Molecular Variation of the Human Angiotensinogen Core Promoter Element Located between the TATA Box and Transcription Initiation Site Affects Its Transcriptional Activity*

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Recent genetic studies indicate that several molecular variants discovered in angiotensinogen (AG), the precursor of vasoactive octapeptide angiotensin II, could potentially be responsible for inherited predisposition to human blood pressure variation. We have previously shown that a ubiquitously expressed nuclear factor, AGCF1, bound to AGCE1 (AG core promoter element 1 including the core nucleotides, CTCGTG, CTC-type) located between the TATA box and transcription initiation site (positions −25 to −1) is an authentic regulator of human AG transcription. In the present study, we showed that AGCF1 has biologically and immunologically similar properties to those of a helix-loop-helix nuclear factor USF1 and examined the effects of two other naturally occurring molecular variants (ATCGTG, ATC-type and ATTTGT, ATT-type) found in the AGCE1 position on the human AG transcriptional activity. Competitive gel-shift and transfection experiments demonstrated that the transcriptional activity for the CTC- and ATC-type promoters was 2.5 times higher than that for the ATT-type through the alteration of AGCF1-binding affinity. These results suggest the possible involvement of USF1 as a component in AGCF1 formation and the potential importance of AGCE1 variation in blood pressure regulation through human AG expression.

The renin-angiotensin system plays an important role in the regulation of blood pressure and electrolyte homeostasis. The reaction between renin and angiotensinogen (AG)1 is the initial and rate-limiting step of this enzymatic cascade that generates the decapeptide angiotensin I, which is further processed to the functional octapeptide angiotensin II by angiotensin-convertase enzyme (1–3). Because plasma AG concentration is close to the $K_m$ of the renin reaction, variation of plasma AG concentration can influence angiotensin II generation (4). Several observations indicate a direct relationship between plasma AG levels and blood pressure. First, plasma AG concentrations highly correlate with blood pressures in some patients (5), and associations between AG concentrations and hypertension have been demonstrated in families (6). Second, the overexpression of AG leads to elevated blood pressure in transgenic animals (7). Recently, Jeunemaitre et al. (8) showed that a common AG gene variant, M235T, was significantly linked to essential hypertension and was also associated with elevated plasma AG concentration. Whether M235T directly accounts for a physiological effect or acts as a marker for a causative mutation is as yet unresolved, they proposed that some other variants of the AG gene lead to a chronic increase in AG levels and thereby eventually to increased blood pressure.

AG is mainly synthesized in the liver and is secreted into the plasma through the constitutive pathway (9). Therefore, it is possible to suppose that the transcriptional regulation of the AG gene affects its plasma concentration. Kim et al. (10) have generated mice carrying two, three, or four functional copies of the murine wild-type AG gene at its normal chromosomal location and reported that plasma AG levels increased progressively with an increase in blood pressure. A recent study showed that the inhibition of AG transcription resulted in a reduction in plasma AG levels associated with a decrease in blood pressure of spontaneously hypertensive rats, by using synthetic double-stranded oligonucleotides as “decoy” cis-elements to block the binding of nuclear factors to the targeted promoter regions (11). These observations suggest an etiological importance of the transcriptional regulation of the AG gene.

We previously identified several regulatory elements of the human AG gene transcription including the upstream and downstream activating elements (12–14) and recently demonstrated that a ubiquitously expressed nuclear factor, AGCF1, bound to AGCE1 (AG core promoter element 1) including the core nucleotides (CTCGTG), an E box-like motif located between the TATA box and transcription initiation site, is an authentic regulator that mediates the responsiveness to multiple AG regulatory elements (15). Moreover, a recent genetic study found the three types of molecular variants, CTCGTG, ATCTGTG, and ATTTGTG, in the AGCE1 position of the human AG promoters (8). Therefore, we examined whether these variations affect the AGCF1-binding affinity and the human AG transcriptional activity. Here, we suggested the possible involvement of USF1, an E box-binding helix-loop-helix (HLH) transcription factor (16) as a component in AGCF1 formation and discussed the potentially important relationship between the naturally occurring AGCE1 variations and their transcriptional activity.
**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The reconstituted human AG gene, 13B2B(3′+(-)) and 13Am4B2(3′+(-)) were constructed as described previously (15). 13cat were used as templates to construct mutations in AGCE1 by oligonucleotides-directed mutagenesis (15, 17). Once the site-directed mutations were obtained and confirmed by sequencing, the altered 1266-base pair (position 222 to +44) fragments were used for constructions of 13(ATACTC2B3′+(-)) and 13(ATTB2B3′+(-)). Human USF1 cDNA fragment was obtained by reverse transcriptase-polymerase chain reaction as follows. Single-stranded cDNA generated from human hepatoma cell line (HepG2) total RNA using a first-strand cDNA synthesis kit (PharMingen Tech Inc., Uppsala, Sweden) was subjected to polymerase chain reaction amplification using primers synthesized based on the human USF sequence (18): 5′-TCCGGATTCCCTCTACAGAGAGATGAAGGGG-3′ (primer 1; corresponding to nucleotide 97–129) for the full-length fragment, or 5′-GTTCCAATTCCGCGCATTGTCACCCAAAGAAGTACTG-3′ (primer 2; corresponding to nucleotide 607–630) for the truncated fragment, and 5′-GATCCCTCAAGCTTCTGTTGCTGCATTCTGTGACCG-3′ (primer 3; complementary to nucleotides 1029–1053). DNA amplification using a GeneAmp AmpliTag PCR kit (Perkin-Elmer) was performed in a Perkin-

Elmer thermal cycler with 25 cycles of denaturation (94 °C, 1 min), annealing (63 °C, 1 min), and extension (72 °C, 1 min). The obtained fragment was cloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA) and confirmed by sequencing.

**Transient Expression Assays**—HepG2 cells were maintained in minimum essential medium containing 10% fetal bovine serum and nonessential amino acids. The cells were plated at a density of 5 × 10^4 cells/60-mm dish and transfected 24 h later by calcium phosphate co-precipitation with reporter plasmids (3 μg) and a β-galactosidase expression plasmid, pCMV-β-gal (1 μg) to normalize transfection efficiency. In the co-transfection assay, 2 μg of reporter plasmids were transfected with 1, 2, or 4 μg of modulator plasmids and 1 μg of pCMV-β-gal. Total amounts of DNA were adjusted 7 μg by pcDNA3. After 48 h of culture, β-galactosidase activities were measured (19), and cell extracts containing equivalent amounts of β-galactosidase activity were used for CAT assay (20). The extent of conversion of chloramphenicol to its acetylated form was measured with a Bio-imaging analyzer (Model BAS2000; FujiX, Tokyo, Japan).

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts from HepG2 cells were prepared using the protocol of Dignam et al. (21). Double-stranded DNA probe was end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. 5 μg of nuclear extracts were incubated with 1 μg of poly(dI-dC) (Boehringer Mannheim) and end-labeled oligonucleotide (0.5 ng, approximately 15,000 cpm) at 20 °C for 15 min in the presence or absence of the unlabeled oligonucleotides. The binding reactions were carried out in a solution containing 12 mM Hepes, pH 7.9, 60 mM KCl, 4 mM MgCl₂, 1 mM EDTA, 12% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. In the supershift experiments, 2 μl of USF1-specific antibody (Santa Cruz Biotechnology, CA) was added to the reaction mixture. The reaction mixtures were directly loaded onto 4.5% nondenaturing polyacrylamide gels containing 4% glycerol made in 1 × TBE (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA). After electrophoresis was performed at 150 V for 2.5 h at 4 °C, the gels were dried and autoradiographed with an intensifying screen.

**RESULTS**

We previously showed that AGCF1 contained 31-, 33-, and 43-kDa proteins as major components by UV cross-linking (15). In the course of characterizing AGCF1, we noticed that its binding activity was heat-stable and affected by MgCl₂ (data not shown). These observations allowed us to speculate that the 43-kDa proteins identified would be USF1, which is classified as the members of basic HLH/leucine zipper family of transcription factors (16), although the nucleotide residue at position 97 of the human AG core promoter was substituted to the thymidine (CTCGTG) from the adenine (CAGCGT) in a consensus E box for USF. To examine whether USF1, a heat-stable MgCl₂-sensitive 43-kDa transcription factor, is a component of AGCF1, we performed EMSA using the nuclear extracts prepared from HepG2 cells (Fig. 1). The DNA-protein complex formed by AGCF1 binding to AGCE1 was inhibited by molar excess of nonlabeled USF1 binding element of the adenovirus major late promoter (18) as well as AGCE1, although retinoic acid response element 1 of the cellular retinoic acid-binding protein II promoter (CRABP-II) (22) and estrogen response element of vitellogenin promoter (vit-ERE) (23), which partially related sequences to AGCE1 (Fig. 1A), did not compete for this binding at all (Fig. 1B, lanes 1–6). Similar results were obtained by using the adenosinovirus major late promoter as the probes (Fig. 1B, lanes 7–12). Furthermore, the addition of USF1-specific antibody to EMSA reactions generated a supershifted complex (Fig. 1B, compare lane 13 with lane 14), indicating that AGCF1 complex contains USF1.

The ability of USF1 to function as a transcriptional modulator of the human AG gene was analyzed by using a dominant negative form of USF1 protein (dnUSF) that lacked the N-terminal activation domain but possessed its DNA binding domain (24). The bacterially expressed GST-dnUSF fusion protein had the same binding specificity as compared with that of AGCF1 (data not shown). The expression of dnUSF dramatically reduced the transcriptional activity of the reconstituted human AG gene (13B2B(3′+(-)) but had little effect on that of the Am4 mutant (13Am4B2B3′+(-)) that completely disrupted AGCF1 binding in vitro (15) in HepG2 cells (Fig. 2B). To further confirm the participation of USF1 in human AG transcription, an activator form of USF1 was co-transfected

**FIG. 1. Identification of USF1 as a component of AGCF1.** A, the AGCF1 binding region of human AG core promoter. A bracketed line represents the region protected from DNase I (15). The TATA box and transcriptional start site are indicated by a box and an arrow, respectively. Recognition sequences for known eukaryotic transcription factors, estrogen response element (vit-ERE), retinoic acid response element (CRABPII-1), and adenosinovirus major late promoter (AdML), were compared with the sequence of the AG core promoter. A • indicates an identity among their nucleotides. Double-stranded versions of the indicated sequences were used in competition experiments. B, EMSA. The indicated double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase using [γ-32P]ATP. 5 μg of HepG2 nuclear extracts were incubated with 0.5 ng of 32P-labeled probe in the presence or absence of 200-fold molar excess of the unlabeled oligonucleotides. In supershift assays, USF1 antibodies were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1 × TBE electrophoresis. AGCF1 is indicated.
with the human AG reporters (Fig. 3A). In this assay, we used the minimal hAG core promoter (including only TATA box and AGCE1) as a reporter because of the saturated CAT activity in the reconstituted construct and to avoid the effects of hAG regulatory elements other than AGCE1. The expression of full-length USF1 activated the human AG minimal promoter (DM12cat), but had little effect on that of the Am4 mutant (DM12Am4cat) in HepG2 cells (15). These results suggest that USF1 could regulate the human AG gene transcription in the AGCE1-dependent manner.

Interestingly, a previous genetic study identified the three types of molecular variants, CTCGTG, ATCGTC, and ATTGTG (8), localized to AGCE1 position of the human hAG promoters. In contrast, these E box-like motifs in AGCE1 were not conserved in the rodent angiotensinogen promoters (Fig. 4A) (25). These differences prompted us to examine the possibility that the AGCF1 binding affinity might be affected by the naturally occurring molecular variants by using competition assays. Although the DNA-protein complex formed by AGCF1 binding to the CTC-type AGCE1 was inhibited by a molar excess of unlabeled CTC-type AGCE1, ATC-type AGCE1 partially prevented this complex formation. In particular, the ATT-type and rodent counterparts hardly competed for this binding (Fig. 4B and C). These results indicate that the AGCF1 binding to AGCE1 is a species-specific interaction and that the molecular variation in AGCE1 alters the AGCF1 binding affinity.

AGCE1 plays an important role in mediating the responsiveness of multiple upstream and downstream cis-acting elements of the human AG gene to activate its promoter (15). As the disruption of AGCF1 binding, which resulted in the functional attenuation of the human AG regulatory elements, dramatically reduced the AG transcriptional activity (15), we examined the possibility that the alteration of AGCF1 binding affinity caused by the three types of naturally occurring mutations affected the AG transcription (Fig. 5). Although the transcriptional activity of the ATC-type in the reconstituted human AG gene (13(AGCF1)B2(3')(++) was not significantly different from that of the CTC-type, the ATT-type represented about 40% of the transcriptional activity compared with that of the CTC-type. Although the ATC-type in the reconstituted human AG gene (13(AGCF1)B2(3')(++) was not significantly different from that of the CTC-type, the ATT-type represented about 40% of the transcriptional activity compared with that of the CTC-type. Taken together, these results demonstrate that molecular variation of the human AG core promoter, AGCE1, affects its transcriptional activity by alteration of the AGCF1-binding activity.

DISCUSSION

In the present study, we showed that AGCF1 has a DNA binding specificity similar to that of a HLH nuclear factor.
USF1 (26–28) (Fig. 1). Furthermore, co-transfection experiments demonstrated that USF1 could regulate transcriptional activity of the human AG gene in the AGCE1-dependent manner (Figs. 2 and 3). Next, we examined the effects of naturally occurring molecular variants (CTCGTG, CTC-type; ATCGTG, ATC-type; and ATTTGTG, ATT-type) localized to AGCE1 on the human AG transcriptional activity. Competitive EMSA and site-directed mutagenesis experiments demonstrated that the transcriptional activity for the CTC- and ATC-type mutations was significantly higher than that for the ATT-type by alteration of transcriptional regulation (16). This factor is also shown to be involved in the regulation of cellular genes, including the adenovirus major late promoter (16). This factor is also shown to be involved in the regulation of cellular genes, including the

murine metallothionein I gene (29), the rat γ-fibrinogen gene (30), the human growth hormone gene (31), the p53 gene (32), and the cardiac ventricular myosin light chain 2 gene (33). Furthermore, USF acts not only as a classical upstream activator, but also as a factor that interacts with initiator elements of a variety of core promoters, which can lead to markedly enhanced levels of basal transcription (34). We previously demonstrated that human AG promoter functioned without TATA box in the presence of AGCE1 (15). This initiator-like activity of AGCE1 may be explained by the presence of USF as a component of AGCF1 (Fig. 1), because USF could activate the basal level of transcription of the human AG core promoter in the AGCE1-dependent manner (Fig. 3).

Recently, Caullied et al. (35) have shown significant linkage between hypertension and chromosomal regions including and close to the human AG gene, but they could not confirm association with the M235T mutation as a candidate marker for essential hypertension, probably due to ethnic differences in its allele frequency. As the functional effect of the M235T on essential hypertension was unclear, Lifton (36) pointed out the possible existence of functional variant(s) other than this mutation at AG locus. For example, we considered that the transcriptional regulation is one of the candidate control mechanism accounting for the variation of human angiotensinogen expression and provided the functional evidence that the transcriptional activities for the CTC- and ATC-type AG promoters were 2.5 times higher than that for the ATT-type by alteration of the AGCF1 binding affinity (Figs. 4 and 5). On the basis of the present results, it is suggested that the transcriptional activities of the CTC/CTC and ATC/ATC homozygotes or the CTC/ATC heterozygotes are 2.5 times higher than that of the ATT/ATT homozygote and that the CTC/ATT and ATC/ATT heterozygotes is 1.75 times higher than that of the ATT/ATT homozygote. As the functional effect of the M235T on essential hypertension, probably due to ethnic differences in its allele frequency. As the functional effect of the M235T on essential hypertension was unclear, Lifton (36) pointed out the possible existence of functional variant(s) other than this mutation at AG locus. For example, we considered that the transcriptional regulation is one of the candidate control mechanism accounting for the variation of human angiotensinogen expression and provided the functional evidence that the transcriptional activities for the CTC- and ATC-type AG promoters were 2.5 times higher than that for the ATT-type by alteration of the AGCF1 binding affinity (Figs. 4 and 5). On the basis of the present results, it is suggested that the transcriptional activities of the CTC/CTC and ATC/ATC homozygotes or the CTC/ATC heterozygotes are 2.5 times higher than that of the ATT/ATT homozygote and that the CTC/ATT and ATC/ATT heterozygotes is 1.75 times higher than that of the ATT/ATT homozygote. As the functional effect of the M235T on essential hypertension was unclear, Lifton (36) pointed out the possible existence of functional variant(s) other than this mutation at AG locus. For example, we considered that the transcriptional regulation is one of the candidate control mechanism accounting for the variation of human angiotensinogen expression and provided the functional evidence that the transcriptional activities for the CTC- and ATC-type AG promoters were 2.5 times higher than that for the ATT-type by alteration of the AGCF1 binding affinity (Figs. 4 and 5). On the basis of the present results, it is suggested that the transcriptional activities of the CTC/CTC and ATC/ATC homozygotes or the CTC/ATC heterozygotes are 2.5 times higher than that of the ATT/ATT homozygote and that the CTC/ATT and ATC/ATT heterozygotes is 1.75 times higher than that of the ATT/ATT homozygote.
been reported (37–42). In addition to the systemic action of angiotensin II, the tissue function of this peptide is now considered to play an important role in local tissue regulation because components of the renin-angiotensin system have been demonstrated in a variety of tissues, such as adrenal glands, kidney, heart, and brain (43). Since AGCF1 is a ubiquitously expressed transcription factor, variants in AGCE1 may affect the regulation of AG levels. Here we present the first clue of the statistical significance that incriminates the AG gene locus induced by the AGCE1 polymorphisms. As discussed to date, the little direct evidence regarding the mechanism by which molecular variants of the AG gene affect the regulation of AG levels. Here we presented the first clue of human AG variations associated with the alteration of their expression. However, there was little direct evidence regarding the mechanism by which molecular variants of the AG gene affect the regulation of AG levels. Here we presented the first clue of human AG variations associated with the alteration of their expression.

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