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Research Article

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Molecular Mechanism of Transcriptional Activation of Angiotensinogen Gene by Proximal Promoter

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Abstract

Angiotensinogen is shown to be produced by the liver and the hepatoma cell line HepG2. As a first step for understanding the molecular relationship between the transcriptional regulation of the angiotensinogen gene and the pathogenesis of hypertension, we have analyzed the basal promoter of the angiotensinogen gene. Chloramphenicol acetyltransferase (CAT) assays with 5'-deleted constructs showed that the proximal promoter region from -96 to +22 of the transcriptional start site was enough to express HepG2-specific CAT activity. Electrophoretic mobility shift assay and DNase I footprinting demonstrated that the liver- and HepG2-specific nuclear factor (angiotensinogen gene-activating factor [AGF2]) and ubiquitous nuclear factor (AGF3) bound to the proximal promoter element from -96 to -52 (angiotensinogen gene-activating element [AGE2]) and to the core promoter element from -6 to +22 (AGE3), respectively. The site-directed disruption of either AGE2 or AGE3 decreased CAT expression, and the sequential titration of AGF3 binding by in vivo competition remarkably suppressed HepG2-specific CAT activity. Finally, the heterologous thymidine kinase promoter assay showed that AGE2 and AGE3 synergistically conferred HepG2-specific CAT expression. These results suggest that the synergistic interplay between AGF2 and AGF3 is important for the angiotensinogen promoter activation. (*J. Clin. Invest.* 1994; 93:1370-1379.) Key words: angiotensinogen • transcription • proximal promoter • nuclear factor • hepatocyte

Introduction

Hypertension is a complex pathological state with a predisposed genetic background involving the autonomic nervous system and the various hormonal vasoactive peptides. Although a large number of important regulatory genes on the circulation systems had been identified, little information was available on their roles in the development of hypertension. By contrast, recent studies have demonstrated that the renin-angiotensin system (RAS)¹ plays an important role in the regula-

tion of blood pressure, fluid volume balance, and other biological responses through generation of angiotensin II, which has a variety of actions such as vasoconstrictor activity and stimulation of the production and release of aldosterone (1). The participation of RAS in several disease states including hypertension has now been proposed (2-15).

Angiotensinogen is the unique substrate of renin in RAS, and the primary source of plasma angiotensinogen is the liver (16, 17). We previously demonstrated that the 5'-flanking region of the human angiotensinogen gene was important for tissue- and cell type-specific expression of the gene in vitro (18) and in vivo (19). We also disclosed that the promoter region of the mouse angiotensinogen gene was able to direct transcription in HepG2 cells (20), and other investigators showed that the 750-bp promoter element from the immediate 5'-flanking region was capable of directing most, but not all, tissue-specific and hormonal regulation of the angiotensinogen minigene in transgenic mice (21). Moreover, we have recently indicated that the proximal promoter region is involved in the adipogenic differentiation-induced expression of the angiotensinogen gene (22). All of these studies indicated that the 5'-flanking sequences played a major role in the regulation of the angiotensinogen gene expression.

The synthesis and release of angiotensinogen into the circulation is regulated in response to a number of different stimuli such as steroid hormones (23-27), cytokines (28), and angiotensin II (29, 30) in the liver. For example, recent studies on the regulation of the gene in the liver during the acute phase response led to the identification of a hormonally induced enhancer unit consisting of two glucocorticoid responsive elements and a cytokine responsive element in the far upstream 5'-flanking region (31, 32), although the exact role of angiotensinogen in inflammation is uncertain (33).

On the other hand, recent transgenic studies using the rat and human angiotensinogen genes (34-37) and genetic linkage analyses of the human angiotensinogen gene with high blood pressure (38, 39) propose that the basal transcriptional mechanism of the angiotensinogen gene is involved in the pathogenesis of hypertension. At present, little information is available concerning the basal transcriptional machinery of the angiotensinogen gene, and the molecular relationship between the regulation of transcription of the gene and the pathogenesis of hypertension is unclear.

In this study, as a first step to approach this question and identify regulatory factors that play a role in the control of angiotensinogen gene transcription in the liver, we performed transient transfection assays using the mouse angiotensinogen promoter linked to chloramphenicol acetyltransferase (CAT) gene in hepatic HepG2 and other extrahepatic cell lines. The

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1. Abbreviations used in this paper: AGE, angiotensinogen gene-activating element; AGF, angiotensinogen gene-activating factor; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility

shift assay; RAS, renin-angiotensin system; TBP, TATA box-binding protein; TK, thymidine kinase.

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hepatoma cell line HepG2 cells have retained several properties of hepatocytes (40), express angiotensinogen mRNA (16), and are considered to be a suitable model system to investigate the molecular mechanism of the angiotensinogen gene expression. We first demonstrated that the proximal promoter region from -96 to +22 was able to confer HepG2-specific transcriptional activity and identified two *cis*-acting elements that contributed to this specificity, angiotensinogen gene-activating element 2 (AGE2) (-96 to -52) and AGE3 (-6 to +22). Electrophoretic mobility shift assay (EMSA) identified two nuclear factors, angiotensinogen gene-activating factor 2 (AGF2) and AGF3. AGF2 bound to AGE2 in HepG2-specific manner, whereas AGF3 interacted with AGE3 in all cell lines examined. DNase I footprint analysis indicated that the palindromic sequences were involved in AGF2 binding and that the exon 1 region was critical for AGF3 binding. Furthermore, substitution mutation analysis and heterologous promoter assay demonstrated that the cooperative interaction between these proximal and core promoter elements was essential for transcriptional activation of the angiotensinogen gene.

Methods

Cell culture. Hepatoma HepG2 cells, cervical carcinoma HeLa cells, and glioblastoma T98G cells were maintained in MEM containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Embryonic fibroblast NIH3T3 cells were cultured in DME supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. These cell lines were kept in 5% CO₂/95% air at 37°C and were passed twice in a week after trypsin-EDTA detachment.

Plasmid constructions. Genomic DNA cloning was performed from C57BL/6 mouse liver as described previously (20). The angiotensinogen promoter-CAT hybrid genes were constructed as follows: Ag501, Ag399, Ag138, Ag96, Ag70, Ag51, and Ag6 contain 523-bp (-501 to +22) HinPI, 421-bp (-399 to +22) PvuII/HinPI, 160-bp (-138 to +22) HaeIII/HinPI, 118-bp (-96 to +22) Sau3AI/HinPI, 92-bp (-70 to +22) RsaI/HinPI, 73-bp (-51 to +22) Sau3AI/HinPI, and 28-bp (-6 to +22) MvaI/HinPI fragments, respectively, and these DNA fragments were subcloned into the BglII/HindIII sites of pUCSV0CAT (41).

A competitive plasmid (pC-AGE3) contained six tandem copies of the 28-bp (-6 to +22) MvaI/HinPI fragment inserted into the SmaI site of pUC19.

Site-directed mutagenesis. The Ag138 hybrid gene was used as a template to construct mutations in AGE2 or AGE3 by oligonucleotide-directed mutagenesis (42). The sequences of the oligonucleotide used to create AGEΔ2 were 5'-CCCCAGCTGAGGTTTAGAGTA-GCCCA-3', while those for AGEΔ3 were 5'-CAGGGGAT-GTTGATGCGAGCCTAGGTTG-3'. Once the site-directed mutations (underlined) were obtained and confirmed by sequencing, the altered 160-bp (-138 to +22) fragments were subcloned into the BglII/HindIII sites of pUCSV0CAT.

DNA transfections and CAT assay. 1 d before transfection, 7×10^5 cells were plated on 60-mm dishes. The cell medium was changed 3 h before transfection. A calcium phosphate coprecipitate containing 4 µg of DNA was added to the cultured cell lines. Cells were incubated for 12 h, washed, and incubated for 36 h in fresh medium. Cells were collected, and cell extracts were prepared by freezing and thawing as described (18). The extracts were heated at 60°C for 10 min, and the precipitate was removed by centrifugation. The protein concentration was determined using BSA as a standard (18). The reaction mixture contained 140 mM Tris-HCl, pH 7.8, 0.2 µCi of [¹⁴C]-chloramphenicol, 4 mM acetylcoenzyme A (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and 40 µg of cell extract in a final volume

of 150 µl. The mixture was incubated at 37°C for 2 h and then extracted with cold ethyl acetate. The solution was dried, and the pellet redissolved in 15 µl of ethyl acetate. The labeled chloramphenicol and acetylated derivatives were separated by ascending TLC using chloroform/methanol (95:5, vol/vol). The chromatograms were subjected to autoradiography at -70°C. CAT activity was quantitated by counting scraped regions of the chromatograms in a liquid scintillation spectrometer.

Preparation of nuclear extracts. Nuclear extracts from HepG2, HeLa, T98G, and NIH3T3 cells were prepared using a modification of the protocol of Dignam et al. (43, 44). The final protein concentration was 5–7 mg/ml. Nuclear extracts from mouse liver were prepared essentially according to the procedure of Gorski et al. (45). The final protein concentration was 2–3 mg/ml.

EMSA. Double-stranded angiotensinogen promoter sequences (-96 to -52, -51 to +22, or -6 to +22) were phosphorylated at both ends using T4 polynucleotide kinase and [γ -³²P]ATP. Nuclear extracts were preincubated for 15 min on ice in a 20-µl reaction mixture containing 12 mM Hepes, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 12% glycerol, and 500 ng of double-stranded poly(dI-dC) in the presence or absence of 50- or 100-fold excess of a specific double-stranded competitor DNA. 0.1–0.5 ng (~15,000 cpm) of a radiolabeled DNA probe was added, and the incubation continued for 30 min at room temperature. The incubation mixture was loaded on a 5% polyacrylamide gel in 1 × TBE (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA), and electrophoresed at 140 V for 3 h followed by autoradiography. Oligonucleotides containing the consensus binding sequences for AP-1, NF-1, and Sp-1 were obtained from Stratagene (Gelshift[®] Kit; La Jolla, CA). Oligonucleotides with consensus binding motifs for C/EBP (46), HNF-1 (47), and CRE (48) were synthesized on an oligonucleotide synthesizer (Cyclone[®] Plus; MilliGen/Bioscience, Burlington, MA), and purified on OPC columns (Applied Biosystems, Inc., Foster City, CA) as described by the manufacturer.

DNase I footprint analysis. Fragments of the angiotensinogen promoter spanning the regions from -501 to +22 and -399 to +22 relative to the transcriptional start site were end labeled with T4 polynucleotide kinase and [γ -³²P]ATP, followed by digestion with PvuII (-399) and HaeIII (-138), respectively, to generate probes suitable for DNase I footprinting. After gel purification, the probe (~20,000 cpm) was incubated with nuclear extracts in a 50-µl reaction volume containing 12 mM Hepes, pH 7.9, 60 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, and 1 µg of double-stranded poly(dI-dC). The mixture was incubated for 30 min on ice, followed by 1 min at room temperature by the addition of 50 µl of a solution containing 12 mM Hepes, pH 7.9, 5 mM CaCl₂, 5 mM MgCl₂, and 5–250 ng of DNase I. The reaction was stopped by the addition of 100 µl of 12 mM Hepes, pH 7.9, 0.6 M sodium acetate, pH 7, 0.5% SDS, 0.1 mM EDTA, and 20 µg of transfer RNA (tRNA). The DNA was extracted with phenol-chloroform (1:1, vol/vol), and was precipitated with 2.5 vol of ethanol before electrophoresis on a 6% polyacrylamide/8 M urea sequencing gel. To define the position of the protected region, G + A sequence ladders were prepared (49).

Results

Angiotensinogen proximal promoter directs transcription in HepG2-specific manner. We previously demonstrated that HepG2 cells derived from differentiated hepatoma expressed angiotensinogen mRNA, and no detectable levels of angiotensinogen mRNA were found in extrahepatic HeLa, T98G, and NIH3T3 cells by Northern blot analysis (18, 50). To define DNA sequences responsible for specific transcription in the angiotensinogen promoter in HepG2 cells, a 523-bp HinPI fragment of the gene containing the 501-bp 5'-flanking region, the transcriptional start site, and the 22-bp exon 1 at positions -501 to +22 was inserted to the BglII/HindIII sites of

pUCSV0CAT in the sense orientation with respect to the CAT gene (Ag501), and a series of 5'-deletion mutants extending from -501 to -6 were constructed and tested for their ability to promote transcription (Fig. 1). These chimeric constructs were transiently introduced into HepG2, HeLa, T98G, and NIH3T3 cells. The promoterless plasmid pUCSV0CAT (SV0) was used as a background reference, and pUCSV3CAT (SV3) was used as a positive control including the SV40 enhancer-promoter region (41). All results were corrected for variations in transfection efficiency by reference to pUCSV3CAT.

In HepG2 cells, Ag501 was able to direct CAT activity to a significant level, and deletion of the promoter to position -96 (Ag96) resulted in a slight increase (1.8 ± 0.4 -fold) in CAT expression relative to the undeleted construct, Ag501. Deletion to position -70 (Ag70) and -51 (Ag51), however, reduced the promoter-CAT hybrid gene expression to 28 ± 4.2 and $8.5 \pm 1.8\%$ of Ag96, respectively. Further deletion up to position -6 (Ag6) decreased CAT activity almost to the background level demonstrated in the negative control, pUCSV0CAT. These results indicate that the proximal promoter region from -96 to +22 (Ag96) is sufficient to confer efficient CAT expression in HepG2 cells, and suggest that the proximal element from -96 to -52 (AGE2) is important for high level CAT activity in these cells and that the basal promoter sequences from -51 to +22 can still mediate minimal promoter activity in HepG2 cells, although the level of CAT activity is low.

None of the deletion mutants, on the other hand, were expressed upon transfection into HeLa, T98G, and NIH3T3 cells. This complete inactivity in these nonhepatoma cell lines was not because of a lower transfection efficiency, since expression directed by the positive control pUCSV3CAT was almost at the same level in these cells as in HepG2 cells.

AGE2, the proximal element (-96 to -52), binding to HepG2-specific factor is important for efficient transcriptional activity of angiotensinogen promoter. The 5'-deletion analysis suggested that the sequences between -96 and -52 (AGE2) were important for the high level CAT expression in HepG2 cells. To identify nuclear factors binding to this region, we performed EMSA using the end-labeled angiotensinogen pro-

motor fragment from -96 to -52 (AGE2) as the probe. Nuclear extracts were prepared from HepG2, HeLa, T98G, and NIH3T3 cells, and from mouse liver. Incubation of AGE2 with either HepG2 or liver nuclear extract produced a single retarded complex (Fig. 2 A, *arrowhead*), which represented a sequence-specific interaction between AGE2 and nuclear factors in the extracts since these were competed out with 100-fold molar excess of the unlabeled AGE2. DNA fragment from -96 to +22, including both AGE2 (-96 to -52) and AGE3 (-6 to +22), also competed for this binding with the liver nuclear extract, although sequences from -51 to +22, which contained AGE3 but not AGE2, failed to compete (Fig. 2 A).

In addition, a high molecular weight band in the liver extract lanes was observed at the top of the gel. This formation could be partially competed with 100-fold molar excess of the unlabeled AGE2. However, this binding could not be competed out completely by 500-fold molar excess of the unlabeled AGE2 (data not shown), and a promoter DNA fragment from -96 to +22, which contained the AGE2 and its downstream sequences through the exon 1 region, did not compete for this high molecular weight band (Fig. 2 A). Thus, we considered that this band formation was due to nonspecific binding. On the other hand, nuclear extracts from HeLa, T98G, and NIH3T3 cells were not able to form DNA-protein complex with this element (Fig. 2 A). These results indicate that the AGE2-binding activity is HepG2 specific and is not detectable in other extrahepatic cell lines. Furthermore, double-stranded oligonucleotides containing the consensus binding sequences for C/EBP, HNF-1, and Sp-1 failed to compete with this AGE2-binding activity (Fig. 2 A), and oligonucleotides with binding motif for neither NF-1, AP-1, nor CRE competed with the binding (data not shown).

DNase I footprint analysis was performed to confirm the recognition site of this AGE2-binding factor (AGF2). A labeled DNA fragment from -399 to +22 was incubated with liver nuclear extracts followed by DNase I digestion. Fig. 2 B demonstrated that a sequence from -79 to -53 in AGE2 is protected from digestion by DNase I (*hatched box*).

From the results of deletion analysis and EMSA, AGE2

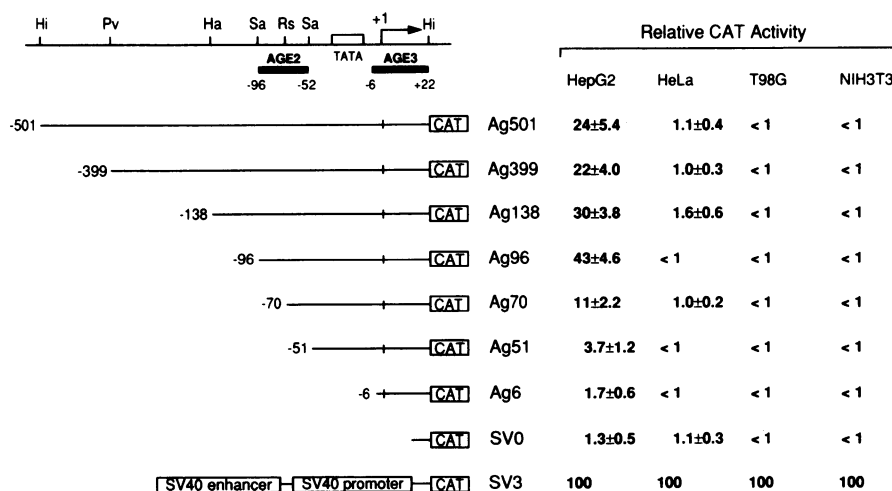


Figure 1. Schematic representation of the mouse angiotensinogen promoter, construction of the angiotensinogen promoter-CAT hybrid genes, and basal expression of these hybrid genes in transfected HepG2, HeLa, T98G, and NIH3T3 cells. Restriction endonuclease cleavage sites are indicated as follows: *Hi*, *HinPI*; *Ha*, *HaeIII*; *Sa*, *Sau3AI*; and *Rs*, *RsaI*. The TATA box is located at nucleotide positions -30 to -25, and the transcriptional start site is indicated by +1. AGE2 (-96 to -52) and AGE3 (-6 to +22) are shown by the solid boxes. Transfection was performed using 4 μ g of plasmid DNA as a CaPO_4 coprecipitate. Cells were harvested 48 h after transfection, and aliquots of cell extract containing equal amounts of total protein (40 μ g) were used in CAT assay. pUCSV3-

CAT (SV3) and pUCSV0CAT (SV0) were used as positive and negative controls, respectively. The CAT activity of each construct was calculated by comparing the percentage conversion of [^{14}C]chloramphenicol with its acetylated forms relative to the activity achieved with pUCSV3CAT in each cell line. These relative CAT activities are averages \pm SEM of six independent experiments.

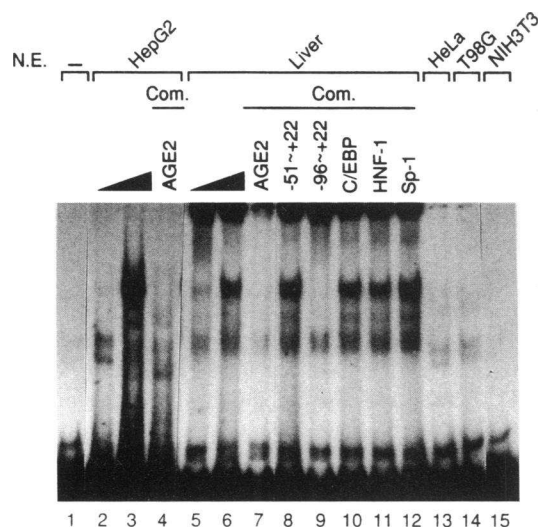
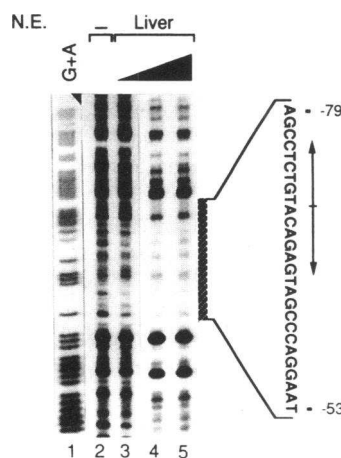
A**B**

Figure 2. Identification of a cell type-specific nuclear factor (AGF2) that binds to AGE2 (-96 to -52). (A) Electrophoretic mobility shift and competition analyses of complexes formed by nuclear factors with AGE2 (-96 to -52). AGE2 was gel purified and end labeled with T4 polynucleotide kinase using [γ -³²P]ATP. Nuclear extracts from HepG2 (lane 2, 5 μ g; lanes 3 and 4, 10 μ g), mouse liver (lane 5, 5 μ g; lanes 6–12, 10 μ g), HeLa (lane 13, 10 μ g), T98G (lane 14, 10 μ g), and NIH3T3 (lane 15, 10 μ g) were incubated with 0.4 ng of the probe. In electrophoretic shift competition assay, 100-fold molar excess of unlabeled DNA fragments (lane 7, AGE2; lane 8, -51 to +22; lane 9, -96 to +22) or oligonucleotides (lane 10, C/EBP; lane 11, HNF-1; lane 12, Sp-1) were added to the

reaction mixture. Arrow points to specific retarded complexes. (B) Lane 1, guanosine (G) and adenosine (A) sequencing-reaction molecular weight markers. DNase I footprint analysis of AGE2 with increasing amounts of liver nuclear extracts. A 421-bp PvuII-HinPI fragment containing angiotensinogen promoter sequences from positions -399 to +22 was labeled on the noncoding strand at the HinPI site. The DNA probe (2 × 10⁴ cpm) was incubated with increasing amounts (lane 3, 10 μ g; lane 4, 20 μ g; lane 5, 40 μ g) of mouse liver nuclear extracts and then was subjected to DNase I digestion. Lane 2 contains no nuclear extract. The protected region is denoted by the hatched box to the right with its nucleotide sequences. The 12-bp palindrome is indicated by arrows.

seems to exert a critical influence on the promoter activity of the angiotensinogen gene. Thus, to evaluate the functional role of AGE2 in the promoter activity, 8-bp sequence substitution mutations were placed in AGE2 to interrupt a perfect 12-bp palindrome at positions -76 to -65 (AGE Δ 2, Fig. 3A). The resulting AGE Δ 2 was used for electrophoretic shift competition assay and transient transfection assay to assess the functional significance of AGF2 in the context of angiotensinogen

promoter. As shown in Fig. 3A, nonlabeled AGE2 competed effectively for the complex formation, whereas AGE Δ 2 containing the 8-bp mutations in the palindrome did not compete at all. The angiotensinogen promoter-CAT chimeric gene with this mutated AGE Δ 2 (Ag138- Δ 2) was then transiently transfected into HepG2 cells and assayed for induction of CAT activity (Fig. 3B). The mutation of the palindromic sequences in AGE2 resulted in a substantial reduction in the level of CAT

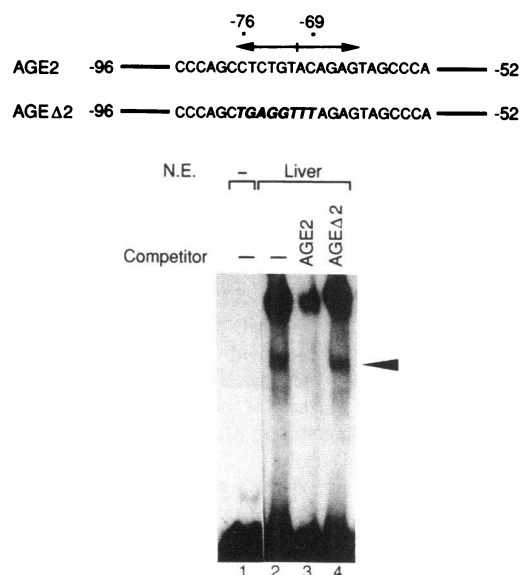
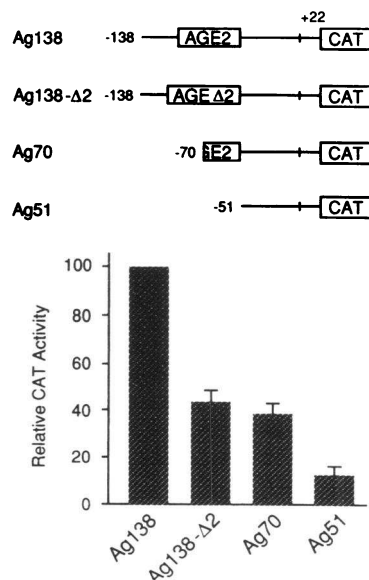
A**B**

Figure 3. Effect of AGE2 mutation on nuclear factor (AGF2) binding and on the transcriptional activity. (A) Electrophoretic shift competition assay using AGE2 and mutated AGE2 (AGE Δ 2) as cold competitors. Site-directed mutations of the palindromic sequences in AGE2 that interfered with nuclear factors binding were indicated by bold and italic letters. Binding reaction was performed with 10 μ g of nuclear extracts from mouse liver (lanes 2–4). Lane 1 contains no nuclear extract. Lane 2 includes no competitor. Lanes 3 and 4 show the effect of native AGE2 and mutated AGE2 (AGE Δ 2) as cold competitors (100-fold molar excess) on the DNA–nuclear factor interaction. Protein–DNA complexes are indicated by the arrowhead. (B) CAT assay with the construct that contain AGE Δ 2. The angiotensinogen promoter–CAT chimeric construct with mutated AGE2 (Ag138- Δ 2) was transiently transfected into

HepG2 cells as well as the parental Ag138. Relative CAT activities were determined as described in the legend of Figure 1 and were demonstrated by striped bars. The values represent the average \pm SEM of four independent experiments.

expression ($43 \pm 4.6\%$) relative to the wild-type construct, Ag138. These results indicate that AGE2, especially the palindromic sequence from -76 to -65 , is important for high level expression in HepG2 cells and that the activator property of AGE2 is dependent on nuclear factor (AGF2)-binding to the element in the angiotensinogen promoter context.

AGE3, the core element (-6 to $+22$), binding to ubiquitous factor is necessary for the transcriptional activity of angiotensinogen promoter. To identify nuclear factors binding to the minimal promoter sequences (-51 to $+22$), we carried out EMSA with this region as the probe and used fragments AGE2 (-96 to -52), A (-51 to $+4$), and AGE3 (-6 to $+22$), spanning different regions of the proximal promoter, for competition experiments (Fig. 4). A specific shifted complex was observed with nuclear extracts from liver, HepG2, and extrahepatic cell lines examined (Fig. 4A). In the extracts from mouse liver, AGE3 competed for the formation of the complex, although fragment A containing the TATA box consensus sequence (TATAAA) and AGE2 was unable to compete with this binding. Similar results were obtained by using the 28-bp double-stranded DNA probe spanning -6 to $+22$ bp (AGE3) of the angiotensinogen promoter. A single specific band, which was competed by the unlabeled AGE3, was observed with nuclear extracts from mouse liver, HepG2, and other extrahepatic cell lines (Fig. 5A). These results demonstrated that a ubiquitously expressed nuclear factor (AGF3) binds to the core promoter region from -6 to $+22$ (AGE3).

DNase I footprint analysis using nuclear extracts from mouse liver showed a region of DNase I protection from -1 to $+14$ in AGE3 (Fig. 5B, *hatched box*). The basal promoter elements around the TATA box (position -33 to -19) and its downstream region (position -16 to -3) were also protected from the digestion with DNase I (Fig. 5B, *open boxes*). To investigate the possible relationship between AGF3 and general transcription factors, several core promoter fragments (thymidine kinase [TK] promoter, -109 to $+19$; SV40 promoter,

-43 to $+64$; and mouse renin promoter, -47 to $+16$ [51, 52]) containing the TATA elements and the transcriptional start sites were used for electrophoretic shift competition assay (Fig. 6). All of the promoter fragments tested failed to compete effectively for binding to AGE3. Furthermore, oligonucleotide with consensus binding motif for either C/EBP, HNF-1, Sp-1, NF-1, AP-1, or CRE could not compete with this binding (data not shown).

To evaluate the functional significance of AGE3 in HepG2-specific angiotensinogen promoter activity, we first assayed the effect of a mutation that disrupted nuclear factors binding to AGE3. As shown in Fig. 7A, the DNA-protein complex formed by the AGE3-binding activity could be competed out by unlabeled AGE3. However, the DNA fragment that contains substitution mutations in AGE3 of the exon 1 region (Fig. 7A, AGE Δ 3) was not able to compete for this binding. In transiently transfected HepG2 cells (Fig. 7B), the angiotensinogen promoter-CAT hybrid gene with this mutated AGE3 (Ag138- Δ 3) gave the greatly reduced promoter activity ($14 \pm 3.2\%$ of the level with the native Ag138 construct). We next performed *in vivo* competition experiments to confirm the functional role of AGE3 as a positive regulator. Six tandem copies of AGE3 were inserted into the pUC19 plasmid. This plasmid, named pC-AGE3, was cotransfected with Ag501 into HepG2 cells, and the CAT activities were analyzed (Fig. 8). The amount of transfected DNA was normalized by pUC19 to $10 \mu\text{g}$. The results obtained in this experiment showed that the CAT activity decreases with increasing amounts of pC-AGE3. Therefore, these functional assays suggest that the core element overlapping the exon 1 region from $+3$ to $+14$ in AGE3 is important as the recognition sequences for AGF3, and that AGE3 could, at least in part, support HepG2-specific promoter activity of the angiotensinogen gene.

Combination of AGE2 and AGE3 confers synergistic activation on a heterologous promoter. To further address how AGE2 and AGE3 alone or together function in conferring HepG2

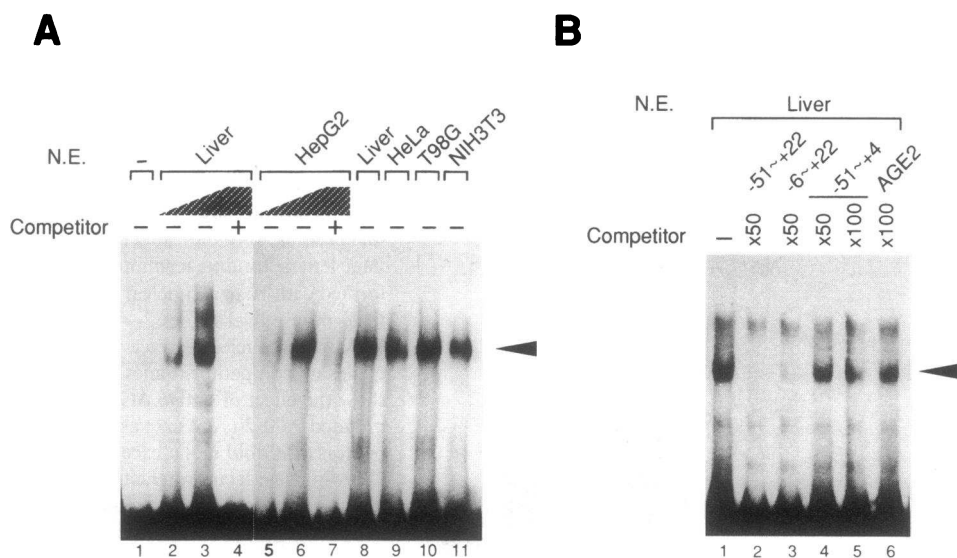


Figure 4. Analysis of nuclear factor binding to the basal promoter sequences from -51 to $+22$ of the angiotensinogen gene. (A) EMSA using ^{32}P -labeled angiotensinogen promoter sequences from -51 to $+22$ revealed ubiquitous DNA-binding activity to this basal promoter region. Nuclear extracts from mouse liver (lane 2, $5 \mu\text{g}$; lanes 3, 4, and 8, $10 \mu\text{g}$), HepG2 (lane 5, $5 \mu\text{g}$; lanes 6 and 7, $10 \mu\text{g}$), HeLa (lane 9, $10 \mu\text{g}$), T98G (lane 10, $10 \mu\text{g}$), and NIH3T3 cells (lane 11, $10 \mu\text{g}$) were incubated with 0.3 ng of the probe. In competition assay, 100-fold molar excess of the unlabeled DNA fragment (-51 to $+22$) was added to the reaction mixture. Lane 1 contains no nuclear extract. Arrow points to specific shifted complexes. (B) Electrophoretic shift competition

assay with DNA fragments spanning different basal promoter regions. Binding reactions were performed with mouse liver nuclear extracts as in A. As competitors, 50- or 100-fold molar excess of unlabeled DNA fragments (lane 2, -51 to $+22$; lane 3, -6 to $+22$ [fragment B]; lanes 4 and 5, -51 to $+4$ [fragment A]; lane 6, -96 to -52 [AGE2]), as indicated for each lane, was added to the reaction mixture. Lane 1 contains no competitor. Arrow points to specific retarded complexes.

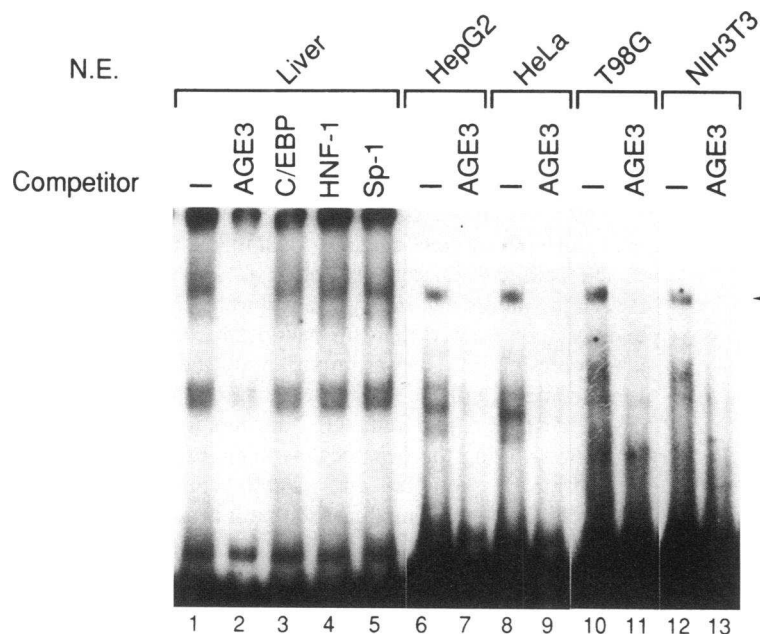
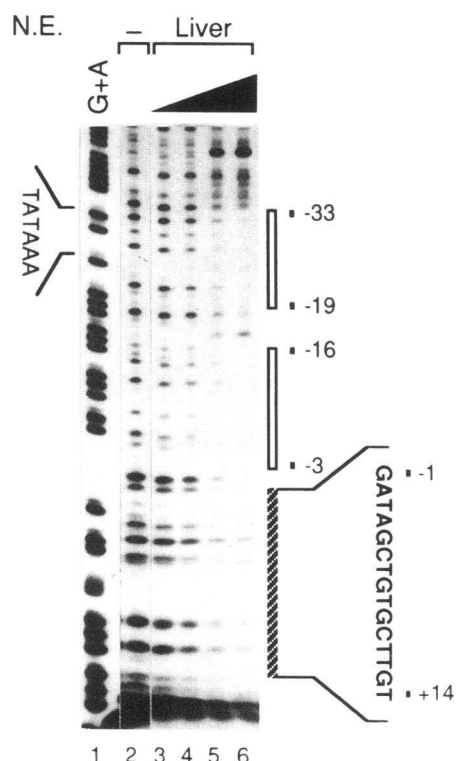
A**B**

Figure 5. Identification of a ubiquitous nuclear factor (AGF3) that interacts with AGE3 (−6 to +22). (A) Electrophoretic mobility shift and competition analyses of complexes formed by factors in liver and cultured cell nuclear extracts with AGE3. Nuclear extracts from mouse liver (lanes 1–5, 10 μ g), HepG2 (lanes 6 and 7, 10 μ g), HeLa (lanes 8 and 9, 10 μ g), T98G (lanes 10 and 11, 10 μ g), and NIH3T3 cells (lanes 12 and 13, 10 μ g) were incubated with 0.2 ng of 32 P-labeled AGE3. In competition assay, 100-fold molar excess of unlabeled AGE3 or double-stranded oligonucleotides (C/EBP, HNF-1, and Sp-1) was added to the reaction mixture. Arrow points to specific DNA–nuclear factor complexes. (B) DNase I footprint analysis of AGE3 with increasing amounts of liver nuclear extracts. A 160-bp HaeIII–HinfI fragment containing angiotensinogen promoter sequences from positions −138 to +22 was 32 P-labeled on the noncoding strand at the HinfI site. The DNA probe was incubated with mouse liver nuclear extracts (lane 3, 5 μ g; lane 4, 10 μ g; lane 5, 20 μ g; lane 6, 40 μ g) and then subjected to DNase I digestion. The hatched box to the right represents the protected region (−1 to +14) within AGE3. Nucleotide sequence of this protected region is shown, and other footprint sequences are denoted by the open boxes. The TATA box is indicated on the left. Maxam–Gilbert sequencing reactions (lane 1, G + A) of the same probe were used as markers for nucleotide positions.

specificity on the angiotensinogen gene, the AGE2 and/or AGE3 were linked upstream of the Herpes simplex virus TK promoter–CAT hybrid gene. The results of transient expression experiments in cultured cells are shown in Fig. 9. AGE2 alone functioned solely in HepG2 cells, in which CAT activity was moderately stimulated and always significantly higher than in other cells. AGE3 alone was not able to activate the TK promoter in all cell lines tested, even though this element was multimerized up to four copies. A construct combining AGE2 with AGE3 ([AGE2 + AGE3] \times 2/TK–CAT) failed to function in HeLa, T98G, and NIH3T3 cells. In contrast, transcription was activated synergistically to a high level (13 ± 2.4 -fold) in HepG2 cells. These results suggest that a combined action of AGE2 and AGE3 promotes transcriptional activity of the angiotensinogen gene in a cell type–specific manner.

Discussion

In the classical endocrine RAS, the primary source of plasma angiotensinogen is the liver, and the regulation of hepatic angiotensinogen synthesis has been investigated (16, 17). How-

ever, the basal transcriptional mechanism of the angiotensinogen gene in hepatocytes is not completely understood. In this study, we show that the cell type–specific activation of this gene transcription results from the cooperative interaction of a proximal promoter element (AGE2) with a novel *cis*-acting element named AGE3 that resides directly around the transcriptional start site in the core promoter region. This conclusion is based on the following evidence. First, a transient transfection study indicates that the proximal promoter region from −96 to +22 of the angiotensinogen gene is sufficient to confer the cell type specificity on CAT reporter gene expression. Second, EMSA revealed that HepG2-specific AGF2 and ubiquitous AGF3 bind to AGE2 and AGE3, respectively. Third, experiments designed to test the involvement of these two elements in the promoter activity demonstrate that a synergistic interaction of AGE2 and AGE3 contributes to HepG2-specific expression of the angiotensinogen promoter.

In EMSA, HepG2 and liver nuclear extracts produced a specific retarded complex with AGE2 due to the binding of AGF2, whereas the nuclear extracts from HeLa, T98G, and NIH3T3 cells lacked detectable binding activity. Inspection of the DNA sequence of this element reveals a G–C rich 5′-left

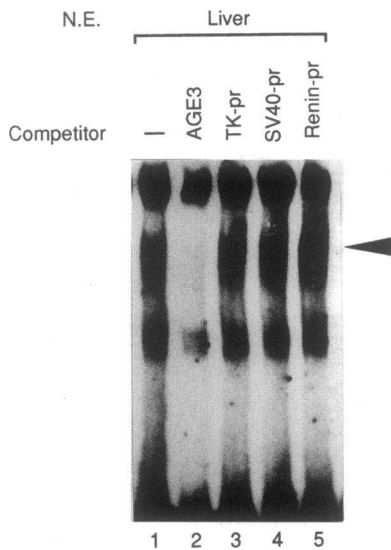


Figure 6. Electrophoretic shift competition analysis of AGF3 using DNA fragments encoding different core promoter regions. Binding reactions were performed as in Figure 5 using 0.2 ng of 32 P-labeled AGE3 as the probe with mouse liver nuclear extracts (10 μ g) per reaction. Several core promoter fragments containing consensus TATA sequences were prepared and these were added at a 100-fold molar excess relative to the probe. DNA fragments used as competi-

tors were: none (lane 1); AGE3 (lane 2); HSV-TK promoter (-109 to +16, lane 3); SV40 promoter (-43 to +64, lane 4); and mouse renin promoter (-47 to +16, lane 5). Arrow denotes specific DNA-nuclear factor complexes.

arm region and a perfect 12-bp palindrome (CTCTGTACA-GAG; -76 to -65) in the center region. The G-C box sequences, one of the most common regulatory DNA elements of eukaryotic genes, are often recognized by Sp-1 transcription factor (53). Sp-1 binds to the G-C rich sequences within a few hundred base pairs upstream of the transcriptional start sites and activates transcription of genes encoding housekeeping proteins such as H-ras (54), *c-myc* (55), insulin receptor (56), and dihydrofolate reductase (44). However, our DNase I foot-

print analysis revealed a protected region from -79 to -53, overlapping the palindromic sequences but not the G-C rich region, and electrophoretic shift competition assay using the consensus binding motif for Sp-1 suggested that AGF2 was distinct from Sp-1 family nuclear proteins. Although there is no apparent homology (> 80%) with the binding motifs of other known transcription factors in AGE2, further experiments are needed to determine whether AGF2 may be a novel transcription factor.

A substitution mutation in this palindrome that disrupted protein binding to AGE2 significantly reduced the transcriptional activation of CAT gene in HepG2 cells, and AGE2 by itself was able to moderately activate the heterologous TK promoter in HepG2-specific manner when linked up to four copies. The palindromic sequences of AGE2 are well conserved between the mouse and rat angiotensinogen genes (20), and a previous study showed that this palindromic sequence of the rat angiotensinogen gene was important for the formation of a specific complex with HepG2 nuclear extracts (57). Therefore, this palindromic region and AGF2 appears to be directly involved in dictating the hepatocyte-specific expression of the angiotensinogen promoter, and in extrahepatic cells AGF2 may be either absent or modified so that it is no longer able to bind to AGE2.

DNase I footprint analysis with liver nuclear extracts disclosed the protected sequences from -1 to +14 overlapping the first exon of the angiotensinogen gene, and substitution mutations within the exon 1 sequences from +3 to +14 abolished the binding ability of AGF3, suggesting that the exon 1 region is involved in AGF3 binding. EMSA concerning the distribution of AGE3-binding activity show that AGF3 is ubiquitously expressed at relatively uniform levels in various cell types examined. Although AGF3 by itself was unable to activate the native angiotensinogen and the heterologous TK promoters, the se-

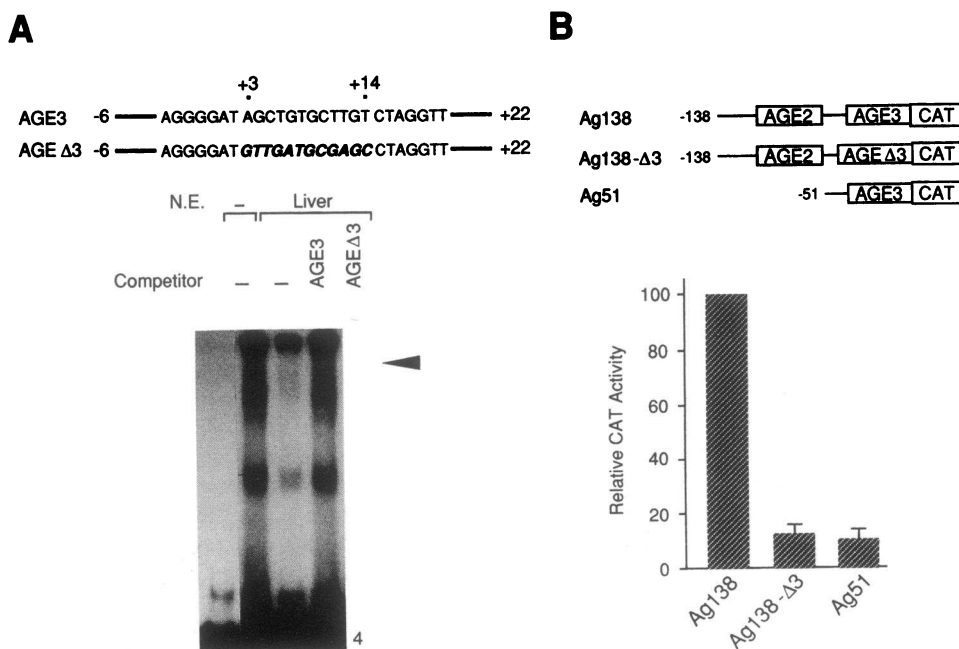


Figure 7. Effect of AGE3 mutation on nuclear factor (AGF3) binding and on transcriptional activity. (A) Electrophoretic shift competition assay using AGE3 and mutated AGE3 (AGEΔ3) as cold competitors. Site-directed mutations of the exon 1 region in AGE3 that interfered with protein binding were indicated by bold and italic letters. Binding reaction was performed with 10 μ g of nuclear extracts from mouse liver (lanes 2-4). Lane 1 contains no nuclear extract. Lane 2 includes no competitor. Lanes 3 and 4 show the effect of the native AGE3 and the mutated AGE3 (AGEΔ3) as cold competitors (100-fold molar excess) on the DNA-nuclear factor interaction. The retarded complexes are indicated by the arrowhead. (B) CAT assay with the construct that contains AGEΔ3. The angiotensinogen promoter-CAT chimeric construct with mutated AGE3 (Ag138-

Δ3) was transiently transfected into HepG2 cells as well as the parental Ag138. Relative CAT activities were determined as described in the legend of Figure 1 and were demonstrated by striped bars. The values represent the average \pm SEM of four independent experiments.

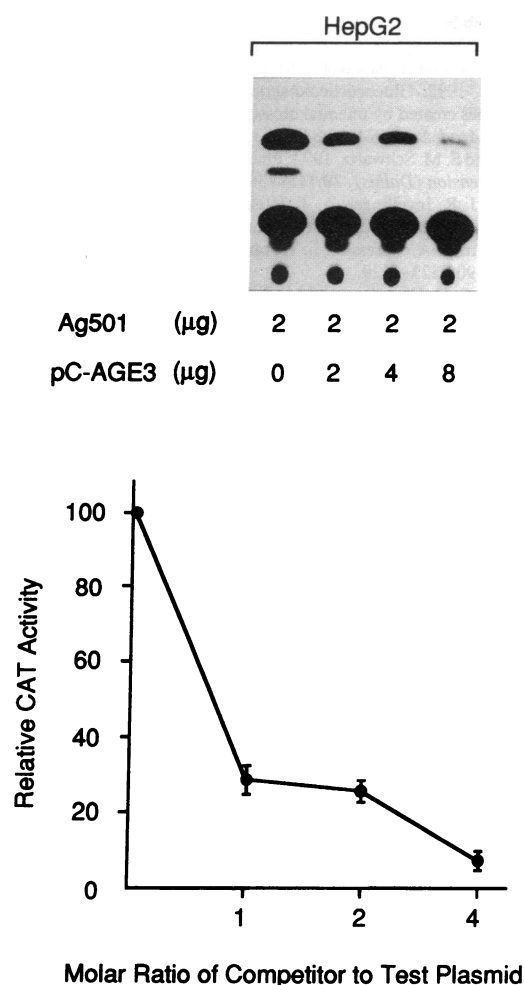


Figure 8. In vivo competition analysis of AGE3 (–6 to +22). A competitive plasmid (pC-AGE3) containing six tandem copies of the sequences from –6 to +22 was used in this experiment. The angiotensinogen promoter–CAT chimeric construct, Ag501 (2 µg), was transiently cotransfected with 8 µg of either competitor (pC-AGE3) or plasmid pUC19 or both into HepG2 cells. CAT assay was performed, and relative CAT activities were determined as described in the legend of Figure 1. The values are averages±SEM of four independent experiments.

quential titration of AGF3 binding by means of the in vivo competition in HepG2 cells led to the significant reduction in the activity of the longest angiotensinogen promoter (Ag501), containing both AGE2 and AGE3 regions, to $6.3 \pm 2.4\%$ of the original activity. Moreover, AGE2 alone was shown to be inefficient in fully activating the heterologous TK promoter in HepG2 cells, thereby demonstrating that a combination of AGE2 and AGE3 synergistically promoted CAT gene transcription under the control of TK promoter in HepG2-specific manner. These observations suggest that AGF3 binding to AGE3 is required for AGF2 to exert its cell type-specific activation property to a full extent in the native angiotensinogen and TK promoter contexts, despite the widespread expression of AGF3 activity. Angiotensinogen mRNA is expressed in a variety of tissue besides liver, such as brain, heart, adrenal gland, kidney, and vascular tissue (16, 17). Therefore, AGF2 may be important for high level expression in liver, and AGF3 may be involved in basal level expression in these extrahepatic tissues.

Of general transcription factors identified to date, TATA box-binding protein (TBP) is now shown to play a central role in the formation of preinitiation complex, TFIID, of RNA polymerase-directed transcription (58). Given that AGF3 is important for determination of the magnitude of cell type-specific transcriptional activity by AGF2, an interesting question can be raised concerning the possible implication of TBP in AGF3 binding to AGE3, because this element is closely located to the TATA box (–30 to –25). In previous analyses regarding TATA-binding activity, variation has been observed in the extent to which the footprints cover the transcription initiation region (59, 60), and a downstream initiation element is reported to be important for efficient TATA box binding and in vitro function of TFIID (61), in part because of its functional heterogeneity (62) and its multicomponent complexity (63), but the TATA site itself is invariably recognized in such footprints. In this sense, the footprint-protected region (–33 to –19) containing TATA sequences of the angiotensinogen gene might be a recognition element for TBP. Nevertheless, TFIID is not usually detectable in crude nuclear extracts (64) and promoter fragments of several genes containing TATA sequences fail to prevent AGF3 binding, suggesting that TBP does not directly support AGF3 binding to AGE3. The func-

		Fold Activation			
		HepG2	T98G	HeLa	NIH3T3
	TK-CAT	1	1	1	1
	AGE2/TK-CAT	3.5 ± 0.8	1.3 ± 0.3	1.2 ± 0.4	1.1 ± 0.3
	AGE3/TK-CAT	1.3 ± 0.4	1.1 ± 0.3	1.2 ± 0.2	1.0 ± 0.2
	[AGE2] _{x4} /TK-CAT	4.8 ± 1.6	1.3 ± 0.5	1.3 ± 0.2	1.1 ± 0.3
	[AGE3] _{x4} /TK-CAT	1.3 ± 0.3	1.2 ± 0.4	1.5 ± 0.5	1.2 ± 0.2
	[AGE2+AGE3] _{x2} /TK-CAT	13 ± 2.4	1.1 ± 0.3	1.9 ± 0.4	1.1 ± 0.3

Figure 9. Synergistic action of AGE2 and AGE3 on the heterologous TK promoter to direct cell type-specific activation. The AGE2 (solid box) or AGE3 (hatched box) fragments together with or without the other fragment were linked upstream to TK promoter in 5'–3' orientation, and transient transfection experiments were performed as described in the legend of Figure 1. Each construct is schematically shown on the left. CAT activities were calculated relative to the level achieved with TK-CAT included in each experiment, and fold activation for each construct is calculated by the CAT activity divided by the CAT activity of TK-CAT. Values are averages±SEM of at least three independent experiments.

tional importance of an additional protected region (-16 to -3) of the angiotensinogen gene demonstrated by DNase I footprinting remains unclear.

Recent investigations concerning the transcriptional regulation of the promoter elements demonstrated that the basal promoter regions of several genes are able to confer cell type specificity (65-70) and indicated that novel transcription factors bind to the elements located directly around the cap sites of several genes, such as terminal deoxynucleotidyltransferase (71), proopiomelanocortin (72), and cytochrome *c* oxidase subunit IV (73) genes. These factors were suggested to be especially important for promoting transcription by the basal promoter elements. Further study will be needed to identify the nature of these kinds of factors, including AGF3, interacting with the exon 1 regions.

In conclusion, the experiments reported here indicate that the proximal promoter region is important for cell type-specific activation of the angiotensinogen gene transcription and suggest that the proximal element AGE2 and the core element AGE3 act in concert to ensure this activation property. It is likely that the regulation of angiotensinogen synthesis in hepatocytes may be influenced by the actions of several cytokines and steroid hormones under physiological conditions. Experiments now in progress will define more precisely the modes of action of both AGF2 and AGF3 and will provide a molecular basis for the relationship between the binding activities of these nuclear factors and steroid hormone receptors or nuclear factors such as those activated by inflammatory responses.

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