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CCAAT/Enhancer-Binding Protein δ Gene Expression Is Mediated by APRF/STAT3[1]

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The CCAAT/enhancer-binding protein δ (C/EBPδ) transcription factor is known to be rarely expressed but sharply induced at an early stage of the acute phase response. To investigate the regulation mechanisms for this induction, the 5′-flanking region of the rat C/EBPδ gene was isolated. Functional analyses involving transfection and footprinting indicated that the upstream region up to −175 bp is sufficient for the full basal activity in rat fibroblast 3Y1 cells. At least three *cis*-elements including a GC box are involved in this activity. When HepG2 cells were treated with interleukin-6 (IL-6), C/EBPδ mRNA was rapidly induced. Transfection and gel shift analyses identified the binding site for the acute phase response factor/signal transducers and activators of transcription (APRF/STAT3). These findings strongly indicate that C/EBPδ gene expression is mediated by APRF/STAT3, which is phosphorylated for the activation through the IL-6 receptor when cells are treated with IL-6, and *trans*-activates the other acute phase response genes.

Key words: acute phase response, APRF/STAT3, CCAAT/enhancer-binding protein, interleukin-6, transcription.

The CCAAT/enhancer-binding protein (C/EBP) was originally identified as a *trans*-activator protein in the nuclear extract of rat liver (*1*). This protein has a basic leucine zipper region (bZIP) containing a DNA-binding domain that is also found in the transcription factors, Jun and GCN4 (*2, 3*). The dimeric forms of C/EBP proteins are active in the transcription regulation of the target genes. Related genes have recently been identified, and it is now well known that C/EBP proteins comprise a gene family consisting of at least six members (*1, 4-7*).

The functions of four C/EBPs have been well studied: C/EBPα (previously designated as C/EBP), C/EBPβ (also termed NF-IL6, LAP/LIP, IL6-DBP, AGP/EBP, CRP2, and SF-B) (*5, 6, 8-12*), C/EBPδ (also termed NF-IL6β, CRP3, and CELF) (*5, 6, 13, 14*), and CHOP10 (also termed GADD153) (*7*). C/EBPα activates the transcription of liver-specific and adipose-specific genes (*15*), and C/EBPβ and C/EBPδ contribute to the acute phase response (*16*). C/EBPβ also regulates the genes in liver both positively and negatively (*5, 6, 8-12, 17, 18*), and CHOP10 functions as a negative regulator through substitutions in the bZIP region (*7*). Although it was thought that the C/EBPγ protein (also termed Ig/EBP-1 and GPE-BP) (*4, 5, 19*) is non-functional, recent reports have revealed that C/EBPγ is expressed to a limited degree during B lymphocyte development and acts as a dominant negative inhibitor (*20, 21*). The function of C/EBPε (also termed CRP1) (*5, 6*) remains unclear.

It is well known that the expression levels of C/EBP family members change dramatically during the acute phase response (*22, 23*): the mRNA level of C/EBPα decreases, and those of C/EBPβ, C/EBPδ, and CHOP10 increase significantly. It is of interest that C/EBPδ is more rapidly and more strongly induced compared with C/EBPα and CHOP10 in the response to lipopolysaccharide (LPS) treatment in the rat (*23*), strongly suggesting that C/EBPδ contributes the initial step of the regulation of expression of acute phase plasma protein genes. Moreover, Ramji *et al.* reported that C/EBPβ and C/EBPδ are induced by interleukin 6 (IL-6) *via* different mechanisms (*24*). To clarify the regulation of expression of these transcription factors, characterization of the promoter region is necessary. The promoter sequences of C/EBPα (*25-28*), C/EBPβ (*9, 29, 30*), and CHOP10 (*31*) have been reported, and it is suggested that both C/EBPα and C/EBPβ are regulated through autoregulation (*25-27, 29*). CHOP10 also has a C/EBP site and is activated by C/EBPβ (*23*). Although a genomic clone of C/EBPδ was isolated from mouse and human, the promoter sequences of this gene were identified only up to −280 bp and −129 bp in mouse and human, respectively (*5, 13, 32*), and the promoter activity has not been analyzed yet.

We report here cloning of the promoter region of the rat

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Abbreviations: APRE, acute phase response element; APRF/STAT3, acute phase response factor/signal transducers and activators of transcription 3; C/EBP, CCAAT/enhancer-binding protein; bZIP, basic region and leucine zipper region; IL-6, interleukin-6; LPS, lipopolysaccharide; PCR, polymerase chain reaction.

C/EBPδ gene, functional analyses of the basal activity, and identification of a *cis*-element modulating the expression of the C/EBPδ gene. The data suggest that the basal activity of the C/EBPδ gene is regulated by multiple *cis*-elements including an Sp1 binding site, and in the response to IL-6 treatment, this gene is activated through the acute phase response element (APRE) which is recognized by the acute phase response factor/signal transducers and activators of transcription 3 (APRF/STAT3).

MATERIALS AND METHODS

Isolation of C/EBPδ Genomic Clones—An EMBL3 SP6/T7 genomic DNA library from adult Sprague-Dawley male rat liver (Clontech Lab., Palo Alto, CA, USA) was screened with genomic DNA corresponding to the open reading frame of the mouse C/EBPδ (kindly provided by Dr. S.L. McKnight) (*5*). Two isolated clones were further analyzed. The fragments containing the promoter and the protein coding region of the C/EBPδ gene were subcloned into pBluescript KS+ (Stratagene Cloning Systems, La Jolla, CA, USA) and sequenced by the dideoxy chain termination method (*33*).

Plasmid Construction—The fragment comprising -2.7 kb to $+42$ bp, and various deletion fragments were inserted into the *Xho*I and *Hin*dIII sites in a promoter-less luciferase vector, PGV-B (Toyo Ink Mfg., Tokyo) according to the standard protocol (*34*). Mutants of various lengths were constructed by means of polymerase chain reaction techniques (PCR) (*35*) or by deletion of the 5′ end by exonuclease III and mung bean nuclease digestions. The internal deletion mutants lacking the $\Delta\delta$IV site, -2700-$\Delta\delta$IV/$+42$, and $-175\Delta\delta$IV/$+42$, were constructed by deoxyoligonucleotide-directed mutagenesis with the following primer: 5′-CAGGGAGGGACCGGAGACCCGCCC-TCTGCC-3′, according to the method of Kunkel *et al.* (*36*). All constructs used here were checked by sequencing with the dideoxy method using denatured plasmid templates (*33*).

Cell Culture and DNA Transfection—3Y1 cells, a rat fibroblast cell line, and HepG2 cells, a human hepatoma cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) and minimal essential medium (MEM), respectively, containing 10% fetal bovine serum. The cells were transfected by the calcium phosphate co-precipitation technique described by Chen and Okayama (*37*). The cells were harvested at 40 h incubation after transfection, and the luciferase activity and protein concentration were determined with Pikka Gene (Toyo Ink Mfg.) and a lumiphotometer, and by the method of Bradford (*38*), respectively. The activities are expressed as relative light unit (RLU)/μg protein. All the transfection experiments were performed at least three times using two or three different preparations of DNA, and the results are presented as mean values. For cells treated with IL-6, 500 U/ml of IL-6 was added to the medium 16 h after the transfection.

RNA Isolation and Northern Blot Analysis—HepG2 cells at 80% confluence were treated with 500 U/ml of IL-6 in serum-free medium, and the cells were harvested at the indicated times. Total RNA was extracted using TRIzol (Gibco BRL Life Technologies, Grand Island, NY, USA). For Northern blot analysis, 50 μg of total RNA was electrophoresed on a 1.0% agarose gel containing 2% formaldehyde, and then transferred to a nitrocellulose filter (Schleicher & Schuell GmBH, Germany). The mouse C/EBPδ genomic DNA was used for the hybridization as a probe.

Animals—Male Wistar rats (8-week-old) received lipopolysaccharide (LPS) (5 mg/kg) from *Escherichia coli* (Difco Lab., Detroit, MI, USA) intraperitoneally for 1 h, and the livers were used for the preparation of a nuclear extract.

Preparation of Nuclear Extracts—Nuclear extracts of 3Y1 and HepG2 cells were prepared according to the method of Dignam *et al.* (*39*). For partial purification of the nuclear extracts of rat liver and 3Y1 cells, the extracts were passed through a heparin-agarose column (Pharmacia Biotech, Uppsala, Sweden) and the bound proteins were eluted with 0.4 M KCl in the buffer described above. For the preparation of the nuclear extract of HepG2 cells, 80% confluent cells were treated with 500 U of IL-6 per ml for 15 min in the serum-free medium, and the extract was prepared in the presence of both protease inhibitors [1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.3 μg/ml antipain A] and phosphatase inhibitors [1 mM NaF, 1 mM $Na_4P_2O_7$, and 0.1 mM sodium orthovanadate].

DNA Binding Analyses—DNase I footprinting analysis was performed as described previously (*40*). The DNA fragment (*Kpn*I/*Hin*dIII; -352 to $+42$) was labeled for the non-coding strand at the 3′ end of the *Hin*dIII site. For the coding strand, the DNA fragment (*Bss*HII/*Hin*dIII; -226 to $+42$) was labeled at the 3′ end of the *Bss*HII site. Gel shift analysis was performed as described previously (*41*). The sequences of the synthetic oligonucleotides of the δIV site in the C/EBPδ gene promoter and the APRE core site in the rat α_2-macroglobulin gene promoter are as follows:

δIV site: 5′-ctagTCGTTCCCAGCAGCACT -3′
3′- AGCAAGGGTCGTCGTGAgatc-5′

APRE core site: 5′-ctagCTTCTGGGAATTCCTA -3′
3′- GAAGACCCTTAAGGATgatc-5′

Nuclear extracts (10 μg protein/reaction) were mixed with 6.25 μl of 20 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, 20 mM EDTA, 1 μg of poly(dI-dC), and 0.2 ng radiolabeled double-stranded oligonucleotides. The binding reaction was continued at 4°C for 1 h. Each reaction mixture was loaded on a 4% non-denaturing polyacrylamide gel, electrophoresed at 150 V for 2 h, fixed with 10% methanol and 10% acetic acid, vacuum dried, and then autoradiographed. For supershift analysis, 1 μl of the STAT3 antibody (New England Biolabs., MA, USA) was added to the nuclear extracts, followed by incubation for 30 min at 4°C, with a subsequent binding reaction with labeled oligonucleotides for 1 h at 4°C.

RESULTS

Cloning of the Rat C/EBPδ Gene—To study the regulation mechanisms for the C/EBPδ gene, we first isolated the C/EBPδ gene from 6×10^5 rat genomic DNA clones with mouse C/EBPδ genomic DNA as a probe. Two clones (λ110, with the promoter region, and λ60, including the open reading frame and 3′ region) were further analyzed after appropriate subcloning. Sequence analysis of the open reading frame region has identified the rat C/EBPδ gene as an intron-less gene, as seen for the human C/EBPδ gene

(*13*), and also for C/EBPα (*24*), and C/EBPβ (*9*) (data not shown). The transcription start site was determined by primer extension analysis using a synthesized oligonucleotide positioned 30 bp downstream from the ATG translation start site, with total RNA prepared from the tissues of a rat treated with LPS, as a primer and an RNA source, respectively (data not shown). In Fig. 1, the nucleotide sequence of the 5′ flanking region of the rat C/EBPδ gene from −693 to the translation start codon, ATG, and the putative binding sites for *trans*-acting factors identified using the transcription factor database (The National Library of Medicine) are shown. A TATA box-like sequence, TAGAAA, was identified at 30 bp upstream from the transcription start site.

Functional Analysis of the Basal Activity of the C/EBPδ Gene Promoter—To identify the region of the rat C/EBPδ which is important for the basal activity of transcription of this gene, the fragment comprising −2.7 kb to +42 bp, and various deletion fragments were joined to a luciferase gene, and then transfection analysis was performed with rat 3Y1 fibroblast cells. As shown in Fig. 2, the region comprising −2.7 kb to +42 bp exhibited even higher basal activity than that of the SV40 promoter in rat fibroblast cell line 3Y1. The various mutants with deletions to −175 bp showed no significant decrease in luciferase activity. However, the −79/+42 construct exhibited very low activity, indicating that there are positive regulatory elements between −175 and −79.

To detect the protein binding region in the promoter, we next performed DNase I footprint analysis using the fragment (−352 to +42) labeled at the 3′ end of the *Hin*dIII site and the nuclear extract of 3Y1 cells (Fig. 3). Three protected sites were observed in the −175/−112 region (named δI, δII, and δIII). This protected region with hypersensitive sites is also shown in Fig. 1. Whereas the third protected region (−127/−112) is probably due to the Sp1 binding because of a typical GC box, GGGCGG, unknown factors may bind to the first two protected regions (−175/−148 and −148/−127).

For determination of whether or not these three binding sites really contribute to the basal promoter activity of the C/EBPδ gene, we next prepared another three deletion mutants, −148/+42 (lacking δI), −127/+42 (lacking δI

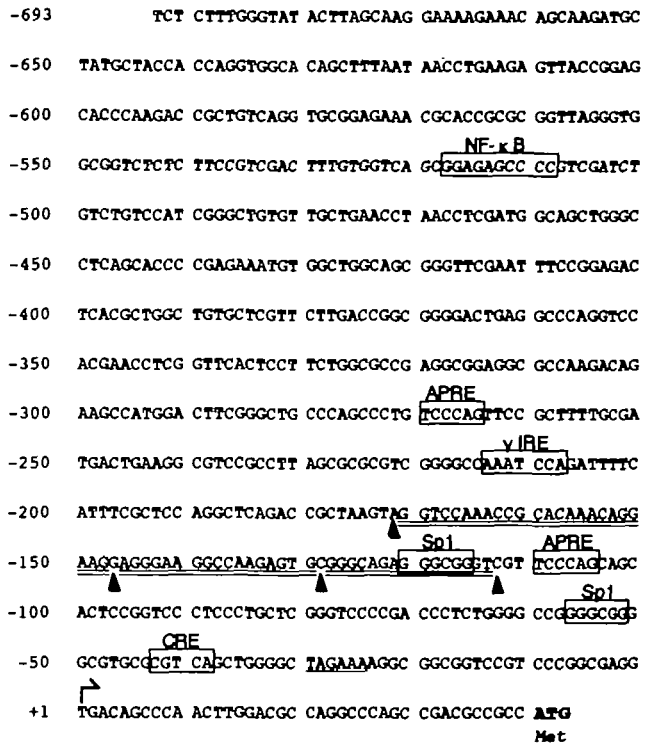


Fig. 1. **Nucleotide sequence of the promoter region of the rat C/EBPδ gene.** The transcription start site and the translation start codon are shown as +1 and in boldface, respectively. The TATA box-like sequence is underlined. The putative binding sites for *trans*-acting factors are indicated. The protected regions and the hypersensitive sites found on DNase I footprint analyses are double-underlined and indicated by arrowheads, respectively.

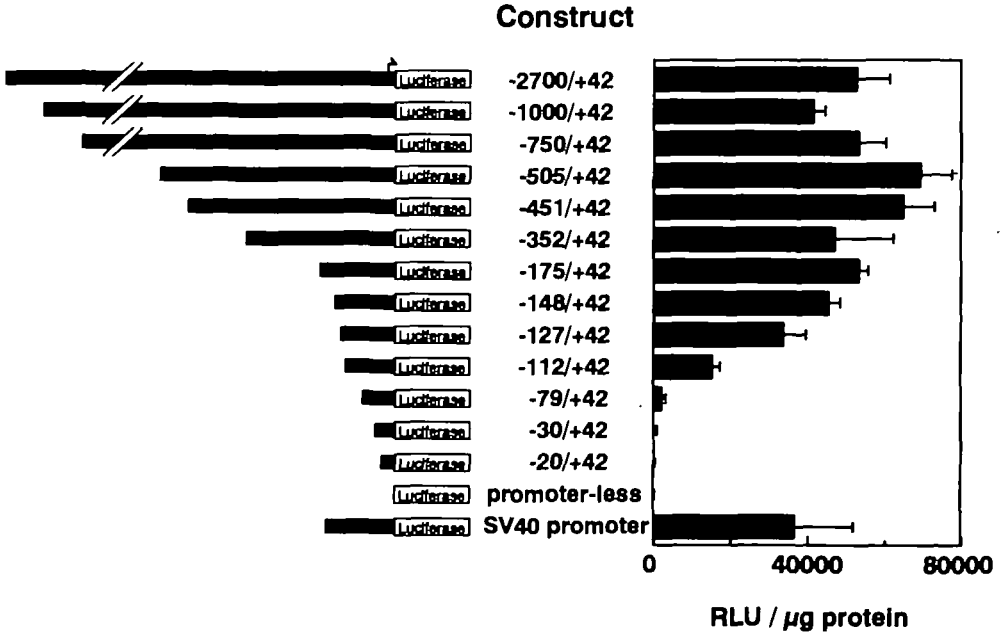


Fig. 2. **Functional analysis of the promoter activity of the C/EBPδ gene.** The left and right panels show the schematic structures of the constructs and the results of the transfection experiment involving the luciferase assay, respectively. Various deletion mutants of promoter regions upstream of +42 were connected to the promoterless luciferase gene, PGV-B. These reporter plasmids (4.5 μg/6 cm dish) were transfected into 3Y1 cells by the calcium phosphate co-precipitation technique, and the luciferase activities were determined with a luminometer. The data are the mean values for RLU/μg protein on four independent transfection analyses. The error bars indicate standard deviations.

and δII), and −112/+42 (lacking δI, δII, and δIII), and performed transfection analysis. As shown in Fig. 2, the luciferase activity gradually decreased with each deletion, strongly indicating that these three binding regions could contribute to the basal transcription activity. The protein bound to the −112/−79 region remains to be investigated.

Compared with the −79/+42 construct with low transcription activity, the −30/+42 construct showed further lower activity and almost complete loss of the transcription activity (Fig. 2). It is possible that the GC box between −57 and −52 also contributes to this weak activity in this region, although footprint analysis did not reveal clear Sp1 binding. The −20/+42 construct exhibited the complete loss of the activity (Fig. 2), probably due to the deletion of the TATA box-like sequence.

Induction of C/EBPδ mRNA in HepG2 Cells Treated with IL-6—It is well known that the mRNA of the C/EBPδ gene increases strongly, even more and earlier than C/EBPβ mRNA does, in the liver during the acute phase response, *e.g.*, on LPS treatment (*22, 23*). Since the acute phase response is a result of very complicated events, and the human hepatoma cell line, HepG2 cells, has proven to be a good cell line for studying IL-6 induced gene expression (*24*), we tried to use this cell line for characterization of the C/EBPδ gene promoter. First, we investigated whether or not the mRNA of the C/EBPδ gene increases on IL-6 treatment. As shown in Fig. 4, when HepG2 cells were treated with IL-6, the level of C/EBPδ gene mRNA increased within 30 min. On the other hand, the mRNA level of C/EBPδ in 3YI cells was unaffected by the IL-6 treatment (data not shown).

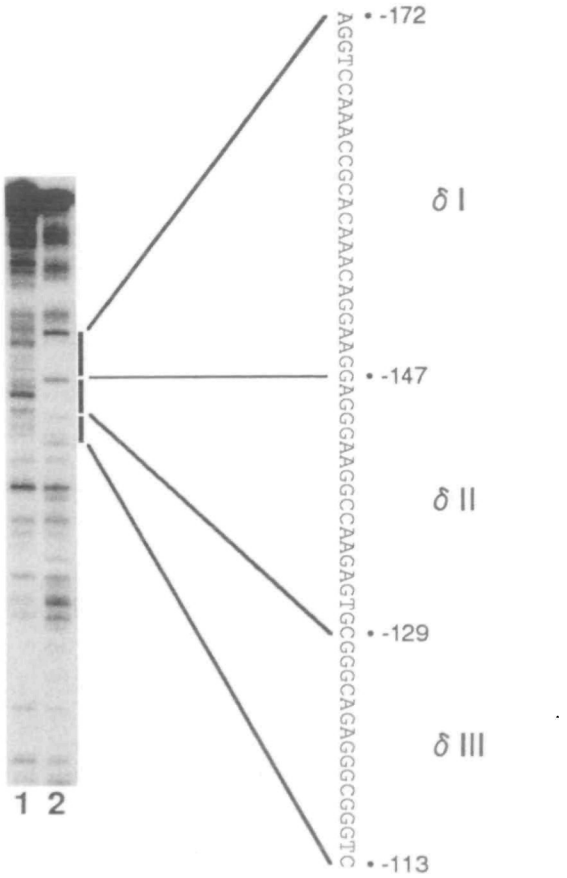
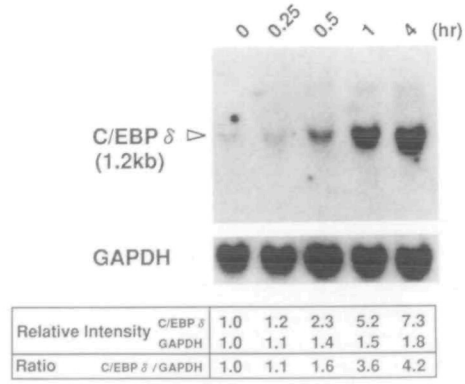


Fig. 3. **DNase I footprint analysis of the 3Y1 nuclear extract with the C/EBPδ gene promoter.** The fragment (−352 to +42) labeled at the 3′ end of the *Hin*dIII site was incubated with the 0.4 M KCl heparin-agarose fraction of the nuclear extract of 3Y1 cells (lane 2) or bovine serum albumin as a control (lane 1). The protected regions, δI, δII, and δIII, are indicated by vertical bars, with the sequence is shown at the right.



Relative Intensity	C/EBP δ	1.0	1.2	2.3	5.2	7.3
	GAPDH	1.0	1.1	1.4	1.5	1.8
Ratio	C/EBP δ /GAPDH	1.0	1.1	1.6	3.6	4.2

Fig. 4. **Northern blot analysis of C/EBPδ in HepG2 cells treated with IL-6.** The top panel shows Northern blot analysis of C/EBPδ using total RNAs (50 μg per lane) prepared from HepG2 cells. Cells were treated with 500 U/ml of IL-6, and harvested after the indicated incubation times. The same filter was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The radioactivities of the corresponding bands of C/EBPδ (shown by the arrowhead) and GAPDH mRNAs were determined with an image analyzer BAS2000 as relative intensity, and the normalized intensities (C/EBPδ/GAPDH) of the mRNA levels at each stage as to no treatment (0 h) are shown.

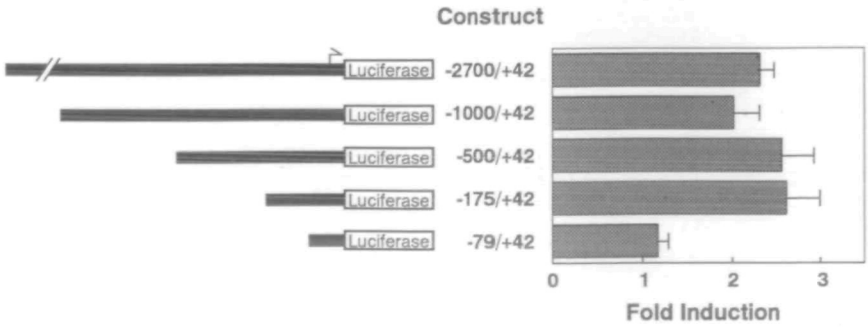


Fig. 5. **Functional analysis of the IL-6 inducible activity of the C/EBPδ gene.** The left and right panels show the schematic structures of the constructs and the results of the transfection experiment involving the luciferase assay, respectively. Various deletion mutants of promoter regions upstream of +42 were connected to the promoter-less luciferase gene, PGV-B. These reporter plasmids were transfected into HepG2 cells by the calcium phosphate co-precipitation technique, and the luciferase activities were determined with a luminometer. The fold induction by IL-6 is shown for four independent transfection analyses. The error bars indicate standard deviations.

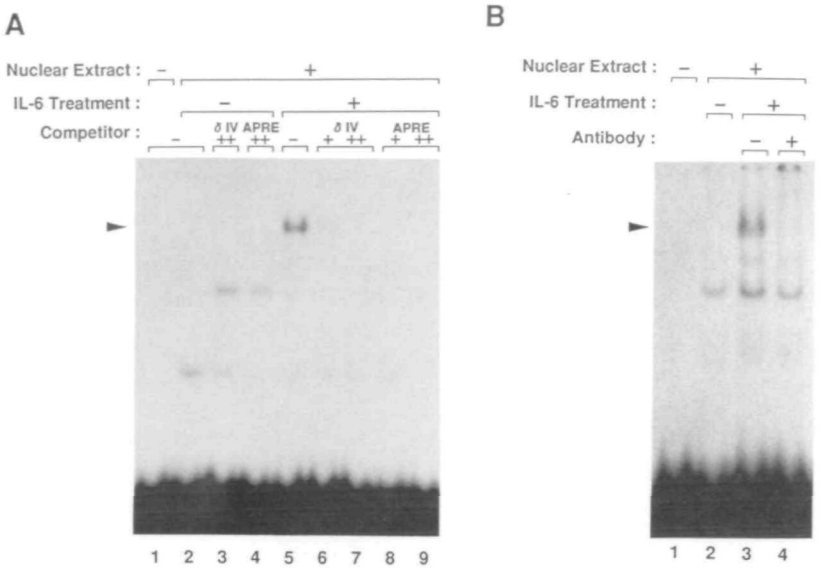


Fig. 6. **Gel shift analysis of APRE binding activity using nuclear extract of HepG2 cells treated with IL-6.** Double strand oligonucleotides, δIV of the C/EBPδ promoter, were used as probes for binding analyses. The probes were incubated with 10 μg of the nuclear extract of HepG2 cells untreated (lanes 2-4 in A and lane 2 in B) or treated with IL-6 (500 U/ml) for 15 min (lanes 5-9 in A and lanes 3 and 4 in B). Lanes 1 in A and B: bovine serum albumin as a control. A 50-fold (+) or 250-fold (++) molar excess of non-labeled oligonucleotides was used for competition. The competitors, δIV of the C/EBPδ promoter and APRE from the rat α_2-macroglobulin promoter, are indicated as δIV or APRE, respectively, at the top of the lanes. Anti-APRF/STAT3 antibodies were used for super-shift analysis (lane 4 in B). The arrowheads indicate the specific binding by APRF/STAT3.

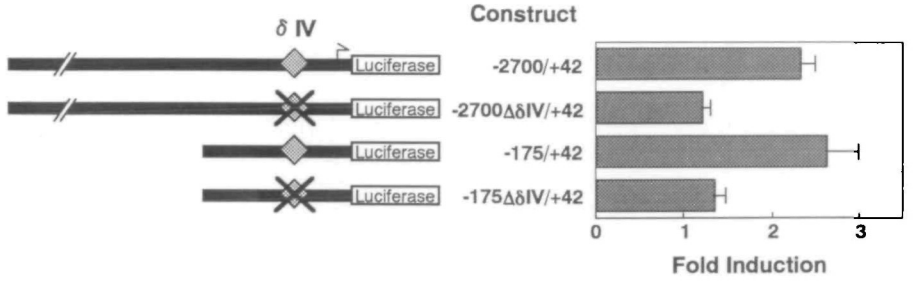


Fig. 7. **Identification of the IL-6 inducible element in the promoter of the C/EBPδ gene.** The left and right panels show the schematic structures of the constructs and the results of the transfection experiment involving the luciferase assay on HepG2 cells, respectively, using internal deletion mutants. The fold induction by IL-6 is shown for four independent transfection analyses. The error bars indicate standard deviations.

Identification of the IL-6 Responsive Element in the Promoter Region of the C/EBPδ Gene—Using the various deletion mutants shown in Fig. 1, the IL-6 responsive element in the promoter region was identified by transfection analysis with HepG2 cells. The −2.7 kb/+42 construct showed IL-6 inducibility, and the 5′ deletion to −175 bp exhibited similar activity, indicating that the region between −175 bp and +42 bp is sufficient for IL-6 responsiveness (Fig. 5). The −79/+42 construct exhibited no inducibility by IL-6.

Next we performed DNase I footprint analysis using the nuclear extract of HepG2 cells treated with IL-6. However, we obtained the same footprint pattern, with three protected regions (δI, δII, and δIII) (data not shown), as seen for the nuclear extract of 3Y1 cells. Next, when a rat was treated with LPS, δI, δII, and δIII were protected the same as in the case of non-treated rat liver, though the nuclear extract gave another protected region, δIV, at a site just downstream of δIII (data not shown). Interestingly, the nucleotide sequence of δIV is quite similar to that of APRE, to which APRF/STAT3 binds (*42-44*) (Fig. 1).

Trans-Activation of the C/EBPδ Gene Promoter through APRF/STAT3 Binding—Although the reason why we could not obtain the protected pattern at the δIV site using the nuclear extract of HepG2 cells treated with IL-6 is not known, it is possible that the sensitivity of DNase I footprint analysis is not enough to obtain reveal clear protection. For determination of whether or not (a) this δIV region responds to IL-6 treatment and (b) APRF/STAT3 binds to this site, we next performed a gel mobility shift assay and transfection analyses. First, to clarify whether the binding of a specific protein for δIV is induced by IL-6, we performed a gel mobility shift assay. We used the δIV site in the C/EBPδ gene promoter as a probe. When the nuclear extract was prepared from untreated HepG2 cells, no binding was detected. However, when the nuclear extract of IL-6 treated cells was used, a shifted band was observed, and this band disappeared on the addition of both the non-labeled δIV site in the C/EBPδ gene promoter and the APRE core sequence from the rat α_2-macroglobulin promoter, indicating that this binding activity is specifically induced by IL-6 treatment (Fig. 6A).

To further determine whether this complex is composed of APRF/STAT3, super-shift analysis was performed. The addition of a specific antibody to APRF/STAT3 clearly inhibited the DNA-protein complex formation (Fig. 6B), whereas preimmune serum or IgG gave an unchanged pattern (data not shown).

Next, we constructed internal deletion mutants lacking the δIV site, and performed transfection analysis. As shown in Fig. 7, two kinds of δIV-deletion mutants, −2700-ΔδIV/+42 and −175ΔδIV/+42, showed a complete lack of IL-6 responsiveness in HepG2 cells. Taken together, these findings demonstrated that on the induction of the C/EBPδ gene by IL-6 treatment, the δIV site in the promoter is the most important *cis*-element, and the binding of APRF/STAT3 to this site triggers the activation of transcription of the C/EBPδ gene.

DISCUSSION

The gene expression in eukaryotes is mainly regulated at the transcriptional level, and numerous transcription factors have been identified for tissue-specific, stage-specific and/or constitutive expression (*45*). Recent studies characterized the promoters of transcription factor genes and clarified the transcription mechanisms for the transcription factors themselves. These findings revealed that the transcription of many transcription factors, including c-jun (*46*), c-fos (*47*), MyoD (*48*), serum response factor (*49*), ets-1 (*50*), and hepatocyte nuclear factor 1 (*51*), is regulated by the factors' own gene products, that is, through autoregulation. The problem of how transcription starts, *i.e.*, by means of a transcription factor's own product or by other transcription factors, may be explained by activation caused by the factor's own product, which is weakly expressed and then modified by phosphorylation or dephosphorylation, for example (*52*). It is also possible that the translocation from the cytosol to the nucleus occurs through the signal (*53*), and that autoregulation is not involved; instead, another transcription factor(s) stimulates the initial step of the transcription.

In the present study, we characterized the promoter activity of the C/EBPδ gene when cells were treated with IL-6 as one of the models of the acute phase response. The acute phase response is mainly regulated by two classes of transcription factors, the C/EBP family and APRF, which each bind to a *cis*-element. Among the C/EBPs, both C/EBPβ and C/EBPδ activate the acute phase responsive genes, while CHOP10 heterodimerizes with these C/EBPs and represses the transcription because of the inability of DNA binding. During the acute phase response, these transcription factors themselves are positively regulated and C/EBPα is negatively regulated. The regulation of the C/EBPα and C/EBPβ genes has been well characterized. Some specific transcription factors for these two genes have been identified, and these genes are also known to be regulated through autoregulation (*25-27, 29*), but little is known about the regulation of the C/EBPδ gene.

As a first step for elucidating the regulation mechanisms for C/EBPδ gene expression, we isolated a rat C/EBPδ genomic clone and characterized its promoter region. At least three *cis*-elements, including a GC box, are involved in the basal activity. When the cells are treated with IL-6, APRF seems to bind to APRE in the C/EBPδ gene promoter, and thereby trigger the transcription. APRF (also termed STAT3) is a key protein, as is the C/EBP family, in inflammation (*42, 43*). APRF is constitutively expressed in a variety of tissues including liver, but lacks DNA binding ability when dephosphorylated (*43*). In response to IL-6 or other cytokine stimulation, this protein is phosphorylated at a tyrosine residue within 5 min, becomes capable of binding to DNA and triggers gene transcription, the phosphorylated APRF/STAT3 disappearing within 1 h (*43*). For maximal activation of transcription by APRF/STAT3, the phosphorylation of a serine residue is also required (*54*). Since these events appear to occur very rapidly, the C/EBPδ gene may be activated quite quickly compared with the induction of C/EBPβ and CHOP10 (*23*). For characterization of the precise functions of APRF/STAT3 in C/EBPδ gene expression, experiments involving dominant negative or knock-out cells will be required.

The mRNA level of the C/EBPδ gene is maintained at a high level compared with of that C/EBPβ (*23*). It is unlikely that APRF/STAT3 continues to activate the gene expression, since APRF/STAT3 is dephosphorylated within 1 h and loses its DNA binding ability (*43*). A possible explanation lies in the stability of mRNA of the C/EBPδ gene, but this was not examined in the present study. Another possibility is that the C/EBPδ gene is also regulated by its own gene product, as observed for C/EBPα and C/EBPβ, as described above. In a preliminary experiment, the C/EBP responsive region was not identified up to -6 kb of the promoter region of the C/EBPδ gene. However, it is possible that other regions, including a 3′-untranslated region and a far upstream region of the promoter, may respond to the gene's own product. This remains to be investigated.

It was reported that C/EBP is required for embryonic development of *Drosophila* (*55*), and for the consolidation of stable long-term synaptic plasticity in *Aplysia* (*56*). Since the DNA binding specificity among C/EBPα, C/EBPβ, and C/EBPδ is quite similar (*41*), the characterization of the specific expression mechanisms for these *trans*-acting factors in the above events is also necessary.

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