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ATF-like Element Contributes to Hepatic Activation of Human Angiotensinogen Promoter

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Angiotensinogen is the precursor protein of angiotensin II that is involved in regulating blood pressure and electrolyte homeostasis, and it is mainly synthesized in the liver. In the present study, we analyzed the human angiotensinogen proximal promoter region by means of Chloramphenicol acetyltransferase assays, and suggested that the region from −106 to +44 is sufficient for hepatoma cell line (HepG2)-specific expression. Electrophoretic mobility shift assays using ALE (ATF-like element, −102 to −87) fragment identified CREB/ATF family nuclear factors and novel ones, ALF (ALE-binding factor). The deletion and *in vivo* competition of ALE decreased the human angiotensinogen promoter activity. Furthermore, the heterologous promoter analysis demonstrated that ALE acts as a HepG2-dependent activating element. These results indicate that ALE plays an important role in hepatic expression of human angiotensinogen gene. © 1997 Academic Press

Angiotensinogen is the precursor protein of angiotensin II that acts as a physiologically important regulator of blood pressure and electrolyte homeostasis as well as a growth factor of cardiac myocytes (1-4). Recently, association between molecular variation of the angiotensinogen gene and diseases including pre-eclampsia, coronary atherosclerosis, myocardial infraction, and nephropathy in insulin-dependent diabetes have been reported (5-10). Especially, genetic linkage analyses proposed association between hypertension and molecular variants of the human angiotensinogen (hAG) gene promoter which may affect its transcriptional regulation (11).

Our previous studies have shown that *cis*-acting elements located at nucleotide positions −1222 to +44 are sufficient for the hAG gene expression in transiently transfected human hepatoma HepG2 cells, and in the liver of the transgenic mice (12, 13). In particular, the 150-bp proximal promoter region is sufficient for hepatic hAG promoter activity, suggesting the presence of *cis*-acting elements (14). Furthermore, we recently identified several regulatory elements of the hAG gene including the upstream region (hASR), the core promoter element (AGCE1), and the downstream enhancer elements (15-18). Although the downstream enhancer acts in a HepG2-dependent manner (17, 18), no exact cell type-dependent *cis*-elements in the promoter region have been reported. In the present study, to further define regulatory elements and *trans*-factors that participate in the control of hepatic hAG expression, we have analyzed its proximal promoter region and identified CREB/ATF family nuclear factors and novel ones, ALF (*AL*E-binding *f*actor), that bound to ALE (*A*TF-*l*ike *e*lement, −102 to −87).

MATERIALS AND METHODS

Plasmid constructions. The hAG promoter-chloramphenicol acetyltransferase (CAT) chimeric constructs were made as follows: DM10cat, DM10.5cat, DM11cat, and DM12cat contained 150-bp (−106 to +44), 118-bp (−74 to +44), 103-bp (−59 to +44), and 76-bp (−32 to +44) fragments, respectively, and these DNA fragments were subcloned into the *Bgl*II/*Hin*dIII sites of SV0cat (14). DM10cat was used as a template to construct internal deletion of ALE by oligonucleotides-directed mutagenesis (19). After the internal deletion mutation was obtained and confirmed by sequencing, the altered 140-bp fragment was subcloned into the *Bgl*II/*Hin*dIII sites of SV0cat. A competitive plasmid, pUC-ALE, was constructed by fusing the eight head-to-tail tandem repeats of ALE (−102 to −87) fragment into *Bam*HI site of pUC119. Herpes simplex virus (HSV) thymidine kinase (tk) promoter (−109 to +19)-CAT chimeric gene, tkcat, was

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Abbreviations: bp, base pair; CAT, chloramphenicol acetyltransferase; hAG, human angiotensinogen; EMSA, electrophoretic mobility shift assay; C/EBP, CCAAT enhancer-binding protein; CREB, cyclic adenosine monophosphate response element-binding protein; ATF, activating transcription factor; ALE, ATF-like element; ALF, ALE binding factor.

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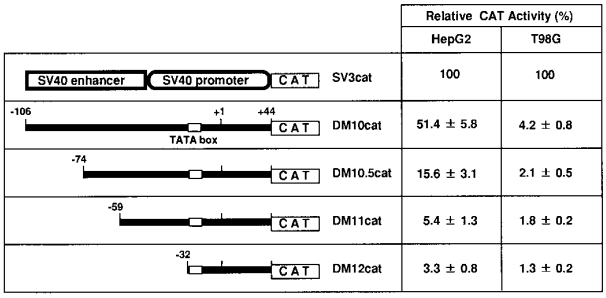

	Relative CAT Activity (%)	
	HepG2	T98G
SV3cat	100	100
DM10cat	51.4 ± 5.8	4.2 ± 0.8
DM10.5cat	15.6 ± 3.1	2.1 ± 0.5
DM11cat	5.4 ± 1.3	1.8 ± 0.2
DM12cat	3.3 ± 0.8	1.3 ± 0.2

FIG. 1. 5′-Deletion analysis of the human angiotensinogen proximal promoter. *Left,* 5′-deletion constructs of the hAG promoter-CAT hybrid genes. *Thick horizontal lines* and *open boxes* marked by *CAT* represent the promoter sequences and CAT vector sequences, respectively. The constructs used are listed to the right. *Right,* HepG2 and T98G cells were transfected with 3 μg of the indicated CAT vectors and 1 μg of the β-galactosidase expression plasmid (pCH110) as an internal control for transfection efficiency. After 48-h culture, β-galactosidase activities were measured and extracts containing equivalent amounts of β-galactosidase activities were used for CAT assays. The CAT activity of SV3cat is designated as 100 and each value of CAT activity represents the mean ± S. E. for at least four independent experiments.

constructed as described previously (20). ALE4tkcat was constructed by inserting four copies of ALE fragment at the upstream of the tk promoter.

Cell culture and transient expression assays. HepG2 and T98G cells were maintained as described previously (21). Transient transfection of DNA by the calcium phosphate precipitation using CellPhect Transfection Kit (Pharmacia), CAT assay, and normalization of transfection efficiency by β-galactosidase assay were carried out essentially as described previously (16). The extent of conversion of chloramphenicol to its acetylated form was measured with a Bioimaging analyzer (Model BAS2000; Fujix, Tokyo, Japan). All experiments were performed at least six times for each construct.

Preparation of nuclear extracts. Nuclear extracts from HepG2 cells were prepared using the protocol of Dignam *et al.* (22). The final protein concentration was about 5 μg/μl.

Electrophoretic mobility shift assays (EMSA). EMSAs were performed as described previously (16). Sources of the oligonucleotide sequences for competition analysis are as follows: the palindromic CREB/ATF site (23), the asymmetric CREB/ATF site (24), C/EBP site (25) and AP-1 site (GELSHIFT KIT, Stratagene).

RESULTS

To identify potential elements that contribute to hepatic hAG gene expression, we analyzed its proximal promoter coupled to the CAT gene. The 150-bp region is functional in transiently transfected HepG2 cells which endogenously express hAG mRNA, but not in T98G cells which do not endogenously express it (21) (FIG. 1). There was a reduction in CAT activity, when the upstream sequence was eliminated further up to −74 bp. These results suggested that the proximal promoter region from −106 bp to +44 bp was sufficient for cell type-specific hAG promoter activity and that at least one positive regulatory element existed at the position from −106 bp to −75 bp.

To investigate the binding activities of nuclear factors for the hAG promoter, EMSA was performed using the dissected DNA fragments that cover with the upstream region, as the probes. The upstream region contained *A*TF-*l*ike *e*lement (ALE), CT-rich sequences, and palindromic sequences (FIG. 2A). Incubation of ALE fragment with the nuclear extracts produced from HepG2 cells produced several retarded complexes, which represented a sequence-specific interaction, since the formation of these complexes was specifically reduced with molar excess of unlabeled competitors (FIG. 2B, lanes 1, 2 and 8). However, no specific complex was produced with incubation of CT+P fragment (data not shown). Because some basic region/leucine zipper (bZIP) classes of DNA binding proteins (26, 27) could bind to the same motif in ALE (24, 28, 29), we characterized ALE-binding factors using competition

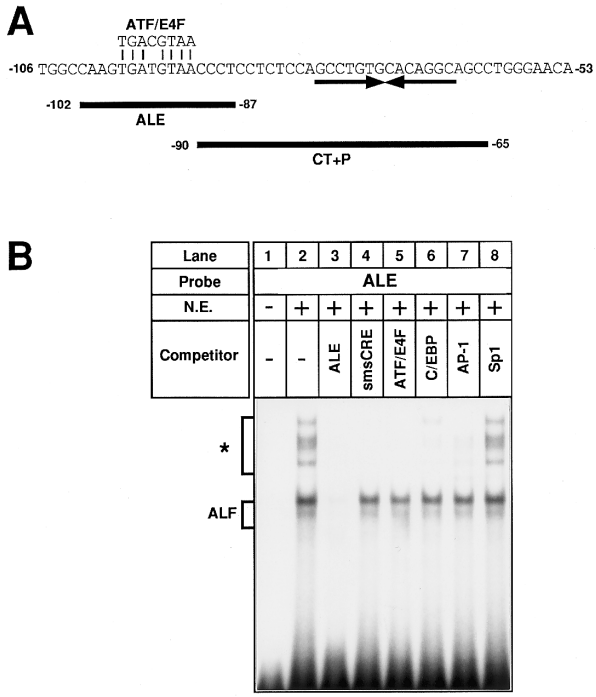

FIG. 2. Identification of sequence-specific nuclear factors using different portions of the upstream promoter sequences. *A,* On the *top,* the upstream promoter sequences of hAG gene, ATF-like motif is represented. *Below,* the oligonucleotides used to detect specific interactions between the upstream promoter sequences and nuclear factors are indicated by *thick horizontal lines. B,* EMSA. The indicated double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP. Five μg of HepG2 nuclear extract were incubated with 0.5 ng of ^{32}P-labeled probe. In a competition assay, 200-fold molar excess of the unlabeled oligonucleotides, as indicated for each lane, was added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1 × TBE electrophoresis. A specific nuclear factor, ALF and *, are indicated.

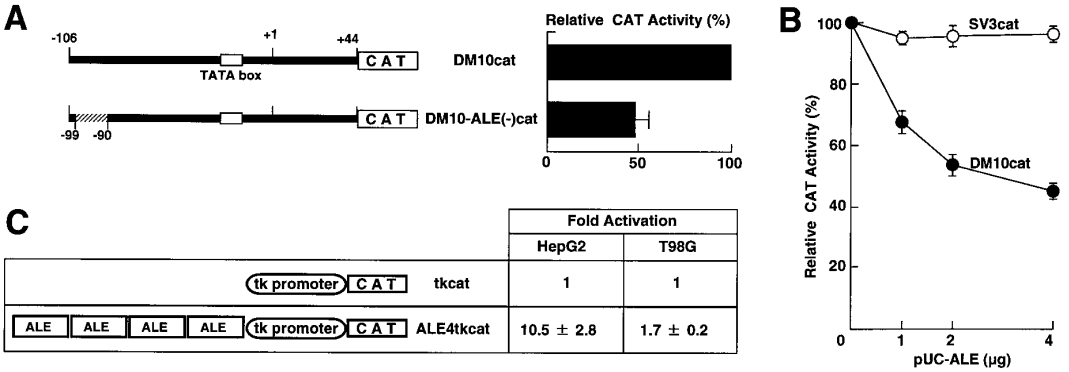

FIG. 3. Functional characterization of ALE-binding factors. *A,* Internal deletion of ALE. *Thick horizontal lines* and *hatched line* represent wild-type promoter sequences and internal deletion whose positions are indicated below the line, respectively. *Right,* HepG2 cells were transfected with the indicated CAT vectors, and CAT assays were performed as described in FIG. 1. The CAT activities are presented relative to that of the DM10cat construct. *Thin lines* represent the S. E.; n = 8 to 10. *B, In vivo* competition analysis of the ALE. A competitive plasmid, pUC-ALE, containing eight head-to-tail tandem repeats of the ALE fragment was used in this experiment. Two micrograms of reporter plasmids [SV3cat or DM10cat] were cotransfected with 1, 2, or 4 μg of pUC-ALE. Total amounts of DNA were adjusted to 6 μg by pUC119, and CAT assays were performed as described in FIG. 1. The CAT activity of each reporter plasmid, cotransfected with 4 μg of pUC119, is designated as 100 and each value of CAT activity represents the mean ± S. E. for at least four independent experiments. *C,* Cell type-specific function of ALE. *Oval* represent HSV-tk promoter. Transfection and CAT assays were performed as described in FIG. 1. The CAT activity of tkcat is designated as 1, and each value of CAT activity represents the mean ± S. E. for at least four independent experiments.

assays. The formation of minor complexes marked by an asterisk could be prevented completely by palindromic CREB/ATF site (smsCRE), asymmetric CREB/ATF site (E4/ATF), C/EBP site (C/EBP), and AP-1 consensus sequences (AP-1). Interestingly, the major complexes marked by ALF (*AL*E binding *f*actor) were not dissociated by smsCRE, E4/ATF, C/EBP and AP-1 sites (FIG. 2B, lanes 4-7). ALF-binding activity was also detected in mouse liver nuclear extract (data not shown). These results suggested that several bZIP superfamily bound to ALE as minor complexes, but ALF represented a novel binding activity.

To assess the role of ALE in the native promoter context, the internal deletion was introduced into the proximal promoter region. We could not use substitution mutation in order to avoid the generation of new binding sequences for the other bZIP-superfamily. As shown in FIG. 3A, the deletion of ALE significantly decreased the proximal promoter activity in HepG2 cells. To confirm the functional importance of ALE-binding factors, we performed *in vivo* competition experiments (FIG. 3B). The various amounts of pUC-ALE, which included 8 tandem copies of ALE in pUC119, were cotransfected with DM10cat or SV3cat. The CAT activity derived from DM10cat decreased with sequential titration of ALE-binding factors, although the activity driven from a negative control, SV3cat, was little influenced. Next, we conducted Northern blottings and CAT assays to obtain the information about bZIP proteins binding to ALE on the human angiotensinogen transcriptional activity. However, cyclic AMP and 12-*O*-tetradecanoylphorbol-13-acetate did not affect the endogenous angiotensinogen mRNA expression and the CAT activity of DM10cat in HepG2 cells (unpublish results). Furthermore, C/EBP expression vector had no effect on the CAT activity of DM10cat, although it could activate the CAT activity of the C/EBP8DM12cat which contained eight copies of C/EBP consensus binding site in DM12cat (data not shown). These results indicate that CRE, AP-1, and C/EBP were not major effectors on human angiotensinogen transcription. Finally, to evaluate the role of ALE on the cell-type dependent hAG promoter activity, four copies of ALE were linked upstream of the HSV tk promoter-CAT hybrid gene. ALE could activate the tk promoter in HepG2 cells, but failed to function in T98G cells (FIG. 3C). Taken together, these results suggest that ALE plays a key role in hepatic activation of the hAG promoter.

DISCUSSION

It has been reported that several bZIP classes of DNA-binding proteins, such as ATF-1, ATF-2, C/EBP, E4F, and E4BP4 can bind to TGATGTAA (ALE), as a homodimer. However, these factors are different from ALF in the point that they also bind to TGACGTAA (ATF/E4F) (24, 28, 29) (FIG. 2). The bZIP superfamily functions as not only homodimers but also heterodimers, and variation in dimer pairing may differentially affect target gene transcription or allow recognition of different DNA-binding specificity. For example, Vallejo *et al.* (30) have shown that the C/ATF-C/EBPβ heterodimer bound to asymmetric CREs that are not recog-

nized by homodimers of C/ATF or C/EBPβ. From this point of view, ALF is likely to possess a novel binding activity which is generated by a heterodimerization among the members of known or unknown bZIP superfamily.

Current models for regulation of transcriptional activation involve the protein-protein interactions between sequence-specific transcription factors and cofactors which have no intrinsic DNA-binding activity. CREB is activated through phosphorylation by protein kinase A, and this phosphorylation allows the recruitment of coactivator, CBP, which then activates basal transcription machinery (31). Furthermore, another coactivator, OBF-1, which is expressed only in B cells, interacts with ubiquitously expressed Oct-1, and give a B cell-specific activation function to Oct-1 (32). We indicated the cell-type dependent function of ALE (FIG. 3C), but ALE-binding activities were detected in all cell lines tested, regardless of whether hAG is expressed or not (data not shown). Based on these principles, we propose the possibility that ALE may have an important function in the HepG2-dependent expression through either a cell-type dependent modification or the interaction of a cell-type specific cofactor or both.

Human ALE motif was compared with the proper position of rat and mouse homologues of angiotensinogen promoter, and revealed that ALE was not conserved in these homologues (33). We previously identified a ubiquitously-expressed nuclear factor, AGCF1, bound to AGCE1 located between the TATA box and transcription initiation site is an authentic regulator that mediates the responsiveness to multiple regulatory elements. Interestingly, AGCE1 was also not conserved in these homologues. These results suggest that human angiotensinogen transcription may be regulated by the different mechanisms from rat and mouse ones.

In conclusion, the experiments reported here indicate that ALE functions as a cell-specific activating element of hAG promoter, and the major binding factor, ALF, has a novel binding activity. Experiments now in progress will define, by means of cloning cDNAs encoding ALE-binding factors, the mechanisms of action of ALE and will provide a molecular basis for the hAG transcriptional regulation.

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