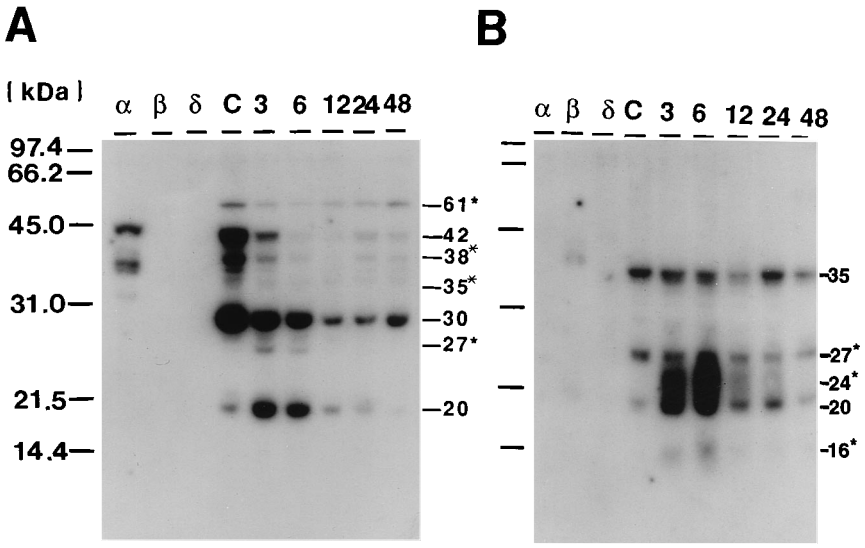

FIG. 3. Western analysis of the levels of C/EBPα and C/EBPβ isoforms in liver nuclei in response to LPS treatment. Nuclear extracts (30 μg) from control (C) and LPS-injected (3-, 6-, 12-, 24-, and 48-h postinjection) BALB/c mice were loaded in individual lanes and subjected to SDS-PAGE. Immunoblots were incubated with monospecific polyclonal antibodies against either C/EBPα (A) or C/EBPβ (B) or with preimmune serum as a control. ^{125}I-labeled protein A was used to detect antigen-antibody complexes in each immunoblot as described in Materials and Methods. Nuclear extracts from COS-1 cells transfected with C/EBPα (lane α), C/EBPβ (lane β), and C/EBPδ (lane δ) expression vectors were used as controls for each protein. No bands were detected with preimmune serum with liver nuclear extracts or COS-1 nuclear proteins (data not shown). The locations of the molecular size standards are indicated on the left of each panel. The asterisks indicate bands which have no binding activity with the APRE oligonucleotide.

blot analyses. The Southwestern data in panel 1 of Fig. 4A show the binding activities of C/EBPα and C/EBPβ isoforms in control (lane 1) and LPS-treated (lane 2) nuclear extracts. Southwestern analyses of the C1, C2, and C3 complexes revealed the presence of 42- and 35-kDa binding proteins (Fig. 4A, panels 1 [lane 3] and 2 [lane 1]); the Western analysis shows that these proteins are the 42-kDa C/EBPα (Fig. 4B, panels 1 [lane 3] and 2 [lane 1]) and the 35-kDa C/EBPβ (Fig. 4C, lane 3). The high-molecular-weight band at ~61 kDa reacts with C/EBPβ antibody (Fig. 4C, lanes 1 and 2). Southwestern analyses of the C3 and C4 complexes from LPS-treated nuclear extract show that the 20-kDa protein has the highest level of binding activity (Fig. 4A, panels 1 [lanes 5 and 6] and 2 [lanes 3 and 4]), and Western analyses identify this protein as the 20-kDa C/EBPβ (Fig. 4C, lanes 5 and 6). These data and the supershift data suggest that the C1 and C2 complexes in control nuclear extract consist of the 42-kDa C/EBPα, the C3 complex consists of the 42-kDa C/EBPα and the 35-kDa C/EBPβ, and the C3 and C4 complexes of LPS-treated nuclear extract consist of the 20-kDa C/EBPβ. (The data also show the shift to the 20-kDa C/EBPβ in the C3 and C4 complexes with LPS treatment.) These results are consistent with our observation that the anti-C/EBPα displaces the C1 and C2 complexes in super gel shift analyses, that anti-C/EBPα and anti-C/EBPβ displace the C3 complex, and that only the anti-C/EBPβ shifts the C4 complex (Fig. 1B).

Identification of C/EBPα and C/EBPβ isoforms in polysomes of control and LPS-treated livers. In order to demonstrate that the low-molecular-weight C/EBPα and C/EBPβ isoforms are products of differential translation of AUG initiation codons, we conducted experiments to analyze the binding activity and molecular weights of nascent chains on C/EBP polysomes. We argue that since nascent chains are usually not susceptible to proteolytic digestion or processing, these studies could directly demonstrate the presence of C/EBP isoforms on the polysomes, suggesting that they are formed by differential initiation. The Southwestern analysis results shown in Fig. 5 indicate that proteins isolated from purified polysomes exhibit a pattern of DNA-binding activity similar to that of liver nuclear extracts. The polysomal proteins from control livers (Fig. 5, lane 3) exhibit binding activity at 42, 38, 35, 30, 20, and 14 kDa. In the polysomal proteins of LPS-treated livers (Fig. 5, lane 4), the binding activities of the 42-, 35-, and 30-kDa proteins are decreased while the activity of the 20-kDa protein increases. This pattern of binding activity is similar to the one we observe with nuclear proteins, suggesting that these nascent chains are the products of differential initiation of translation (Fig. 5, lanes 1 and 2).

Transcriptional regulation of C/EBPα and C/EBPβ genes during the APR. Our studies have suggested that LPS treatment results in qualitative and quantitative changes in C/EBPα and C/EBPβ protein pool levels and that these changes could be due to differential initiation of translation from their respective mRNAs. However, we have shown previously that C/EBPα and C/EBPβ mRNA levels are also affected by LPS treatment, suggesting that transcription and/or mRNA stability may also be regulated (2, 4). We therefore conducted a time course analysis to determine whether LPS affects the rates of transcription of these intronless genes. Nuclear run-on assays were done with nuclei from fresh livers of control and LPS (50 μg)-treated mice (Fig. 6). In uninduced liver nuclei, the rate of transcription of C/EBPα was slightly higher than that of C/EBPβ. Transcription of the C/EBPα gene decreased by 60% by 3 h after LPS treatment. On the other hand, transcription of the C/EBPβ gene increased approximately twofold within 1 h after LPS treatment and returned to the control level by 24 h after treatment. These data suggest that LPS stimulates factors that regulate the rates of transcription of the C/EBPα and C/EBPβ genes.

Changes in C/EBPα and C/EBPβ mRNA levels during LPS induction. To determine if the changes in levels of C/EBP proteins in control and LPS-treated mice correlate with mRNA levels, we performed Northern blot hybridization with total RNA from livers of control and LPS-treated young male

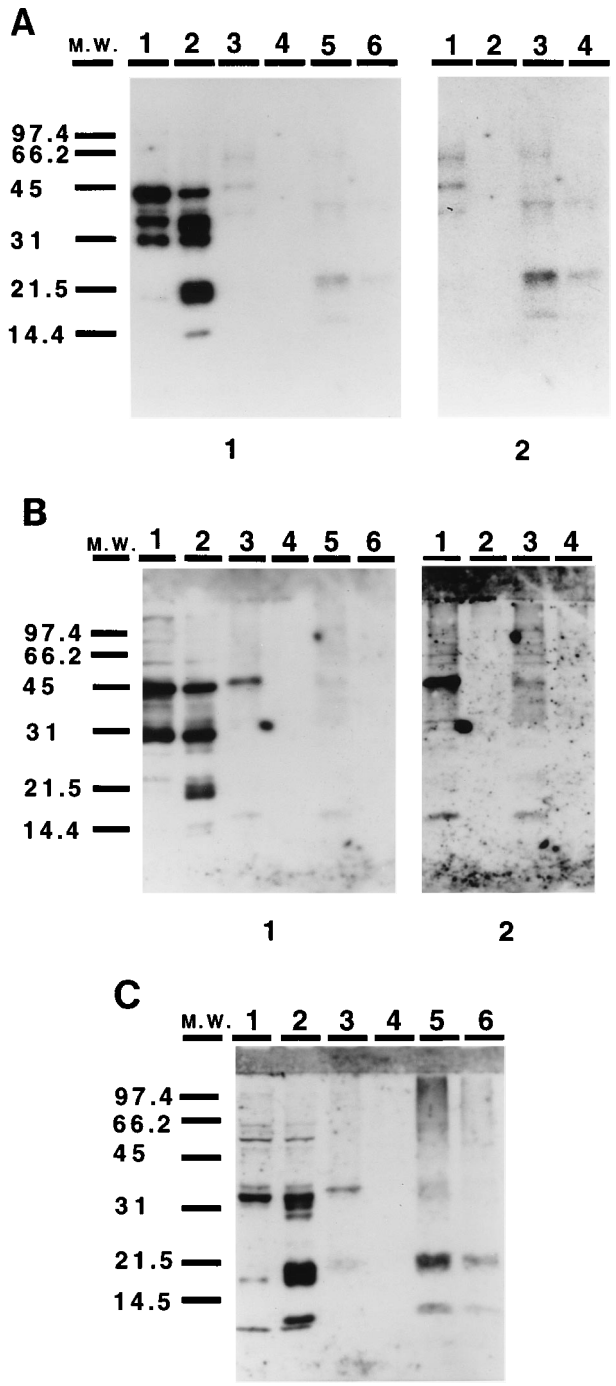

FIG. 4. Identification of the C/EBP proteins that interact with the APRE oligonucleotide to form C1, C2, C3, and C4 complexes in response to LPS. The C1 to C3 and C4 complexes formed with control or LPS-treated (6 h) nuclear extracts were resolved by gel shift electrophoresis. The bands were excised from the gel, eluted, concentrated, and subjected to Southwestern and to ECL-Western immunoblot analyses. (A) Southwestern analyses of the binding activities of proteins isolated from C1 to C3, C3, and C4 DNA-protein complexes. Probe, APRE of AGP-1. Panel 1: lane 1, control; lane 2, LPS-treated nuclear extract; lane 3, C1 to C3 complexes formed with control nuclear extract; lane 4, C4 complex formed with control nuclear extract; lanes 5 and 6, C3 and C4 complexes, respectively, formed with mouse liver nuclear extract after 6 h of LPS treatment. Panel 2: lanes 1 to 4, same as lanes 3 to 6 in panel 1 but with longer exposure times. (B) Western analyses with antibody to C/EBPα; samples for panels 1 and 2 are the same as for panels 1 and 2 of Fig. 4A. (C) Western analysis with antibody to C/EBPβ; the lanes are the same as for panel 1 of Fig. 4A. The locations of the molecular weight (M.W.) standards are shown on the left.

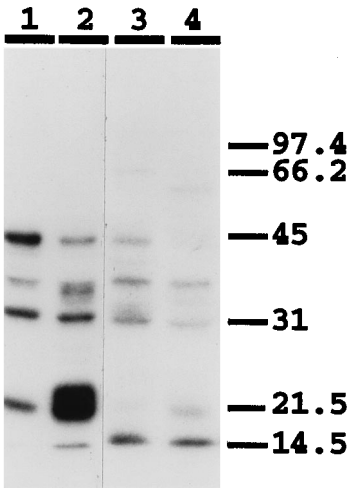

FIG. 5. Analysis of binding of mouse liver polysomal proteins to the APRE oligonucleotide. Liver polysomes from control and LPS-treated mice were prepared for Southwestern analysis as described in Materials and Methods. The ^{32}P-labeled APRE oligonucleotide was prepared as described in Materials and Methods. Lane 1, control liver nuclear extract; lane 2, LPS-treated liver nuclear extract; lane 3, control liver polysomal proteins; lane 4, LPS-treated liver polysomal proteins. The locations of the molecular mass standards are shown on the right.

mice and with C/EBPα and C/EBPβ cDNA probes (Fig. 7). The C/EBPα mRNA level decreased 20% after 3 h of treatment with 50 μg of LPS, and recovery was detected between 12 and 24 h.

Up-regulation of C/EBPβ mRNA levels was observed in the same LPS-treated mice. The constitutive level of C/EBPβ mRNA is relatively low (Fig. 7). LPS-induced levels of C/EBPβ mRNA increased 2.4-fold by 3 h and reached a peak at 6 h; this was followed by a decline to control levels at 24 h (Fig. 7B).

DISCUSSION

In this study we have detected multiple forms of C/EBPα and C/EBPβ proteins in mouse liver and demonstrated that their DNA-binding activities and protein pool levels change dramatically in response to LPS treatment. More specifically, the level of $p42^{C/EBP\alpha}$ is reduced and the levels of $p20^{C/EBP\alpha}$ and $p20^{C/EBP\beta}$ are stimulated by LPS treatment. By using the APRE oligonucleotide which corresponds to the C/EBP-binding site of the mouse AGP-1 promoter, we demonstrated the existence of multiple forms of C/EBPα and C/EBPβ proteins with DNA-binding activities (15, 37). The combination of immunological and DNA-binding activity analyses showed that there are families of both C/EBPα and C/EBPβ DNA-binding proteins of various molecular weights. Similar C/EBP isoforms have been detected in rat liver (37, 38), preadipocytes (22), adipocytes (41), and HepG2 cells (44). Our studies show that the formation of these proteins occurs in vivo in the mouse liver and suggest that LPS mediates the differential initiation of translation at multiple AUG sites within the single C/EBPα and C/EBPβ mRNAs.

The question of whether the C/EBP isoforms are synthesized by differential initiation of translation or are formed by specific posttranslational processing, i.e., specific proteolysis, poses two important mechanisms. To address this question, we argue that analysis of the binding activity of polysomal nascent peptide chains would eliminate the problem of posttranslational processing or degradation and would strongly support our hypothesis that differential initiation of translation occurs.

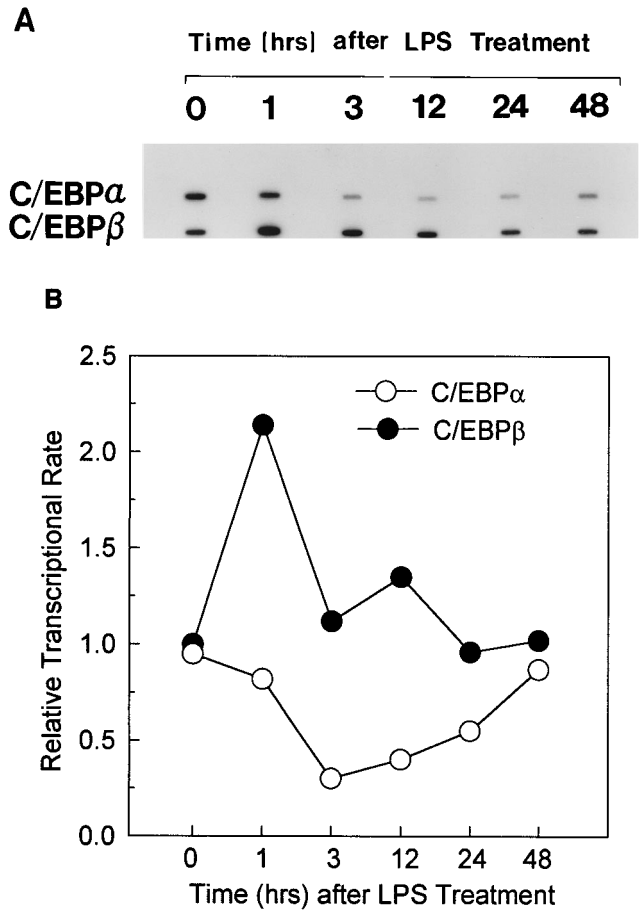

FIG. 6. Time course of rates of transcription of the C/EBPα and C/EBPβ genes in response to LPS treatment. (A) Nuclear run-on transcriptional analyses were performed with fresh liver nuclei isolated from control mice and from LPS (50 μg)-injected mice at various times after LPS treatment, as indicated. The cDNA clones corresponding to the two C/EBP genes indicated on the left were used to hybridize to ^{32}P-labeled nascent nuclear RNA. (B) Relative rates of transcription of the two genes, quantitated as described in Materials and Methods.

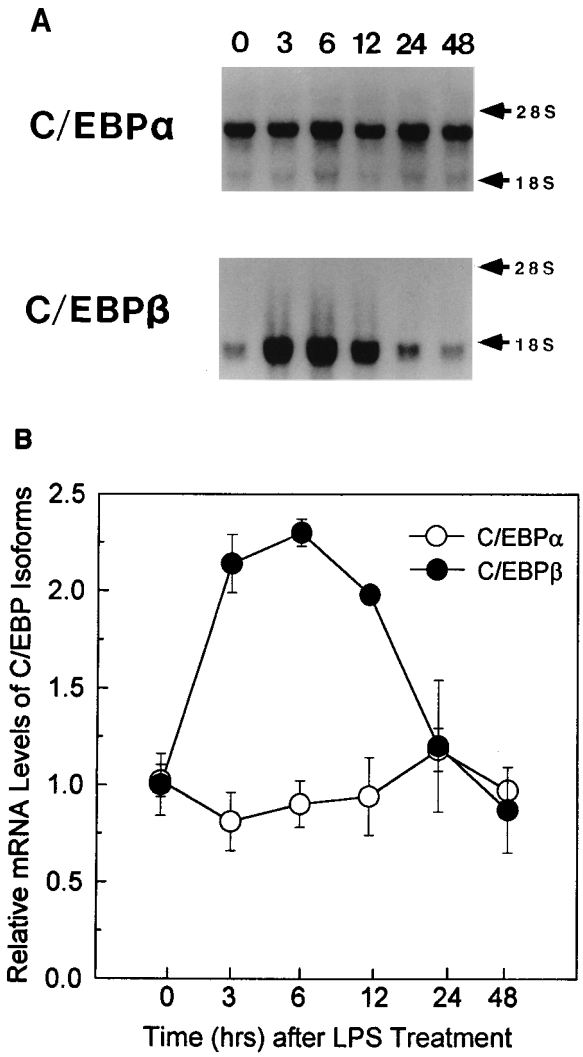

FIG. 7. Time course of C/EBPα and C/EBPβ mRNA levels in response to LPS treatment. (A) Total liver RNA from control and LPS (50 μg)-treated 4-month-old mice were hybridized with the cDNA fragments corresponding to C/EBPα and C/EBPβ as described in Materials and Methods. The sizes of mouse C/EBPα and C/EBPβ mRNAs are approximately 2.7 and 1.5 kb, respectively, on the basis of the positions of the 28S and 18S rRNAs, which are indicated by arrowheads. (B) Relative mRNA levels. The signals on the autoradiogram were quantitated as described in Materials and Methods.

Our analyses clearly support this translational mechanism, since the results of Southwestern analyses of nuclear protein extracts and polysomal proteins are identical. However, our experiments do not completely rule out the occurrence of an equally interesting mechanism, i.e., very specific processing or proteolytic cleavage of the nascent chain of a high-molecular-weight isoform at designated AUG (Met) sites. To begin addressing this, we prepared an antibody to the C/EBP NH-terminal region downstream of the 35-kDa C/EBPβ start site in order to detect the product of proteolytic cleavage at either the 30- or 20-kDa start site. If proteolysis occurs, this antibody would detect a 6-kDa and/or a 16-kDa NH-terminal fragment. Our results indicate that only the 35-kDa C/EBPβ isoform is present in both the nuclear and cytoplasmic extracts (58a) (data not shown). These results suggest that the C/EBPβ 30- and 20-kDa isoforms are not products of proteolytic cleavage at the second and third AUG start sites. Preparation of antibodies to the NH-terminal region of C/EBPα for use in similar analyses is under way.

The occurrence of differential translation of the C/EBPβ mRNA is supported by the studies of Sears and Sealy (52), who show that there is no precursor-product relationship between the high-molecular-mass isoforms (38 and 34 kDa) and the low-molecular-mass isoform (20 kDa). They interpret their results to indicate that the 20-kDa C/EBPβ is not a product of proteolytic cleavage of the high-molecular-weight isoforms. Further supporting evidence is provided by Calkhoven et al. (14), who showed that an increase in synthesis of the 42-kDa C/EBPα caused a decrease in the synthesis of the 30-kDa isoform. These results suggest that there is no precursor-product relationship in the production of the 30-kDa C/EBPα isoform. In a similar series of experiments, Lin et al. (41) demonstrated that the 30-kDa C/EBPα expressed by 3T3-L1 adipocytes and by adipocyte and liver tissues is a product of the alternative translation of the C/EBPα mRNA. The studies of Ossipow et al. (44) also conclude that the 30-kDa C/EBPα and the 20-kDa C/EBPβ (LIP) are products of the differential initiation of translation of the C/EBPα and C/EBPβ mRNAs, respectively. On the basis of these experiments as well as our

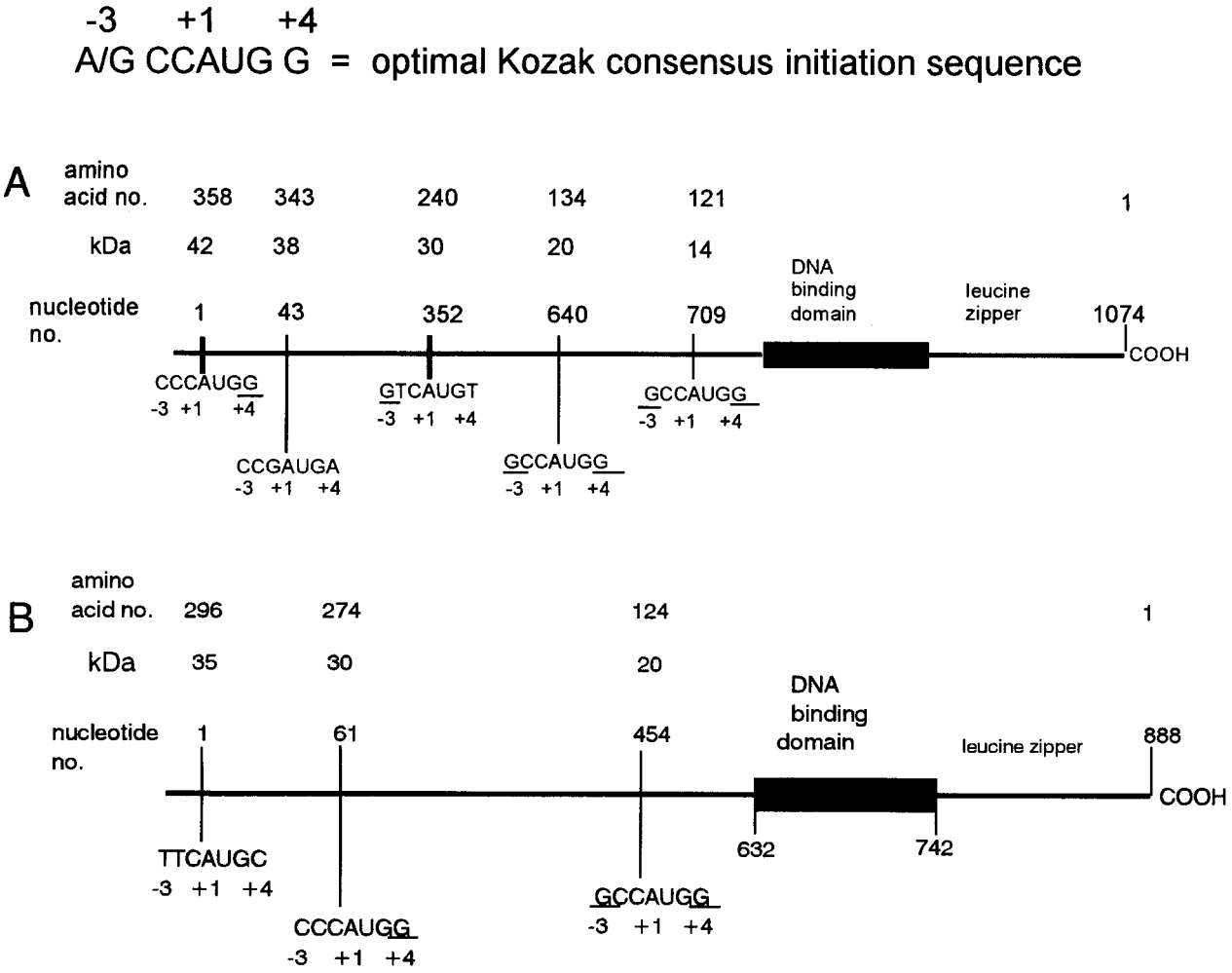

FIG. 8. Map of the translation start sites in C/EBPα (A) and C/EBPβ (B) mRNAs. The positions of the AUG initiation sites in each mRNA are indicated. The nucleotide number indicates the position of the A in each initiation codon. The molecular masses of the proteins that can be formed by initiation at the designated sites are shown below the numbers of amino acids in the isoforms.

own, we propose that the changes in C/EBPα and C/EBPβ isoform production in livers of LPS-treated mice are products of the differential initiation and repression of specific AUG start sites in their respective mRNAs.

Our experiments indicate that the LPS-induced increase in the newly synthesized $p20^{C/EBP\beta}$ (Fig. 5, lane 4) is far less than the increase in the nuclear level of this protein (Fig. 5, lane 2). These data suggest that in addition to the increase in translation there is a stabilization effect on $p20^{C/EBP\beta}$ during the APR which results in an increased nuclear pool level of this protein. We speculate that posttranslational modification in the cytoplasm, such as phosphorylation or protein-protein interactions associated with nuclear translocation, and the formation of DNA-protein complexes in the nucleus may be important factors in the stabilization of this protein and the elevation of its pool level in the nucleus.

Specific antiserum recognized multiple forms of C/EBPα with molecular masses ranging from 61 to 20 kDa. The constitutive levels of the 42-, 38-, and 30-kDa forms were high in control nuclear extracts and decreased significantly after LPS treatment. The protein pool of the 30-kDa isoform also decreases after LPS treatment, but not as rapidly as that of the 42-kDa form. In contrast, protein levels of the 20-kDa isoform were low in controls and increased dramatically with LPS treatment. This is the first indication that the protein pool levels of some C/EBPα isoforms can be stimulated by LPS while others are decreased by this treatment. The reduction in pool levels of some isoforms and the increase in levels of others during the APR suggest that the levels of C/EBPα isoforms are differentially regulated. During differentiation of 3T3-L1 preadipocytes to adipocytes and during hepatocyte development, the ratio of $p42^{C/EBP\alpha}$ to $p30^{C/EBP\alpha}$ changes, raising the possibility of a regulatory role for these isoforms. We also observe significant changes in C/EBPα and C/EBPβ isoform ratios after LPS treatment in vivo, and we propose that this may be a part of the regulatory processes during the APR to LPS. Our data also show a significant reduction of C/EBPα transcription but only a slight reduction of its mRNA levels. The persistence of a large C/EBPα mRNA pool is not consistent with the nearly complete loss of high-molecular-weight C/EBPα protein. Thus, we propose that this may represent translational regulation in which initiation at the AUGs at positions +1 and +352 is down-regulated while initiation at the AUG at +640 is up-regulated (Fig. 8A). This suggests that the C/EBPα isoforms may play an important role in gene regulation during the inflammatory response to LPS. On the basis of studies of

hormone-mediated differentiation of preadipocytes to adipocytes (22, 41) and liver-specific (albumin) gene regulation (44), we propose that the genetic response to LPS, i.e., the APR, may involve differential inhibition of translation of the 42-kDa isoform and induction of translation of the 20-kDa isoform. A possible role for C/EBPα isoforms may be to attenuate the control activities of target genes, such as the APR genes. Attenuator domains have been identified within the transactivation domain of $p42^{C/EBP\alpha}$ and shown to be functional in the viral genome (45, 46).

Antiserum against C/EBPβ recognized isoforms of 35 and 20 kDa. The 35- and 20-kDa isoforms may be formed by differential initiation of translation of the C/EBPβ mRNA. The 35-kDa form, which is in the size range reported for C/EBPβ (NF-IL6) (1, 15, 18, 19, 25, 48, 58), showed no significant change in pool level during the APR. This result was unexpected, as the supershift with C/EBPβ antibody showed a dramatic increase in the C/EBPβ-containing DNA-protein complex (C4). The 20-kDa form shows a dramatic increase after LPS treatment, which suggests that in nuclear extracts from LPS-treated livers the $p20^{C/EBP\beta}$ isoform is preferentially bound to the APRE oligonucleotide (Fig. 1) even in the presence of significant levels of $p35^{C/EBP\beta}$ (Fig. 3). The diagrams in Fig. 8 show that there are initiation codons within the mRNA sequences that could give rise to these isoforms. We propose that the C/EBPβ isoforms arise via a mechanism involving the differential regulation of the initiation of translation. Thus, we propose that initiation of translation of $p20^{C/EBP\beta}$ is a regulatory process associated with the APR.

Interestingly, the Western and Southwestern analyses show that the 42-kDa C/EBPα isoform is the protein that forms the C1 and C2 complexes; we detect no 30-kDa C/EBPα in these complexes and very little 20-kDa C/EBPα. This observation was unexpected, since the 30-kDa C/EBPα pool level is just as high as the 42-kDa C/EBPα pool level. The lack of formation of a 30-kDa C/EBPα-DNA complex suggests that these proteins have some specificity for the AGP-1 APRE. Similarly, even though there is a relatively high level of 35-kDa C/EBPβ in control and LPS-treated nuclear extracts, this isoform is only detected in the DNA-protein complex formed with the control nuclear extract. Analysis of the C3 and C4 complexes from LPS nuclear proteins showed that they contain the $p20^{C/EBP\beta}$ isoform. No $p20^{C/EBP\alpha}$ isoform was detected in this complex. Thus, even though there were significant protein pool levels of $p20^{C/EBP\alpha}$ and $p35^{C/EBP\beta}$, these proteins did not bind to the APRE oligonucleotide in the presence of $p20^{C/EBP\beta}$. This specificity may be due to differential binding affinity or to protein interactions that play a role in the determination of the compositions of these DNA-protein complexes.

The occurrence of multiple isoforms of each C/EBP suggests that these proteins may represent modifications needed for their function or may be degradation products. However, the observation that LAP ($p35^{C/EBP\beta}$) and LIP ($p20^{C/EBP\beta}$) are translated from two AUGs within the same reading frame of a single mRNA species suggests that some of the proteins that we detect are functional molecules formed by differential initiation of translation in response to LPS (26). The existence of multiple isoforms of the C/EBP family has been observed by other investigators. For example, at least six DNA-protein complexes are formed by interaction of the minimal element of the hemopexin gene promoter with nuclear extracts from Hep3B cells (48). Moreover, multiple proteins can bind to a 45-bp IL-1-responsive element of the human C3 promoter; two complexes, C1 and C2, were formed in control nuclear extracts of Hep3B2 cells, but these complexes were decreased and three new IL-1-induced complexes (S1, S2, and S3) were formed with IL-1 stimulated extracts (33).

Landschulz et al. (37, 38) have shown that 42-, 30-, 20-, and 14-kDa species of C/EBPα protein are detectable in rat liver nuclei. They also point out that the 20- and 14-kDa isoforms represent products of degradation of a larger polypeptide that are formed when the livers are frozen and thawed (37). In order to avoid such degradation in mouse livers, we always prepare our nuclear proteins from fresh livers. Under these conditions, we do not detect low-molecular-weight isoforms in control livers. It has also been reported that 35- and 20-kDa isoforms of C/EBPβ were detected in nuclear extracts from IL-1–IL-6–dexamethasone-stimulated H-35 cells (8, 47). Furthermore, three protein bands with molecular masses of 43, 39, and 16 kDa have been detected in control adipocytes and observed to decrease upon treatment with tumor necrosis factor alpha (53, 54). Thus, previous investigators have reported the occurrence of several protein species of lower molecular weight as degradation products (37, 38, 53, 54) or as repressors (44). However, we propose an alternative explanation for the existence of these isoforms. For example, the three proteins detected with C/EBPβ (LAP) antiserum are translation products initiated at the first, second, and third in-frame AUGs of LAP full-length mRNA rather than products of proteolysis (26). Similarly, the isoforms detected with C/EBPα antibody are translational products of the multiple AUG sites of the full-length C/EBPα mRNA. Moreover, Stephens and Pekala (54) have shown that the existence of truncated forms of C/EBPα can be predicted from alternative translation start sites in the cDNA sequence of C/EBPα. Therefore, it is likely that various C/EBPα and C/EBPβ isoforms detected with monospecific antibodies are alternative products translated from several in-frame AUG initiation codons of each full-length mRNA by a leaky ribosome-scanning mechanism (34). In this process, the small subunit of the ribosome first recognizes the 5′ terminal cap structure of an mRNA and then scans the mRNA sequence in a 5′-to-3′ direction for potential AUG initiation codons. However, the first AUG is not always utilized for the initiation of protein synthesis. The nature of the sequence surrounding it determines whether this AUG is selected. A nine-nucleotide consensus sequence, CCA/GC CAUGG, is optimal, and the underlined positions at −3 and +4 are particularly important to ensure a high efficacy of translation initiation. Ribosomal subunits that fail to initiate at the first AUG can continue their search for an AUG in a more favorable sequence context and, if successful, commence protein synthesis at this downstream initiation codon (26, 34). Inspection of the C/EBPα and C/EBPβ cDNA sequences revealed potential AUG start sites in both C/EBPα and C/EBPβ coding regions that could account for the multiple isoforms we observed (Fig. 8). However, we also note that the optimal sequences are not at the first AUG of either mRNA but are at their 30- and 20-kDa sites (Fig. 8) and that these are the sites that are repressed in control liver and stimulated by LPS treatment. We propose, therefore, that another factor(s) that regulates these initiations must be involved in preventing their initiation in control liver and augmenting their initiation in response to LPS.

The qualitative and quantitative changes in C/EBPα and C/EBPβ isoform pool levels suggest that LPS or an LPS-stimulated factor can regulate the selection of an AUG start site. In a recent study, Calkhoven et al. (14) showed that a small 5′ ORF located just upstream of the first C/EBPα AUG codon is the start site for the synthesis of a pentapeptide that is essential for generating translation start site multiplicity within the mRNA and that this is the basis for the formation of C/EBPα

isoforms. In this mechanism, the small 5′ ORF serves as a start site for the synthesis of an oligonucleotide (MPGEL) which conditions ribosomes for leaky scanning by allowing some of the ribosomes to ignore the first C/EBPα AUG codon and proceed by the ribosome-scanning mechanism to the next initiation site. These investigators also showed in a transient expression assay that conversion of the 42-kDa C/EBPα AUG site to a perfect Kozak sequence or elimination of oligopeptide formation by mutation of the 5′ ORF increases the formation of the 42-kDa isoform and suppresses the formation of the 30-kDa isoform. These observations suggest that translational start site multiplicity by the leaky ribosome-scanning mechanism occurs and is assessed by two important factors: (i) the sequence context of the initiation codon, i.e., the presence of a perfect Kozak sequence, and (ii) the 5′ ORF's ability to initiate and terminate synthesis of an oligopeptide and its distance from the downstream 42-kDa C/EBPα AUG start site. Similarly, a 5′ ORF occurs upstream of the second C/EBPβ AUG codon, i.e., the 30-kDa start site, which could cause some of the ribosomes to initiate protein synthesis at the downstream 20-kDa C/EBPβ start site. Our studies show that LPS mediates dramatic changes in the initiation of C/EBPα and C/EBPβ isoforms at these sites. This mechanism must, therefore, offer an explanation for the LPS-mediated down-regulation of the first AUG codon of C/EBPα and the dramatic up-regulation at the fifth AUG codon of the 20-kDa C/EBPα and the third AUG codon of the 20-kDa C/EBPβ. These regulatory events suggest the existence of regulatory proteins that may act as translational *trans*-acting factors, as well as processes that involve leaky ribosomal scanning.

The 61- and 27-kDa C/EBPα isoforms and the 27- and 24-kDa C/EBPβ proteins do not exhibit DNA-binding activity with the APRE oligonucleotide. Although the 61- and 27-kDa C/EBPα proteins exhibit down-regulation and up-regulation, respectively, their very low pool levels relative to those of the other C/EBPα isoforms suggest that they may not play a major role in the APR. The 38-kDa C/EBPα exhibits a high pool level, and its kinetics parallel those of DNA-binding activity. However, Southwestern analyses indicate that this isoform has very weak binding activity. As with the 42-kDa C/EBPα, this isoform is present mainly in control nuclear extracts and disappears with LPS treatment. Interestingly, its failure to exhibit strong binding activity indicates that the 38-kDa isoform may be a member of the bZIP family, with potential repressor activity, or alternatively that its binding activity is altered by SDS treatment.

The fact that the 27- and 24-kDa C/EBPβ isoforms are also not detected by Southwestern analysis indicates that they do not have DNA-binding activity. The 24-kDa isoform is absent in control cells, is strongly induced during the early phases of LPS treatment, and is gradually lost. The 27-kDa isoform shows a similar pattern, except that it is also present in the control liver. Since these isoforms show strong responses to LPS, they may play a role in regulation during the APR. Furthermore, the fact that they have no detectable DNA-binding activity suggests that they could function as repressors. Similar repressor-type proteins such as Id, CHOP-10, and GADD153, do not have DNA-binding properties but do have dimerization properties (27, 50). Both CHOP-10 and GADD153 are of major relevance to our study; CHOP-10 belongs to the C/EBP family and has been proposed to function as a dominant negative modulator of gene transcription by forming heterodimers with C/EBPα and LAP in certain terminally differentiated cell types (50). GADD153 is a hamster homolog of CHOP-10 and is induced by DNA-damaging agents (27).

Studies by several laboratories have shown that C/EBPα and C/EBPβ isoforms have specific transcriptional regulatory properties. For example, the 42-kDa C/EBPα has antiproliferative activities for preadipocytes (40, 41) and mouse hepatoma cells (32a) (data not shown); the 42-kDa C/EBPα activates many adipocyte-specific genes and has repressor activity for human hepatitis B virus and simian virus 40 (42, 45); the 30-kDa C/EBPα loses its antiproliferative activity and maintains the ability to partially activate the adipocyte differentiation program (41); and the 20-kDa C/EBPβ acts as a repressor with the albumin promoter region D binding site, while both the 35- and 20-kDa C/EBPβs repress transcription of Rous sarcoma virus (52). We have previously shown that C/EBPβ is associated with activation of the transcription of the AGP-1 gene (2). Our present studies show that when transcription of the AGP-1 gene is activated, it is the 20-kDa C/EBPβ that binds to the APRE, even in the presence of other C/EBPs. Mutation of this site in an expression vector prevents binding of C/EBP and activation of the reporter gene by IL-6 (2). On the basis of these studies, we conclude that $p20^{C/EBP\beta}$ is a potential activator of the AGP-1 gene. This is the first indication that $p20^{C/EBP\beta}$ may function as a transactivator. In order to demonstrate directly that $p20^{C/EBP\beta}$ is a transactivator, we have shown that the recombinant isoform synthesized in bacterial cells exhibits some activity in the in vitro transcription of an APRE-adenovirus major late promoter-chloramphenicol acetyltransferase expression vector (58a) (data not shown). Studies to characterize this transcriptional activity of $p20^{C/EBP\beta}$ with the AGP-APRE promoter binding site are in progress.

It has been shown that the promoter of the C/EBPα gene has a binding site with which the C/EBPα protein may interact to up-regulate its own transcription (21). The ability of C/EBPα to activate its own gene would explain how a decrease in the C/EBPα protein pool level would slow down transcription of the C/EBPα gene. It is also possible that $p20^{C/EBP\alpha}$ plays a role in this transcriptional down-regulation. Furthermore, the possibility that $p42^{C/EBP\alpha}$ is required for the reactivation of the C/EBPα gene in the recovery from LPS treatment raises the interesting question of how recovery of transcription of the C/EBPα gene occurs. Since this mechanism would require the reappearance of $p42^{C/EBP\alpha}$, the question of how the translation of this isoform is reinitiated becomes relevant. Answering these questions will be an important future goal of our research.

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