

Role of C/A Polymorphism at -20 on the Expression of Human Angiotensinogen Gene

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Abstract—Angiotensinogen is the glycoprotein precursor of 1 of the most potent vasoactive hormones, angiotensin II. Human angiotensinogen gene contains a C/A polymorphism at -20 located between the TATA box and transcriptional initiation site. We show here that when nucleoside A is present at -20, this sequence binds to the estrogen receptor. We also show that transcriptional activity of reporter constructs containing human angiotensinogen gene promoter with nucleoside A at -20 is increased on cotransfection of an expression vector containing human estrogen receptor- α coding sequence in human hepatoma cells (HepG2) followed by estrogen treatment. On the other hand, adenoviral major late transcription factor binds preferentially to this region of the promoter when nucleoside C is present at -20. We also show that reporter constructs containing human angiotensinogen gene promoter with nucleoside C at -20 have increased basal promoter activity on transient transfection in HepG2 cells as compared with reporter constructs with nucleoside A at -20. Our data suggest that C/A polymorphism at -20 may modulate the expression of human angiotensinogen gene in a sex-specific manner. (*Hypertension*. 1999;33:108-115.)

Key Words: angiotensinogen ■ genes ■ polymorphism ■ estrogen ■ gene regulation ■ regulation, hormonal ■ major late transcription factor

The renin-angiotensin system plays an important role in the regulation of blood pressure, fluid balance, and electrolyte homeostasis. Angiotensin II, which is 1 of the most potent vasoactive hormones, is obtained from its precursor molecule, angiotensinogen, by the combined proteolytic action of renin and angiotensin-converting enzyme. Angiotensinogen is primarily synthesized in the liver, although recent studies have shown that its mRNA is also present in fat, brain, kidney, heart, and aorta of rats¹ and humans.² Because plasma concentration of angiotensinogen is close to the Michaelis constant of the enzymatic reaction between renin and angiotensinogen,³ a rise or fall in plasma angiotensinogen levels can lead to a parallel change in the formation of angiotensin II, and an increase in plasma angiotensin II may lead to hypertension. Previous studies have shown a highly significant correlation between plasma concentrations of angiotensinogen and blood pressure,⁴ higher plasma angiotensinogen concentrations in hypertensive parents and their offspring,⁵ and elevations of blood pressure in transgenic animals overexpressing the angiotensinogen gene.^{6,7} Recent studies have suggested that the angiotensinogen gene locus is involved in human essential hypertension⁸ and pregnancy-induced hypertension.⁹

Human angiotensinogen gene contains a C/A polymorphism at -20 located between the TATA box and transcrip-

tional initiation site.⁸ We show here that this region of the promoter binds to estrogen receptor- α when nucleoside A is present at -20. We also show that a reporter construct, pHAG1.2CAT (-20A), is transactivated by cotransfection of the mammalian expression vector pSG5 containing the coding sequence of the human estrogen receptor- α (HEO) in human hepatoma cells (HepG2) followed by estrogen treatment. Our transient transfection assay shows that a reporter construct pHAG40CAT (-20A) containing only 40 bp of the 5'-flanking sequence contains a functional estrogen responsive element (ERE) when nucleoside A is present at -20. On the other hand, reporter constructs with nucleoside C at -20 are transactivated by HEO to a lesser extent. We also show that adenoviral major late transcription factor (MLTF) binds preferentially to this region of the promoter when nucleoside C is present at -20. Reporter constructs pHAG1.2CAT and pHAG47CAT have increased basal transcriptional activity on transfection in HepG2 cells when nucleoside C is present at -20 compared with reporter constructs when nucleoside A is present at -20.

Methods

Materials

Expression vectors pSVoCAT, pGem-T, and RSV-gal were obtained from Promega Biotec (Madison, WI); the expression vector contain-

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ing the coding sequence of chloramphenicol acetyl transferase gene attached to 80 bp of the herpes virus thymidine kinase promoter (TK-CAT) was provided by Dr Anuradha Ray (Yale University, New Haven, CT); HEO was provided by Dr P. Chambon (INSERM, France); and the expression vector containing the MLTF coding sequence attached to the cytomegalovirus promoter (CMV-MLTF) and MLTF antibody were provided by Dr Robert Roeder (Rockefeller University, New York, NY). Restriction enzymes were purchased from New England Biolab (Cambridge, MA) or GIBCO/BRL (Gaithersburg, MD). 17- β estradiol was obtained from Sigma Chemical (St Louis, Mo). Qiagen mini and midi plasmid kits were obtained from Qiagen (Chatsworth, Calif). Monoclonal antibody against human estrogen receptor (hER) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif).

Oligonucleotides

The CA (with nucleoside A at -20) and CC (with nucleoside C at -20) oligonucleotides were obtained by annealing 5'-CTAGTAGGGCATCGTGACCCGGCCAGGGG and CTAGTAGGGCCTCGTGACCCGGCCAGGGG with their complementary sequences. These oligonucleotides correspond to -2 to -26 of the human angiotensinogen gene promoter and contain an extra *Xba*I restriction site (CTAG) at their 5' ends. Two copies of these oligonucleotides were also attached in front of TK-CAT to produce reporter constructs (CA)2TKCAT and (CC)2TKCAT. The MLTF and vitellogenin ERE oligonucleotides were obtained by annealing 5'-CTAGTGTAGGCCACGTGACCGGG and AAAGTCAGGTCA-CAGTGACCTGATCAAAGA with their complementary sequences. The reporter construct p(mCA)2TK-CAT was synthesized by annealing 5'-CTAGTAGGATCTCGTGACCCGGCCAGGGG and its complementary sequence and by attaching 2 copies in TK-CAT (mutation in the ERE is underlined). The double-stranded oligonucleotide used for the mutation of nucleoside A to C at -20 in pHAG1.2CAT was obtained by annealing 5'-GCTATAAATAGGGCCTCGTG ACCCGG and its complementary sequence.

Plasmid Construction

The reporter construct pHAG1.2-CAT was synthesized by attaching ~1.2 kb of 5'-flanking region of the human angiotensinogen gene^{10,11} in front of the chloramphenicol acetyl transferase (CAT) gene in the expression vector pSVoCAT. The ~1.2 kb region of the promoter contained 1223 bp of the 5'-flanking region and 44 bp of the first exon of the human angiotensinogen gene that were obtained by polymerase chain reaction of human genomic DNA and that contained nucleoside A at -20. The reporter construct pHAG47CAT (containing only 47 bp of the 5'-flanking region) was obtained as a deletion mutant from pHAG1.2CAT. The reporter construct pHAG40CAT, containing 40 bp of the 5'-flanking region and 36 bp of the first exon, was constructed by polymerase chain reaction using pHAG1.2CAT as a template. Nucleotide sequences of the reporter constructs were confirmed by restriction and sequence analysis. Plasmid DNAs for transfection were prepared by Qiagen column, and the quality of plasmid DNAs was checked by gel electrophoresis. Site-specific mutagenesis was performed in the expression vector pHAG1.2CAT to mutate nucleoside A to C at -20 with a Quick change site-directed mutagenesis kit by Stratagene (La Jolla, Calif) as suggested by the manufacturer.

Cell Culture and Transient Transfection

Human hepatoma cells (HepG2) were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin in an atmosphere of 5% CO₂. Transient DNA transfections were performed by the calcium phosphate precipitation method using supercoiled plasmid DNA at 10 to 20 μ g/plate and the expression vector containing the coding sequence of the β -galactosidase (β -gal) gene attached to Rous sarcoma virus promoter (pRSV-gal; 2 μ g) as an internal control to normalize efficiency of transfection. After a 4-hour treatment with the DNA precipitate, cells were washed with phosphate-buffered saline and incubated with fresh medium. Cells

were harvested after 48 hours of transfection, total extract was prepared by 3 cycles of freezing and thawing in liquid nitrogen, an aliquot (5 λ) was used for β -gal assay, and the rest of the extract was heated at 65°C for 5 minutes. After centrifugation, an aliquot of the extract (after normalization with the β -gal activity) was used to perform CAT assay using ¹⁴C-chloramphenicol as a substrate followed by separation of acetylated products by thin-layer chromatography using silica gel plates. After autoradiography, spots corresponding to ¹⁴C-chloramphenicol and its acetylated derivatives were scraped from thin-layer chromatography 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 nonacetylated spots). For transfection experiments in which the effect of estrogen was analyzed, cells were cotransfected with HEO (2 μ g). For these experiments cells were grown in phenol red-free medium in the presence of charcoal-treated serum. After 24 hours of transfection, cells were treated with 17- β estradiol (100 nmol/L), and promoter activity was analyzed after 24 hours of hormone treatment. Transient transfections were performed at least 3 times using at least 2 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 -³²P] ATP. The radiolabeled oligonucleotide (20 000 to 50 000 cpm), 1 to 2 μ g of poly(dI-dC), and 5 to 10 μ g of the protein extract were incubated in a solution containing 10 mmol/L HEPES (pH 7.5), 50 mmol/L KCl, 5 mmol/L MgCl₂, 0.5 mmol/L EDTA, 1 mmol/L dithiothreitol, 12.5% glycerol in ice for 30 minutes and separated on a 5% to 8% polyacrylamide gel in a cold room. After 2 to 3 hours, the gel was dried under vacuum and protein-nucleic acid complexes were identified by autoradiography. For supershift experiments, monoclonal antibody against hER (1 μ L) was added to the reaction mixture, which was then incubated for 30 minutes. Nuclear extracts for gel mobility shift assay were prepared by a previously described method.¹² Whole-cell extracts from COS- and HEO-transfected COS cells were obtained by freeze-thawing of cells as previously described.¹³

Results

1.2-kb 5' Flanking Region of Human Angiotensinogen Gene With Nucleoside A at -20 Contains an Estrogen-Responsive Element

To understand the regulation of human angiotensinogen gene expression by estrogen, we constructed the expression vector pHAG1.2CAT, which contains nucleoside A at -20 (Figure 1A), and cotransfected it with HEO in HepG2 cells (because HepG2 cells do not contain functional estrogen receptor [ER]). After transfection, cells were treated with 17- β estradiol, and promoter activity was analyzed by the CAT assay. Results of this experiment (Figure 1B) indicate that cotransfection of HEO and estrogen treatment increased the promoter activity of pHAG1.2CAT by 5- to 6-fold. Because the putative ERE is located adjacent to the TATA box in human angiotensinogen gene (Figure 1A), it was of interest to determine whether binding of liganded ER to this site interferes with the formation of the preinitiation complex that may ultimately result in transcriptional downregulation. To answer this question, we constructed an expression vector pHAG40CAT, which was then cotransfected with HEO in HepG2 cells. The promoter activity was then analyzed after estrogen treatment. Results of this experiment, shown in Figure 1C, indicate that estrogen treatment increased the promoter activity of pHAG40CAT by 15- to 20-fold and

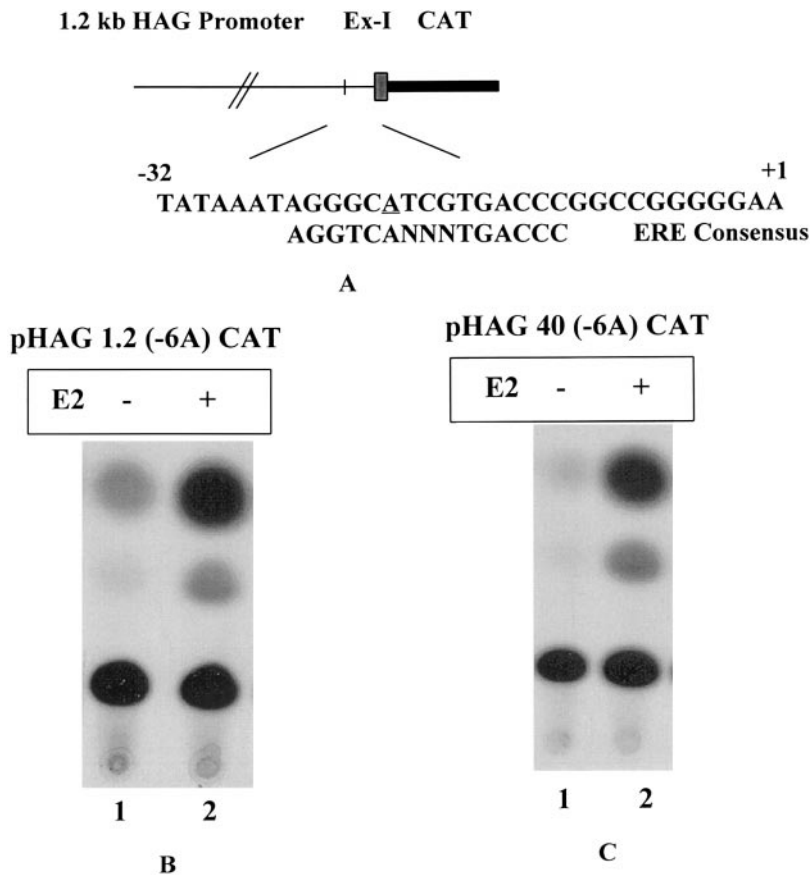


Figure 1. The promoter activity of reporter constructs pHAG1.2CAT and pHAG40CAT (with nucleoside A at -20) is increased on cotransfection of HEO and estrogen treatment in HepG2 cells. A, The nucleotide sequence containing 1223 bp of the 5'-flanking region and 44 bp of exon-I of the human angiotensinogen gene was ligated in pSVoCAT to produce reporter construct pHAG1.2CAT. The insert shows nucleotide sequence located between the TATA box and transcriptional initiation site (-32 to -1) and its homology with consensus ERE. The reporter construct pHAG1.2CAT (B) or pHAG40CAT (C) ($20 \mu\text{g}$) was transiently cotransfected with HEO and RSV-gal in HepG2 cells. After 24 hours, cells were treated with 17β -estradiol (E2). After 24 hours of hormone treatment, cell extract was prepared by 3 cycles of freeze-thawing, and CAT activity was determined after normalization with the β -gal activity.

suggest that binding of the ligand-bound ER to this site does not interfere with the formation of preinitiation complex.

Palindromic Sequence Located Between the TATA Box and Transcriptional Initiation Site of the Human Angiotensinogen Gene Binds to the ER

To examine whether the palindromic sequence located between the TATA box and transcriptional initiation site binds with ER, we performed a gel shift assay with CA oligo and protein extract (HEO) obtained from COS cells that were transfected with HEO and treated with 17β -estradiol. Results of this experiment (Figure 2A) show that HEO formed a major complex with the CA oligo (lane 2) and that mobility of this protein:DNA complex was different when extract obtained from COS cells alone was used (lane 1). The intensity of the shifted band was reduced in the presence of cold CA oligo (lane 3) and in the presence of vitellogenin estrogen responsive element (vit-ERE) (lanes 4 and 5).

To further confirm that the complex obtained with the CA oligo and HEO is due to binding with the ER, we performed a gel shift assay with CA and vit-ERE oligonucleotides in the presence and absence of a monoclonal antibody against hER. Results of this experiment, which are shown in Figure 2B, indicate that electrophoretic mobility of the protein:DNA complex obtained with the vit-ERE (lane 1) is almost identical to that of the complex obtained with CA oligo (lane 3). Furthermore, ER monoclonal antibody produced a supershift with vit-ERE (lane 2) similar to that of the CA oligo (lane 4).

Reporter Construct Containing 2 Copies of CA Oligonucleotide Attached to Heterologous TK-CAT Promoter Is Transactivated by HEO and Estrogen Treatment

To confirm the functional role of putative ERE located between the TATA box and the transcriptional initiation site of the human angiotensinogen gene, we cotransfected reporter constructs p(CA)₂-TKCAT and p(mCA)₂-TKCAT with HEO in HepG2 and COS cells. After transfection, cells were treated with 17β -estradiol and promoter activity was analyzed by CAT assay. Results of transient transfection in COS cells (Figure 3A) indicate that, although cotransfection of HEO and estrogen treatment increased the promoter activity of reporter construct p(CA)₂TKCAT by 5- to 6-fold, it actually decreased the promoter activity of the reporter construct p(mCA)₂TKCAT. Similar results were obtained on transient transfection in HepG2 cells (data not shown).

Cotransfection of HEO and Estrogen Treatment Increases the Promoter Activity of Reporter Constructs Containing Nucleoside A at -20 as Compared With Reporter Constructs Containing Nucleoside C at -20

Because human angiotensinogen gene contains a C/A polymorphism at -20 , we next studied the effect of this polymorphism on estrogen-induced promoter activity of the human angiotensinogen gene. Transient cotransfection of pHAG1.2CAT and pHAG47CAT with nucleoside A or C at -20 was therefore performed with HEO in HepG2 cells, and

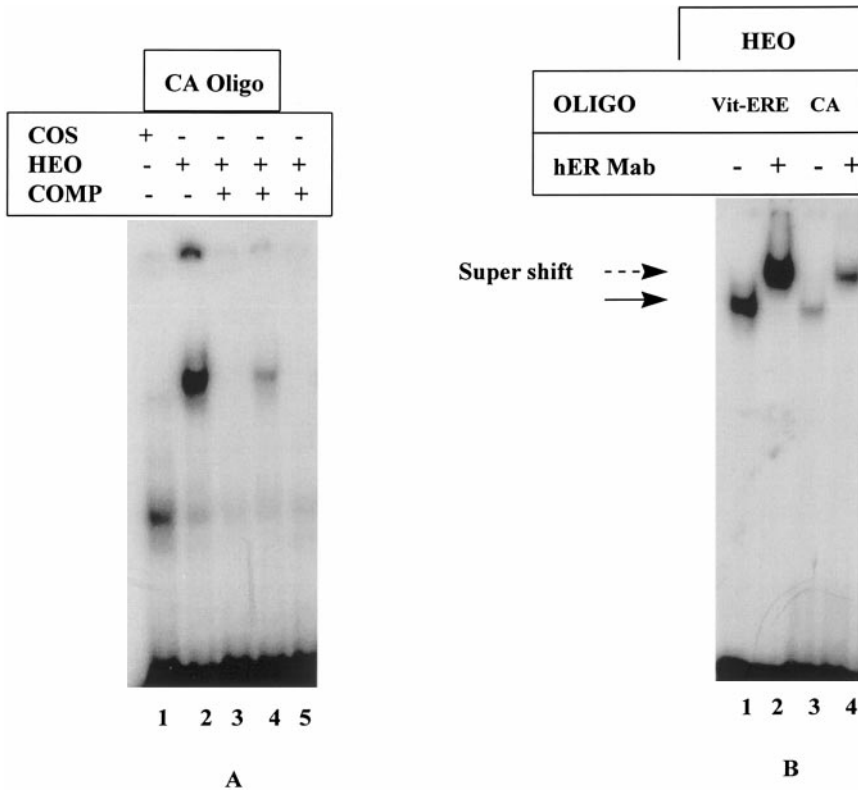


Figure 2. Nucleotide sequence located between the TATA box and transcriptional initiation site of the human angiotensinogen gene binds to the recombinant ER. A, Radiolabeled CA oligonucleotide was used in a gel shift assay in the presence of protein extract obtained either from COS cells alone or COS cells transfected with HEO and treated with estrogen (HEO). Lane 1, COS extract; lane 2, HEO extract; lane 3, 50-fold excess of cold CA oligo; lanes 4 and 5, 50- and 100-fold excess of vit-ERE. B, A gel shift assay was performed with CA and vit-ERE using HEO extract in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of monoclonal antibody against hER (1 μL). The shifted band is shown by a solid arrow, and the supershifted band is shown by a broken arrow. COMP indicates competitor oligonucleotide.

promoter activity was analyzed after 17-β-estradiol treatment. Results of this experiment, shown in Figure 3B, indicated that estrogen-induced promoter activity of pHAG1.2CAT and pHAG47CAT were drastically reduced when nucleoside A was changed to C at -20.

Reporter Constructs With Nucleoside C at -20 Have Increased Promoter Activity Compared With Reporter Constructs With Nucleoside A at -20
Because the nucleotide sequence of the human angiotensinogen gene located between TATA box and transcriptional

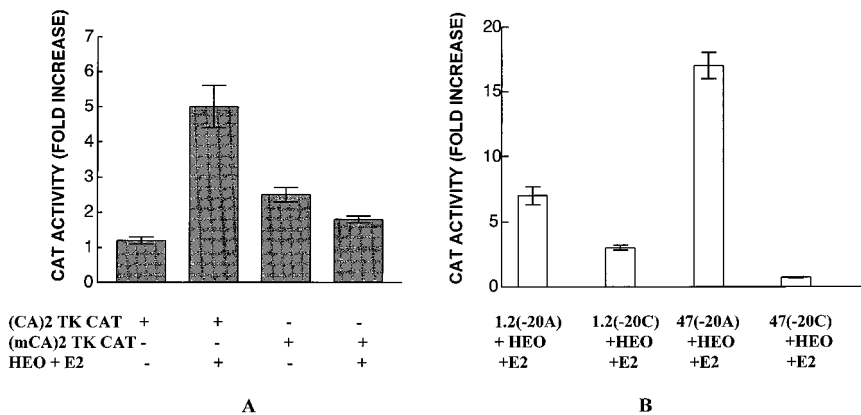


Figure 3. A, The promoter activity of reporter construct (CA)2TK-CAT is increased on cotransfection of HEO and estrogen treatment. Reporter construct (CA)2TK-CAT or (mCA)2TK-CAT was cotransfected with RSV-gal in the presence of HEO in COS-7 cells. After 24 hours of transfection, cells were treated with 17-β-estradiol (E2) for 24 hours, and CAT activity was analyzed after normalization with β-gal activity as described in text. Results represent an average of 3 experiments. Fold increase in the promoter activity was calculated by assuming the basal promoter activity of (CA)2TK-CAT as 1. B, Mutation A to C at -20 drastically reduces the estrogen-induced promoter activity of reporter constructs pHAG1.2CAT and pHAG47CAT. An equal amount of reporter constructs pHAG1.2CAT and pHAG47CAT containing nucleoside A or C at -20 was cotransfected with HEO. After transfection, cells were treated with 17-β-estradiol (E2) for 24 hours, and promoter activity was analyzed by CAT assay. Fold increase in the promoter

ERE

H: TATAAATAGGGCATCGTGACCCGGCCGGGGGA
***** ** * * * * *
R: TATAAATAAGGCTGCTTGGTTCA---CCAGGGGA
M: TATAAATAAGCCTGCTTGGCTCA---CCAGGGGA

activity in different experiments was calculated by assuming the basal promoter activity of pHAG47CAT(-20C) as 1. C, A comparison of nucleotide sequences of the human (H), rat (R), and mouse (M) angiotensinogen genes located between TATA box and transcriptional initiation site shows that ERE (line appears above the human sequence) is not present in rat and mouse genes.

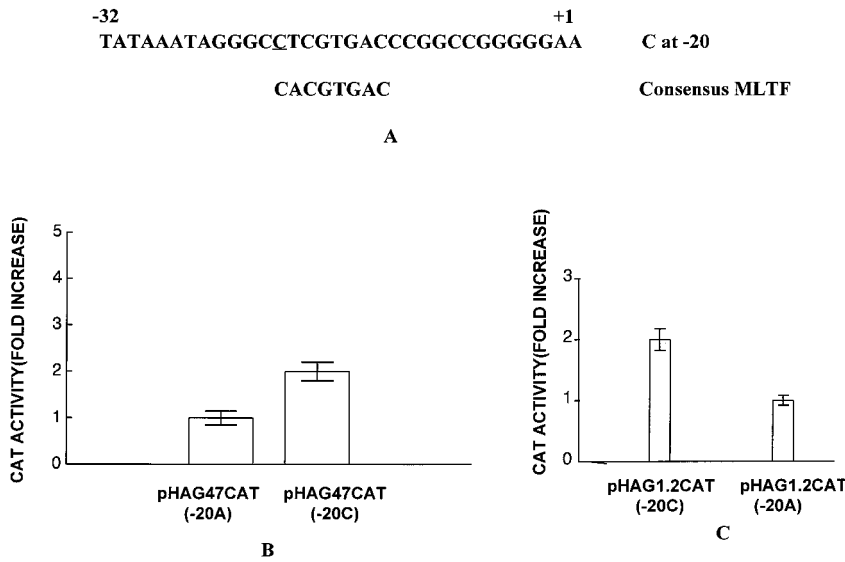


Figure 4. A, Sequence homology between MLTF binding site with nucleotide sequence located between TATA box and transcriptional initiation site containing nucleoside C at -20. B and C, Reporter constructs pHAG47CAT (-20C) and pHAG1.2CAT (-20C) have increased basal promoter activity as compared with the reporter construct pHAG47CAT (-20A) and pHAG1.2CAT (-20A) on transient transfection in HepG2 cells. An equal amount (20 μ g) of reporter constructs and RSV-gal (2 μ g) was transiently transfected in HepG2 cells under identical conditions. After 48 hours of transfection, an equal amount of cell extract (based on β -gal assay) was used for CAT assay. Quantitation of the CAT assay was based on an average of 3 experiments.

initiation site has homology with the MLTF binding site when nucleoside C is present at -20 (Figure 4A), and because MLTF plays an important role in basal expression of many liver specific genes, we next examined the effect of nucleoside C at -20 on the basal promoter activity of this gene. We therefore transiently transfected equal amounts of reporter constructs pHAG47CAT and pHAG1.2CAT containing either nucleoside A or C at -20 in HepG2 cells under identical conditions. After 48 hours of transfection, the promoter activity was analyzed by CAT assay after normalization with the β -gal assay. Results of this experiment (Figure 4B and 4C) show that reporter constructs containing C at -20 have 2- to 3-fold increased promoter activity as compared with reporter constructs containing A at -20.

Nucleotide Sequence Located Between the TATA Box and Transcriptional Initiation Site of Human Angiotensinogen Gene With Nucleoside C at -20 Binds to the MLTF

Because the reporter construct containing nucleoside C at -20 had increased promoter activity on transient transfection in HepG2 cells, we chose to examine whether CC oligo binds more strongly to the HepG2 nuclear extract as compared with the CA oligo. For this purpose, we performed a gel shift assay using equal amounts of radiolabeled CA and CC oligonucleotides and an equal amount of HepG2 nuclear extract. Results of this experiment (Figure 5A) indicated that, indeed, CC oligo formed a stronger complex with HepG2 nuclear extract as compared with the CA oligo (compare lanes 3 and 1). Moreover, the protein:DNA complex formed with these oligonucleotides was competed out by MLTF oligonucleotide (lanes 2 and 4).

We next performed a reverse gel shift assay using radiolabeled MLTF oligonucleotide and HepG2 nuclear extract in the presence of cold CC and CA oligonucleotides. Results of this experiment, shown in Figure 5B, indicate that consensus MLTF oligo formed a complex (lane 1) that was removed by selfcompetition (lane 2) and supershifted in the presence of MLTF antibody (lane 3). Moreover, this complex was com-

peted out efficiently by the cold CC oligo (lane 5) but not by the cold CA oligo (lane 4).

To further confirm that the transcription factor MLTF is involved in binding with the CC oligonucleotide, we performed a gel shift assay in the presence of specific and nonspecific antibodies. Results of this experiment, shown in Figure 5C, indicated that the complex obtained with CC oligonucleotide and HepG2 nuclear extract (lane 1) was removed in the presence of cold oligonucleotide containing consensus MLTF binding site (lane 2) and supershifted in the presence of MLTF antibody (lane 3) but not in the presence of nonspecific NF-1 antibody (lane 4). The electrophoretic mobilities of protein:DNA complexes shown in Figure 5B and 5C are different because the reaction mixture in Figure 5C was analyzed by 4% polyacrylamide gel and that in Figure 5B was analyzed by 8% polyacrylamide gel.

Cotransfection of CMV-MLTF Transactivates the Reporter Construct Containing 2 Copies of the CC Oligonucleotide Attached to a Heterologous Promoter

To examine the functional significance of mutation A to C at -20 on transcriptional regulation, we cotransfected reporter constructs (CA)₂TKCAT and (CC)₂TKCAT in HepG2 cells with an expression vector CMV-MLTF. Results of this experiment, shown in Figure 6A, indicated that cotransfection of CMV-MLTF increased the promoter activity of (CC)₂TKCAT by 3- to 4-fold but had no appreciable effect on the promoter activity of (CA)₂TKCAT. Cotransfection of CMV-MLTF also increased the promoter activity of pHAG47CAT containing nucleoside C at -20 (Figure 6B).

Discussion

In the present study, we have analyzed the role of C/A polymorphism on transcriptional regulation of the human angiotensinogen gene. We have shown the presence of a functional ERE located between the TATA box and transcriptional initiation site when nucleoside A is present at -20. Previously, thyroid¹⁴ and glucocorticoid hormone receptor

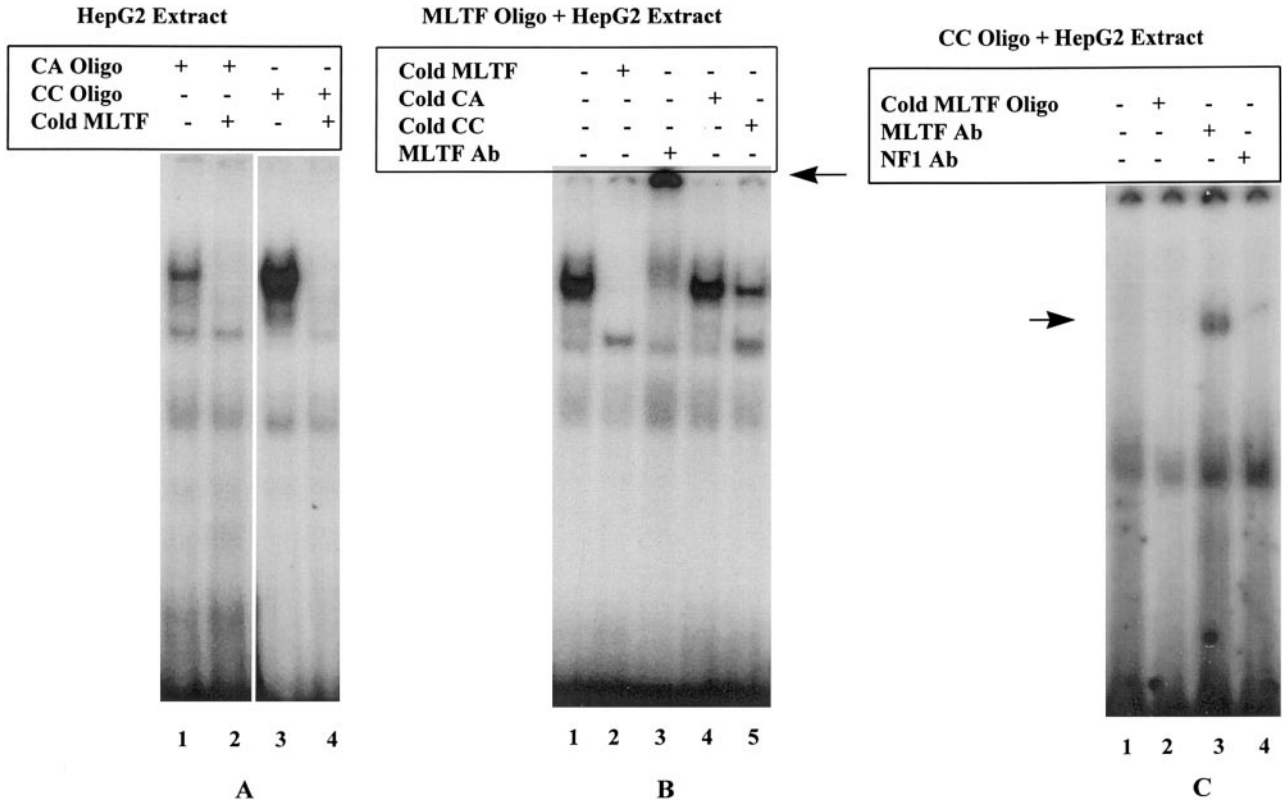


Figure 5. The CC oligonucleotide binds strongly to the transcription factor MLTF as compared with CA oligonucleotide. A, A gel shift assay was performed with equal amounts of radiolabeled CA and CC oligonucleotides using an equal amount of HepG2 nuclear extract under identical conditions in the presence and absence of MLTF oligonucleotide. B, A gel shift assay was performed with radiolabeled MLTF oligonucleotide and HepG2 extract in the presence and absence of cold CA and CC oligonucleotides and MLTF antibody (MLTF Ab). Supershifted band in lane 3 is shown by an arrow. All competition experiments were performed in the presence of a 100-fold excess of the cold oligonucleotide. C, The protein:DNA complex obtained by radiolabeled CC oligonucleotide and HepG2 extract is supershifted by MLTF antibody and not by NF-1 antibody. A gel shift assay was performed with radiolabeled CC oligonucleotide and HepG2 extract in the presence of MLTF-specific antibody (lane 3) and a nonspecific NF-1 antibody (lane 4). The supershifted band in lane 3 is shown by an arrow.

binding sites¹⁵ have been identified close to the transcriptional initiation site. However, to the best of our knowledge, human angiotensinogen gene is the first gene in which an ERE is located close to the TATA box. In the case of osteocalcin gene promoter,¹⁵ glucocorticoid responsive element overlaps with the TATA box and glucocorticoid treatment reduces its gene expression most probably by interfering with the bind-

ing of TATA box binding proteins. However, our data suggest that transfection of HEO followed by estrogen treatment actually increases the promoter activity of pHAG40CAT containing only 40 bp of the human angiotensinogen promoter.

The nucleotide sequences of human, rat, and mouse angiotensinogen genes, located between the TATA box

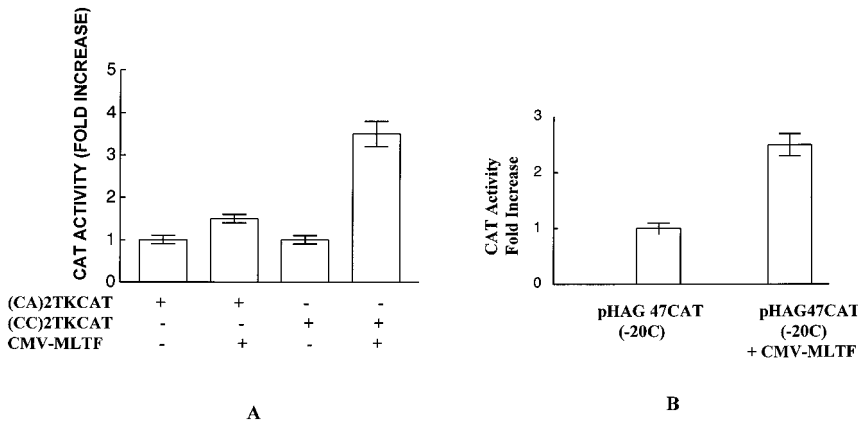


Figure 6. Transient cotransfection of CMV-MLTF increases promoter activity of the reporter constructs (CC)2TK-CAT and pHAG47CAT (-20C) in HepG2 cells. A, An equal amount of reporter constructs (CC)2TK-CAT and (CA)2TK-CAT was transiently cotransfected with CMV-MLTF in HepG2 cells, and CAT activity was analyzed after 48 hours of transfection after normalization with the β -gal activity. Results represent an average of 3 experiments. The promoter activity in different experiments was calculated by assuming the basal promoter activity of (CA)2TK-CAT as 1. B, The reporter construct pHAG47CAT (-20C) and RSV-gal were transfected in HepG2 cells in the presence and absence of CMV-MLTF. After 48

hours of transfection, the promoter activity was analyzed after normalization with the β -gal activity. Results show an average of 3 experiments. Fold increase in the promoter activity was calculated by assuming the basal promoter activity of pHAG47CAT as 1.

and transcriptional initiation site, show very little homology (Figure 3C). Unlike the human gene, rat and mouse genes do not contain a palindromic ERE in this region of the promoter, and this alteration may be responsible for differential species-specific regulation of this gene in rodents and primates. Transient transfection of expression vectors containing 5'-deletion mutants of the rat angiotensinogen gene promoter has suggested a single half palindromic sequence (GGGTCC) located between 87 and 91 bp upstream from the transcriptional initiation site as a potential ERE.¹⁶ The human angiotensinogen gene does not contain a half palindromic sequence at this site, although a sequence AGGTCC is located around -160. We have confirmed the results of previous studies that the promoter activity of a reporter construct containing 1.6-kb 5'-flanking region of the rat angiotensinogen gene was increased only by 1.5-fold on estrogen treatment.¹⁶ On the other hand, promoter activity of pHAG1.2CAT (-20A) was increased by 5- to 6-fold on estrogen treatment. We suggest that the presence of a palindromic ERE located close to the TATA box in the human angiotensinogen gene containing nucleoside A at -20 may be responsible for its increased transactivation by estrogen as compared with the rat gene.

In this article, we also show that when nucleoside C is present at -20, transcription factor MLTF binds preferentially to this site in place of the estrogen receptor. Reporter constructs containing either ~1.2 kb or only 47 bp of the human angiotensinogen gene promoter with C at -20 have increased basal transcriptional activity on transient transfection in human hepatoma cells. In addition, we show that transient cotransfection of an expression vector containing MLTF coding sequence increases the expression of (CC)2TK CAT and pHAG47CAT(-20C) on transient transfection. The presence of a role for nucleoside C at -20 in transcriptional activity of the human angiotensinogen gene is also substantiated by a recent report by Yanai et al,¹⁷ who have shown that the nucleotide sequence AGCE1 (TAGGGCCTCGTGACCCAGGGG) located between -1 and -25 of the human angiotensinogen gene plays an important role in the expression of this gene. They have shown that (1) mutation of CGT (underlined in the sequence) to ATG alters the binding of HepG2 nuclear extract to this region of the promoter, (2) a reporter construct containing 106 bp of the 5'-flanking region with this mutation had only 10% of the transcriptional activity as compared with the wild-type sequence on transient transfection in HepG2 cells, and (3) transcriptional activity of a reporter construct containing 1.2 kb of the promoter was reduced about 50% by this mutation. Because we now show that MLTF binds to this region of the promoter, and because CGT is part of the putative MLTF binding site CTCGTGAC, its mutation to ATG will disrupt the binding of MLTF, which will result in reduced promoter activity.

Previous studies have shown that the angiotensinogen gene locus is involved in human essential hypertension, and hypertensive patients with an M235T mutation have increased plasma angiotensinogen levels.⁸ However, because this mutation is in the coding region, it is difficult to explain

increased plasma angiotensinogen levels by this mutation. Recently, Inoue et al¹⁸ have shown that G/A polymorphism located at -6 in the promoter of human angiotensinogen gene is in complete association with M235T and that reporter constructs containing nucleoside A at -6 have increased promoter activity. This observation may explain increased plasma angiotensinogen levels in patients with threonine at 235. However, the transcription factor that binds to the -6 region of the promoter has not been identified, and the mechanism involved in the transcriptional regulation by polymorphism at -6 is not known. On the other hand, Sato et al¹⁹ have shown that M235T and A-20C show significant linkage disequilibrium, suggesting an association between nucleoside C at -20 with amino acid threonine at 235. Our data that MLTF binds strongly to the promoter when nucleoside C is present at -20 and that reporter constructs containing human angiotensinogen gene with C at -20 have increased transcriptional activity in HepG2 cells may also explain increased plasma angiotensinogen levels in patients with threonine at 235 (if -20C is associated with 235T as suggested by Sato et al). The interactive relationship between polymorphisms at -20 and -6 on transcriptional regulation of the human angiotensinogen gene remains to be examined.

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