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CCAAT/Enhancer-Binding Protein δ Gene Expression Is Mediated by Autoregulation through Downstream Binding Sites

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CCAAT/enhancer-binding protein δ (C/EBPδ) transcription factor is sharply induced at the early stage of the acute phase response. We previously reported that the C/EBPδ gene expression is induced by the acute-phase response factor/signal transducers and activators of transcription 3 (APRF/STAT3). However, the expression level of the C/EBPδ gene is relatively high up to several hours after the stimulation, whereas APRF/STAT3 is inactivated within one hour. In this report, we identified the two C/EBPδ binding sites at the downstream region of this gene. The binding analysis revealed that both of these sites bound recombinant C/EBPδ protein. A cotransfection analysis identified these sites as the *cis*-elements for the autoregulation. We conclude that the C/EBPδ gene is activated by APRF/STAT3, and the expression level is then maintained by an autoregulation mechanism. © 1998 Academic Press

The expression levels of the CCAAT/enhancer-binding protein (C/EBP) family change markedly during the acute phase response: the mRNA level of C/EBPα decreases, and those of C/EBPβ, C/EBPδ and CHOP10 (also termed GADD153) increase significantly (1, 2). It is of interest that C/EBPδ is more rapidly and more strongly induced compared with C/EBPβ and CHOP10 in the response to lipopolysaccharide (LPS) treatment in rats (2), strongly suggesting that C/EBPδ contributes the initial step of the regulation of gene expression in acute-phase plasma protein genes. We previously reported the cloning of the promoter region of the rat C/EBPδ gene, the functional analyses of the basal activity, and the identification of *cis*-elements modulating the expression of the C/EBPδ gene (3). These data suggested 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 inflammation, 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) (4–6).

APRF/STAT3 is quickly phosphorylated in response to interleukin-6 (IL-6), and this phosphorylated form binds to APRE and activates the transcription of the C/EBPδ gene. In contrast, the activation of APRF/STAT3 is transient and the phosphorylated APRF/STAT3 is dephosphorylated within one hour after stimulation (5), and a high expression level of C/EBPδ mRNA is maintained for several hours (2).These observations indicated that other factors are involved, such as mRNA stability and the contribution of other *trans*-acting factors.

It is well known that the regulation of many *trans*-acting factors is mediated by an autoregulation mechanism; for example, Jun is positively autoregulated (7) and Fos is negatively autoregulated (8). It was also reported that the C/EBPα and C/EBPβ genes are autoregulated (9–12). We therefore tried to identify the autoregulation sites of the C/EBPδ gene, and we found two typical C/EBPδ binding sites in the downstream region of this gene.

MATERIALS AND METHODS

Plasmid constructions. The fragment containing −3.5 kb to +42 bp and various deletion fragments were inserted into *Xho*I and *Hin*dIII sites in a promoter-less luciferase vector, PGV-B (Toyo Ink Mfg. Co., Ltd. Tokyo, Japan) according to the standard protocol (13).

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Abbreviations used: APRE, acute phase response element; APRF/STAT3, acute phase response factor/signal transducers and activators of transcription 3; C/EBP, CCAAT/enhancer-binding protein; GADD153, growth arrest and DNA damage inducible gene 153; IL-6, interleukin 6; LPS, lipopolysaccharide; PCR, polymerase chain reaction.

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Some fragments in the upstream and downstream regions were also inserted at −167 of the −167C/EBPδ-luciferase or *Bam*HI site, which is 3 kb away from the promoter of the -167C/EBPδ-luciferase. The various lengths of mutants were made by polymerase chain reaction (PCR) techniques (14) or by the deletion at the 5′ end by exonuclease III and mung bean nuclease digestions. The internal deletion mutants were constructed by the deoxyoligonucleotide-directed mutagenesis according to the method of Kunkel *et al.* (15). The primers used are as follows, and (*) are the points deleted: $\Delta\delta$A: 5′-TATTAAGTAAGCTAC(*)TGAGTCAGCATATGT-3′; $\Delta\delta$B: 5′-GTTCGTTGGCAAAGT(*)CTACATTGGGAAATT-3′; $\Delta\delta$A & $\Delta\delta$B: 5′-GTTCGTTGGCAAAGT(*)TGAGTCAGCATATGT-3′. All constructs used here were checked by sequencing with the dideoxy method using denatured plasmid templates (16).

Cell culture and DNA transfection. HepG2 cells, a human hepatoma cell line, were cultured in minimal essential medium (MEM) containing 10% fetal bovine serum. The cells were transfected by the calcium phosphate co-precipitation techniques described by Chen and Okayama (17). Two μg of luciferase reporter plasmid, 2 μg of C/EBPδ expression plasmids (MSV-C/EBPδ), and 0.2 μg of β-galactosidase expression plasmid (pRSVGAL) were transfected into the cells. The total amount of plasmids transfected were adjusted to 4.5 μg with pBluescript. The cells were harvested after a 40-hours incubation following the transfection, and the luciferase activity and protein concentration were determined with Pikka Gene (Toyo Ink) and a lumiphotometer, and by the method of Bradford (18), respectively. The β-galactosidase activity was measured as described (19). The transfection efficiencies were normalized by the β-galactosidase activities. The fold stimulations by C/EBPδ are shown from four independent transfection analyses. All the transfection experiments were performed by using two or three different preparations of DNA.

Production of C/EBPδ protein in bacteria. The production of C/EBPδ protein in *E. coli* was described previously (20). In brief, the DNA binding domain of C/EBPδ was subcloned into pQE-30 expression vector, and this recombinant plasmid was transformed into M15[pREP4]. The resultant transformant was grown and the C/EBPδ expression was induced with isopropyl-β-D-thiogalactopyranoside. After harvesting the cells, the cells were suspended in 25 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA, 40 mM KCl, 10 % glycerol and 1 mM DTT, sonicated, and centrifuged, and the supernatant was used for the gel shift analysis.

Gel shift analysis. The gel shift analysis was performed as described previously (20). The sequences of the synthetic oligonucleotides of the δA and δB in the downstream region of the C/EBPδ gene, and of the δIV site as a non-specific competitor, are δA site: 5′-ctagGACTCAATTTCCCAATGTAGCT -3′; 3′- CTGAGTTAAAGGGTTACATCGAgatc -5′; δB site: 5′-ctagATGTAGCTTACTTAATACTTTG -3′; 3′- TACATCGAATGAATTATGAAACgatc -5′; δIV site : 5′-ctagTCGTTCCCAGCAGCACT -3′; and 3′- AGCAAGGGTCGTCGTGAgatc -5′.

RESULTS

Identification of the autoregulation site in the downstream region of the rat C/EBPδ gene. To identify the

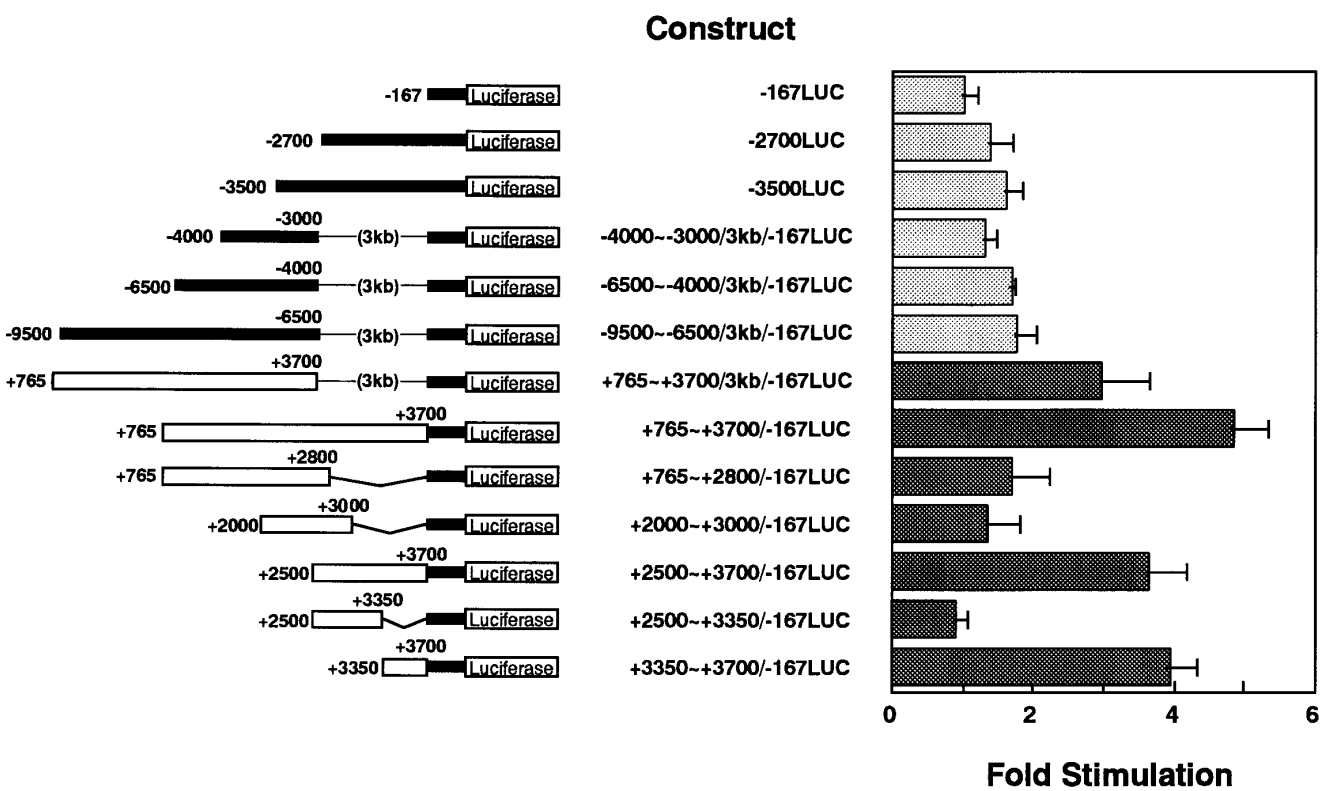

FIG. 1. Identification of the autoregulation site in the upstream and downstream regions of the C/EBPδ gene. The left and right panels show the schematic structures of the constructs and the transfection experiment by luciferase assay, respectively. The various regions in the upstream and downstream of the C/EBPδ gene were connected to the promoter-less luciferase gene, PGV-B or -167C/EBPδ-luciferase. These reporter plasmids with C/EBPδ expression plasmid were transfected into HepG2 cells by a calcium phosphate co-precipitation technique, and the luciferase activities were determined with a luminometer. The fold stimulations by C/EBPδ are shown from four independent transfection analyses. The error bars indicate standard deviations.

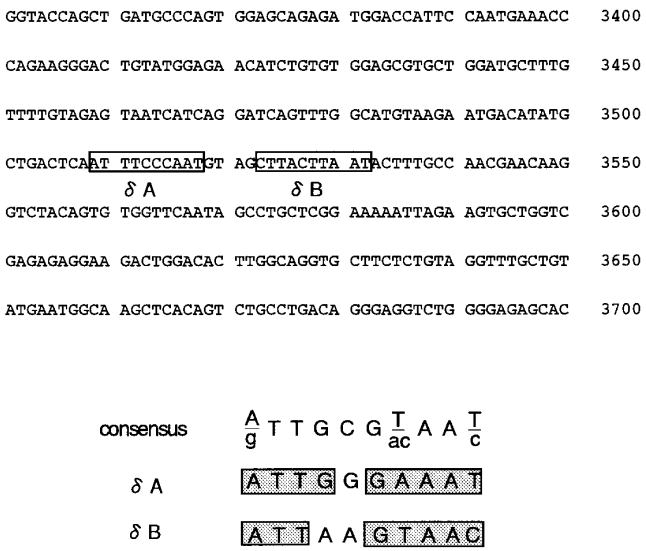

FIG. 2. The nucleotide sequence of the downstream region (+3350 ~ +3700 bp) of the C/EBPδ gene. The locations of the putative C/EBP binding elements, δA and δB, are indicated in the sequence. These two sequences and the consensus sequence of the C/EBP binding element are also shown in the lower panel. The shaded nucleotides are identical to those in the consensus sequence.

region of the rat C/EBPδ gene which contributes to the autoregulation, the various fragments including the upstream region of C/EBPδ gene promoter were joined to a luciferase gene, and the transfection analysis was performed in the HepG2 cells with or without C/EBPδ expression plasmid. The fold stimulations by C/EBPδ are shown in Fig. 1. The fragments including −3500 to +42 bp showed little responsiveness to C/EBPδ. When the fragments from −4000 to −3000 bp, −6500 to −4000 bp and −9500 to −6500 bp were connected to −167C/EBPδ-luciferase with a 3 kp spacer sequence, none showed responsiveness to C/EBPδ, indicating that there is no autoregulation site in the promoter region up to -9500 bp. In contrast, when the downstream fragment containing +765 bp to +3700 bp was connected to just upstream of -167C/EBPδ-luciferase, the luciferase activity increased by 5-fold. When this fragment was joined to the *Bam*HI site which is 3 kb away from the promoter of the −167C/EBPδ-luciferase, 3-fold stimulation was observed (Fig. 1).

Next, we prepared the various deletion fragments in the downstream region of C/EBPδ gene, and the transfection analysis was performed. Whereas the constructs containing +765 to +3350 were not stimulated by the cotransfection of the C/EBPδ expression plasmid, the fragment containing +3350 to +3700 was enough for full activity for the stimulation by the C/EBPδ expression plasmid, strongly indicating that this fragment contains C/EBPδ binding sites (Fig. 1). The sequence of +3350/+3700 is shown in Fig. 2. We previously determined the C/EBPδ binding site, A/gTTGCGT/acAAT/c, by using the PCR-mediated random site selection method (20). The computer analysis revealed two putative C/EBPδ binding sites, named δA and δB, in the +3350/+3700 region. Only one or two bases out of ten bases are mismatched to the consensus sequences, and the most important sequences, TT at positions 2 and 3, and AA at positions 8 and 9, are conserved in both the δA and δB sites (Fig. 2).

Gel-mobility shift analysis of the C/EBPδ site in the downstream region of the rat C/EBPδ gene. To determine whether C/EBPδ protein binds to the δA and δB sites, we next performed a gel-mobility shift analysis using bacterially expressed C/EBPδ protein, and the δA and δB sites as probes. The retarded band was observed when both the δA and δB sites were used as probes (Fig. 3, lane 2 in both panels). This band disappeared with the addition of a 50- or 250- fold molar excess of non-labeled δA and δB sites, while a 250-fold molar excess of unrelated oligonucleotide, δIV, had no effect on the binding, indicating that C/EBPδ protein binds specifically to both the δA and δB sites. In this competition experiment, δA competed more efficiently than did δB, regardless of whether the δA or δB site was used as the probe, indicating that the binding affinity of C/EBPδ protein to the δA site is higher than that to δB site.

Internal deletion analysis of the C/EBPδ site in the downstream region of the rat C/EBPδ gene. For further characterization of the δA and δB sites, we next constructed the internal deletion mutants lacking the δA and/or δB sites, and the transfection experiment was performed. When +3350/+3700 was connected to -167C/EBPδ-luciferase, the construct lacking the δA site partially lost the stimulation activity, while that lacking the δB site almost lost the responsiveness to C/EBPδ protein, as did the construct lacking both δA and δB sites (the lower part of Fig. 4). When the wider fragment (+765/+3700) was used, the exact same result was obtained (the upper part of Fig. 4). These observations strongly suggested that both the δA and δB sites are responsible to C/EBPδ protein, and the C/EBPδ gene expression is mediated by the autoregulation mechanism through these two sites.

DISCUSSION

In this study, we identified the autoregulation site consisting of two C/EBPδ binding sites in the downstream region of the C/EBPδ gene. According to the transfection analysis, the δB site is more critical than the δA site for the autoregulation (Fig. 4), although C/EBPδ protein binds to more strongly to the δA site than the δB site (Fig. 3). It is likely that the surrounding sequence is also important and that the δB site works more efficiently *in vivo*. Since the δA site is only 4 bp away from the δB site, it is also possible that

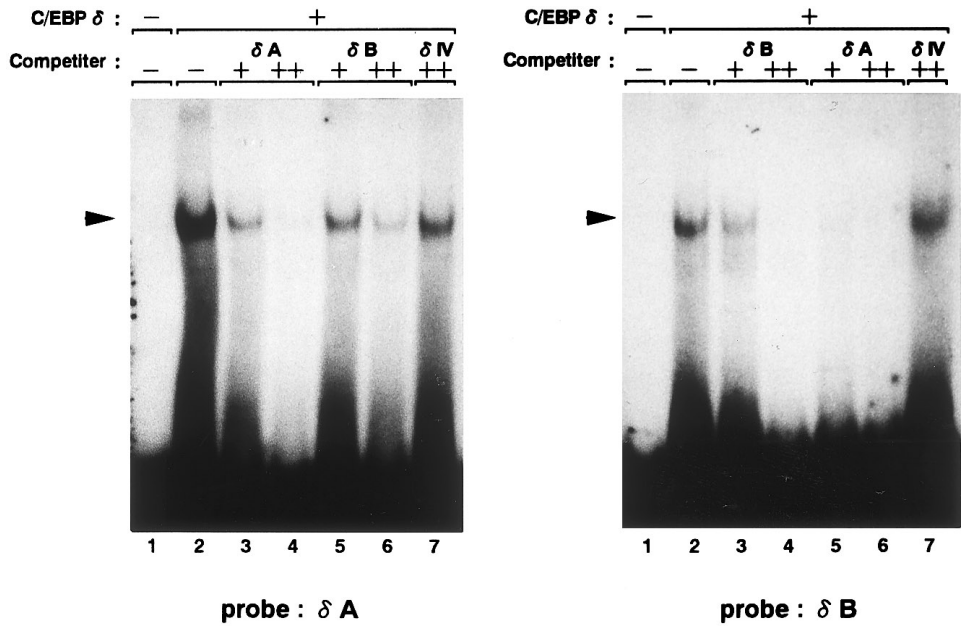

FIG. 3. Gel shift analysis of the putative C/EBPδ binding site using bacterially expressed C/EBPδ protein. Double strand oligonucleotides, δA and δB, were used as probes for binding analysis. Each probe (δA in the left panel and δB in the right panel) was incubated with bacterially expressed C/EBPδ (lanes 2–7). Lane 1, bovine serum albumin, added as a control. A 50-fold (+) or 250-fold (++) molar excess of non-labeled oligonucleotides was used for the competition analysis. The δIV was used as a non-specific competitor.

there are some cooperative interactions between these two sites.

The proposed signaling pathway of gene expression mediated by C/EBPδ protein during the acute phase response is as follows. APRF/STAT3 is constitutively expressed in a variety of tissues including the liver, but lacks DNA binding ability when dephosphorylated (5). During the acute phase response, such as that induced by IL-6 or other cytokines stimulation, APRF/STAT3 is phosphorylated within 5 min, becomes capable of binding to DNA and activates the transcription of the C/EBPδ gene (3). The phosphorylated APRF disappears within 1 hr (5). However, once the C/EBPδ gene is induced, the C/EBPδ protein itself binds to the

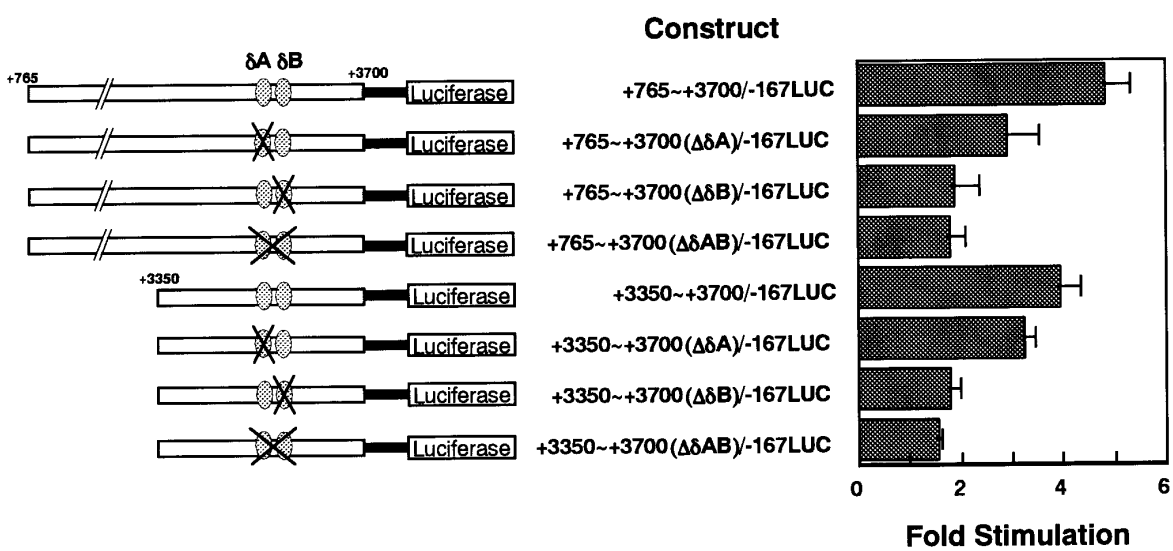

FIG. 4. Internal deletion analysis of the autoregulation site in the downstream region of the C/EBPδ gene. The left and right panels show the schematic structures of the constructs and the transfection experiment results by luciferase assay, respectively. The fragments of +765~+3700 and +3350~3700 downstream of the C/EBPδ gene were connected to the -167C/EBPδ-luciferase. Internal deletion constructs with δA, δB, or both δA and δB were also tested. The transfection analysis was performed as shown in Fig. 1.

downstream C/EBP site of the C/EBPδ gene, and activates the transcription of its own gene. Thus, the expression level is maintained at a high level for several hours. Although it is also possible that the stability of the mRNA of the C/EBPδ gene is another critical factor, this was not examined in the present study.

The expression of the C/EBPδ gene decreases several hours after stimulation (2). It was reported that the C/EBPβ gene is also induced during the acute phase response (2), and that C/EBPβ activates CHOP10 through the C/EBP site (21). CHOP10 is a member of the C/EBP family and a negative regulator, since CHOP10 heterodimerizes with other C/EBPs, but has no DNA binding ability (21, 22). The shorter form of C/EBPβ, LIP, lacks the transactivation domain and functions as a negative regulator (23). It is likely that the homodimer form of LIP or the heterodimer form with C/EBPδ binds to the downstream C/EBP site of the C/EBPδ gene, since the binding sequences of the C/EBP family are quite similar each other (20). CHOP10 and/or LIP may heterodimerize with C/EBPδ protein and inactivate the C/EBPδ gene expression. These possibilities are now under investigation.

It has been reported that many *trans*-acting factors are regulated by their own product (7–12, 24–28). In all cases, the binding sites which bind to their own products are located within several hundred bp from the transcription start site in the promoter region of the genes. In contrast, the autoregulation sites of the C/EBPδ gene are found 3 kp away from the transcription start site in the downstream region of the C/EBPδ gene. These elements might therefore function as enhancer elements (29). However, the stimulation of the C/EBPδ gene by its own product is relatively low (3-fold) when these elements are located 3 kb away from the promoter in the luciferase reporter plasmid, compared when they are joined at the just upstream region of the reporter plasmid (5-fold) (Fig. 1). It is possible that these weak interactions of the gene's own products are helpful for the complex regulation circuit, e.g., the quick activation by APRF/STAT3, the activation by the C/EBPδ's own product and the repression by the related gene family product, during the acute phase response.

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