Structure and Expression of the Human Angiotensinogen Gene
IDENTIFICATION OF A UNIQUE AND HIGHLY ACTIVE PROMOTER*

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We have isolated the human angiotensinogen gene from a genomic library and determined the exon-intron junction sequences. The gene is 12 kilobases long and consists of five exons interrupted by four introns, as a single copy in the human genome. Of particular interest are the positions of the introns in the human angiotensinogen gene which are identical to those in the highly homologous human α, antitrypsin and α1-antichymotrypsin genes, as well as rat and mouse angiotensinogen genes. Northern blot analysis showed that human hepatoma cells (HepG2) produce a large amount of angiotensinogen mRNA but not human glioma cells (T98G). To assay the promoter activity, the 1.3-kilobase genomic fragment containing the 5'-flanking region, first exon, and a part of first intron at positions -1222 to +44 was fused upstream to the chloramphenicol acetyltransferase gene, then transfected into HepG2 and T98G cells. The gene sequence was active only in HepG2 cells, suggesting the presence of a functional promoter. Analysis of deletion mutants demonstrated that the 76-base pairs region from -32 to +44 containing the TATA box and first exon is the minimal promoter, whose activity is as high as that of the SV40 enhancer-promoter. Since the basal expression of the human angiotensinogen gene is much higher in HepG2 than T98G cells, these results may reflect cell-specific differences in the gene transcription.

One striking effect of angiotensin II is to cause arteriolar vasoconstriction (3). The renin-angiotensin system therefore plays an important role in the regulation of blood pressure and in the pathogenesis of hypertension (4). In addition to this peripheral effect, angiotensin II in the central nervous system (5) has important actions including stimulation of thirst, alteration of sympathetic outflow, and release of several pituitary hormones such as vasopressin, corticotropin, and prolactin. Still other important roles of angiotensin II are its effect on stimulation of new vessel formation (6) and reproductive functions such as follicular development and ovulation (7).

Although a number of in vivo studies with rats (8-13) revealed that angiotensin production is regulated by several factors including steroid hormones, as well as by certain pathophysiological conditions, the regulatory mechanisms of the human angiotensinogen gene expression have not been clarified. In the present study, in order to understand the basal regulation of the gene expression, we determined the structure of the human angiotensinogen gene and identified the minimal promoter region required for the cell-specific expression of the gene.

EXPERIMENTAL PROCEDURES

Materials-Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo, Nippon Gene, Toyobo, and New England Biolabs. BglII and HindIII linkers were obtained from Takara Shuzo. [α-32P]dCTP (400 and 300 Ci/mmol) was purchased from Amersham Corp. [35S]Chloramphenicol (50-60 mCi/ml) was from DuPont-New England Nuclear.

Screening of Human Genomic Library.—Human genomic DNA was obtained from placenta as described (14). A genomic library was constructed in λ phage vector Charon 28 from a partial Sau3AI digest of genomic DNA using standard methods (15). Approximately, 6 × 109 phage plaques were screened with probe A as described below. Hybridization was carried out at 65 °C for 16 h in 5 × SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M NaH2PO4, H2O, 1 mM EDTA, pH 7.4), 1 × Denhardt’s solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 5 × 105 cpm/ml labeled probe A. After hybridization, filters were washed twice with 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature, twice with 2 × SSC and 0.1% SDS at 65 °C for 30 min, and twice with 0.1% SSC and 0.1% SDS at 65 °C for 30 min. Dried filters were subjected to autoradiography at -70 °C with an intensifying screen.

Subcloning of Genomic DNA Fragments.—The 3.5-kb EcoRI/BamHI, 5.0-kb BamHI, and 9.5-kb BgIII fragments from the recombinant phAG-1 were subcloned into the polylinker region of plasmid pUC19 and were designated phAG35EB, phAG90EB, and phAG95G, respectively. The 2.7-kb BamHI fragment from phAG95G was inserted into the BamHI site of pUC19 to generate phAG27B.

Preparation of Probes,—A 129-bp BalI fragment, designated probe A, was isolated from phAG3 (1) and used for plaque screening. A 129-bp ApolI/Apal fragment (the 5′-end probe, probe B) was excised from phAG35EB and used for Southern blot analysis. A 413-bp RsaI fragment (the exon 2 probe, probe C) was from phAG27B and used for Southern and Northern blot analyses. These probes were labeled with [α-33P]dCTP by the random primer method (16).

Southern Blot Analysis — Human placenta DNA (10 μg) and the cloned bacteriophage DNA (0.5 μg) were digested with a variety of

Human angiotensinogen is a hormone precursor of M, 61,400. The mature form consists of a single polypeptide chain of 452 amino acid residues with 14% carbohydrate content (1, 2). Angiotensinogen is synthesized primarily by the liver and released into the circulation, where it is cleaved by renin (EC 3.43.15), an aspartyl proteinase, to generate a decapeptide, angiotensin I. It is subsequently converted to a multifunctional octapeptide, angiotensin II by angiotensin-converting enzyme.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X19325, X19324, X19329, X19328, and X19327.

1 The abbreviations used are: kb, kilobase; bp, base pair; SDS, sodium dodecyl sulfate.
restriction enzymes, electrophoresed on 0.7% agarose gels, and transferred to GeneScreen Plus (nylon) membrane (Du Pont-New England Nuclear) as described (17). Hybridization was conducted at 65 °C for 16 h in 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 1 × 10^6 cpm/ml labeled probe B or C. Filters were washed twice with 2 × SSC at room temperature for 5 min, twice with 2 × SSC and 1% SDS at 65 °C for 30 min, and twice with 0.1 × SSC at room temperature for 30 min. Filters were then subjected to autoradiography at -70 °C with an intensifying screen.

Preparation of RNA and Northern Blot Analysis—Total RNA was isolated from human liver, HepG2, and T98G cell lines by the guanidinium-cesium chloride method (18). Total RNA was denatured with 1 M glyoxal and 50% dimethyl sulfoxide (19), electrophoresed on a 1.2% agarose gel, and transferred to GeneScreen Plus membrane. Hybridization was carried out at 60 °C for 16 h in 1× SSC, 5% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 1 × 10^6 cpm/ml labeled probe C. The filter was washed twice with 2 × SSC at room temperature for 5 min, twice with 2 × SSC and 1% SDS at 60 °C for 30 min, and twice with 0.1 × SSC at room temperature. The filter was then subjected to autoradiography at -70 °C with an intensifying screen.

DNA Sequence Analysis—DNA fragments carrying exons and the 5'- and 3'-flanking regions were identified by restriction mapping. After subcloning into M13 or pUC19 vectors, the sequence of exon/exon/intron boundaries, and flanking regions were determined by the dideoxynucleotide chain termination method (20).

Plasmid Constructions—pSV2cat (21) was digested with AccI, made blunt-ended by treatment with Escherichia coli DNA polymerase I (Klenow subunit) and ligated to BglII linker. The DNA was then digested with BglII and BamHI to generate a 2159-bp fragment. pUC19 was digested with HindIII, converted to blunt ends by treatment with E. coli DNA polymerase I (Klenow subunit) and ligated to HindIII linker. This vector DNA was digested with BglII and BamHI.

The vector and the BglII/BamHI fragment from pSV2cat were ligated to construct pUCSV2cat.

A 1651-bp HindIII/BamHI fragment derived from pSV2cat was inserted into HindIII/BamHI sites of pUC19 to construct pUC0cat. A 1268-bp Apal fragment from phAG35EB was treated with T4 DNA polymerase I to make blunt ends and was then ligated to HindIII linker. This fragment was digested with HindIII and ligated into HindIII site of pUC0cat. Using this procedure, insertions representing both orientations were obtained to construct pUCHAG(-1222)cat (sense orientation) and pUCHAG(-1222)catRv (reverse orientation). Deletion mutants were produced by digestion with Exonuclease III as described (22), and the resulting end points were determined by sequencing.

Cell Culture, DNA Transfection, and Chloramphenicol Acetyltransferase Assay—Human hepatoma cells (HepG2) and human glioma (T98G) cells were maintained in minimum essential medium (GIBCO) containing 10% fetal bovine serum and nonessential amino acids.

One day before transfection, 1 × 10^6 and 0.5 × 10^6 cells for HepG2 and T98G cells, respectively, were plated on 60-mm dishes. The cell medium was changed 3 h prior to transfection. A calcium phosphate coprecipitate containing 3 μg of DNA was added to the cultured cell lines. Cells were incubated for 6 h, washed, and incubated for 36 h in fresh medium. Cells were collected and cell extracts were prepared by freezing and thawing as described (21). The extracts were heated at 60 °C for 10 min, and the precipitate was removed by centrifugation. The protein concentrations were determined using bovine serum albumin as a standard (23). The reaction mixture contained 140 mM Tris-HCl, pH 7.8, 0.2 μCi of [3H]chloramphenicol, 4 mM acetyl-coenzyme A (Pharmacia LKB Biotechnology Inc.), and 40 μg of cell extract in a final volume of 150 μl. The mixture was incubated at 37 °C for 1 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 as-
The human angiotensinogen gene. The termination codon is marked by asterisks. TATA box and the putative polyadenylation signals are underlined by dashed lines. Filled and open circles indicate possible glucocorticoid and estrogen responsive elements, respectively. The 14-bp sequence with perfect dyad symmetry is indicated by arrows. Filled and open squares indicate the consensus sequences for RNA polymerase III promoters and the heat shock element, respectively. The nucleotide sequence (second line) is shown with the deduced amino acid sequence.

**FIG. 2.** Nucleotide sequence of the 5'-flanking region, all five exons, and the 3' flanking region of the human angiotensinogen gene. The nucleotide sequence (second line) is shown with the deduced amino acid sequence.
cending thin layer chromatography using chloroform/methanol (95:5, v/v). The chromatograms were subjected to autoradiography at −70 °C. Chloramphenicol acetyltransferase activity was quantitated by counting scraped regions of the chromatograms in a liquid scintillation spectrometer.

RESULTS

Isolation and Characterization of the Human Angiotensinogen Gene—A human genomic DNA library (6 × 10⁶ phages) was screened with probe A, resulting in isolation of one positive clone, XhAG-1. This clone, containing an insert of 16 kb, was characterized by a combination of restriction enzymes and Southern blot analyses. Human genomic and the cloned the introns begin with the nucleotides GT and end with AG.

Structure of the Human Angiotensinogen Gene—Nucleotide sequences of all exon-intron junctions have been determined. The human angiotensinogen gene is composed of five exons separated by four introns, which are identified by comparison of the cDNA sequence (1). As in other eukaryotic genes, the introns begin with the nucleotides GT and end with AG.
regions is shown in Fig. 2. The nucleotide numbering was
designated by assigning the proposed transcription initiation
site (1) as nucleotide 1.

The first exon contains 36 bp of untranslated nucleotides
that are present in the 5' region of the transcript (1). The
second exon consists of 899 nucleotides in which the signal
peptide and angiotensin I are encoded. The third and fourth
exons are composed of 266 and 165 nucleotides, respectively.
The last exon, exon 5, consists of 796 nucleotides in which
two possible polyadenylation signals are present.

Comparison of the exon sequences with the cDNA sequence
(1) revealed two differences. In the protein coding region, a T
to C transition at position nucleotide 842 changes a methio-
nine codon in the cDNA to a threonine codon in the genomic
DNA. Another is a C to T transversion at position 2054 in
the 3'-untranslated region. These differences may represent
either a cloning artifact or DNA sequence polymorphisms
because the genomic and cDNA libraries were prepared from
different individuals.

It has been revealed that the amino acid sequences of rat
and mouse angiotensinogens are significantly related to those
of human α1-antitrypsin, antithrombin, and ovalbumin, which
belong to the serine protease inhibitor family (25, 26). Fur-
thermore, the rat angiotensinogen gene showed a striking
structural similarity to the human α1-antitrypsin and human
α1-antichymotrypsin genes with respect to the number and
position of the introns (27). Therefore, we compared the
intron positions of the human angiotensinogen gene with
those of the human α1-antitrypsin (28) and human α1-
antichymotrypsin genes (29), as well as rat (27) and mouse (26)
angiotensinogen genes. All these genes have three features in
common. 1) They consist of five exons and four introns. 2)
The first intron is located in the 5'-untranslated portion of
these genes. 3) The second, third, and fourth introns are in
homologous positions and are interrupted in the same manner
(Fig. 3). These similarities in structure are consistent with a
hypothesis that the serine protease inhibitor gene family
evolved from a common ancestral gene through a series of
gene duplications, insertions, and deletions (29).

Analysis of the Nucleotide Sequence of the 5'-Flanking Region—
Sequence analysis of the 5'-flanking region revealed a number of interesting sequence motifs (Fig. 2). The putative
promoter region contains a classical TATA box (24) at posi-
tions −31 to −34. However, there is neither a CAAT box (30)
nor a GC box (31). Further upstream from the TATA box,
we found potential regulatory sequences including glucocorticoid (−130 to −125 and −675 to −670) (32, 33), estrogen (−337 to
−324) (34), acute phase (−278 to −270) (35, 36), cAMP (−839
to −833) (37), and heat shock (−574 to −561) (38) responsive
elements. At positions −78 to −65, there is a 14-nucleotide
sequence with perfect dyad symmetry, GCCTGTGCA-
CAGGC. Around this region, a sequence (TGTGCA-
CAGGCTGG) is located at positions −68 to −81 in the
complementary strand, which is very similar to the consensus
sequence of the first promoter for RNA polymerase III,
TRNYNNRRNGG (R, purine residue; Y, pyrimidine residue)
(39).

Cell-specific Minimal Promoter of the 5'-Flanking Region—
Angiotensinogen is synthesized primarily by the liver. To
examine the expression of human angiotensinogen mRNA,
RNAs from human liver, human hepatoma (HepG2), and
human glioma (T98G) cells have been isolated and analyzed
by Northern blot hybridization. As shown in Fig. 4, human
liver and HepG2 cells contain a considerable quantity of the
angiotensinogen mRNA. On the other hand, it was not
detected in T98G cells, suggesting that there must be cell-
specific control mechanisms responsible for the specificity
of angiotensinogen gene expression.

To identify a promoter region required for the cell-specific
expression of the angiotensinogen gene at the basal level, a
1.3-kb A/pol fragment (Fig. 1A) of the gene containing 1222
bp of the 5'-flanking region, 36 bp of exon 1 (Fig. 2), and 8
bp of intron 1 (Fig. 2) at positions −1222 to +44 was inserted
at a unique HindIII site of pUC0cat. pUCAG(−1222)cat has
the 1.3-kb fragment in the sense orientation to the chloram-
phenicol acetyltransferase gene, and pUCAG(−1222)catRv
has the sequence in the reverse orientation. A series of dele-
tion mutants extending from −1222 to −33 and from +44 to
−16 were constructed. These constructions were then intro-
duced into HepG2 and T98G cells (Fig. 5).

In T98G cells, chloramphenicol acetyltransferase transcrip-
tion did not exceed the background level for pUC0cat which
was less than 1% relative to pUCSV2cat. This complete
inactivity of all deletion mutants in T98G cells was not due
to a lower transfection efficiency, since expression directed
by pUCSV2cat was higher in these cells than in HepG2 cells.

In HepG2 cells, pUCAG(−1222)cat elicited a high level of
chloramphenicol acetyltransferase activity. In contrast,
pUCAG(−1222)catRv failed to express the enzyme activity,
indicating that this expression is dependent upon the orien-
tation of the gene promoter. Deletion from −1222 to −33
resulted in no significant change in the promoter activity.
The removal of the region between −16 and +44 containing
exon 1 from pUCAG(−1222)cat resulted in a 95% reduction
of the promoter activity, showing that this domain represents
a region of functional importance for the efficient gene expres-
sion. Together with the fact that all deletion mutants are not
transcribed in T98G cells, these findings suggest that the 76-
bp region from −32 to +44 containing the TATA box and
exon 1 is the minimal promoter required for cell-specific
FIG. 5. Basal expression of the human angiotensinogen-chloramphenicol acetyltransferase hybrid genes in HepG2 and T98G cells. A series of plasmids containing deletions in the 5'-flanking region DNA were constructed and their activities in HepG2 and T98G cells determined as described under “Experimental Procedures.” The TATA box and exon 1 are located at positions −31 to −24 and +1 to +36, respectively. Activities are expressed relative to those achieved with pUCSV2cat in each cell line and represent the average of four separate transfections. The eight gene constructs used in this assay are designated as follows: pUChAG(-1222)catRv, pUChAG(-1222)cat, pUChAG(-747)cat, pUChAG(-516)cat, pUChAG(-243)cat, pUChAG(-106)cat, pUChAG(-32)cat, and pUChAG(-1222/-16)cat.

expression of the human angiotensinogen gene in this transient assay.

DISCUSSION

Nucleotide sequence and Southern blot analyses revealed that the human angiotensinogen gene consists of five exons and four introns distributed over 12 kb and that it exists as a single gene in the human genome.

Northern blot analysis showed that the human angiotensinogen mRNA is detected in human hepatoma cells (HepG2) but not in human glioma cells (T98G), implying that the expression of the human angiotensinogen gene is regulated in a cell-specific manner. As a first approach for elucidating the cell-specific regulation of the gene expression at the basal level, we have identified the minimal promoter of the human angiotensinogen gene by deletion analysis using transient chloramphenicol acetyltransferase assay.

It has been shown that the upstream elements of genes transcribed by RNA polymerase II are required for maximum levels of gene expression and are involved in tissue- or cell-specific expression of genes (40). In most cases thus far examined, the loss of transcriptional activities has been shown to be associated with progressive deletions of the 5'-flanking region. Unexpectedly, our results indicated that successive deletions of the upstream region from −1222 to −106 have little effect on the high level of expression of the human angiotensinogen-chloramphenicol acetyltransferase hybrid genes in HepG2 cells. It was even more surprising to find that the high level of chloramphenicol acetyltransferase expression is still observed in a minimum gene construct, pUChAG(-32)cat, containing the TATA box (at positions −31 to −24), exon 1 (at positions +1 to +36), and a part of intron 1 (at positions +37 to +44). On the other hand, the same deletion mutant did not express the chloramphenicol acetyltransferase activity in T98G cells. Further analysis showed that deletion of the region between −16 and +44 containing exon 1 leads to significant reduction of the chloramphenicol acetyltransferase transcriptional activity even though the TATA box and 1.2-kb upstream sequences are retained.

Our results can raise two questions: why is the expression of pUChAG(-32)cat as efficient as pUCSV2cat in HepG2 cells, and why is it suppressed in T98G cells? One possible explanation for these questions is that a cell-specific enhancer protein or a positive regulatory factor may exist, which can act on sequences within the short regulatory region only in HepG2 cells. However, no sequence homology could be found between consensus sequences of any known viral enhancer and the region from −32 to +44. Another is that a cell-specific activating protein can stimulate transcription by facilitating the formation of the preinitiation transcription complex. In any case, it is unique that the basal expression of the human angiotensinogen gene is directed only by the 76-bp sequences at positions −32 to +44.

Although we found a number of tentative regulatory sequences, including glucocorticoid, estrogen, acute phase,
cAMP, and heat shock-responsive elements in the 5'-flanking region of the human angiotensinogen gene, it should be emphasized that none of the above sequence motifs have yet been shown to function in the expression of the angiotensinogen gene at the basal level. However, this information will serve as a guide in studying the mechanisms that underlie the regulation of the gene transcription.

A more detailed characterization of the 76-bp regulatory region at the basal level and of the putative upstream elements at the regulatory level will be required for a more thorough understanding of the regulation of the human angiotensinogen gene. It would be also interesting to investigate how a protein factor(s) interacts with the short regulatory region and enhances the transcriptional activity.

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