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DBP Binds to the Proximal Promoter and Regulates Liver-Specific Expression of the Human Angiotensinogen Gene

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Angiotensinogen is the glycoprotein precursor of one of the most potent vasoactive hormones, angiotensin-II. It has been shown recently that an ATF like element (ALE) located between bases -102 and -87 of the human angiotensinogen gene plays an important role in liver specific expression of this gene and binds to CREB/ATF family of transcription factors and a novel factor (ALF). We show here that this sequence binds to the liver enriched transcription factor DBP and cotransfection of expression vector CMV-DBP increases the expression of reporter constructs containing this sequence. In addition, we show that transcription factor C/EBP-δ binds to this sequence and an expression vector containing C/EBP-δ coding region increases the expression of reporter constructs containing this sequence. Since DBP is involved in circadian rhythm, our studies suggest that this sequence may be involved in circadian expression of the human angiotensinogen gene.

Key Words: **DBP; C/EBP; circadian rhythm; angiotensinogen gene; transcriptional regulation; HepG2.**

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. The octapeptide, angiotensin-II, is one of the most active vasopressor agents and is obtained by the proteolytic cleavage of a larger precursor molecule, angiotensinogen, which is first converted by renin to produce a decapeptide, angiotensin-I. Angiotensin-converting enzyme (ACE) then removes a C- terminal dipeptide from this decapeptide to produce angiotensin-II (1). The plasma concentration of angiotensinogen is close to the Michaelis constant of the enzymatic reaction between renin and angiotensinogen (2). For this reason, a rise in plasma angiotensinogen levels can lead to a parallel increase in the formation of angiotensin-II which may ultimately result in hypertension. Plasma angiotensinogen levels are increased in hypertensive patients (3,4) and recent linkage studies have suggested that angiotensinogen gene locus is involved in human hypertension (5), pregnancy induced hypertension (6,7) and coronary artery disease (8).

Angiotensinogen is primarily expressed in the liver and to some extent in adipose, kidney, brain, heart, and vascular walls of the rat (9) and human (10). Yanai et al (11) have recently shown that human angiotensinogen gene fragment ALE (located between nucleotide sequence -102 to -87) plays an important role in its liver specific expression and binds to CREB/ATF family of transcription factors and to a novel factor ALA. Although, the protein : DNA complex formed with radiolabeled ALE and HepG2 extract was competed out by a cold oligonucleotide containing C/EBP consensus binding site, expression vector containing C/EBP coding region did not increase the promoter activity of a reporter construct containing 106 bp of the 5′-flanking region of the human angiotensinogen gene (12). We show here that this sequence binds to the liver enriched transcription factor DBP (13). We also show that transcriptional activity of reporter constructs containing either 122 bp of the 5′-flanking region of the human angiotensinogen gene attached to the CAT gene (pHAG122CAT) or two copies of ALE attached to the heterologous TK-CAT gene (ALE)2TK-CAT is increased on co-transfection of CMV-DBP in HepG2 cells. Our studies also show that transcription factor C/EBP-δ binds to this sequence and co-transfection of expression vector MSV- C/EBP-δ increases the expression of these reporter constructs in HepG2 cells.

MATERIALS AND METHODS

Expression plasmid *pSVoCAT*, *pGem-T*, and *RSV-gal* were obtained from Promega Biotec (Madison, WI), CMV-DBP, bacterial expression vector containing DBP coding sequence in bluescript , and

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Abbreviations: ab, antibody; CAT, chlormphenicol acetyl transferase; PCR, polymerase chain rection; PMSF, phenyl methyl sulfonyl flouride; TK-CAT, CAT gene attached to 80 bp of herpes virus thymidine kinase promoter; CMV-DBP, expression vector containing DBP coding sequence attached to CMV promoter; MSV-C/EBP-δ, expression vector containing C/EBP-δ coding sequence attached to MSV promoter.

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DBP antibody were provided by Dr. Uri Schibler (University of Geneva, Switzerland), MSV-C/EBP-δ was provided by Dr. Steve McKnight (Southwestern Medical Center, Dallas, TX), expression vector containing $(His)_6$-C/EBP-δ was provided by Dr. Peter F. Johnson (Frederick Cancer Center, Frederick, MD), and TK-CAT was provided by Dr. Anuradha Ray (Yale University, New Haven, CT). Restriction enzymes were purchased from New England Biolab (Cambridge, MA) or Gibco/BRL (Gaithesburgh, MD). Qiagen mini and midi plasmid kits were obtained from Qiagen (Chatsworth, CA). C/EBP-δ, Sp1, NF1, and Egr antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid constructions. The reporter construct *pHAG1.2-CAT* was synthesized by attaching ~1.2 kb of 5′-flanking region of the human angiotensinogen gene (14,15) in front of the CAT gene in the reporter construct *pSVoCAT*. The ~1.2 kb region of the promoter contained 1223 bases of the 5′-flanking region and 44 bases of the first exon of the human angiotensinogen gene that were obtained by PCR of human genomic DNA (our amplified fragment had nucleoside A at −20). 5′-Deletion mutants were obtained from pHAG1.2CAT by Bal31 treatment. Nucleotide sequence of reporter constructs and deletion mutants were confirmed by sequence analysis. Plasmid DNAs for transfection were prepared by Qiagen column. The quality of plasmid DNA was checked by gel electrophoresis and restriction analysis.

Cell culture and transient transfection. Human hepatoma cells (HepG2) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2. Transient DNA transfections were performed by the calcium phosphate precipitation method using supercoiled plasmid DNA at 10 μg/plate and *pRSV-gal* (2 μg) as an internal control to normalize efficiency of transfection. Expression vectors CMV-DBP and MSV-C/EBP-δ were co-transfected at 4μg per plate and total weight of the transfected DNA was kept constant at 20 μg by the addition of bluescript DNA. After a 4 h treatment with the DNA precipitate, cells were washed with PBS and incubated with fresh medium. Cells were harvested after 48 h of transfection, total extract was prepared by three cycles of freezing and thawing in liquid nitrogen, an aliquot (5 λ) was used for β-gal assay and rest of the extract was heated at 65°C for 5 min. After centrifugation, an aliquot of the extract (after normalization with the β-gal activity) was used to perform CAT assay using ^{14}C-chloramphenicol as a substrate followed by separation of acetylated products by TLC using silica gel plates. After autoradiography, spots corresponding to ^{14}C-chloramphenicol and its acetylated derivatives were scraped from TLC plates and radioactivity in each spot was measured using a liquid scintillation counter. CAT activity was determined by dividing the counts in acetylated spots with the total number of counts (present in acetylated and non-acetylated spots). Transient transfections were performed at least three times using at least two different preparations of plasmid DNAs.

Gel mobility shift assay. The probes for gel mobility shift analysis were chemically synthesized, annealed and radiolabeled at the 5′-ends by polynucleotide kinase and [γ-^{32}P] ATP. The radiolabeled oligonucleotide (0.1 nmole), 1-2 μg of poly(dI-dC), and 5-10 μg of the protein extract were incubated in a solution containing 10 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 12.5% glycerol in ice for 30 min and separated on a 5-8% polyacrylamide gel at room temperature. After 2-3 h of electrophoresis, the gel was dried under vacuum and protein-nucleic acid complexes were identified by autoradiography. For supershift experiments, 1 μl of the DBP antibody was added to the reaction mixture which was then incubated for 30 min before gel electrophoresis. Nuclear extracts for gel mobility shift assay were prepared as previously described (16). Recombinant DBP was obtained by in vitro coupled transcription-translation using TNT kit (Promega Biotec) according to the instructions provided by the manufacturer.

Oligonucleotides. The nucleotide sequence of forward and reverse primers used for the construction of *pHAG 1.2CAT* were 5′-GAGTCCCTATCTATAGGAACA and 5′-CATACCCT TCTGCTGTAGTAC. The double stranded oligonucleotide ALE was obtained by annealing CTAGGGCCAAGTGATGTAACCC and its complementary sequence (this oligonucleotide corresponds to the nucleotide sequence −88 to −105 of the human angiotensinogen gene and contain an extra Xba-1 restriction site CTAG at its 5′-end). This oligonucleotide was dimerized and inserted in TK-CAT through Xba restriction site to produce the reporter construct (ALE)2TK-CAT. The double stranded oligonucleotide mut-ALE was obtained by annealing GGCCAAGTGCCATGGCCC and its complementary oligonucleotide (mutated residues are underlined in this sequence). This sequence was also used to perform site specific mutagenesis in reporter construct *pHAG1.2CAT* to produce *mut-pHAG1.2CAT*. The double stranded oligonucleotides containing consensus C/EBP, NF-1, and HNF-1 binding sites were obtained by annealing CATGAGATTGTGCAATGT, CCTTTGGCATGCTGCCAATATG, and CTGTGGTTAATGATTAACCC respectively with their complementary sequences.

RESULTS

Since nucleotide sequence located between −90 and −100 of the human angiotensinogen gene shows homology with the DBP binding site (13) (Fig. 1A) (nine out of ten bases are homologous), we were interested in examining whether this transcription factor is involved in the liver specific expression of the human angiotensinogen gene. To answer this question, we first co-transfected 2, 4, and 8 μg of CMV-DBP with 10 μg of pHAG1.2CAT in HepG2 cells as described in Materials and Methods section. The CAT activity was then analyzed after normalization with β-gal activity. Results of this experiment showed that 4μg of CMV-DBP produced maximum increase in the promoter activity of pHAG1.2CAT and therefore this amount of CMV-DBP was co-transfected in future experiments (data not shown). We then co-transfected reporter constructs pHAG1.2CAT and its 5′-deletion mutants pHAG122CAT and pHAG83CAT (containing 122 and 83 bp of the promoter sequence attached to the CAT gene) in the presence and absence of CMV-DBP (4 μg) in HepG2 cells. Results of this experiment shown in Fig. 1B indicated that whereas co-transfection of DBP increased the promoter activity of reporter constructs pHAG1.2CAT and pHAG122CAT, it had no effect on the promoter activity of pHAG83 CAT. These data confirm that nucleotide sequence located between −122 and −83 was responsible for DBP induced promoter activity. We then synthesized a reporter construct (ALE)2TK-CAT by attaching two copies of the nucleotide sequence located between −88 and −105 in front of reporter construct TK-CAT. This construct was then co-transfected in the presence and absence of CMV-DBP in HepG2 cells and promoter activity was analyzed by the CAT assay. Results of this experiment (Fig. 1C) indicated that co-transfection of DBP increased the expression of (ALE)2 TK-CAT by about fifteen fold. On the other hand promoter activity of

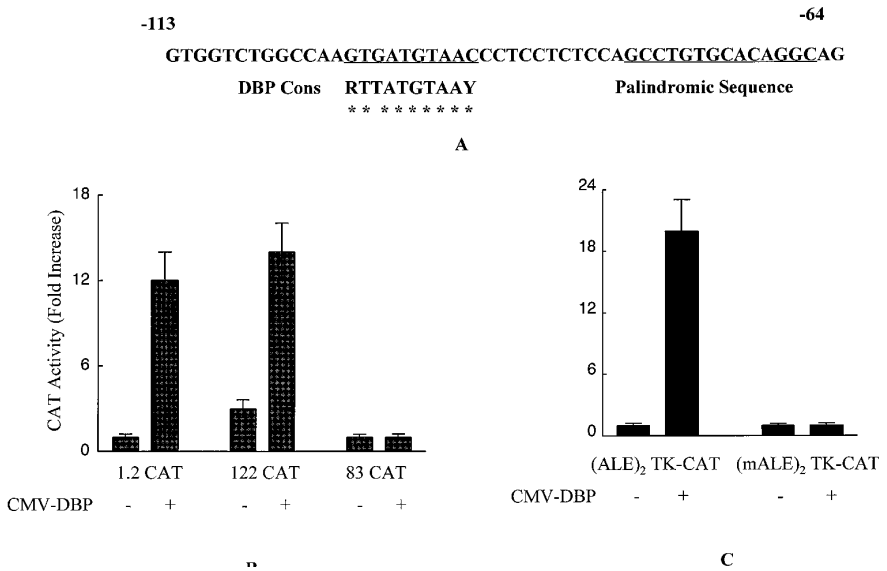

FIG. 1. Promoter activity of reporter constructs containing nucleotide sequence located between −90 and −100 of the human angiotensinogen gene is increased on cotransfection of CMV-DBP. (A) Nucleotide sequence homology between human angiotensinogen gene promoter and consensus DBP binding site (identical bases are shown by asterisks). (B) CAT activity of reporter constructs containing 1.2 kb, 122 bp, and 83 bp of the 5′-flanking region and 44 bp of exon-1 of the human angiotensinogen gene in the absence and presence of CMV-DBP. CAT activity was calculated by assuming basal promoter activity of pHAG1.2CAT as one, and the mean value of three experiments is shown. (C) CAT activity of reporter constructs containing two copies of ALE and mut-ALE attached to the TK-CAT gene in the presence and absence of CMV-DBP. CAT activity was calculated by assuming basal promoter activity of (ALE)2 TK-CAT as one, and the mean value of three experiments is shown.

(mALE)2 TK-CAT (where DBP binding site was mutated by site specific mutagenesis) was not increased by co-transfection of DBP.

We next performed a gel shift assay to demonstrate that oligonucleotide ALE binds to DBP like transcription factor present in HepG2 cells. Results of our gel shift assay using radiolabeled ALE and HepG2 nuclear extract in the presence of different competitor oligonucleotides are shown in Fig 2A. The protein : DNA complex (lane 1) was competed out with a 100 fold excess of cold ALE (lane 2), oligonucleotides containing consensus DBP and C/EBP binding sites (lanes 3 and 4 respectively), but not with oligonucleotides containing consensus HNF-1 and NF-1 binding sites (lanes 5 and 6 respectively). Multiple C/EBP like transcription factors may bind to this sequence as homo and heterodimers giving rise to multiple bands observed in lane 1 of this experiment.

To confirm that DBP binds to the oligonucleotide ALE, we performed a gel shift assay using recombinant DBP (rDBP) obtained by in vitro coupled transcription-translation of the DBP cDNA in bluescript. Results of this experiment (Fig. 2B) show that the protein : DNA complex obtained with radiolabeled ALE and rDBP (lane 1) was competed out by a 100 fold excess of cold ALE and consensus DBP oligonucleotide (lanes 2 and 3), but not with cold oligonucleotides containing consensus NF-1 or HNF-1 binding site (lanes 6 and 7). Moreover, the intensity of this complex was reduced and partially supershifted by DBP antibody (lane 4). On the other hand a non-specific EGR antibody neither supershifted nor reduced the intensity of this complex (lane 5). We also performed a reverse gel shift assay where radiolabeled DBP oligonucleotide was used in the presence of rDBP. Results of this experiment shown in Fig. 2C indicated that the protein : DNA complex (lane 1) was reduced in the presence of cold ALE and consensus DBP oligonucleotide (lanes 2 and 3 respectively), but not in the presence of cold oligonucleotides containing consensus NF-1 or HNF-1 sites (lanes 6 and 7 respectively). In addition, the intensity of this complex was reduced and the complex was partially supershifted in the presence of DBP antibody (the supershift was more prominent on longer exposure of the film) (lane 4) but EGR antibody had no effect on the intensity of the protein : DNA complex (lane 5). Taken together results of these experiments confirm that DBP binds to the nucleotide sequence located between -90 and -100 and activated the expression of human angiotensinogen gene in HepG2 cells.

Since C/EBP family of transcription factors are known to bind to some of the DBP binding sites (17), it was of interest to determine whether members of C/EBP family of transcription factors bind to ALE. To answer this question, we first performed a control gel shift assay with radiolabeled oligonucleotide contain-

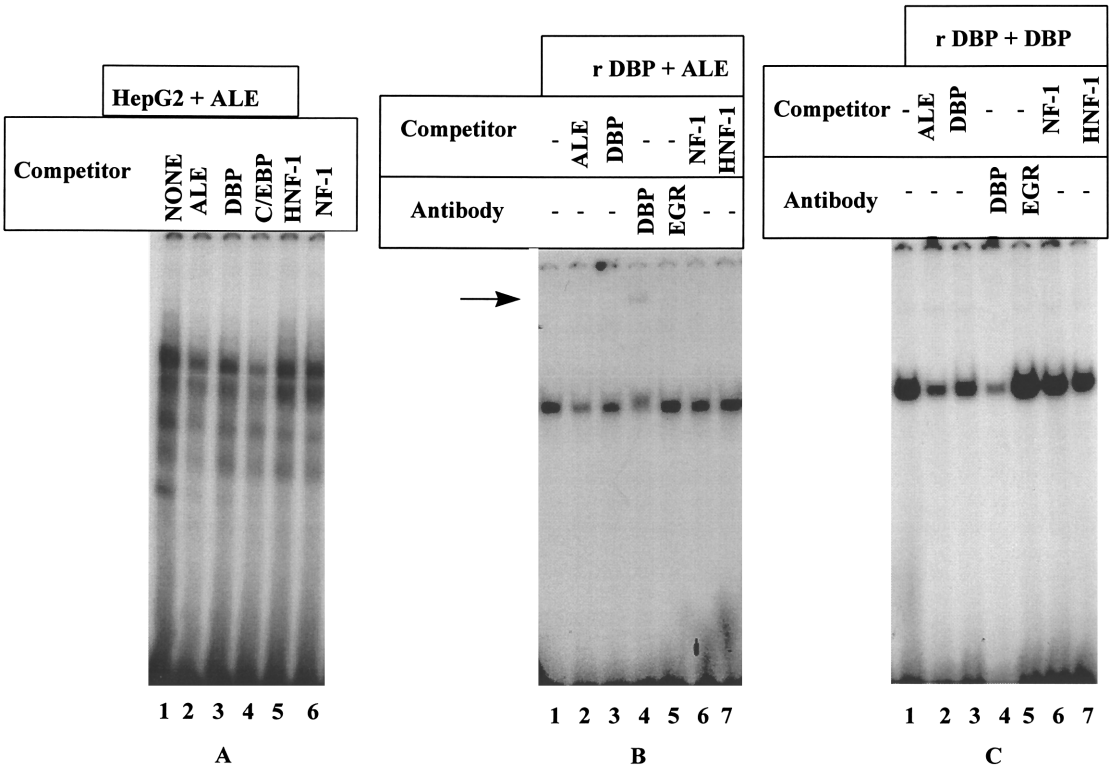

FIG. 2. Nucleotide sequence located between −90 and −100 of the human angiotensinogen gene binds to DBP like transcription factor. (A) Radiolabeled ALE was used in a gel shift assay using HepG2 nuclear extract in the absence and presence of a 100-fold excess of specific and nonspecific oligonucleotides. Lane 1: without competitor; lanes 2, 3, and 4: in the presence of cold ALE, DBP, and C/EBP oligonucleotides; lanes 5 and 6: in the presence of cold HNF-1 and NF-1 oligonucleotides. (B) Radiolabeled ALE was used in a gel shift assay using *in vitro* translated rDBP in the presence of cold competitor oligonucleotides and antibodies. Lane 1: no competitor; lanes 2 and 3: in the presence of cold ALE and DBP oligonucleotides; lanes 6 and 7: in the presence of cold NF-1 and HNF-1 oligonucleotides; lanes 4 and 5: in the presence of DBP and EGR antibodies. (C) Radiolabeled DBP consensus oligonucleotide was used in a gel shift assay using *in vitro* translated rDBP in the presence of cold competitor oligonucleotides and antibodies. Lane 1: no competitor; lanes 2 and 3: in the presence of cold ALE and DBP oligonucleotides; lanes 6 and 7: in the presence of cold NF-1 and HNF-1 oligonucleotides; lanes 4 and 5: in the presence of DBP and EGR antibodies.

ing consensus C/EBP binding site and recombinant C/EBP-δ (containing six histidine residues and purified by nickel sepharose beads). Results of this experiment (presented in Fig. 3A) show that the protein : DNA complex (lane 1) was competed out by cold C/EBP oligonucleotide (lane 2) and ALE (lane 3), and supershifted in the presence of C/EBP-δ antibody. We then performed a gel shift assay with radiolabeled ALE and recombinant C/EBP-δ in the presence of different competitor oligonucleotides. Results of this experiment shown in Fig. 3B indicated that the protein : DNA complex (lane 1) was competed out by cold ALE (lane 2) and an oligonucleotide containing consensus C/EBP binding site (lane 3). Moreover, this complex was supershifted in the presence of C/EBP-δ antibody (lane 5). In another experiment, we mutated nucleoside bases in ALE that are supposedly involved in binding with DBP and used mALE as a competitor. Results of this experiment (lane 4) showed that mALE did not remove the protein : DNA complex obtained with radiolabeled ALE and rC/EBP-δ. Taken together, results of these experiments show that ALE also binds to C/EBP-δ and mutated bases in mALE are involved in binding with C/EBP family of transcription factors.

To understand the functional role of ALE in C/EBP induced promoter activity, we co-transfected MSV-C/EBP-δ and pHAG1.2CAT, pHAG122CAT, and pHAG83CAT in the presence and absence of C/EBP-δ in HepG2 cells and promoter activity was analyzed by the CAT assay. Results of this experiment indicated that C/EBP-δ increased the expression of pHAG1.2CAT and pHAG122CAT but not that of pHAG83CAT (Fig. 4A). Co-transfection of MSV-C/EBP-δ also increased the expression of reporter construct (ALE)2 TK-CAT but not of (mALE)2 TK-CAT (Fig. 4B) These data confirmed that nucleotide sequence located between −90 and −100 bp of the human angiotensinogen gene binds to DBP as well as C/EBP-δ and reporter constructs containing this sequence are transactivated by DBP as well as C/EBP-δ.

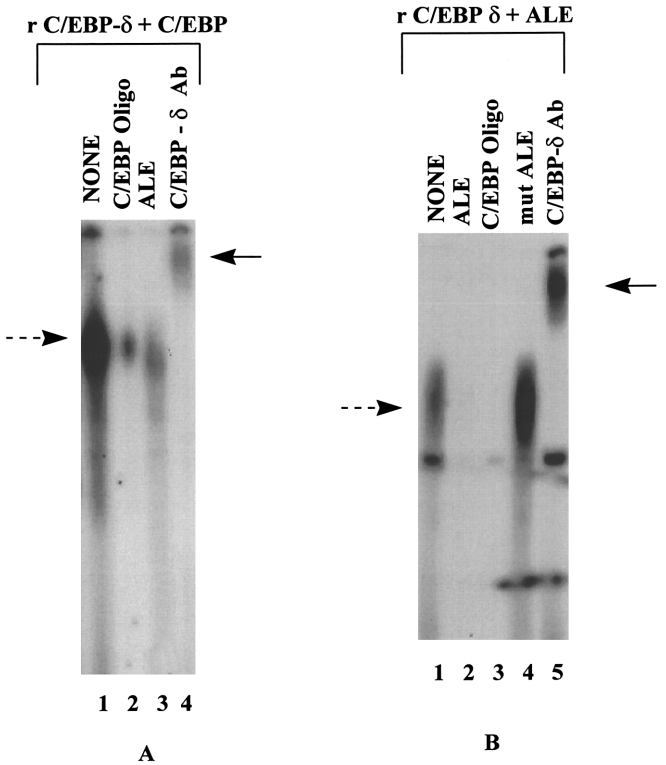

FIG. 3. Nucleotide sequence located between −90 and −100 of the human angiotensinogen gene also binds to C/EBP-δ. (A) A control gel shift assay was performed with radiolabeled oligonucleotide containing consensus C/EBP binding site and recombinant C/EBP-δ attached to six histidine residues in the presence of cold competitor oligonucleotides and C/EBP-δ antibody. Lane 1: without competitor or antibody; lanes 2 and 3: in the presence of C/EBP and ALE oligonucleotide respectively; lane 4: in the presence of C/EBP-δ antibody. (B) A gel shift assay was performed with radiolabeled ALE oligonucleotide and recombinant C/EBP-δ in the presence of cold competitor oligonucleotides and C/EBP-δ antibody. Lane 1: without competitor or antibody; lanes 2 and 3: in the presence of ALE and C/EBP oligonucleotide respectively; lane 4: in the presence of mutated ALE oligonucleotide; lane 5: in the presence of C/EBP-δ antibody. Protein–DNA complex with rC/EBP-δ is shown by a broken arrow and supershifted complex is shown by a solid arrow.

DISCUSSION

In this paper we show that transcription factor DBP binds to the nucleotide sequence located between −95 and −102 of the human angiotensinogen gene and transactivates the expression of reporter constructs containing this sequence in HepG2 cells. DBP is a liver enriched transcription factor and belongs to basic leucine zipper family of proteins (13). Members of this family of transcription factors dimerize by carboxy terminal leucine zipper domain and bind DNA through a domain rich in basic amino acids. Although DBP mRNA is present in most tissues, the protein accumulates to high levels only in the nuclei of adult liver. The most remarkable aspect of DBP is that its expression follows a stingent circadian rhythm (18). The daily cycle of DBP protein level in rat changes ~100 fold in magnitude with maximum level around 8 pm (19). The DBP binding site has been identified in a number of liver specific genes such as albumin (13), phosphoenol pyruvate carboxy kinase (PEPCK) (20)), and cholesterol 7-α hydroxylase (17,21). However, the physiological relevance of DBP modulated circadian expression of a particular gene depends on the half life of the protein that it codes. Since half life of the albumin is comparatively long, DBP induced expression of the albumin gene does not play an important role on its plasma level. However, DBP induced expression plays an important role on the plasma level of cholesterol 17-α hydroxylase which is highest around 10 pm.

It has been shown previously that DBP binding sites are also recognized by C/EBP family of transcription factors although DBP recognizes more stringent binding site. There are multiple sites in the promoter of albumin gene that are recognized by the C/EBP family of transcription factors, however only site D is recognized by DBP. Similarly, in cholesterol 7-α hydroxylase gene only one of the three C/EBP binding sites is recognized by DBP. We show here that ALE is also recognized by C/EBP-δ and promoter activity of reporter constructs containing ALE is increased by co-transfection of expression vector containing C/EBP-δ coding sequence. C/EBP-δ is involved in cytokine induced expression of liver specific genes (22-24). C/EBP-δ is normally present in very small amount in mouse, rat, and human liver cells. However cytokine treatment drastically increases the expression of C/EBP-δ in hepatic cells which then binds to C/EBP binding sites either as homo- or hetero-dimers and increases the expression (25,26). It is possible that DBP is involved in basal expression of the human angiotensinogen gene in the liver and IL-6 treatment increases the expression of C/EBP-δ which then displaces DBP and becomes involved in IL-6 induced expression of this gene. Experiments are in progress now to determine whether C/EBP group of transcription factors co-operatively interact with DBP and increase DBP induced expression of the human angiotensinogen gene.

The nucleotide sequence located between TATA box and transcriptional initiation site of the human angiotensinogen gene has been shown to be crucial for its basal expression (12) and we have recently shown that transcription factor MLTF binds to this region of the promoter (unpublished). In this paper, we show that liver enriched transcription factor DBP binds to the nucleotide sequence located between −95 and −102 of the human angiotensinogen gene promoter and plays an important role in its liver specific expression. Surprisingly both of these sites are unique to the human angiotensinogen gene and are not present at these positions in the promoter of either mouse or rat gene. This strongly suggests that ALE may play an important role in species specific expression of the human

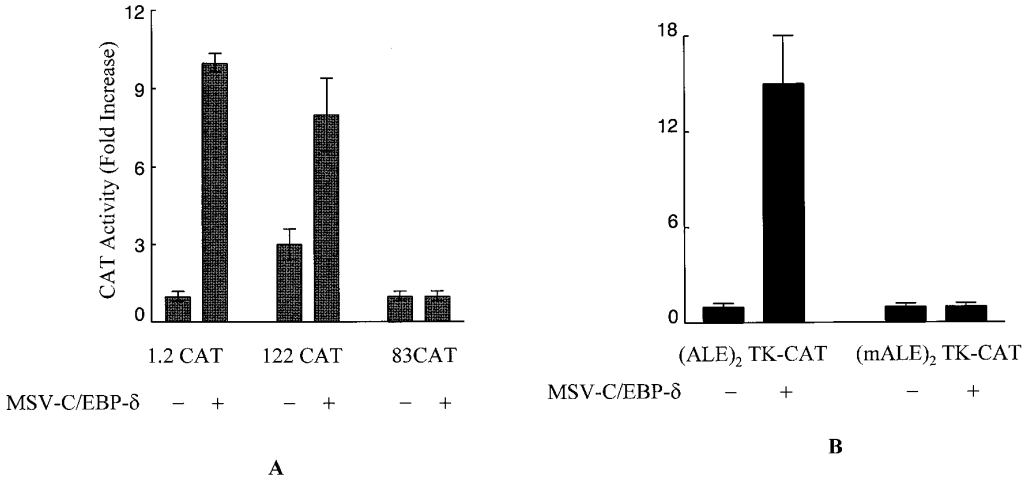

FIG. 4. Promoter activity of reporter constructs containing ALE is increased by cotransfection of MSV-C/EBP-δ. (A) CAT activity of reporter constructs pHAG1.2CAT, pHAG122CAT, and pHAG83CAT in the presence and absence of MSV-C/EBP-δ. Reporter constructs (10 μg) and RSV-gal (2 μg) were transfected in the presence and absence of MSV-C/EBP-δ (4 μg) in HepG2 cells and promoter activity was analyzed after 48 h of transfection after normalization with the β-gal assay. CAT activity was calculated by assuming basal promoter activity of pHAG1.2CAT as one, and the mean value of three experiments is shown. (B) CAT activity of reporter constructs containing two copies of ALE and mut-ALE attached to the TK-CAT gene in the presence and absence of MSV-C/EBP-δ. Reporter constructs (10 μg) and RSV-gal (2 μg) were transfected in the presence and absence of MSV-C/EBP-δ (4 μg) in HepG2 cells and promoter activity was analyzed after 48 h of transfection after normalization with the β-gal assay. CAT activity was calculated by assuming basal promoter activity of (ALE)2 TK-CAT as one, and the mean value of three experiments is shown.

angiotensinogen gene. It remains to be examined whether plasma level of human angiotensinogen is altered by circadian rhythm and plays any role in hypertension.

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