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Transcriptional Activation of C/EBPβ Gene by c-Jun and ATF2

WEI-CHIN LIN,[1] BIN-JON SHEN,[2] YEOU-GUANG TSAY,[3] HSUEH-CHI YEN,[2] SHENG-CHUNG LEE,[1,3] and CHING-JIN CHANG[1]

ABSTRACT

C/EBPβ is one of the key transcription factors responsible for the induction of a wide array of genes. Like many proto-oncogenes and transcription factors, transcription of C/EBPβ gene can be induced by multiple extracellular signals. Using nuclear extracts from lipopolysaccharide (LPS)-stimulated mouse liver, five *trans*-acting factor-binding motifs, URE1 (−376 to −352), URE2 (−253 to −223), URE3 (−220 to −190), URE4 (−123 to −103), and URE5 (−72 to −45) were identified by DNAse I footprinting assays. Competition and supershift analysis of the complexes formed at the URE2 and URE4 indicated that they contain CREB/ATF and AP-1 family factors. Furthermore, recombinant ATF2 and c-Jun proteins from mammalian and bacterial cells can bind to URE2 and URE4 but not URE1. Cotransfection experiments showed that ATF2 and c-Jun activate the C/EBPβ gene expression cooperatively through URE2 and URE4, and this activation was greatly increased under the treatment of low concentration of anisomycin. During acute phase response, the phosphorylation of c-Jun and ATF2 was found to correlate with C/EBPβ gene expression. Taken together, our results provide the evidences that both c-Jun and ATF2 are the regulators of C/EBPβ gene.

INTRODUCTION

C/EBPβ BELONGS TO THE BASIC-LEUCINE ZIPPER (bZIP) protein family. It has been shown to be an important transcriptional regulator during immune responses (Akira and Kishimoto, 1992). It is responsible for the induction of a variety of acute-phase proteins and cytokines (Akira *et al.*, 1990; Chang *et al.*, 1990; Matsusaka *et al.*, 1993; Pope *et al.*, 1994). Consistent with these observations, the C/EBPβ-deficient mice also revealed that the lack of C/EBPβ may lead to defective immune responses, including impaired bactericidal activity of macrophage and increasing susceptibility to infections (Tanaka *et al.*, 1995).

There exist a multitude of extracellular signals that can modulate C/EBPβ activity through its phosphorylation (Metz and Ziff, 1991; Wegner *et al.*, 1992; Katz *et al.*, 1993; Nakajima *et al.*, 1993; Trautwein *et al.*, 1993). In addition to post-translation modification, the transcription level of C/EBPβ was found to be modulated during cellular activation and differentiation (Akira *et al.*, 1990; Bretz *et al.*, 1994; Chang *et al.*, 1995; Yeh *et al.*, 1995). The regulation of C/EBPβ gene transcription is complex and, depending on the stimuli, numerous transcription factors are involved. CREB/ATF, SP1, NF-κB, as well as C/EBPβ itself have been implicated in the regulation of C/EBPβ gene expression (Chang *et al.*, 1995; Shen *et al.*, 1997; Niehof *et al.*, 1997; Berrier *et al.*, 1998).

In addition to C/EBPβ, other bZIP proteins including AP-1 (Jun/Fos) and CREB/ATF also are important targets of extracellular signals upstream of the gene activation events associated with growth, differentiation, and cellular stresses (Karin and Huntor, 1995). AP-1 and CREB/ATF proteins control gene expression by binding to the TPA response element (TRE) and cyclic AMP response element (CRE), respectively (Ziff, 1990). Some members of both CREB/ATF and AP-1 families are able to form interfamily heterodimers that possess altered DNA binding specificity different from their respective homodimers (Hai and Curran, 1991; van Dam *et al.*, 1993). Furthermore, their activities could be modulated by protein phosphorylation.

[1]Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan.
[2]Institute of Molecular Medicine, Dr. H.L. Tsai Memorial Laboratory.
[3]Institute of Clinical Medicine, College of Medicine, National Taiwan University.

For example, cAMP-dependent protein kinase (PKA), ribosomal S6 kinase 2 (RSK2), and MAP-kinase-activated protein kinase (PKA), ribosomal S6 kinase 2 (RSK2), and MAP-kinase-activated protein kinase 2 (MAPKAP-K2) have been reported to target CREB (Gonzales and Montminy, 1989; Tan *et al.*, 1996; Xing *et al.*, 1996). Moreover, c-Jun N-terminus kinases (JNKs) and p38MAPKs, the subgroups of mitogen activated protein kinases (MAPK), can phosphorylate c-Jun at Ser 63 and Ser 73, and also phosphorylate ATF2 at Thr 69 and Thr 71 (Derijard *et al.*, 1994; Gupta *et al.*, 1995). JNK and p38MAPK are mediators of pro-inflammatory cytokines TNF and IL-1 as well as stress stimuli such as UV-irradiation, heat shock, translation inhibitor anisomycin, and hyperosmolarity (Freshney *et al.*, 1994; Lee *et al.*, 1994; Han *et al.*, 1994; Cano *et al.*, 1996). The phosphorylation induces an increased affinity for the transcriptional coactivator CBP and enhances the CREB and c-Jun transactivation activities (Chrivia *et al.*, 1993; Bannister *et al.*, 1995).

Because C/EBPβ gene expression is enhanced during acute-phase response, we have previously studied how its promoter activity is regulated under such condition (Chang *et al.*, 1995; Shen *et al.*, 1997). Here, we set out to further characterize some *cis*-elements and *trans*-acting factors present within the C/EBPβ promoter and their roles in the gene regulation. As reported herein, c-Jun and ATF2 were found to bind C/EBPβ promoter and positively modulate C/EBPβ gene activity.

MATERIALS AND METHODS

Plasmids and constructs

The C/EBPβ promoter–CAT constructs have been described previously (Chang *et al.*, 1995). Briefly, an *Eco*RI-*Sph*I fragment spanning from -3 to $+82$ kb was isolated from genomic C/EBPβ DNA and cloned into the pGEM4 vector (Promega, Madison, WI). This fragment was used for generating a series of 5′ deletion fragments using *Bal*-31 digestion. The constructs $-250/+82$–CAT, $-156/+82$–CAT, and $-44/+82$–CAT were obtained by inserting the deletion fragments to pCAT-basic (Promega).

URE2($-253/-223$), URE4($-123/-103$), URE1($-376/-352$) oligonucleotides (sequence was shown in Fig. 2A) were kinase-treated, ligated, and cloned into M13mp18 (New England Biolabs, Beverly, MA). Plasmids with insertion were sequenced. BamHI fragments containing tetrameric inserts were subcloned into $-44/+82$–CAT reporter to obtain $(URE2)_4$–CAT, $(URE4)_4$–CAT, and $(URE1)_4$–CAT constructs. pCMV–CBP was generously given by Dr. Shengcai Lin. pRSV–Jun and pCMV–ATF2 were generously provided by Dr. Young-Sun Lin. Full-length human ATF2 and c-Jun cDNA was inserted to pCMV–Tag2 vector (Stratagene, Cambridge, UK) independently to express Flag-tagged ATF2 and c-Jun in mammalian cells. c-Jun cDNA was also inserted to pcDNA4/His-Max (Invitrogen, San Diego, CA) to express recombinant His-tagged Jun protein.

Expression of recombinant proteins

The *Bsa*AI–*Pst*I fragment of c-Jun (amino acids 1–222) was cloned into pGEX1 (Amersham Pharmacia, Buckinghamshire, UK), and the *Bam*HI–*Xba*I fragment of ATF2 (amino acids 1–108) was ligated to pGEX2, from which GST–Jun and GST–ATF2 fusion proteins were expressed according to the manufacturer's instruction. To generate the recombinant proteins for the binding assay, the *Bsa*AI–*Rsa*I fragment of Jun (amino acids 1–330), and the *Bam*HI fragment of the full-length ATF2 (amino acids 1–506) were individually ligated to pRSET (Invitrogen). Recombinant proteins were expressed by BL21 (DE3) cells and purified by nickel affinity chromatography.

Cell culture, transient transfection, and CAT assay

Human renal 293T cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Paisley, Scotland), supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin and 10 mg ml^{-1} streptomycin. DNA transfection was performed based on the calcium phosphate precipitation method. For transfection, cells were grown in 6-well plates to 30–40% confluency. The amounts of CAT reporter plasmid DNA and expression plasmid DNA used in each experiment are described in the figure legends. pSV–βGal (0.25 μg) was included in each reaction as an internal control. The total amount of plasmid DNA of each reaction was adjusted to the same level by the addition of control noncoding plasmid. The cells were harvested 48 h post-transfection. Cells were extracted with 100 μl of 0.25 M Tris-HCl (pH 7.8). The acetylated forms of chloramphenicol were separated by TLC and quantified with image analyzer (Fuji, BAS 1000). Relative CAT activity was given as the ratio of the relative CAT conversion to the relative β-galactosidase units. All transfection experiments were done in triplicates.

Nuclear extract preparation, DNase I footprinting assay, and Western blot

BALB/c Mice were treated with 100 μg of lipopolysaccharide (Sigma, Poole, UK) intraperitoneally for 0, 30, 60, and 120 min. Nuclear extracts were prepared from mouse liver as described (Chang *et al.*, 1990). Plasmid containing insertion of C/EBPβ promoter region ($-156/+82$-CAT) was linearized by *Hind*III (5′ end), fill-in-labeled with [α-^{32}P]dCTP by the Klenow enzyme, and digested with *Xba*I. DNA fragments were eluted from agarose gel. DNA probe (1 ng/20,000 cpm) was incubated with nuclear extracts at room temperature for 40 min. Then $CaCl_2$ and $MgCl_2$ were added to the final concentrations of 1 and 5 mM, respectively, and the DNA was subsequently digested with 30 ng of DNase I (for 10 μg nuclear extract) on ice for 3 min. After extraction and precipitation, the samples were loaded onto denaturing polyacryamide gel. For Western blot assay, 30 μg of nuclear extracts were separated on SDS-PAGE and transferred to nitrocellulose membrane. The proteins on membrane were detected by anti-c-Jun (Transduction Lab, Lexington, KY) or anti-ATF2 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA).

Electrophoretic mobility shift assay

Double-stranded oligonucleotides of URE2, URE4, and URE5 (sequence shown in Fig. 2A) were end-labeled with [γ-^{32}P] ATP. Binding reactions were carried out in 10 μl containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 0.1 mM EDTA,

1 mM DTT, 10% glycerol, 1 μg poly[dI-dC], and 3 μg liver nuclear extract and probe (10,000 cpm) and other oligonucleotide competitors at room temperature for 30 min. Supershift experiments were performed by further incubation with antibodies for 30 min at room temperature. Reaction mixtures were resolved by 6% native gel in 0.5 × Tris-glycine buffer at 12 V/cm. The antibody against Fos were purchased from Santa Cruz Biotechnologies.

RNA extraction and Northern blot analysis

Total RNAs from mouse livers were prepared by using Tri-Zol reagent (Life Technology, Gaithersburg, MD). The RNA pellet was dissolved in H_2O, and the concentration of RNA was determined at $A_{260\ nm}$. Total RNA (10 μg) was separated by electrophoresis with 1.5% formaldehyde agarose gel. The separated RNA was transferred to Hybond-N membrane (Amersham Pharmacia) and hybridized with ^{32}P-labeled C/EBPβ and GAPDH cDNA probes.

Solid-phase kinase assay

Mouse liver nuclear extract (150 μg) or 300 μg of 293T whole-cell extract was diluted to a final buffer composition as follows: 20 mM HEPES, pH 7.7, 75 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml papstatin A, 100 μg/ml PMSF. Then extract was incubated with 10 μl of GSH-Sepharose suspension (Amersham Pharmacia) to which 2 μg of either GST–Jun, or GST–ATF2 was bound. The mixture was rotated at 4°C for 3 h and pelleted by centrifugation at 10,000 × *g* for 20 sec. After 4 × 1-ml washes in HEPES binding buffer (20 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100), the beads were resuspended in 30 μl of kinase buffer (20 mM HEPES, pH 7.7, 20 mM $MgCl_2$, 2 mM DTT, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4) containing 20 μM ATP and 5 μCi (γ-^{32}P) ATP. After 20 min at 30°C, the reaction was terminated and washed with HEPES binding buffer. Phosphorylated proteins were eluted with SDS-PAGE sample buffer and resolved on 10% SDS-PAGE.

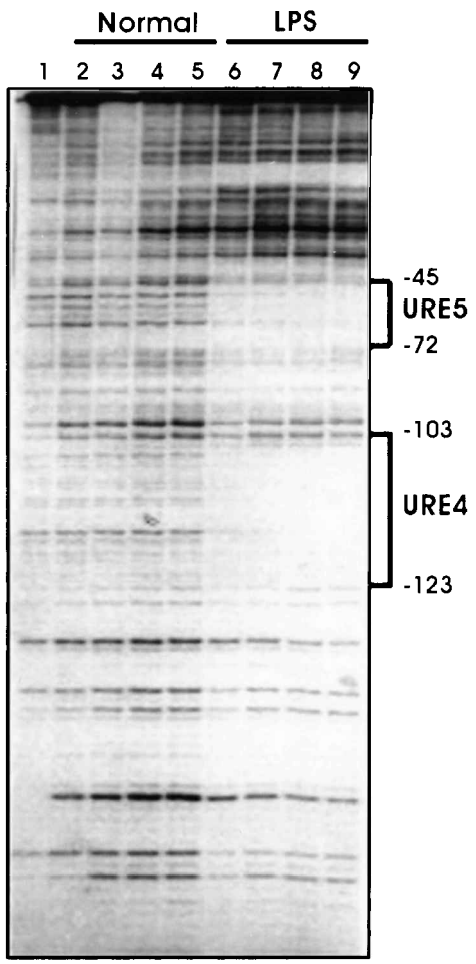

FIG. 1. DNase I footprinting analysis of the 5′ flanking sequence of C/EBPβ with nuclear extracts from normal or LPS-stimulated mouse liver. −156 to +82 fragment of C/EBPβ promoter was used as probes. Lane 1 is 10 μg BSA control, lanes 2–5 and lanes 6–9 are increasing amounts (10, 20, 30, 40 μg) of nuclear extracts from normal and LPS-stimulated mouse liver, respectively. The protein protective regions are indicated with brackets.

RESULTS

Identification and characterization of cis*-acting elements of the C/EBPβ gene*

Induction of the C/EBPβ gene during the acute phase reaction has previously been documented. The *cis*-elements of the distal promoter from nucleotides −390 to −150, which contains one constitutive URE3 (−220 to −190) element and two LPS-stimulated URE1 (−376 to −352) and URE2 (−253 to −223) elements, have already been characterized. C/EBPβ protein is known to bind both URE1 and URE2 to autoactivate its promoter (Chang *et al.*, 1995). We further analyzed the proximal promoter from nucleotides −156 to +82 by performing DNase I footprinting experiments using liver nuclear extracts prepared from normal or LPS-treated mouse (Fig. 1). Two prominent protection regions termed URE4 (−123 to −103) and URE5 (−72 to −45) were observed. In particular, the footprints (except URE3) were greatly enhanced when using nuclear extracts from LPS-treated mice compared to the untreated control mice, indicating that URE1, URE2, URE4, and URE5 are likely the motifs recognized by extracellular signal-regulated transcription factors.

Based on sequence homology comparison, URE2 (TGACGCCA, lower strand), URE4 (TGACGCGC), and URE5 (TGACGCAG) share extensive homology to CRE (TGACGTCA) and AP-1 (TGA(G/C)TCA) elements (Fig. 2A). The binding specificity of factors to these elements was further demonstrated by electrophoretic mobility shift competition assay. As shown in Figure 2B, the slower migrating DNA–protein complexes formed with URE2 and URE4 probes were decreased effectively when increasing amounts of competitors including URE2, URE4, URE5, as well as AP1 and CRE were present. The middle complexes formed with URE5 could also be partially competed by the URE2, URE4, URE5, AP1, and

A.

URE1: -376 to –352
5′-AGGAACGATCTGTTTCCCAAGAGTT-3′
3′-TCCTTGCTAGACAAAGGGTTCTCAA-5′

URE2: -253 to –223
5′-GAAGGGGGCGGGCTGGCGTCACCCGCGTCCGT-3′
3′-CTTCCCCCGCCCGACCGCAGTGGGCGCAGGCA-5′

URE4: -123 to –103
5′-GTGGCCGGGCAATGACGCGC-3′
3′-CACCGGCCCGTTACTGCGCG-5′

URE5: -72 to –45
5′-CCCAGCGTGACGCAGCCCGTTGCCAGGC-3′
3′-GGGTCGCACTGCGTCGGGCAACGGTCCG-5′

AP1:
5′-CGCTTGATGACTCAGCCGGAA-3′
3′-GCGAACTACTGAGTCGGCCTT-5′

CRE:
5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′
3′-TCTCTAACGGACTGCAGTCTCTCGATC-5′

B.

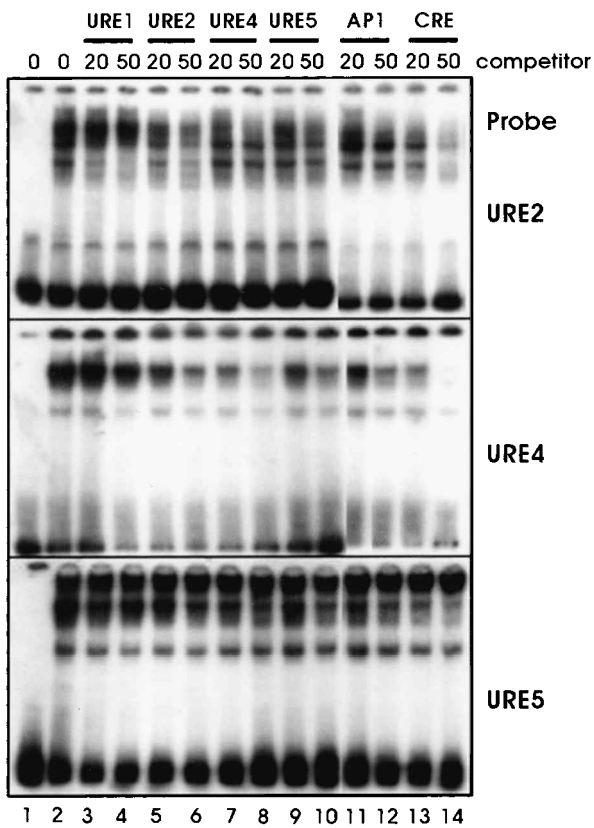

FIG. 2. Binding specificity of URE2, URE4, and URE5 elements. (**A**) The sequence of URE2, URE4, and URE5 and consensus CRE and AP1. (**B**) Electrophoretic mobility shift and competition assay. One nanogram of URE2, or URE4, or URE5 probe was incubated with nuclear extracts from LPS-treated mouse liver, in the absence or presence of 20 or 50 ng of competitors of URE1, URE2, URE4, URE5, AP-1, and CRE, as indicated.

CRE oligonucleotides. In contrast, URE1 oligonucleotide only slightly competes the faster migrating complex, which may be the C/EBP-like complex. These results indicated that the URE2, URE4, and URE5 binding factors are related to the cAMP-responsive factors or AP1.

c-Jun and ATF2 are involved in URE2 and URE4 binding

Previous studies have demonstrated that both URE4 and URE5 are cAMP-responsive elements, and the binding factors are of the CREB/ATF family (Niehof *et al.*, 1997; Berrier *et al.*, 1998). Because the AP-1 consensus oligonucleotide could compete the URE2– and URE4–protein complexes, we further examined whether the Jun/ATF proteins are present in these *cis*-elements binding activity. Due to the much more complicated binding patterns observed in the case of URE5, we will not further pursue analyzing these activities. As shown in Figure 3A, the slower migrating URE2– and URE4–protein complexes (arrowhead indicated) could be supershifted by anti-ATF2 and anti-c-Jun antibodies. This may reflect the presence of c-Jun and ATF2 in the protein complexes that bind to these sites, and recombinant c-Jun and ATF2 proteins from mammalian cells and bacteria were prepared to further confirm these binding activities. The nuclear extracts from 293T cells transfected with plasmids expressing c-Jun or Flag–ATF2 or both were isolated for immunoblotting (Fig. 3B) and for binding assay with the URE2 probe (Fig. 3C). As shown in Figure 3C,

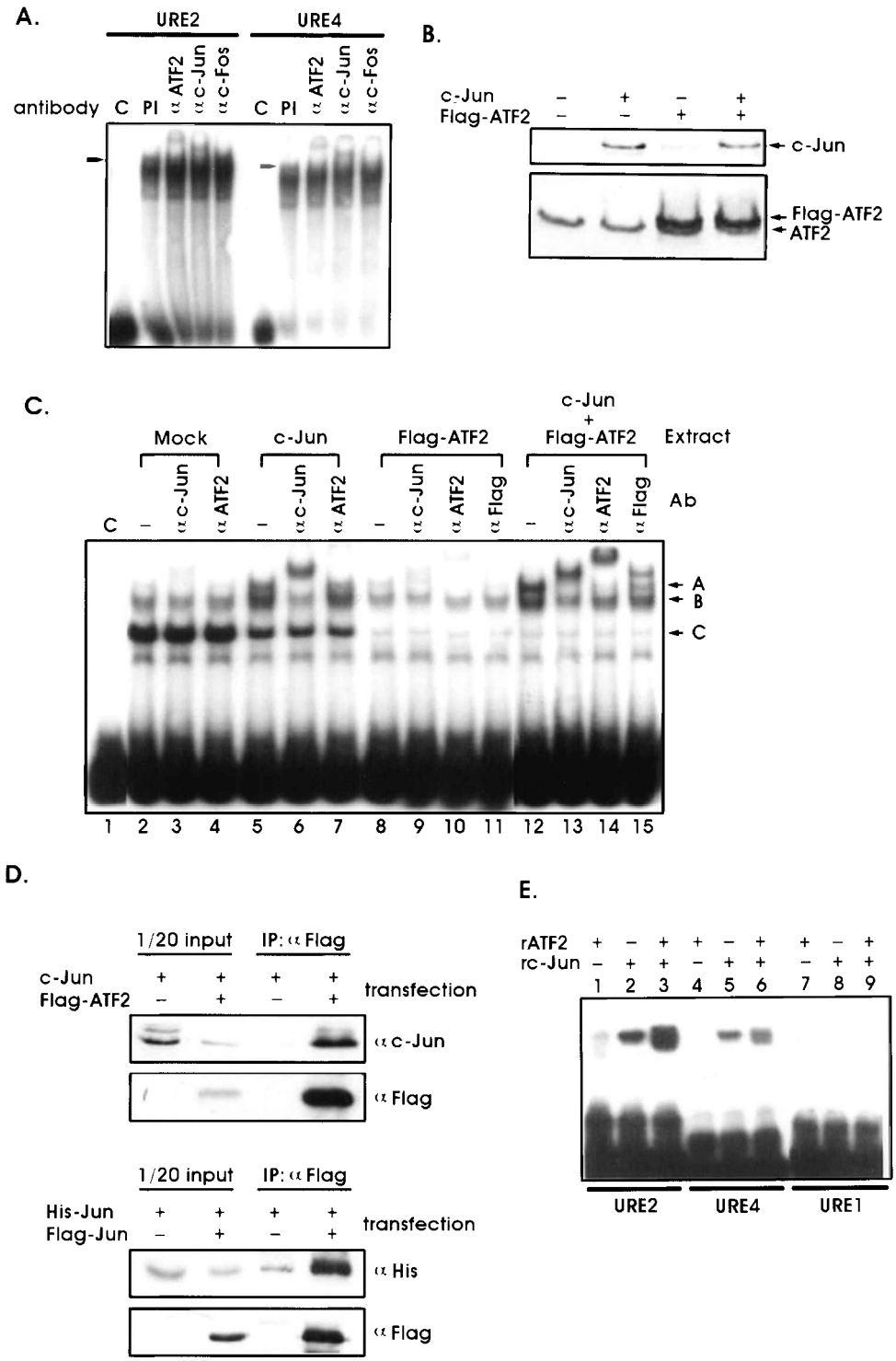

FIG. 3. c-Jun and ATF2 specifically bind URE2 and URE4. (**A**) LPS-treated mouse liver nuclear extracts were incubated with 1 ng of URE2 or URE4 probe, and then different antibodies (indicated on top of each lane) were added to induce DNA–proteins complex supershift. Lane C is probe-only control. The shifted band was indicated by arrowhead. (**B**) 293 T cells were transfected with pRSV–Jun, pCMV–Flag–ATF2, or both. The nuclear extracts were isolated and separated on SDS-PAGE. Western blot analysis was performed using anti-c-Jun or anti-ATF2 antibody. (**C**) The gel shift assay was carried out by using the nuclear extracts from (**B**) and URE2 probe. Anti-c-Jun, anti-ATF2, and anti-Flag antibodies were used for antibody supershift assay as indicated. (**D**) Upper panel: 293T cells were transfected with RSV–Jun or both RSV–Jun and pCMV–Flag–ATF2, and then the cell lysates were isolated and immunoprecipitated by anti-Flag agarose. The precipitated protein complexes were separated on SDS-PAGE and Western blotting with anti-c-Jun and anti-Flag antibodies. Lower panel: immunoprecipitated assay was performed by using cell lysates from pCMV–His–Jun or both pCMV–His–Jun and pCMV–Flag–Jun transfected 293T cells. After anti-Flag agarose precipitation, the protein complexes were detected by anti-His and anti-Flag antibodies. (**E**) Recombinant ATF2 (lanes 1, 4, and 7), c-Jun (lanes 2, 5, and 8), and combination of ATF2 and c-Jun (lanes 3, 6, and 9) were incubated with 1 ng of the URE1 (lanes 7–9), URE2 (lanes 1–3), and URE4 (lanes 4–6) probes.

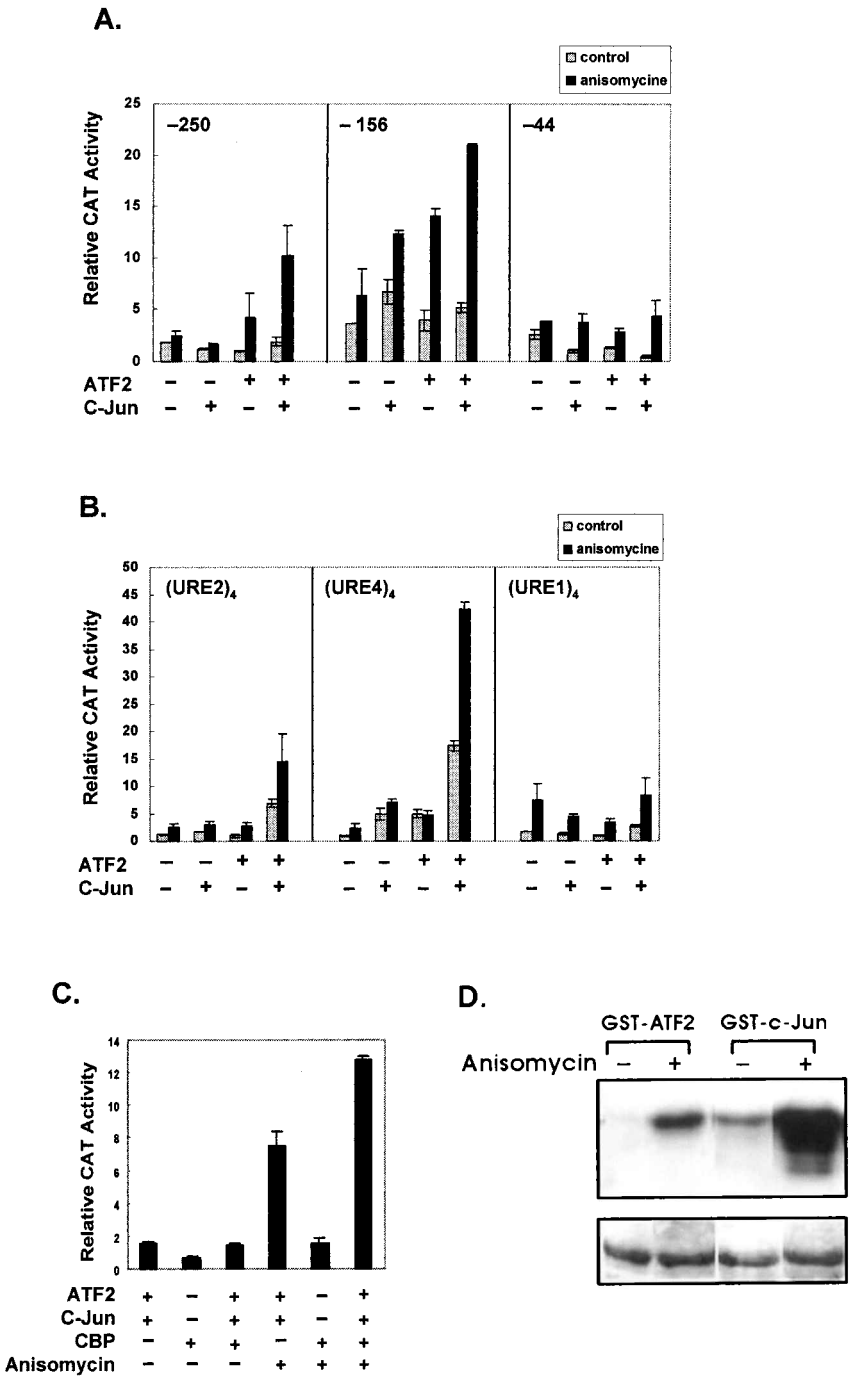

FIG. 4. The effect of c-Jun and ATF2 on C/EBPβ promoter activity. 293T cells were cotransfected with 0.25 μg of −250/+82-CAT, −156/+82-CAT, or −44/+82-CAT (**A**), $(URE1)_4$–CAT, $(URE2)_4$–CAT or $(URE4)_4$–CAT (**B**) together with 0.25 μg of pCMV–Flag–ATF2, or pRSV–Jun, or both. For the stress stimulation, 25 ng/ml of anisomycin was added for 16 h. The results of the relative CAT activity were obtained from two independent experiments. Two fold-amplified CAT relative activity from −44/+82-CAT result was represented. (**C**) 293T cells were cotransfected with 0.25 μg of −250/+82-CAT, 0.25 μg of pRSV–Jun, 0.25 μg of pCMV–Flag–ATF2, and 1 μg of pCMV–CBP as indicated with or without 25 ng/ml anisomycin treatment. The relative CAT activity was analyzed and plotted. (**D**) Beads with bound GST–Jun or GST–ATF2 were incubated with whole cells extract from nonstimulated or anisomycin-stimulated 293T cells. After extensive washes, solid-phase kinase assay was performed on the precipitated protein complexes. The gel was autoradiographed (upper) and Coomassie blue stained (lower).

the anti-c-Jun and anti-ATF2 antibodies supershift assays revealed that the A complex contains predominantly ATF2 and c-Jun, while the B complex contains c-Jun alone. Extracts with overexpressed c-Jun alone or both c-Jun and Flag–ATF2 could bind to the URE2 probe effectively, but the binding activity of the extracts with Flag–ATF2 alone was extremely poor. Moreover, coimmunoprecipitation experiments showed that c-Jun can from complex with itself and ATF2 (Fig. 3D). This suggests that Jun–Jun homodimer or Jun–ATF2 heterodimer can interact with URE2 element of the C/EBPβ promoter. An unidentified C complex was slightly decreased using the nuclear extracts with overexpressed c-Jun, and it was dramatically diminished by nuclear extracts with overexpressed Flag–ATF2. The gel shift results using the URE4 probe are identical to those using URE2 probe (data not shown). The bacterially expressed c-Jun and ATF2 proteins were also used in the gel shift assay. Figure 3E showed that ATF2 alone binds to URE2 and URE4 quite weakly (lanes 1 and 4), c-Jun alone has stronger binding activity (lanes 2 and 5), and both together possess the greatest binding activity (lanes 3 and 6). URE1 probe was served as a negative control that could not interact with either ATF2 or c-Jun or both together (Fig. 3E, lanes 7–9). Taken together, we concluded that c-Jun–ATF2 can specifically interact with both the URE2 and URE4 elements.

c-Jun and ATF2 activate C/EBPβ gene expression

To address the functional consequence of c-Jun and ATF2 binding to URE2 and URE4, transfection-based reporter assays were performed. 293T cells were cotransfected with expression vectors encoding ATF2, c-Jun, and reporter constructs of either −250/+82–CAT, −156/+82–CAT, −44/+82–CAT, $(URE2)_4$–CAT, $(URE4)_4$–CAT, or $(URE1)_4$CAT. c-Jun and ATF2 have been reported to be activated through phosphorylation by stress-activated protein kinases. We thus included usage of low concentration of anisomycin in our transfection experiments as a means of activating c-Jun and ATF2. In Figure 4A, the C/EBPβ promoter −250/+82 (containing URE2 and URE4) and −156/+82 (containing URE4) could be activated by a combination of c-Jun and ATF2 under the treatment of anisomycin. Overexpression of either protein activates the reporter activity less effectively. The basic promoter −44/+82 was unable to respond to both c-Jun and ATF2. On the other hand, the artificial constructs $(URE2)_4$–CAT and $(URE4)_4$–CAT responded to coexpression of c-Jun and ATF2 in the absence of stimulation, and the anisomycin treatment enhanced such promoter response (Fig. 4B). The URE1 reporter was not responsive to the expression of c-Jun and ATF2. To further explore the molecular mechanism underlying c-Jun–ATF2-mediated C/EBPβ activation, the coactivator CBP was included in the transfection assays. CBP functions as a coactivator protein for many transcription factors including c-Jun (Goodman and Smolik, 2000). As shown in Figure 4C, CBP could further enhance the c-Jun–ATF2 activity only under the stimulation of anisomycin. In regard to the effect of anisomycin on the activation of Jun and ATF2, we discovered that, through *in vitro* kinase assay, such drug treatment was able to induce signaling cascade, which ultimately leads to the phosphorylation of c-Jun and ATF2 (Fig. 4D). However, as demonstrated by the gel shift assay, the URE2 and URE4 binding activity present in the nuclear extracts was not altered upon anisomycin treatment (data not shown). Taken together, these results demonstrate that c-Jun and ATF2 cooperatively stimulate C/EBPβ promoter activity through their binding to the URE2 and URE4 elements, and their activity could be enhanced by protein phosphorylation, but via mechanisms other than increasing affinity towards the promoter elements.

The activation of c-Jun and ATF2 during acute phase reaction

Because both c-Jun and ATF2 are involved in C/EBPβ gene activation, we carried out experiments to further explore the expression and activation of c-Jun and ATF2 during the acute phase reaction. Northern blot analysis showed that C/EBPβ was induced in mouse liver upon LPS stimulation (Fig. 5A). The

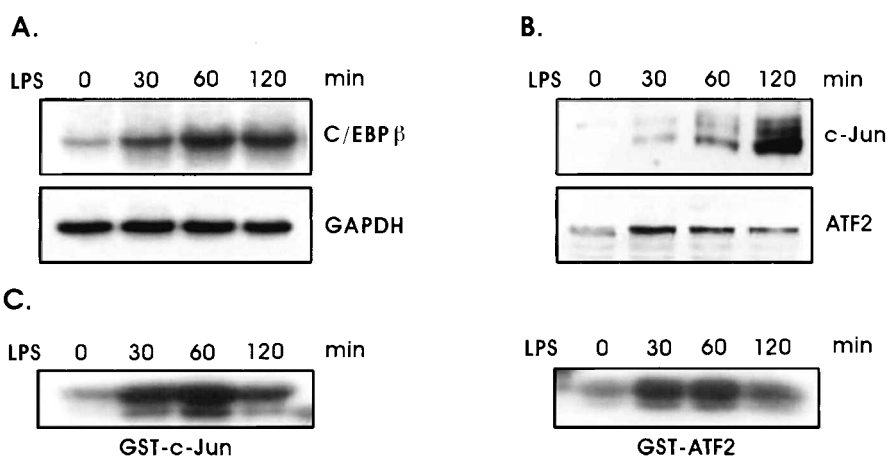

FIG. 5. The phosphorylation of c-Jun and ATF2 is associated with C/EBPβ gene expression during LPS treatment. (**A**) Northern blot analysis of C/EBPβ mRNA expression. Total RNA from mouse livers that were LPS-stimulated for 0, 30, 60, and 120 min, and was extracted and analyzed using ^{32}P-labeled C/EBPβ and GAPDH probes. (**B**) Western blot analysis of mouse liver nuclear extracts (subjected to LPS treatment for 0, 30, 60, 120 min) using anti-Jun or anti-ATF2 antibody. (**C**) Solid-phase kinase assays of c-Jun and ATF2. GST–Jun or GST–ATF2 was incubated with nuclear extracts of mouse liver stimulated with LPS for 0, 30, 60, 120 min. After extensive washes, solid-phase kinase assay was performed on the precipitated protein complexes.

expression levels of c-Jun and ATF2 during acute phase reaction were also examined by Western blot. The level of c-Jun was low in normal liver, but increased after LPS treatment (Fig. 5B). The level of ATF2 was slightly increased after LPS administration (Fig. 5B). Phosphorylation status of these two proteins during acute phase reaction were also examined because it is an indication of protein activation. Solid-phase kinase assay showed that nuclear extracts from LPS-treated mouse liver cold induce the phosphorylation of c-Jun and ATF2 (Fig. 5C).

DISCUSSION

We have provided several lines of evidence that both c-Jun and ATF2 may be involved in C/EBPβ gene expression: (1) gel shift assays showed that URE2 and URE4 can be specifically bound by ATF2 and c-Jun; (2) ATF2 and c-Jun cooperatively activate the C/EBPβ promoter through URE2 and URE4; (3) during acute phase response, the correlation between the expression of C/EBPβ and phosphorylation of ATF2 and c-Jun was observed.

The acute phase response induced by LPS is known to be mediated by certain cytokines such as IL-1, IL-6, and TNF-α. C/EBPβ itself can regulate an array of acute-phase proteins and cytokine genes in response to stress or acute inflammation conditions. The induction of C/EBPβ activity may be coordinated and controlled by gene transcription and protein modification. We present evidence here that the immediate early gene products c-Jun and ATF2 could respond to extracellular signals, and in turn, regulate C/EBPβ gene expression. This offers an explanation as to how C/EBPβ is activated during the acute phase response. Several other genes induced by inflammatory mediators, including *c-Jun, urokinase, E-selectin*, and *β-interferon*, have functional elements for ATF2 or ATF2–c-Jun heterodimers (Du *et al.*, 1993; van Dam *et al.*, 1993; Read *et al.*, 1997; Cirillo *et al.*, 1999). The inflammatory cytokines can activate cellular stress MAP kinases to phosphorylate ATF2 and c-Jun. After phosphorylation, they seem to interact with transcription coactivators such as CBP/p300 family to achieve optimal transactivation functions (Bannister *et al.*, 1995).

In addition to phosphorylation, dimer formation and protein–protein interaction are critical for the regulation of ATF2 and c-Jun activity. Both URE2 and URE4 in C/EBPβ promoter preferentially binds the c-Jun–ATF2 heterodimer but not the ATF2 homodimer (as shown in Fig. 3C). This dimer partner specificity is dependent on the DNA binding site variation. c-Jun–ATF2 and ATF2–ATF2 show differences in their relative affinities for more degenerated 8 bp ATF-like motifs (Benbrook and Jones, 1994). As seen in Figures 3C and E, recombinant c-Jun alone possessed high binding activity towards URE2 and URE4, much like the c-Jun–ATF2 complex. However, transactivation activity of c-Jun alone is weaker than that of the combination of c-Jun and ATF2. Why does the c-Jun–ATF2 heterodimer have higher transactivation activity? There may be a number of reasons: (1) ATF2 transactivates C/EBPβ promoter activity via its intrinsic, phosphorylation-dependent histone acetyltransferase activity (Kawasaki *et al.*, 2000); (2) our transfection assay was performed in 293T, a human renal epithelial cell line that is transformed by adenovirus E1A gene product and SV40 large T antigen. E1A protein has been reported to stimulate the c-Jun–ATF2 activity rather than the c-Jun–c-Jun activity (Hagmeyer *et al.*, 1995), possibly by increasing the transactivating capacity of ATF2 via an as yet unknown mechanism (Duyndam *et al.*, 1999); (3) c-Jun–ATF2 binding to URE2 and URE4 could cause the dissociation of some repressive complexes (such as the C complex in Fig. 3C); (4) ATF2 may interact with other proteins to synergistically activate C/EBPβ gene expression. The signaling crosstalk among other factors and c-Jun–ATF2 constitutes a complex but delicately controlled regulatory loop of C/EBPβ gene expression.

Previous reports showed that C/EBPβ itself and CREB could bind URE2 and URE4, respectively (Chang *et al.*, 1995; Niehof *et al.*, 1997). Thus, the same *cis*-acting elements in the C/EBPβ promoter could be recognized by different *trans*-acting factors. The contribution of each factor may depend on different cell types and physiologic conditions. For example, in macrophage and hepatocyte, CREB was found to modulate C/EBPβ promoter activity via different regulatory elements (Niehof *et al.*, 1997; Berrier *et al.*, 1998). In the case of the phosphoenolpyruvate carboxykinase (PEPCK) promoter, it can exist in altered states of cAMP responsiveness, depending on which of the CREB or C/EBPs proteins occupy specific *cis*-elements in the cAMP-response unit (Roesler, 2000).

In addition to acute phase reaction, C/EBPβ also plays an important role in the control of cellular proliferation and differentiation. It has been implicated in the regulation of proliferation and differentiation of adipocytes, hepatocytes, white blood cells, ovarian granulose cells, and mammary epithelial cells (Tanaka *et al.*, 1997; Robinson *et al.*, 1998; Seagroves *et al.*, 1998). Based on our results described here, there is a definite involvement of CREB, ATF2, and c-Jun in the activation of C/EBPβ gene, and clarification of their roles under various biologic conditions should be a subject of future research.

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Address reprint requests to:
Ching-Jin Chang
Institute of Biological Chemistry
Academia Sinica
No. 1 Sec. 4 Roosevelt Rd.
Taipei, Taiwan

E-mail: chingjin@gate.sinica.edu.tw

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