STRUCTURE OF THE HUMAN ANGIOTENSINOGEN GENE AND ITS REGULATION IN CULTURED CELLS AND TRANSGENIC MICE

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Contents

Chapter I: Preface

Human angiotensinogen, a glycoprotein consisting of 452 amino acids (1,2), is synthesized predominantly in the liver and secreted into the circulation, where the enzyme renin (EC3.4.23.15) cleaves angiotensinogen to generates angiotensin I. Subsequently, angiotensin I is processed to octapeptide angiotensin II, a most potent vasoconstrictor, by angiotensin-converting enzyme. Since the cleavage of angiotensinogen by renin is thought to be the rate-limiting step in the physiological cascade, the renin-angiotensin system plays an important role in the regulation of blood pressure and thus has been implicated in the pathogenesis of hypertension (Fig. 1).

The development of recombinant DNA technology has introduced new directions for the study of the angiotensinogen gene. It has been shown that the structure of the rat angiotensinogen gene shares an identical structural organization with genes for human α_1-antitrypsin and α_1-antichymotrypsin, classified as serine protease inhibitors (3, 4). This observation suggested that these genes were derived from a common ancestral gene (4, 5). The structural relationship of angiotensinogen to the serine protease inhibitor genes raises a possibility that angiotensinogen takes part in an acute phase response because the serine protease inhibitor family is closely related to the acute phase inflammation reaction (6). In fact, it has become clear that angiotensinogen in serum protein level was elevated in humans with acute inflammatory diseases (7), and rat angiotensinogen mRNA level in liver significantly increased following the administration of *E. coli* lipopolysaccharide (8).

Because a number of studies (9-14) have suggested that angiotensin production is regulated by several factors including steroid hormones, as well as by certain pathophysiological conditions, I have been interested in the regulatory mechanisms for the angiotensinogen gene expression. In order to

clarify this mechanism, rat and human angiotensinogen cDNAs (1,15), and their genes (3,16) have been cloned and sequenced. However, their promoter regions associated with the cell-specific expression of these genes have not been identified. The cloning of the angiotensinogen gene offers the opportunity to study this regulation at the transcriptional level.

Angiotensinogen mRNA have shown to accumulate mainly in liver, but in variety of rat extrahepatic tissues including adrenal, brain, kidney, large intestine, lung, ovary, spleen, and stomach (9,10). Although the human gene is expressed in liver, the expression in the other tissues or cell lines have not been identified, because of limited availability of human tissues and the absence of appropriate cell lines. The cell lines derived from extrahepatic tissues which express angiotensinogen gene may become useful to study the biosynthesis and expression of that gene in such tissues.

The introduction of foreign genes into the germ line of mammals has been a practical reality now for a number of years. This form of experimentation allows the creation of lines of animals to answer specific molecular genetic questions. Manipulation of the mammalian embryos has been enormously important in developmental biology in recent years and that experience has brought about the possibility of new experiments allowing the molecular analysis of many biological process. The methodologies involved in constructing transgenic animals have been published extensively in a number of comprehensive reviews. In typical experiments, pronuclear stage (one cell) embryos are collected after fertilization, but prior to the onset of cleavage. Exogenous cloned linearized DNA is injected into one of the two pronuclei by means of a finely drawn injection pipet. The manipulated embryo is transferred into the oviduct or ovarian bursal space of a surrogate mother previously mated with a sterile male. Alternative methods include retroviral transfection of cleavage stage embryos or insertion of genetically engineered embryo-derived embryonal stem cells into blastocysts. Offspring from these procedures are screened by

standard molecular analyses to determine presence of the foreign genetic material. The transgenic approach is very useful to study a specific set of problems : (i) regulation of gene expression *in vivo*, (ii) the establishment of disease models for the study of pathogenesis, (iii) the use of exogenous genetic elements to correct specific genetic defects, (iv) the role of insertional mutagenesis in disruption of normal development.

In this thesis, to examine the regulation of the human angiotensinogen gene, I cloned and determined the structure of the gene including the 5'-flanking region (promoter and putative regulatory regions), and identified the minimal promoter region for its cell-specific expression with *in vitro* transient transfection technique (chapter II). In chapter III, I investigated the extrahepatic expression of the human angiotensinogen gene by Northern blot analysis and studied the influence of changes in ADP-ribosylation on the expression of the human angiotensinogen gene in cultured cells. Furthermore, to investigate the expression of the human angiotensinogen gene *in vivo,* I generated transgenic mice harboring the human angiotensinogen gene and analyzed the tissue-specific expression of transgene in mice (chapter IV).

Chapter II: Structure and Expression of the Human Angiotensinogen Gene

Summary

I 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_1-antitrypsin and α_1-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 position -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 demonstrate 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.

Introduction

Human angiotensinogen is a hormone precursor of Mr 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 3.4.23.15), an aspartyl proteinase, to generate a decapeptide, angiotensin I. It is subsequently converted to a multifunctional octapeptide, angiotensin II by angiotensin-converting enzyme (Fig. 1).

One striking effect of angiotensin II is to cause arteriolar vasoconstriction (18). The renin-angiotensin system therefore plays an important role in the regulation of blood pressure and in the pathogenesis of hypertension (19). In addition to this peripheral effect, angiotensin II in the central nervous system (20) 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 (21) and reproductive functions such as follicular development and ovulation (22).

Although a number of *in vivo* studies with rats (8, 10-14) revealed that angiotensin production is regulated by several factors including steroid hormones, as well as by certain pathophysiological conditions, the regulatory mechanisms of the the human angiotensinogen gene expression have not been clarified. In the present chapter, in order to understand the basal regulation of the gene expression, I determined the structure of the human angiotensinogen gene and identified the minimal promoter region required for the cell-specific expression of the gene.

Materials and Methods

(a) *Materials*

Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo, Nippon Gene, Toyobo, and New England Biolabs. *Bgl*II and *Hin*dIII linkers were obtained from Takara Shuzo. [α-^{32}P]dCTP (400 and 3000 Ci/mmol) was purchased from Amersham Corp. [^{14}C]Chloramphenicol (50-60 mCi/mmol) was from DuPont-New England Nuclear.

(b) *Screening of human genomic library*

Human genomic DNA was obtained from placenta as described (23). A genomic library was constructed in λphage vector Charon 28 from a partial *Sau*3AI digest of genomic DNA using standard methods (24). Approximately, 6 x 10^5 phage plaques were screened with probe A as described below. Hybridization was carried out at 65°C for 16 h in 5 x SSPE (1 x SSPE = 0.15 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.4), 1 x 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 x 10^6 cpm/ml labeled probe A. After hybridization, filters were washed twice with 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature, twice with 2 x SSC and 0.1% SDS at 65°C for 30 min, and twice with 0.1 x SSC and 0.1% SDS at 65°C for 30 min. Dried filters were subjected to autoradiography at -70°C with an intensifying screen.

(c) *Subcloning of Genomic DNA Fragments*

The 3.5-kb *Eco*RI/*Bam*HI, 5.0-kb *Bam*HI, and 9.5-kb *Bgl*II fragments from the recombinant phage λhAG-1 were designated phAG35EB, phAG50B, and phAG95G, respectively. The 2.7-kb *Bam*HI fragment from phAG95G was

inserted into the *Bam*HI site of pUC19 to generate phAG27B.

(d) *Preparation of Probes*

A 1262-bp *Bst*EII fragment, designated probe A, was isolated from pHag3 (1) and used for plaque screening. A 123-bp *Apa*LI/*Apa*I fragment (the 5'-end probe, probe B) was excised from phAG35EB and used for Southern blot analysis. A 413-bp *Rsa*I fragment (the exon 2 probe, probe C) was from phAG27B and used for Southern and Northern blot analysis. These probes were labeled with [α-^{32}P]dCTP by the random primer method (25).

(e) *Southern Blot Analysis*

Human placenta DNA (10μg) and the cloned bacteriophage DNA (0.3 μg) were digested with a variety of restriction enzymes, electrophoresed on 0.7% agarose gels, and transferred to GeneScreen Plus (nylon) membrane (DuPont-New England Nuclear) as described (26). Hybridization was conducted at 65°C for 16 h in 1M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 1 x 10^6 cpm/ml labeled probe B or C. Filters were washed twice with 2 x SSC at room temperature for 5 min, twice with 2 x SSC and 1% SDS at 65°C for 30 min, and twice with 0.1 x SSC at room temperature for 30 min. Filters were then subjected to autoradiography at -70°C with an intensifying screen.

(f) *Preparation of RNA and Northern Blot Analysis*

Total RNA was isolated from human liver, HepG2 (27), and T98G (28) cell lines by the guanidinium-cesium chloride method (29). Total RNA was denatured with 1 M glyoxal and 50% dimethyl sulfoxide (30), electrophoresed on a 1.2% agarose gel, and transferred to GeneScreen Plus membrane.Hybridization was carried out at 60°C for 16 h in 1M NaCl, 1% SDS, 50% dextran sulfate, 100 mg/ml denatured salmon sperm DNA, and 1 x 10^6

cpm/ml labeled probe C. The filter was washed twice with 2 x SSC at room temperature for 5 min, twice with 2 x SSC and 1% SDS at 60°C for 30 min, and twice with 0.1 x SSC at room temperature. The filter was then subjected to autoradiography at -70°C with an intensifying screen.

(g) *DNA Sequence Analysis*

DNA fragment 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 (31).

(h) *Plasmid Constructions*

pSV2cat (32) was digested with *Acc*I, made blunt-ended by treatment with *Escherichia coli* DNA polymerase I (Klenow subunit) and ligated to *Bgl*II linker. The DNA was then digested with *Bgl*II and *Bam*HI to generate a 2159-bp fragment. pUC19 was digested with *Hin*dIII, converted to blunt ends by treatment with *E. coli* DNA polymerase I (Klenow subunit) and ligated to *Bgl*II linker. This vector DNA was digested with *Bgl*II and *Bam*HI. The vector and the *Bgl*II/*Bam*HI fragment from pSV2cat were ligated to construct pUCSV2cat.

A 1651-bp *Hin*dIII/*Bam*HI fragment derived from pSV2cat was inserted into *Hin*dIII/*Bam*HI sites of pUC19 to construct pUC0cat.

A 1268-bp *Apa*I fragment from phAG35EB was treated with T4 DNA polymerase I to make blunt ends and was then ligated to *Hin*dIII linker. This fragment was digested with *Hin*dIII and ligated into *Hin*dIII 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 (33), and the resulting end points were determined by sequencing.

(i) ***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 acid.

One day before transfection, 1 x 10^6 and 0.5 x 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 collected and cell extracts were prepared by freezing and thawing as described (32). The extracts were heated at 60°C for 10 min, and the precipitate was removed by centrifugation. The protein concentration was determined using bovine serum albumin as a standard (34). The reaction mixture contained 140 mM Tris-HCl, pH 7.8, 0.2 μCi of [^{14}C]chloramphenicol, 4mM 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 labeled chloramphenicol and acetylated derivatives were separated by ascending 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 lisquid scintillation spectrometer.

Results

(a) *Isolation and Characterization of the Human Angiotensinogen Gene*

A human genomic DNA library (6 x 10^5 phages) was screened with probe A, resulting in isolation of one positive clone, λhAG-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 bacteriophage (λhAG-1) DNAs were digested with *Apa*LI/*Bgl*II, *Bgl*II, and *Bam*HI; they were analyzed by Southern blotting using probe B or C. As displayed in Fig. 2, hybridization-positive bands are of the same size in both human genomic and λhAG-1 DNAs, indicating that the cloned angiotensinogen gene has not rearranged during cloning procedures. This result also suggests that there is a single gene encoding angiotensinogen in the human genome.

(b) *Structure of the Human Angiotensinogen Gene*

Nucleotide sequences of all exon-intron junctions have been determined. (Fig .3). The human angiotensinogen gene is composed of five exons separated by four introns, which are identified by comparison with the cDNA sequence (1). As in other eukaryotic genes, the introns begin with the nucleotides GT and end with AG (35). The nucleotide sequence of all exons and their flanking region is shown in Fig. 4. 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 859 nucleotides in which the signal peptide and angiotensin I are encoded. The third and fourth exons are composed of 268 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 nucleotide position 842 changes a methionine codon in the genomic DNA. Another is an A to C transversion at position 2054 in the 3'-untranslated region. These differences may represent either a cloning artifact or DNA sequences 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 α_1-antitrypsin, antithrombin, and ovalbumin, which belong to the serine protease inhibitor family (36, 16). Furthermore, the rat angiotensinogen gene showed a striking structural similarity to the human α_1-antitrypsin and human α_1-antichymotrypsin genes with respect to the number and position of the introns (3). Therefore, we compared the intron positions of the human angiotensinogen gene with those of the human α_1-antitrypsin (37) and human α_1-antichymotrypsin gene (4), as well as rat (3) and mouse (16) angiotensinogen genes. All these genes have three features in common. 1) They consists 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. 5 and 6). 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 (4).

(c) *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. 4). The putative promoter region contains a

classical TATA box (35) at position -31 to -24. However, there is neither a CAAT box (38) nor a GC box(39). Further upstream from the TATA box, we found potential regulatory sequences including glucocorticoid (-130 to -125 and -675 to -670) (40, 41), estrogen (-337 to -324) (42), acute phase (-278 to -270) (43,44) cAMP (-839 to -833) (45), and heat shock (-574 to -561) (46) responsive elements. At positions -78 to -65, there is a 14-nucleotide sequence with perfect dyad symmetry, GCCTGTGCACAGGC. Around this region, a sequence (TGTGCACAGGCTGG) 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) (48). Upstream from the putative first promoter, a sequence (GATCGATGC) is present at positions -180 to -188 in the complementary strand, which is completely identical to the consensus sequence of the second promoter for RNA polymerase III, G(A/T)TC(G/A)ANNC (47).

(d) *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. 7, 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 *Apa*I fragment (Fig. 2A) of the gene containing 1222 bp of the 5'-flanking region, 36 bp of exon 1 (Fig. 4), and 8 bp of intron 1 (Fig. 6) at positions -1222 to +44 was inserted at a unique *Hin*dIII site of pUC0cat. pUChAG(-1222)cat has the 1.3-kb fragment in the

sense orientation to the chloramphenicol acetyltransferase gene, and pUChAG(-1222)catRv has the sequence in the reverse orientation. A series of deletion mutants extending from -1222 to -33 and from +44 to -16 were constructed. These constructions were then introduced into HepG2 and T98G cells (Fig. 8).

In T98G cells, chloramphenicol acetyltransferase transcription 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, pUChAG(-1222)cat elicited a high level of chloramphenicol acetyltransferase activity. In contrast, pUChAG(-1222)catRv failed to express the enzyme activity, indicating that this expression is dependent upon the orientation 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 pUChAG(-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 expression. 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 expression of the human angiotensinogen gene in this transient assay.

Discussion

Nucleotide sequence and Southern blot analysis 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, I 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 (48). In most cases thus far examined, the loss of transcriptional activities have 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-CAT hybrid genes in HepG2 cells. It was even more surprising to find that the level of CAT expression is still observed in a minimum gene construct, pUChAG(-32)cat, containing the TATA box (at positions -31 to -24), exon 1 (at position +1 to +36), and a part of intron 1 (at position +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 gene between -16 to +44 containing exon 1 leads to significant reduction of the chloramphenicol acetyltransferase activity even though the TATA box and 1.2-kb upstream sequences are retained.

Our result 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. 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 I found a number of tentative regulatory sequences, including glucocorticoid, estrogen, acute phase, cAMP, and heat shock-responsible 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 enhancers the transcriptional activity.

In summary, I cloned and sequenced the human angiotensinogen gene which consists of five exons and four introns. In the 5'-flanking region, there are putative nucleotide sequences involved in the regulation by steroid hormones and certain pathophysiological conditions. I demonstrated that the upstream sequences of the human angiotensinogen gene play an essential role in the basal regulation of its tissue-specific expression.

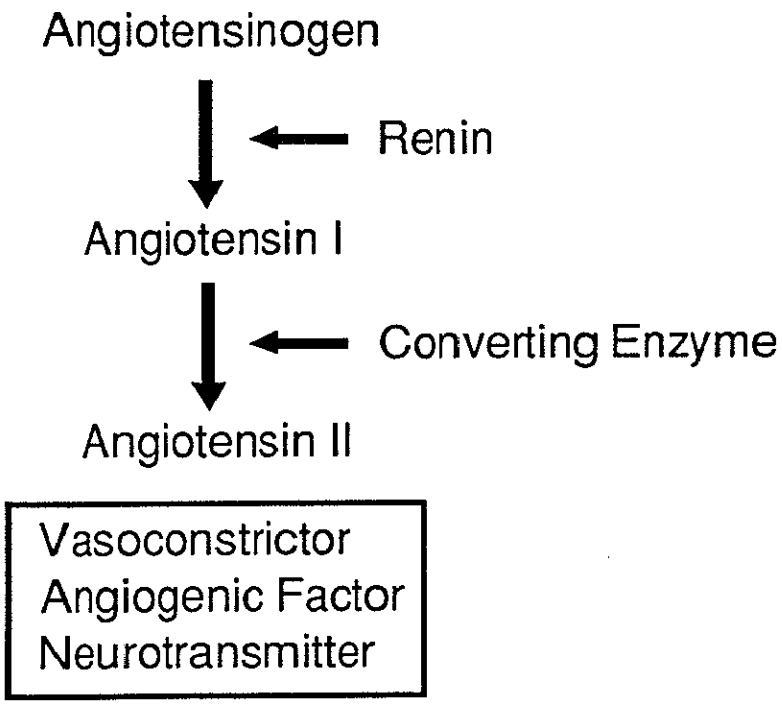


Fig. 1. Illustration of the Renin-Angiotensin System

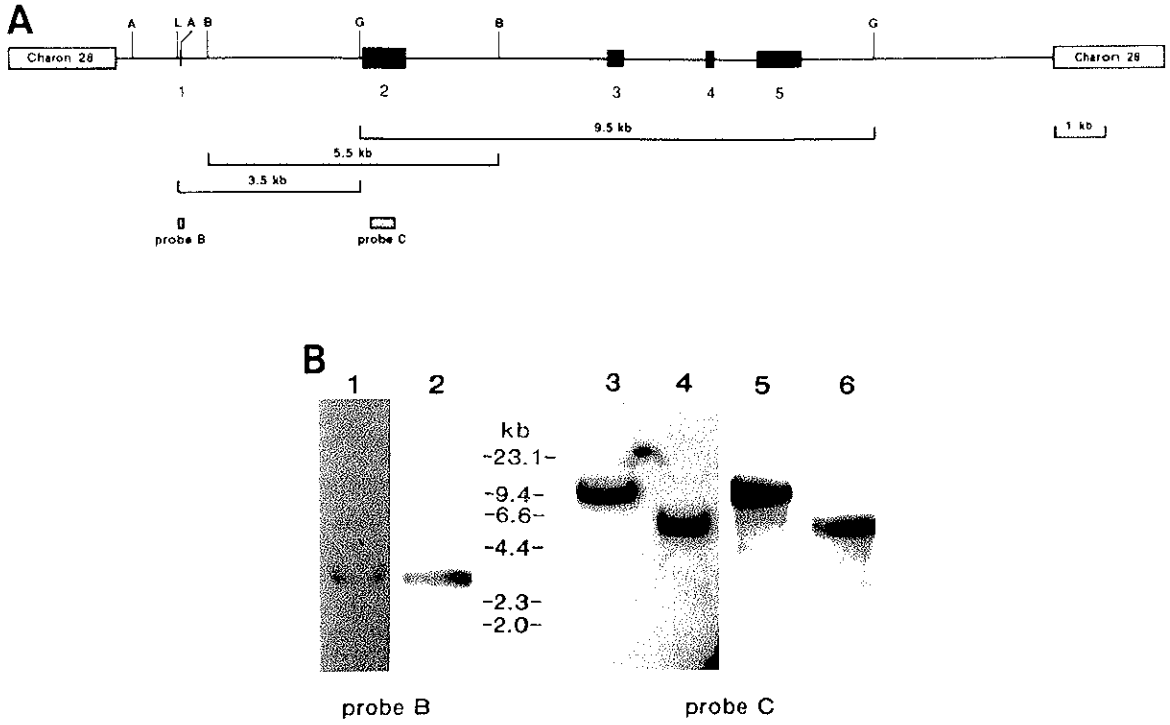


Fig. 2. Structure of the human angiotensinogen gene. ***A***- partial restriction map and relevant restriction sites of λhAG-1 carrying the human angiotensinogen gene are displayed. The five exons are represented by solid bars. Restriction sites are indicated as follows: A, *Apa*I; B, *Bam*HI; G, *Bgl*II; L, *Apa*LI. ***B***- Southern blot analysis. Human genomic DNA (lanes 1, 3 and 4) and λhAG-1 (lanes 2, 5 and 6) were digested with *Apa*LI/*Bgl*II (lanes 1 and 2), *Bgl*II (lanes 3 and 5), and *Bam*HI (lanes 4 and 6). Probe B was used for lanes 1 and 2; probe C was used for lanes 3-6. λDNA digested with *Hind*III served as a size marker (sizes in kilobases).

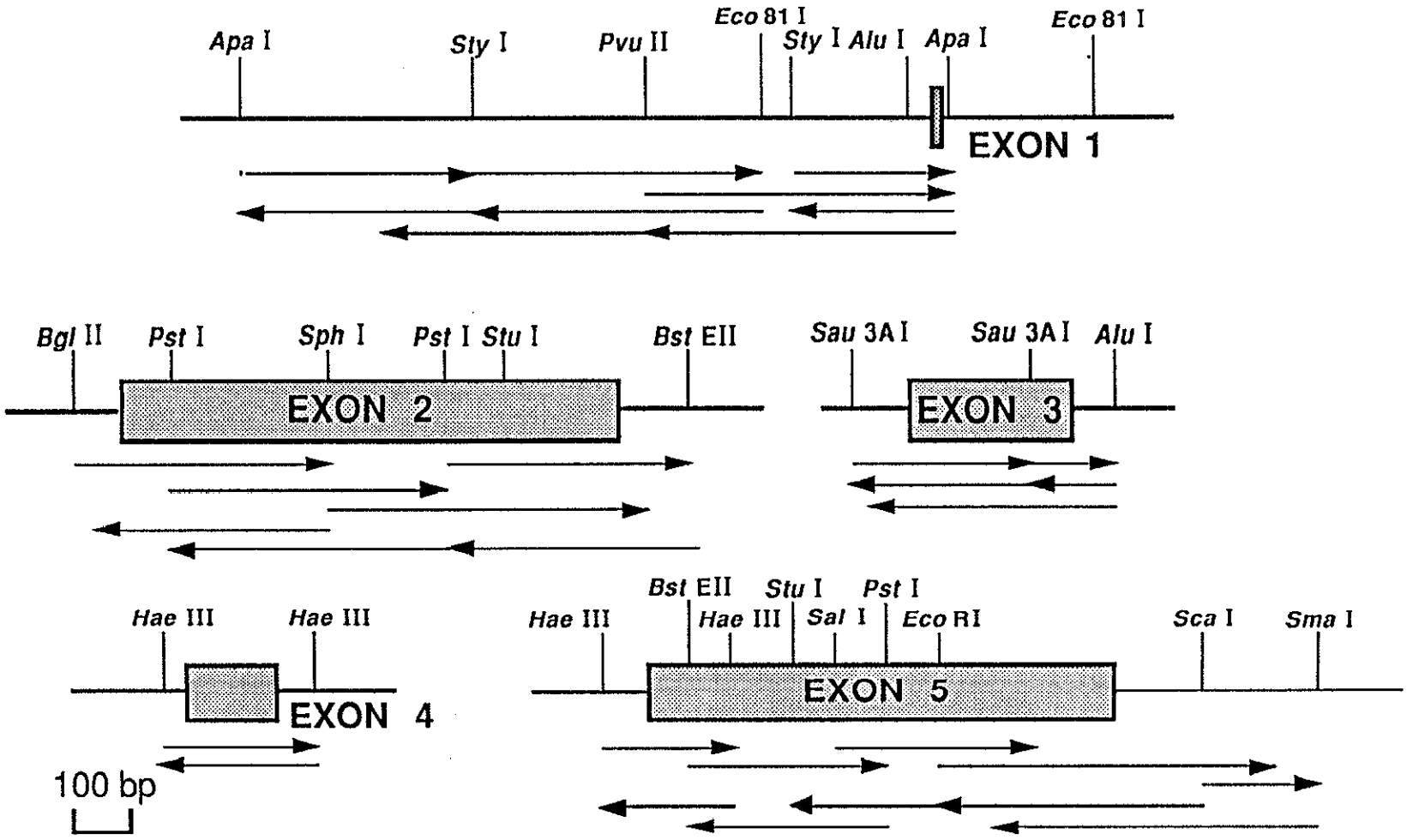
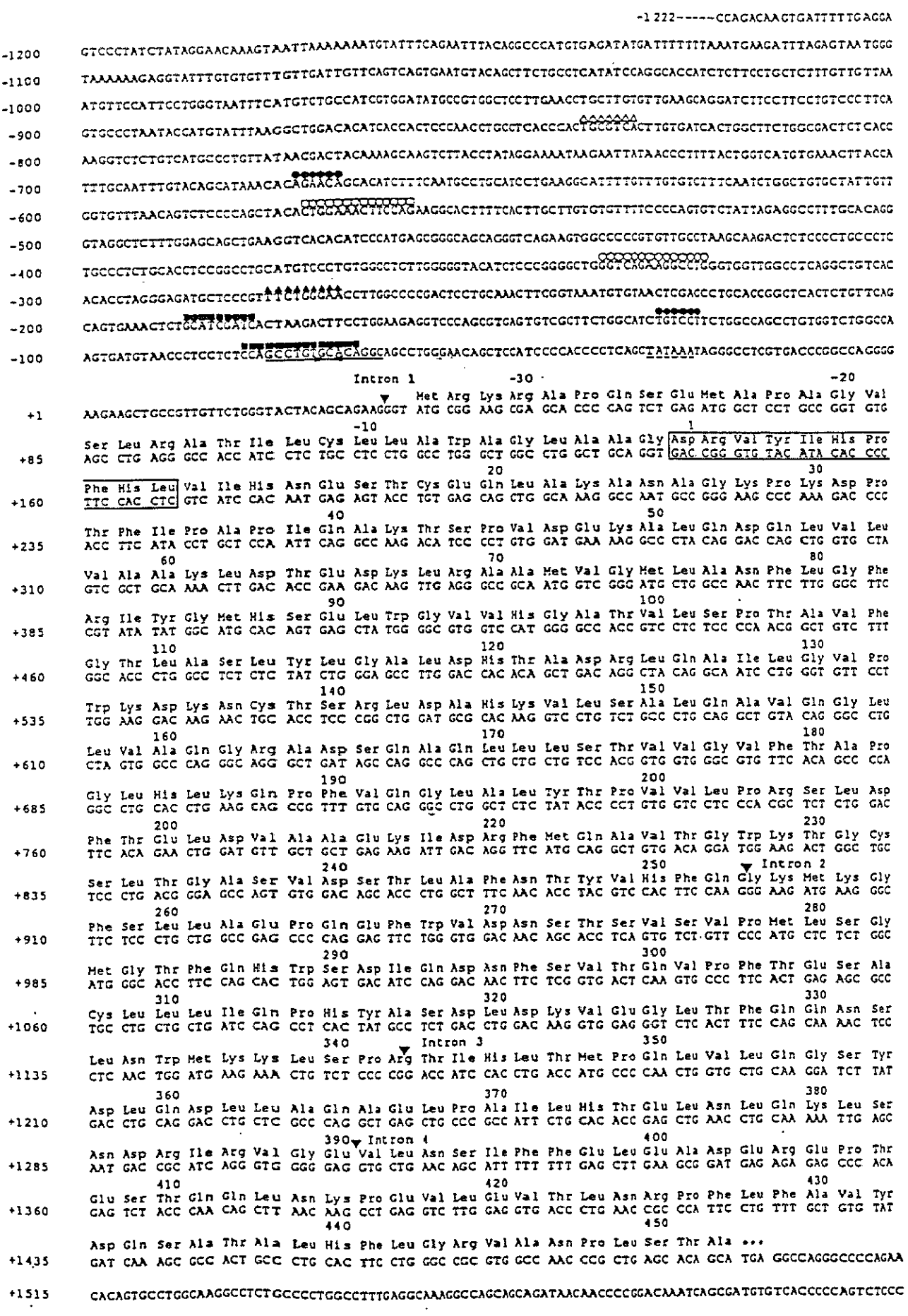


Fig. 3. Restriction map and sequencing strategy for 1.3 kb 5' to exon 1, the five exons with portions of the adjacent introns, and the 3'-flanking regions of the human angiotensinogen gene. Horizontal arrows indicate the direction and length of each fragment of DNA sequenced.



-1222-----CCAGACAAGTGATTTTTGAGGA

-1200 GTCCCTATCTATAGGAACAAAGTAATTAAAAAAATGTATTTCAGAATTTACAGGCCCATGTGAGATATGATTTTTTTAAATGAAGATTTAGAGTAATGGG

-1100 TAAAAAAGAGGTATTTGTGTGTTTGTTGATTGTTCAGTCAGTGAATGTACAGCTTCTGCCTCATATCCAGGCACCATCTCTTCCTGCTCTTTGTTGTTAA

-1000 ATGTTCCATTCCTGGGTAATTTCATGTCTGCCATCGTGGATATGCCGTGGCTCCTTGAACCTGCTTGTGTTGAAGCAGGATCTTCCTTCCTGTCCCTTCA

-900 GTGCCCTAATACCATGTATTTAAGGCTGGACACATCACCACTCCCAACCTGCCTCACCCACTGCCTCACTTGTGATCACTGGCTTCTGGCGACTCTCACC

-800 AAGGTCTCTGTCATGCCCTGTTATAACGACTACAAAAGCAAGTCTTACCTATAGGAAAATAAGAATTATAACCCTTTTACTGGTCATGTGAAACTTACCA

-700 TTTGCAATTTGTACAGCATAAACACAGAACAGCACATCTTTCAATGCCTGCATCCTGAAGGCATTTTGTTTGTGTCTTTCAATCTGGCTGTGCTATTGTT

-600 GGTGTTTAACAGTCTCCCCAGCTACACTGGAAACTTCCAGAAGGCACTTTTCACTTGCTTGTGTGTTTTCCCCAGTGTCTATTAGAGGCCTTTGCACAGG

-500 GTAGGCTCTTTGGAGCAGCTGAAGGTCACACATCCCATGAGCGGGCAGCAGGGTCAGAAGTGGCCCCCGTGTTGCCTAAGCAAGACTCTCCCCTGCCCTC

-400 TGCCCTCTGCACCTCCGGCCTGCATGTCCCTGTGGCCTCTTGGGGGTACATCTCCCGGGGCTGGGTCAGAAGGCCTGGGTGGTTGGCCTCAGGCTGTCAC

-300 ACACCTAGGGAGATGCTCCCGTTTCTGGCAACCTTGGCCCCGACTCCTGCAAACTTCGGTAAATGTGTAACTCGACCCTGCACCGGCTCACTCTGTTCAG

-200 CAGTGAAACTCTGCATCGATCACTAAGACTTCCTGGAAGAGGTCCCAGCGTGAGTGTCGCTTCTGGCATCTGTCCTTCTGGCCAGCCTGTGGTCTGGCCA

-100 AGTGATGTAACCCTCCTCTCCAGCCCTGTGCCAGGCAGCCTGGGAACAGCTCCATCCCCACCCCTCAGCTATAAATAGGGCCTCGTGACCCGGCCAGGGG

Intron 1 -30 -20

+1 AAGAAGCTGCCGTTGTTCTGGGTACTACAGCAGAAGGGT ATG CGG AAG CGA GCA CCC CAG TCT GAG ATG GCT CCT GCC GGT GTG
Met Arg Lys Arg Ala Pro Gln Ser Glu Met Ala Pro Ala Gly Val

-10 1

+85 AGC CTG AGG GCC ACC ATC CTC TGC CTC CTG GCC TGG GCT GGC CTG GCT GCA GGT GAC CGG GTG TAC ATA CAC CCC
Ser Leu Arg Ala Thr Ile Leu Cys Leu Leu Ala Trp Ala Gly Leu Ala Ala Gly Asp Arg Val Tyr Ile His Pro

20 30

+160 TTC CAC CTC GTC ATC CAC AAT GAG AGT ACC TGT GAG CAG CTG GCA AAG GCC AAT GCC GGG AAG CCC AAA GAC CCC
Phe His Leu Val Ile His Asn Glu Ser Thr Cys Glu Gln Leu Ala Lys Ala Asn Ala Gly Lys Pro Lys Asp Pro

40 50

+235 ACC TTC ATA CCT GCT CCA ATT CAG GCC AAG ACA TCC CCT GTG GAT GAA AAG GCC CTA CAG GAC CAG CTG GTG CTA
Thr Phe Ile Pro Ala Pro Ile Gln Ala Lys Thr Ser Pro Val Asp Glu Lys Ala Leu Gln Asp Gln Leu Val Leu

60 70 80

+310 GTC GCT GCA AAA CTT GAC ACC GAA GAC AAG TTG AGG GCC GCA ATG GTC GGG ATG CTG GCC AAC TTC TTG GGC TTC
Val Ala Ala Lys Leu Asp Thr Glu Asp Lys Leu Arg Ala Ala Met Val Gly Met Leu Ala Asn Phe Leu Gly Phe

90 100

+385 CGT ATA TAT GGC ATG CAC AGT GAG CTA TGG GGC GTG GTC CAT GGG GCC ACC GTC CTC TCC CCA ACG GCT GTC TTT
Arg Ile Tyr Gly Met His Ser Glu Leu Trp Gly Val Val His Gly Ala Thr Val Leu Ser Pro Thr Ala Val Phe

110 120 130

+460 GGC ACC CTG GCC TCT CTC TAT CTG GGA GCC TTG GAC CAC ACA GCT GAC AGG CTA CAG GCA ATC CTG GGT GTT CCT
Gly Thr Leu Ala Ser Leu Tyr Leu Gly Ala Leu Asp His Thr Ala Asp Arg Leu Gln Ala Ile Leu Gly Val Pro

140 150

+535 TGG AAG GAC AAG AAC TGC ACC TCC CGG CTG GAT GCG CAC AAG GTC CTG TCT GCC CTG CAG GCT GTA CAG GGC CTG
Trp Lys Asp Lys Asn Cys Thr Ser Arg Leu Asp Ala His Lys Val Leu Ser Ala Leu Gln Ala Val Gln Gly Leu

160 170 180

+610 CTA GTG GCC CAG GGC AGG GCT GAT AGC CAG GCC CAG CTG CTG CTG TCC ACG GTG GTG GGC GTG TTC ACA GCC CCA
Leu Val Ala Gln Gly Arg Ala Asp Ser Gln Ala Gln Leu Leu Leu Ser Thr Val Val Gly Val Phe Thr Ala Pro

190 200

+685 GGC CTG CAC CTG AAG CAG CCG TTT GTG CAG GGC CTG GCT CTC TAT ACC CCT GTG GTC CTC CCA CGC TCT CTG GAC
Gly Leu His Leu Lys Gln Pro Phe Val Gln Gly Leu Ala Leu Tyr Thr Pro Val Val Leu Pro Arg Ser Leu Asp

200 220 230

+760 TTC ACA GAA CTG GAT GTT GCT GCT GAG AAG ATT GAC AGG TTC ATG CAG GCT GTG ACA GGA TGG AAG ACT GGC TGC
Phe Thr Glu Leu Asp Val Ala Ala Glu Lys Ile Asp Arg Phe Met Gln Ala Val Thr Gly Trp Lys Thr Gly Cys

240 250 Intron 2

+835 TCC CTG ACG GGA GCC AGT GTG GAC AGC ACC CTG GCT TTC AAC ACC TAC GTC CAC TTC CAA GGG AAG ATG AAG GGC
Ser Leu Thr Gly Ala Ser Val Asp Ser Thr Leu Ala Phe Asn Thr Tyr Val His Phe Gln Gly Lys Met Lys Gly

260 270 280

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290 300

+985 ATG GGC ACC TTC CAG CAC TGG AGT GAC ATC CAG GAC AAC TTC TCG GTG ACT CAA GTG CCC TTC ACT GAG AGC GCC
Met Gly Thr Phe Gln His Trp Ser Asp Ile Gln Asp Asn Phe Ser Val Thr Gln Val Pro Phe Thr Glu Ser Ala

310 320 330

+1060 TGC CTG CTG CTG ATC CAG CCT CAC TAT GCC TCT GAC CTG GAC AAG GTG GAG GGT CTC ACT TTC CAG CAA AAC TCC
Cys Leu Leu Leu Ile Gln Pro His Tyr Ala Ser Asp Leu Asp Lys Val Glu Gly Leu Thr Phe Gln Gln Asn Ser

340 Intron 3 350

+1135 CTC AAC TGG ATG AAG AAA CTG TCT CCC CGG ACC ATC CAC CTG ACC ATG CCC CAA CTG GTG CTG CAA GGA TCT TAT
Leu Asn Trp Met Lys Lys Leu Ser Pro Arg Thr Ile His Leu Thr Met Pro Gln Leu Val Leu Gln Gly Ser Tyr

360 370 380

+1210 GAC CTG CAG GAC CTG CTC GCC CAG GCT GAG CTG CCC GCC ATT CTG CAC ACC GAG CTG AAC CTG CAA AAA TTG AGC
Asp Leu Gln Asp Leu Leu Ala Gln Ala Glu Leu Pro Ala Ile Leu His Thr Glu Leu Asn Leu Gln Lys Leu Ser

390 Intron 4 400

+1285 AAT GAC CGC ATC AGG GTG GGG GAG GTG CTG AAC AGC ATT TTT TTT GAG CTT GAA GCG GAT GAG AGA GAG CCC ACA
Asn Asp Arg Ile Arg Val Gly Glu Val Leu Asn Ser Ile Phe Phe Glu Leu Glu Ala Asp Glu Arg Glu Pro Thr

410 420 430

+1360 GAG TCT ACC CAA CAG CTT AAC AAG CCT GAG GTC TTG GAG GTG ACC CTG AAC CGC CCA TTC CTG TTT GCT GTG TAT
Glu Ser Thr Gln Gln Leu Asn Lys Pro Glu Val Leu Glu Val Thr Leu Asn Arg Pro Phe Leu Phe Ala Val Tyr

440 450

+1435 GAT CAA AGC GCC ACT GCC CTG CAC TTC CTG GGC CGC GTG GCC AAC CCG CTG AGC ACA GCA TGA GGCCAGGGCCCCAGAA
Asp Gln Ser Ala Thr Ala Leu His Phe Leu Gly Arg Val Ala Asn Pro Leu Ser Thr Ala ***

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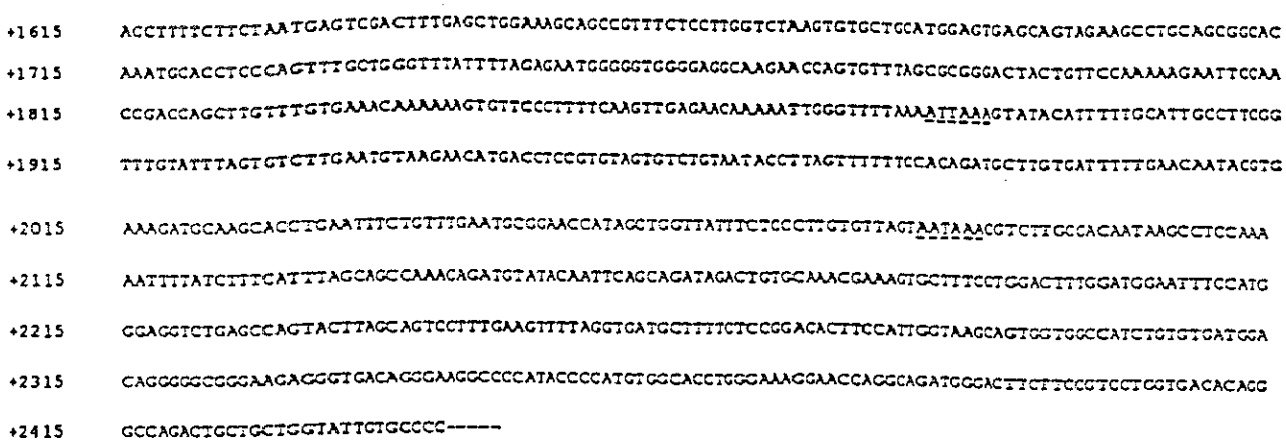

Fig. 4. Nucleotide sequence of the 5'- flanking region, all five exons, and the 3'- flanking region of the human angiotensinogen gene. The nucleotide sequence (second line) is shown with the deduced amino acid sequence (first line). Nucleotides are numbered at the left with the putative initiation site of transcription designated +1. Amino acid residues are numbered beginning with the first residue of angiotensinogen. The angiotensin I sequence is boxed with a solid line. Arrowheads indicate the positions where the four introns are inserted in the 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. Filled and open triangles indicate the acute phase- and the cAMP-responsive elements, respectively. Filled and open squares indicate the consensus sequences for RNA polymerase III promoters and the heat shock element, respectively. The 14-bp sequence with perfect dyad symmetry is indicated by arrows.

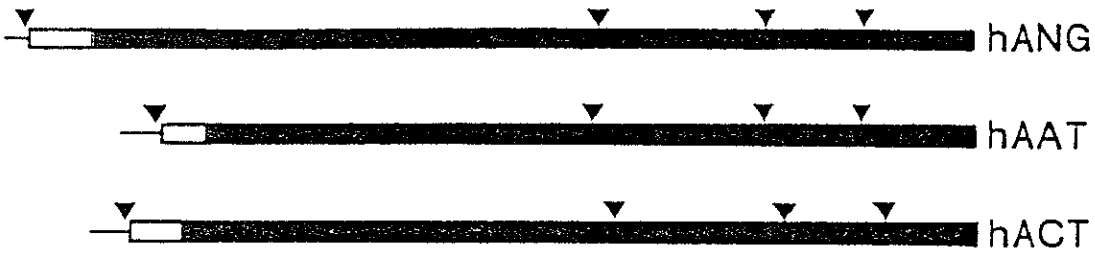

Fig. 5. Schematic representation of the structual organizations of the human angiotensinogen (hANG) gene, human α_1-antitrypsin (hAAT) gene, and human α_1-antichymotrypsin (hACT) gene. Horizontal lines indicate non-coding regions. The coding regions is shown shown by boxes. Open boxes indicate the positions of the signal peptides. The positions of introns is marked by arrowheads.

```
                Exon 1                  Intron 1                Exon 2
hANG ... TAC TAC AGC AGA AG gtaagccg ..... tttcag     GGT ATG CGG ...
                                                          Met Arg
rANG ... GGC TGG AGC TAA AG gtaagcag ..... ttccag     GAC ACA CAG ...

mANG ... GGT TGG CGC TGA AG gtaagcag ..... ttccag     GAC ACA CAG ...

hAAT ... TGG GAC AGT GAA TC gtaagtat ..... ttgcag   G ACA ATG CCG ...
                                                          Met Pro
hACT ... TCC CTG AGG CAG    gtaatcca ..... tcacag TTG AGA ATG GAG ...
                                                          Met Glu

                Exon 2                  Intron 2                Exon 3
hANG ... CAC TTC CAA G      gtaagg ..... tttcag  GG AAG ATG AAG ...
         His Phe Gln G                           ly Lys Met Lys
rANG ... CAC TTC CAA G      gtgagg ..... tttcag  GG AAG ATG AGA ...
         His Phe Gln G                           ly Lys Met Arg
mANG ... CAC TTC CAA G      gtgagg ..... tttcag  GA ACG ATG AGA ...
         His Phe Gln G                           ly Thr Met Arg
hAAT ... TTC TTT AAA G      gtaagg ..... ctccag  GC AAA TGG GAG ...
         Phe Phe Lys G                           ly Lys Trp Glu
hACT ... TTC TTT AAA G      gtgagt ..... cttcag  CC AAA TGG GAG ...
         Phe Phe Lys A                           la Lys Trp Glu

                Exon 3                  Intron 3                Exon 4
hANG ... CTG TCT CCC CG     gtagga ..... ccccag   G ACC ATC CAC ...
         Leu Ser Pro Ar                          g Thr Ile His
rANG ... CCG CCT CCT CG     gtagga ..... ccacag   G GCC ATC CGT ...
         Pro Pro Pro Ar                          g Ala Ile Arg
mANG ... CCG CCT CCT CG     gtagga ..... ccacag   G GCC ATC CGC ...
         Pro Pro Pro Ar                          g Ala Ile Arg
hAAT ... GAA GAC AGA AG     gtgatt ..... cagcag   A TCT GCC AGC ...
         Glu Asp Arg Ar                          g Ser Ala Ser
hACT ... CTG GAG TTC AG     gtgatt ..... ttctag   A GAG ATA GGT ...
         Leu Glu Phe Ar                          g Glu Ile Gly

                Exon 4                  Intron 4                Exon 5
hANG ... AGG GTG GGG GAG    gtatgt ..... ttgcag GTG CTG AAC AGC ...
         Arg Val Gly Glu                             Val Leu Asn Ser
rANG ... CGA GTG GGA GAG    gtgagt ..... ttgcag GTT CTC AAC AGC ...
         Arg Val Gly Glu                             Val Leu Asn Ser
mANG ... CGA GTG GGA GAG    gtgagt ..... ttgcag GTT CTC AAT AGC ...
         Arg Val Gly Glu                             Val Leu Asn Ser
hAAT ... AAG CTC TCC AAG    gtgaga ..... ctccag GCC GTG CAT AGG ...
         Lys Leu Ser Lys                             Ala Val His Lys
hACT ... GCA GTC TCC CAG    gtgagt ..... gacgag GTG GTC CAT AAG ...
         Ala Val Ser Gln                             Val Val His Lys
```

Fig. 6. Exon-intron junction sequences of hAAT, hACT, hANG, rANG, and mANG genes. Exon sequences are in capital letters; intron sequences are in lower case letters. hAAT, human α_1-antitrypsin; hACT, human α_1-antichymotrypsin; hANG, rANG, and mANG are human, rat, and mouse angiotensinogen, respectively.

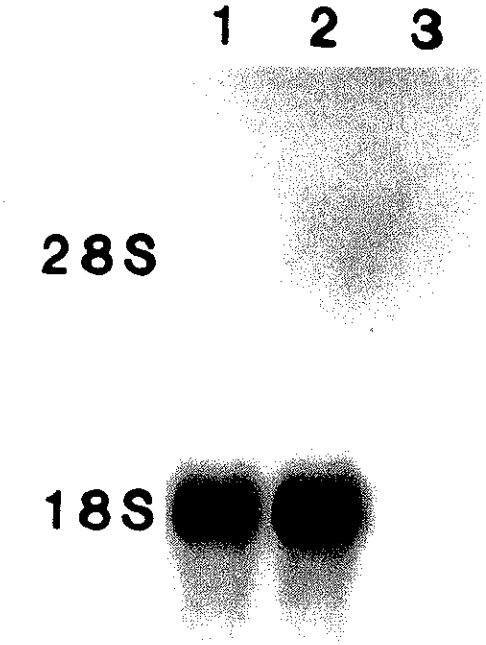

Fig. 7. Expression of human angiotensinogen mRNA. Total RNAs isolated from human liver, HepG2, and T98G cells were denatured and electrophoresed on a 1.2% agarose gel. After transfer to a GeneScreen Plus (nylon) membrane, the angiotensinogen mRNA was detected using probe C. Lane 1, human liver (2.5 µg); lane 2, HepG2 cells (2.5 µg); lane 3, T98G cells (25 µg). The internal size markers were 18 s and 28 s rRNA stained with ethidium bromide.

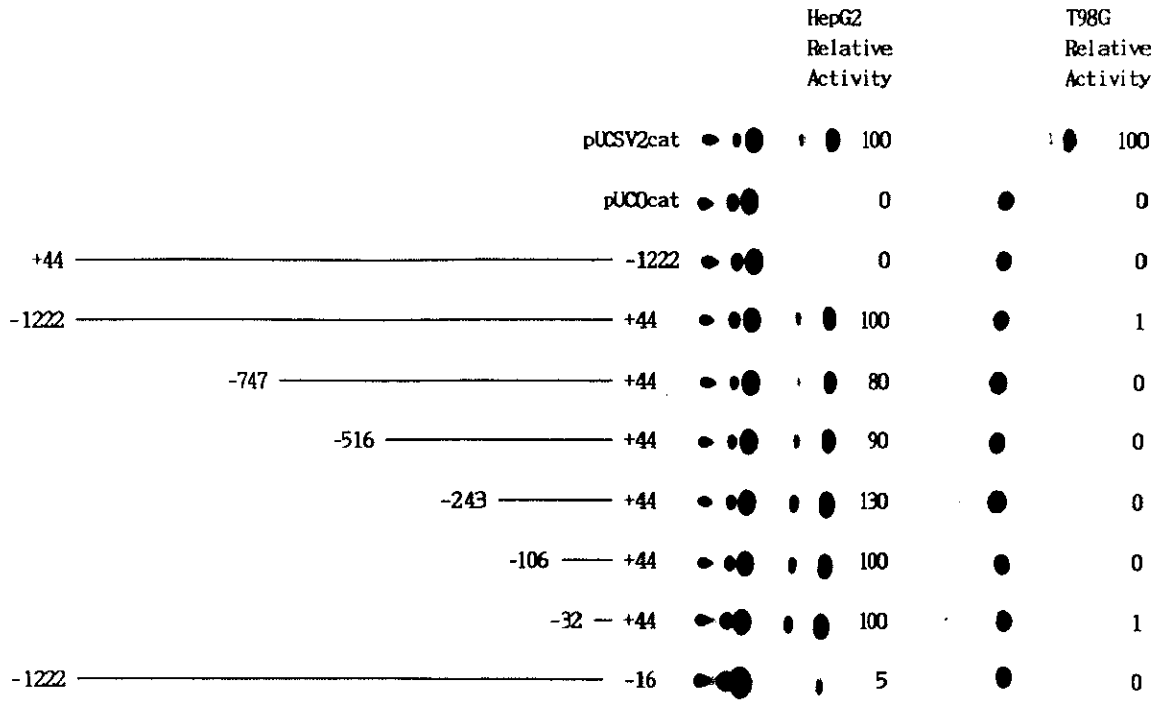

Fig. 8. Basal expression of the human angiotensinogen-chloramphenicol acetyltransferase hybrid genes in HepG2 and T98G cells. A series of plasmid containing deletions in the 5'- flanking region DNA were constructed and their activities in HepG2 and T98G cells determined as described under "Experimental 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.

Chapter III : Expression of the Human Angiotensinogen Gene in Human Cell Lines

Summary

The human angiotensinogen gene is expressed in liver and HepG2 cells derived from human hepatoma. To examine whether the angiotensinogen gene is expressed in extrahepatic cells, RNAs from kidney and several human cell lines have been isolated and analysed by Northern blot hybridization with the cloned gene as a probe. The mRNA for angiotensinogen was detected in human kidney and human glioblastoma (A172) cells. To investigate the endogenous regulation of angiotensinogen gene expression, HepG2 cells were cultured in the presence of 3-aminobenzamide, a specific inhibitor of poly (ADP-ribose) polymerase. The expression of the human angiotensinogen gene was demonstrated to be completely suppressed by 3-aminobenzamide (10mM).

Introduction

Angiotensinogen circulating in plasma is synthesized mainly by the liver, but messenger RNA (mRNA) for angiotensinogen has been localized in a variety of rat extrahepatic tissues such as adrenal, brain, kidney, large intestine, lung, ovary, spleen, and stomach (10,11). Regulation of the angiotensinogen gene expression has been well studied in H4IIE cells derived from rat hepatoma. It has been shown that these cells produce angiotensinogen mRNA and that its expression is regulated by glucocorticoid (12). In human, the angiotensinogen gene is also expressed in liver and a hepatoma cell line (HepG2). However, expression of the human gene in the other tissues or cell lines have not been identified and little has been known about endogenous and cellular regulation of the human angiotensinogen gene expression, because of limited availability of human tissues and the absence of appropriate cell lines. Thus, the discovery of extrahepatic tissues and cell lines which express angiotensinogen gene may be useful to study the biosynthesis and expression of that gene in extrahepatic tissues.

Covalent modification of proteins is important in regulating a number of cellular process. For example, phosphorylation of proteins is involved extensively in mammalian cell regulation (49). Less is known about the role of other types of modifications. One such modification is ADP ribosylation (50). Modification of chromatin proteins with polymers of ADP-ribose (51,52) by the DNA-dependent enzyme polyadenosine diphosphoribose (ADP-ribose) polymerase appears to be a ubiquitous mechanism that modulates chromatin structure (53, 54) in eukaryotic cells. Thus, the ADP-ribosylation of chromatin proteins has been suggested to play a biological role in diverse chromatin functions, *i.e.* DNA replication (55), gene expression (56-58), and repair to DNA damage (59-62).

In the present chapter, I investigated extrahepatic expression of the human

angiotensinogen gene, and examined the influences of changes in ADP-ribosylation on the endogenous expression of the human angiotensinogen gene in HepG2 cells using 3-aminobenzamide (63) as a probe. This compound is an inhibitor of poly (ADP-ribose) polymerase, the enzyme that catalyzes ADP ribosylation of chromosomal proteins (52).

Materials and Methods

(a) *Cell Culture*

HepG2, Chang liver (64), HT1080 (65), and T98G cell lines were maintained using minimum essential medium supplemented with 10% fetal bovine serum. The A172 cell lines (66) was maintained using Dulbecco's modified Eagle's medium with 10% bovine fetal serum. These cells were grown as a monolayers at 37°C with 5% CO_2 in air. One day before treatment, 1 x 10^6 cells were then exposed to 3-aminobenzamide or *p*-aminobenzonic acid in fresh medium for 24 h.

(b) *Northern Blot Analysis*

Total RNA from human liver, kidney, and several cell lines were denatured, electrophoresed on 1.2% agarose gel, and transferred to a GeneScreen Plus membrane (Dupont, Boston, MA, U.S.A.) as recommended by the manufacturer. Hybridization was carried out at 60°C for 16 h in 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 50% dextran sulfate, 150 mg/ml denatured salmon sperm DNA, and 1 x 10^6 cpm/ml labeled probe. THe membrane was washed in 2 x standard sodium citrate (SSC) (1 x SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0) and 1% SDS at 60°C and then 0.1 x SSC at room temperature. The DNA probe used was the second exon from the cloned human angiotensinogen gene (67). It was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham, Buckinghamshire, U.K.) by the random primer method.

Results

Angiotensinogen RNA sequences accumulate in liver, fat, and brain and have been detected, albeit in small amounts, in a variety of tissues including lung, kidney, ovary, adrenal grand, heart, spinal cord, and testes. Similarly, renin and its mRNA were found in these above tissues. Angiotensin II produced by organs independent of the circulating renin-angiotensin system is thought to be an important regulator of tissue function. Although the exact role of the tissue renin-angiotensin system is unclear, such system is of particular interest because of the potential importance of its local function. However, little information is available for the extrahepatic source of human angiotensinogen mRNA production.

To examine whether the human angiotensinogen gene is expressed in cells derived from extrahepatic tissues, RNAs from human liver, kidney, A-172, Chang liver, HepG2, HT1080, and T98G cells were isolated and analyzed by Northern hybridization. As shown in Fig. 9, the mRNA for angiotensinogen is detected in human kidney and A-172 cells derived from human glioblastoma in addition to human liver and HepG2 cells from human hepatoma. But, angiotensinogen mRNA could not be detected when 20 μg total RNA of T98G, Chang liver, and HT1080 cells were analysed.

To investigate the cellular regulation of angiotensinogen gene expression, I monitored changes in the amount of its mRNA in HepG2 cells treated with 3-aminobenzamide, which is a specific inhibitor of poly(ADP-ribose) polymerase and with *p*-aminobenzoic acid, which is structurally similar but is not an inhibitor of the polymerase. As demonstrated in Fig. 10B, a 24-h exposure of HepG2 cells to 10 mM 3-aminobenzamide resulted in complete suppression of the expression of angiotensinogen gene, although there was no effect on the expression with 1 mM 3-aminobenzamide. On the other hand, *p*-aminobenzoic acid had no significant effect on the gene expression.

Discussion

Recent interest has focused on the synthesis of angiotensinogen in the extrahepatic cells, where it may contribute to a local renin-angiotensin system (68). I obtained direct evidence that the human angiotensinogen gene is expressed in the kidney, the major site of renin synthesis. This result therefore adds support to the concept of an intrarenal renin-angiotensin system that may be important for regulation of local hemodynamics.

Of particular interest is the *de novo* synthesis of angiotensinogen in the rat brain (69). However, production of human angiotensinogen in the brain has not been studied because of limited availability of human brain and the absence of appropriate cell lines. I established that the cell line derived from human glioblastoma, i.e., A172 cells, produces angiotensinogen mRNA. Thus, this cell line may be useful as a brain model for studying the biosynthesis and gene expression of human angiotensinogen in the brain.

Cell nuclei contain poly(ADP-ribose) polymerase. This enzyme catalyzes ADP-ribosylation, one of the post-translational modifications of chromosomal proteins in eukaryotic cells (52). The involvement of ADP-ribosylated nuclear proteins in gene expression may be determined by inhibiting the polymerase reaction with a specific inhibitor, 3-aminobenzamide. ADP ribosylation has been shown to stimulate the gene expression of growth hormone and prolactin in GH_3 cells (70), mouse mammary tumor virus in 3T1 cells (71) and phosphoenolpyruvate carboxykinase in H4IIE cells (72). In contrast, the findings of the human angiotensinogen gene in HepG2 cells is suppressed by 3-aminobenzamide. Therefore, it is likely that the down regulation of the expression of this gene is controlled by an ADP-ribosylation pathway different from that of the other genes that have been examined using 3-aminobenzamide as a probe.

In summary, the findings presented herein indicate that the human

angiotensinogen gene is expressed in the extrahepatic cells such as kidn and A-172, and that the gene expression is suppressed when poly(ADP-ribos polymerase is inhibited.

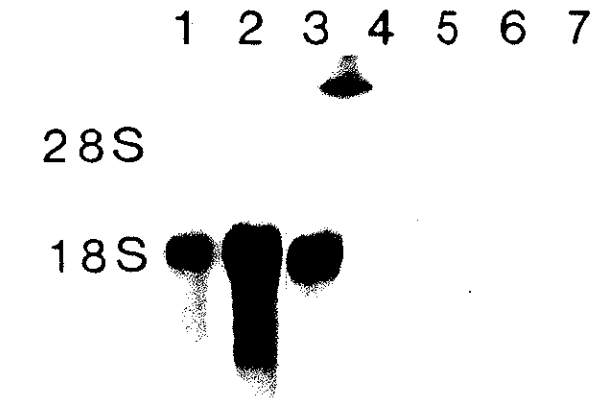

Fig. 9. Expression of the human angiotensinogen mRNA. Total RNAs were subjected to Northern hybridization. Lane 1, human liver (5 μg); lane 2, HepG2 (10 μg); lane 3, A172 (20 μg); lane 4, T98G (20 μg); lane 5, Chang liver (20 μg); lane 6, human kidney (20 μg); and lane 7, HT1080 (20 μg). Lane 1 and 2 were exposed for 16 h, and lanes 3 to 7 were for 96 h at -70°C. The internal size markers were 18 S and 28 S rRNAs stained with ethidium bromide.

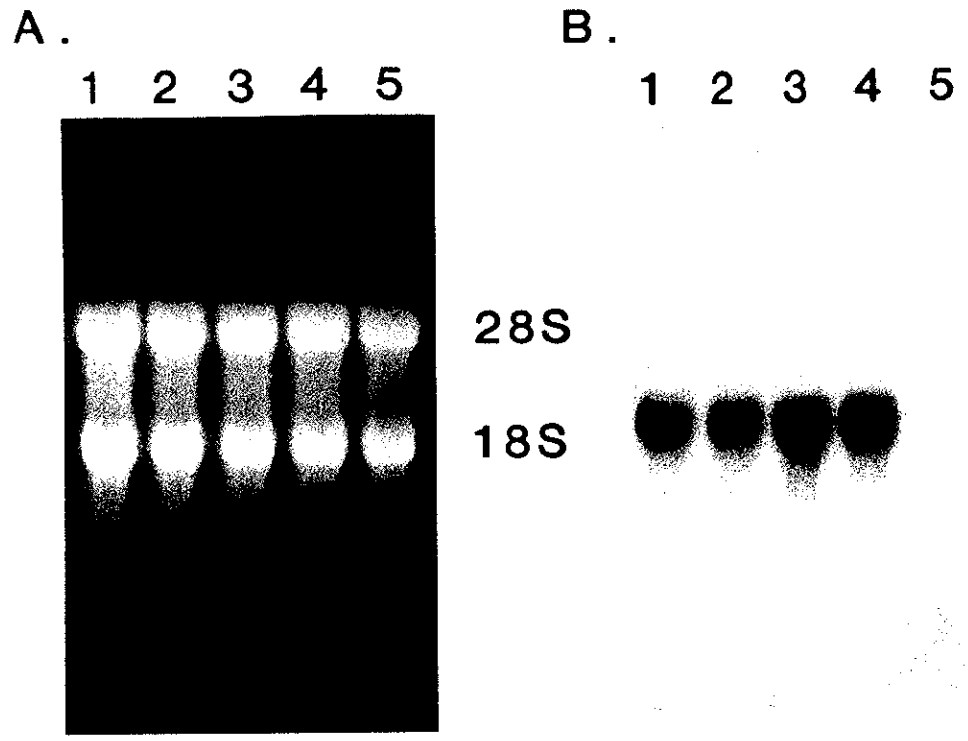

Fig. 10. Effect of 3-aminobenzamide and *p*-aminobenzoic acid on human angiotensinogen gene expression in HepG2 cells. ***A***- This panel shows an ethidium bromide stain (rRNA) of the samples (20 µg/lane) used for Northern hybridization. ***B***- Cells were cultured in the absence (lane 1) and in the presence of 1 mM (lane 2) and 10 mM (lane 3) *p*-aminobenzoic acid as well as 1 mM (lane 4) and 10 mM(lane 5) 3-aminobenzamide for 24 h. Total RNAs from these cells were subjected to Northern hybridization. The positions of 28S and 18S rRNA are indicated.

Chapter IV : Expression of the Human Angiotensinogen Gene in Transgenic Mice and Transfected Cells

Summary

I have generated two lines of transgenic mice with integrated copies of a 14-kilobase pair (kb) human DNA fragment containing the angiotensinogen gene, which includes 1.3 kb of 5'- and 3'-flanking regions. In both transgenic lines, the introduced human gene was able to confer tissue specificity and a considerable quantity of the correctly initiated and processed angiotensinogen mRNA was accumulated in the liver. Notably, mRNA for the transgene was detectable in the kidney, where is normally the minor source of angiotensinogen, to levels comparable to that in the liver. The transient transfection analyses showed that chloramphenicol acetyltransferase (CAT) transcription from several constructs with DNA segments of the 14-kb transgene cloned into downstream of the CAT gene under control of the 1.3-kb angiotensinogen promoter is all activated in human hepatoma HepG2 and human embryonic kidney 293 cells but not in human glioma T98G cells. These *in vivo* and *in vitro* experiments suggested that the 1.3-kb 5'-flanking sequences are essential for expression of the angiotensinogen gene in hepatic and renal cells and that the microinjected DNA lacks key control elements that normally function to repress the gene in renal cells.

Introduction

Human angiotensinogen, a glycoprotein consisting of 452 amino acids (1), is synthesized predominantly in the liver and secreted into the circulation. By Northern blot analysis, in the previous chapters, I have shown that a considerable amount of angiotensinogen mRNA is detected in human liver and human hepatoma HepG2 cells (67) and its mRNA is also detectable but very low in human kidney (73). The human angiotensinogen gene consists of five exons interrupted by four introns (67,74) and maps to chromosome 1 close to the human renin gene within the region of 1q4 (75). The structure of the human angiotensinogen gene, as well as that of the rat gene, with respect to the number and location of the introns is similar to that found in genes for human α_1-antitrypsin and α_1-antichymotrypsin, which are classified as serine proteinase inhibitor family (4). In a transient expression assay, I have also shown that the 1.3-kb 5'-flanking region of the gene can function as a cell-specific promoter (67).

The main objective of the current study was to investigate whether a 14-kb DNA fragment containing the angiotensinogen gene conferred proper tissue-specific expression. For this purpose, I have generated and analyzed two lines of transgenic mice carrying the 14-kb gene construct and examined transcriptional activity of the gene segments of the construct by *in vitro* transfection analysis.

(a) *Generation of transgenic mice*

The 14-kb *Nhe*I DNA fragment containing the human angiotensinogen gene was separated from pUC19 and purified from phAGTM14 by sucrose gradient velocity centrifugation. Microinjection and oviduct transfer procedures were done as described (76). Embryos for microinjection were derived from C57BL/6. Offspring were analyzed for the presence of the injected fragment by Southern blot analysis of tail DNA. Transgenic lines were established by mating to either male and female mice.

(b) *Preparation of probes*

A 1.1-kb *Bam*HI/*Xba*I fragment (probe A) excised from phAG55B (67) and a 413-bp RsaI fragment (probe B) from phAG27B (67) were used for Southern blot analysis. A 298-bp *Apa*I/*Eco*RI fragment (human probe) from pHag3 (1) and a 229-bp *Nco*I/*Cfr*13I fragment (rat probe) from pRag16 (15) were used for detecting the transgenic human mRNA and endogenous mouse mRNA, respectively, on Northern blot analysis. These probes were labeled with [α-^{32}P]dCTP by the random primer method (25).

(c) *Southern blot analysis*

Genomic DNAs from human hepatoma HepG2 and transgenic mice were digested with a variety of restriction enzymes, electrophoresed on 0.7% agarose gels and transferred to GeneScreen Plus membrane (Dupont-New England Nuclear). These blots were hybridized with probe A or B as described previously (67).

(d) ***Preparation of RNA and Northern blot analysis***

Total RNAs isolated from HepG2, 293, and T98G cells or various tissues of transgenic and nontransgenic mice by the guanidinium-cesium chloride method (29) were denatured, electrophoresed on a 1.2% agarose gel and transferred to GeneScreen Plus membrane. Hybridization was carried out using ^{32}P-labelled DNA probe as described (67).

(e) ***Primer extension analysis***

Primer extension analysis was performed using a 20-residue oligonucleotide (5'-dGTACTCTCATTGTGGATGAC-3') synthesized complementary to residues 169 to 188 of human angiotensinogen mRNA (1). After 5'-end labeling, the oligonucleotide was used for primer extension with 4.5 μg of poly(A)+RNA.

(f) ***Plasmid constructions***

pUChAGcat13 was constructed by inserting the 135-bp HpaI-BamHI DNA fragment containing SV40 early polyadenylation signals immediately 5' to the 1.3-kb 5'-flanking sequences of pUChAG(-1222)cat (67). The unique BamHI site 3' of the chloramphenicol acetyltransferase (CAT)-coding domain of pUChAGcat13 was used as an insertion site for the various fragments. The 5.5-kb BamHI DNA fragment (fragment A) was inserted directly in this site, whereas BamHI linkers were used for the insertion of the 6.0-kb BglII-ClaI (fragment B) and 3.8-kb ClaI-BglII (fragment C) fragments with non-complementary restriction sites. Using the resulting fragments, we constructed pUChAGcat13A, pUChAGcat13B and pUChAGcat13C.

(g) ***Cell culture, DNA transfection, and CAT assay***

HepG2 and T98G cells were maintained in minimum essential medium (SIGMA) containing 10% fetal bovine serum and nonessential amino acids. 293 cells were maintained in minimum essential medium (SIGMA) containing 10% heat-inactivated horse serum.

Approximately 5 x 10^5 cells were transfected with 5.2 pmol of plasmid DNA/dish by the calcium phosphate precipitation technique (77). The total amount of DNA was maintained constant at 4 μg by the addition of pUC19. Cells were harvested after 36 h, and extracts were prepared and assayed for CAT activity by standard methods (32).

Results

(a) *Generation of transgenic mice*

Transgenic mice were produced by transferring the 14-kb genomic DNA fragment encompassing the human angiotensinogen gene with the 1.3-kb 5'- and 3'-flanking regions (Fig. 11A) into the male pronuclei of mouse (C57BL/6) fertilized eggs. The DNA from tails of potentially transgenic mice were screened by Southern blot analysis using a human angiotensinogen probe. Of 29 live offspring, one female (hAG2-5) and one male (hAG3-2) were shown to carry the transgene (data not shown). The intensity of bands corresponding to the transgene compared with those corresponding to the cloned gene for copy number suggested that in the founder animals, 100 copies or more of the transgene were present. Both founder animals were used in generating transgenic mouse lines (Fig. 12).

(b) *Structure of human angiotensinogen gene in transgenic mice*

In order to examine whether the integrated human gene is rearranged in the mice, by Southern blot hybridization I analyzed genomic DNAs from two lines of transgenic mice and HepG2 cells which express the angiotensinogen gene (67). As displayed in Fig. 11, the DNA from the three samples showed an identical pattern of the hybridization. The introduced human gene is, therefore, not deleted from both transgenic mouse genomes and appears not to be grossly rearranged, at least within the limits of resolution of this analysis.

(c) *Preparation of differential DNA probes*

In order to distinguish the mouse angiotensinogen mRNA sequence from the human one in the transgenic mice by Northern blot analysis, I have first prepared the differential DNA probes. The DIAGON computer program (78) was used to compare the cDNA sequences of the human and rat which is highly

homologous to mouse one (1, 15, 16) and to obtain a clear representation of the pattern of homology (Fig. 13). As shown by a long main diagonal lines of dots, quite striking homology commences at the start sites of transcription and continue to nucleotide position at 1470; thereafter, a dramatic reduction in sequence homology is observed, as indicated by arrowheads. I, therefore, chose and excised the DNA regions for hybridization probes from the rat and human angiotensinogen genes; 229-bp *Nco*I-*Cfr*13I and 298-bp *Apa*I-*Eco*RI DNA fragments for mouse and human, respectively.

(d) *Expression of human angiotensinogen gene in transgenic mice*

In order to determine the tissue specificity of the human angiotensinogen gene expression in hAG2-5 and hAG3-2 mouse lines, the level of RNA in various tissues was analyzed by Northern hybridization with the human-specific angiotensinogen probe. As judged by comparison with human HepG2 RNA, the expected 2-kb RNA for the human transgene was highest in liver of both mouse lines (Fig. 14A) and generated, at least predominantly, from the normal human angiotensinogen transcriptional site (Fig. 15). These results suggested that initiation of the transgene transcription, processing of the nuclear precursor mRNA and polyadenylation are performed correctly in the mice and that the 1.3-kb 5'-flanking sequence of the transgene can be utilized as an *in vivo* promoter for its efficient expression.

Surprisingly, a significant quantity of human angiotensinogen mRNA were found in transgenic kidney, although levels of mouse angiotensinogen were very low in kidney (Fig. 14B) as well as in human (73). In addition, mRNA for the transgene was detectable in heart. Lower levels of the human transcripts were also seen in every tissue of the two transgenic lines upon prolonged exposure of these autoradiograms. The expression pattern was almost identical in the different mouse lines, implying that expression of the transgene occurs independently of the integration site.

To compare expression of the endogenous mouse angiotensinogen gene between transgenic and nontransgenic mice, I have probed the RNA from these mice with a rat specific-angiotensinogen DNA. As shown in Fig.14B, the pattern of expression was identical in these three mice. This suggested that the transfer of new genes into mice did not disturb the normal regulation of the mouse angiotensinogen gene.

(e) *Expression of human angiotensinogen gene in tissue cultur cells*

Although a number of possible explanations could account for the lack of down regulation of the human angiotensinogen gene in kidney of the transgenic mice, it could be that suppression of the angiotensinogen gene expression in kidney is maintained by negative regulatory sequences that were missing in the microinjected DNA fragment. To examine this possibility, we have chosen human hepatoma HepG2, human embryonic kidney 293 and human glioma T98G cells, as in vitro expression systems, in which the angiotensinogen gene was shown not to be deleted or rearranged by Southern blot analysis (data not shown). The three subfragments (A, B and C fragments) were tested for their capacity following cloning into the BamHI site 3' of pUChAGcat13 that contains 1.3 kb of the 5'-flanking sequences, as a functional promoter, of the human angiotensinogen gene (Fig. 16B and C) and the resulting CAT expression constructs were transiently transfected into the three cell lines. As shown in Fig. 16C, all constructs yielded efficient expression of CAT activity in human embryonic kidney 293 cells despite lack of expression of the endogenous angiotensinogen gene (Fig. 16A). High level of CAT activity was also observed in HepG2 cells, albeit at different levels in each, whereas these constructs were essentially inactive in T98G cells. Also, it is of interest to note that the C fragment is capable of activating CAT activity by 2.4 folds only in

HepG2 cells, as compared with pUChAGcat13. These results suggested that the 1.3-kb upstream sequences are important for efficient expression of the human angiotensinogen gene in hepatic and renal cells but that the injected 14-kb construct lacks a domain responsible for the down regulation of the gene in renal cells.

Discussion

Two characteristics of expression of the human angiotensinogen gene after transfer into mice provide insight into the regulation of this gene. First, the human gene with the correct transcription initiation was expressed at highest levels in transgenic liver and at much lower levels in other tissues except for kidney. Second, we found the high levels of mRNA for the transgene in kidney, where the endogenous angiotensinogen mRNA was usually accumulated at trace levels in both mouse and human (73).

Many genetic elements show tissue-specific expression in transgenic mice but are frequently subjected to ectopic production and are transcribed to relatively low levels. In fact, anomalous expression of rabbit globin genes has been observed in mouse skeletal muscle and testis, presumably because the rabbit gene integrates within a region of chromatin normally "open" for transcription in those tissues (79). However, the level of expression was very low, and it was observed in only one of nine lines generated. In our mice, such an explanation is unlikely since both hAG2-5 and hAG3-2 lines expressed the human angiotensinogen gene in kidney at levels to comparable to that in liver.

Another way to explain this aberrant expression in kidney is that the human gene is separated from a *cis*-acting negative regulatory element during cloning. These elements have been detected in many eukaryotic genes (80-83) and they play an important role in tissue-specific and developmentally regulated expression of the genes (84,85). For example, in transgenic mice carrying the apolipoprotein E gene, which is predominantly expressed in liver, it has been reported that the human gene is expressed in transgenic kidney at higher than in the liver (86,87) and demonstrated that the 23-kb additional regulatory region downstream of the gene is required for its down regulation in the kidney (86). In the case of human α_1-antitrypsin gene, the transgene has also shown to be expressed in kidney at appreciable levels in addition to liver

(88-90). My present *in vitro* experiments using human embryonic kidney 293 cells indicated that transcription from the transiently introduced angiotensinogen-CAT fusion genes was highly active, nevertheless the chromosomal angiotensinogen gene was silent in this cell line. Therefore, together with the above observations, the results presented in this report may support our initial idea that the lack of the down regulation of the introduced angiotensinogen gene in transgenic and cultured kidney cells results from missing a negative regulatory element in the 14-kb constructs.

In summary, I have generated transgenic mice with high levels of hepatic and renal expression of the human angiotensinogen gene. My current study indicates that the 1.3-kb 5'-flanking sequences can function as *in vivo* and *in vitro* promoters and suggests that the promiscuous expression of the human angiotensinogen gene in renal cells results from a lack of negative regulatory region within the 14-kb DNA fragment used for the *in vivo* and *in vitro* introduction. The unexpected expression of the transgene in kidney may thus shed new light upon a novel regulation of the human angiotensinogen gene. Further study will be required for identifying the putative negative regulatory region by cloning extended flanking regions and generating additional transgenic mouse lines.

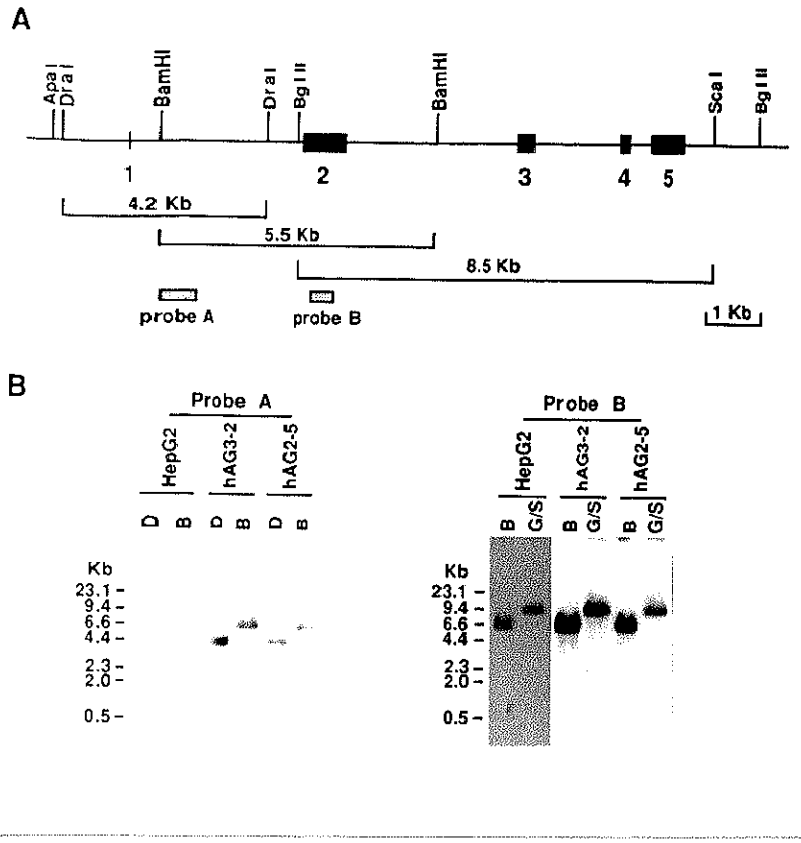

Fig. 11. Southern blot analysis of human angiotensinogen gene. ***A***- Partial restriction map and relevant restriction sites of the human angiotensinogen gene. The predicted genomic DNA fragments hybridized with probe A or B are shown. Restriction sites are indicated as follows: ***B***- *Bam*HI; D, *Dra*I; G, *Bgl*II and S, *Sca*I. B- Southern blot analysis. Genomic DNA isolated from HepG2 cells and transgenic mice (hAG2-5 and hAG3-2) was digested with *Bam*HI (B), *Dra*I (D) and *Bgl*II (G)/*Sca*I (S) as indicated and electrophoresed on a 0.7% agarose gel. After transfer to GeneScreen Plus membrane, blots were hybridized to ^{32}P-labelled probe A or B. DNA digested with *Hind*III served as a size marker (sizes in kb).

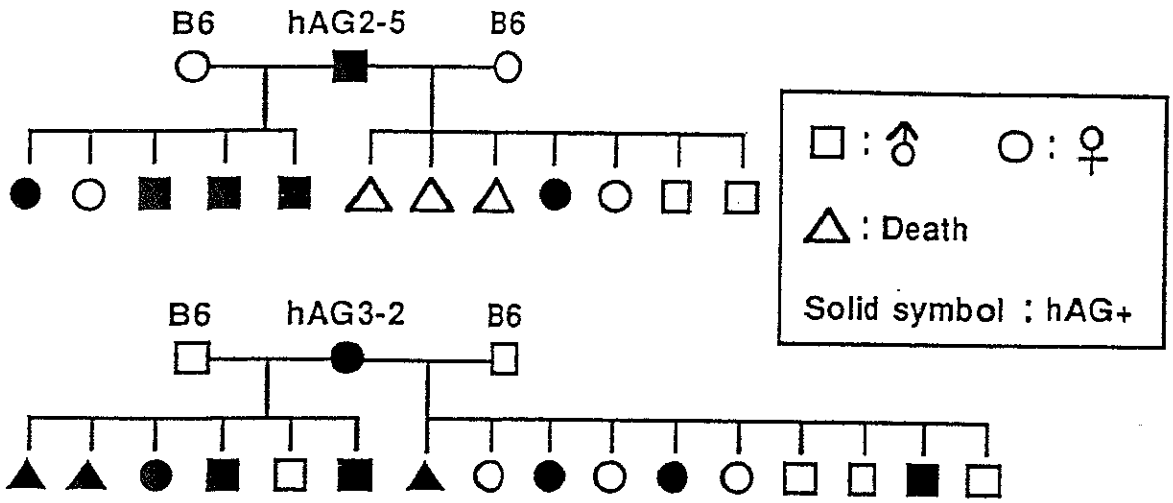

Fig. 12. Pedigree chart of the transgenic mice harboring the human angiotensinogen gene. Females are indicated by circles, males by squares, neonatal death by triangles and solid symbols denote transgenic mice.

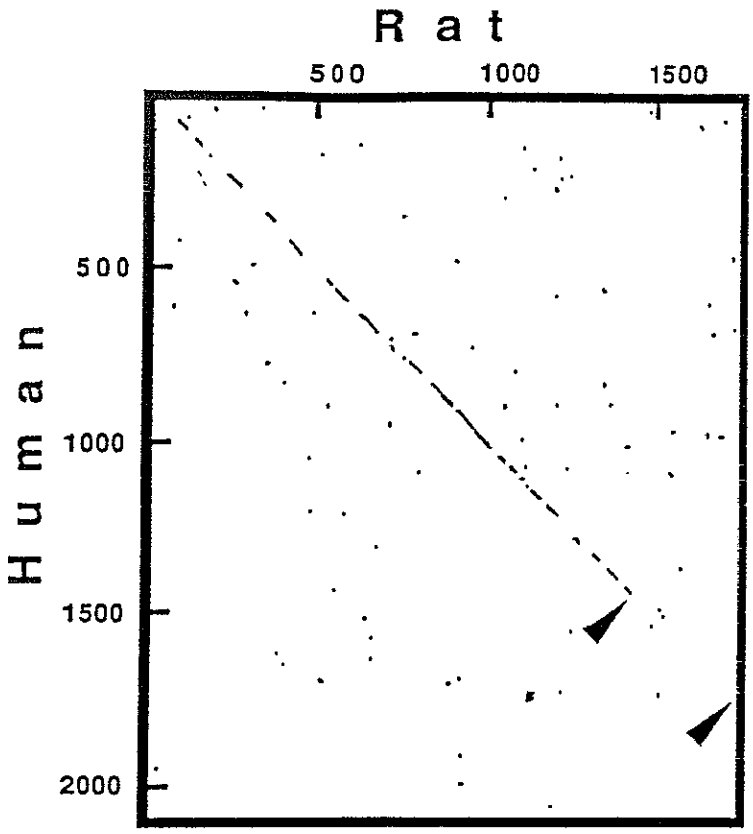

Fig. 13. Dot matrix comparison of the cDNA sequences of rat (horizontal axis) and human (vertical axis) angiotensinogens. The numbering along the axes corresponds to the nucleotide position in the cDNAs. The rat sequence was aligned for maximal match with the human sequence using the DIAGON program. The parameters used were a span length of 15 and a proportional matching score of 12. Diagonal lines indicate homologous regions. The DNA regions used for Northern blot analysis as the differential probes are shown by arrowheads.

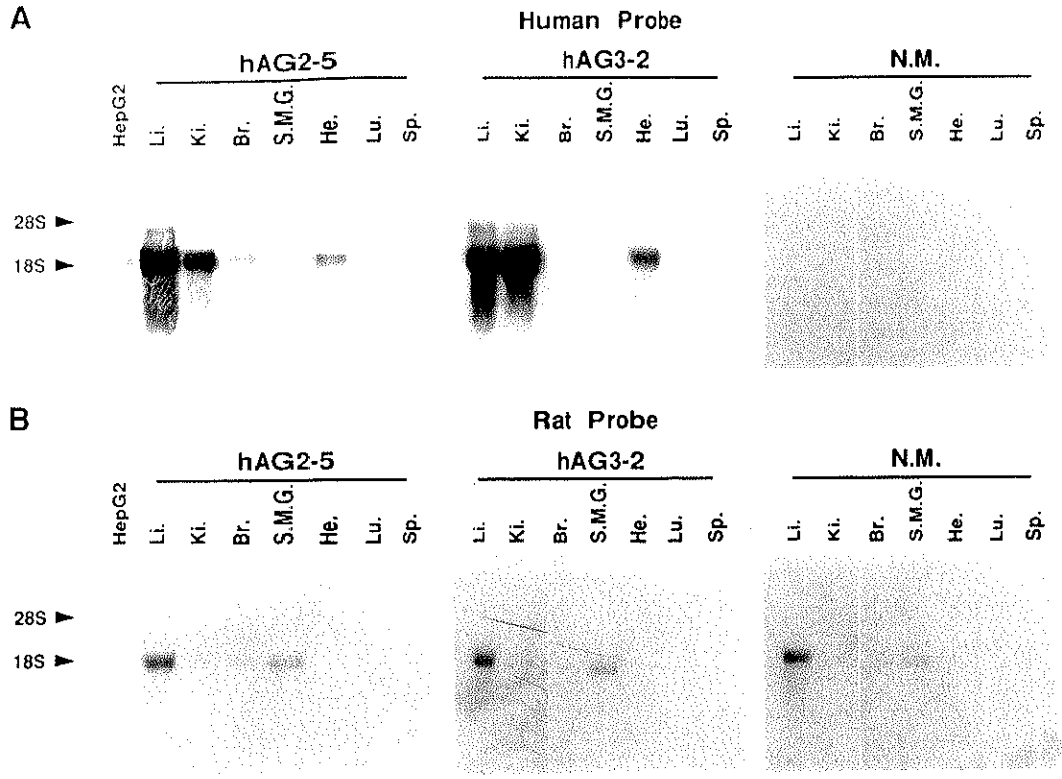

Figure 14. Tissue-specific expression of human angiotensinogen transgene. Total RNA (4 µg/lane) form various tissues of transgenic and nontransgenic mice or HepG2 cells were denatured and electrophoresed on a 1.2% agarose gel. After transfer to GeneScreen Plus membrane, the angiotensinogen mRNA was detected using human (A) and rat (B) angiotensinogen probes. The tissues analyzed were as follows: liver (Li), Kidney (Ki), brain (Br), submandibular gland (SMG), heart (He), lung (Lu), and spleen (Sp). The internal markers were 18S and 28S rRNA stained with ethidium bromide.

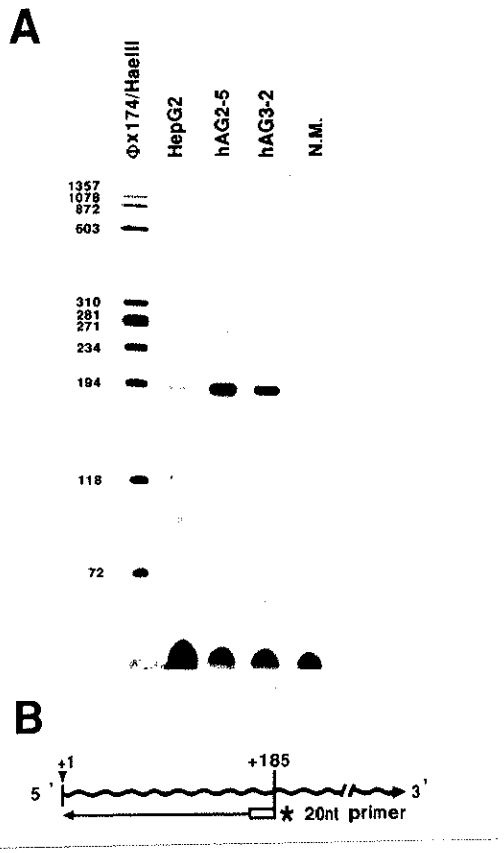

Figure 15. Correct initiation of human angiotensinogen gene in transgenic mice. The 5' end-labeled oligonucleotide primer for the human angiotensinogen mRNA was hybridized, at 55°C for 1 h, with 4.5 µg of poly(A)+RNA from liver of the transgenic (hAG2-5 and hAG3-2) and nontransgenic (NM) mice or HepG2 cells and then extended by Rous associated virus (RAV-2) reverse transcriptase at 37°C for 45 min. ***A***- Autoradiograph of the primer-extended cDNA. The extended cDNA was analyzed on a 5% denaturing polyacrylamide gel followed by autoradiography. Size markers (in nucleotides) were derived from ϕX174 digested with *Hae*III. ***B***- Schematic representation of the primer extension analysis. The asterisk denotes the labeling position; the box represents the primer.

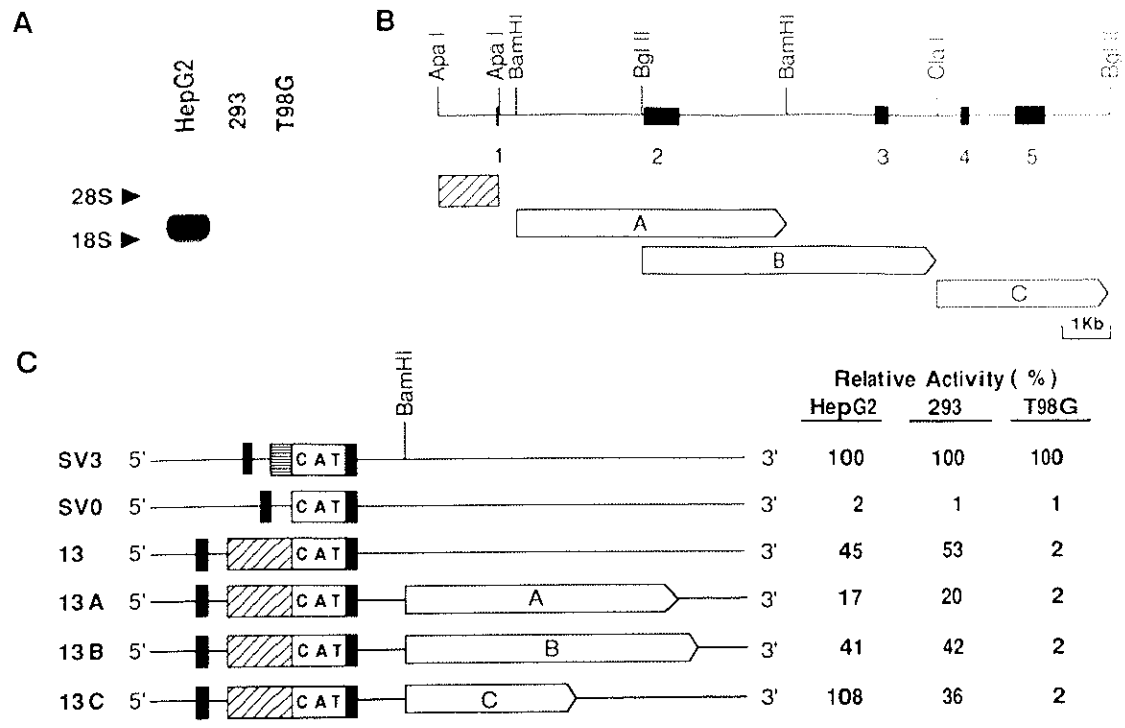

Figure 16. Expression of human angiotensinogen promoter-directed CAT gene in cultured cell lines. ***A***- Northern blot analysis. Total RNAs from HepG2 (5 µg), 293 (20 µg), and T98G (20 µg) were denatured, electrophoresed on a 1.2% agarose gel, and transferred to GeneScreen Plus membrane. Hybridization was carried out as described previously (2). ***B***- Structure of the human angiotensinogen gene and the gene segments (A, B and C) for subcloning into pUChAGcat13. The black boxes represent exons; their numbers are given. The hatched box indicates the 1.3-kb upstream region. ***C***- CAT activities of gene segments under control of the 1.3-kb upstream region (hatched box). The values in the columns on the right represent the means of results that were obtained from four separate experiments. Activities are expressed relative to those achieved with pUCSV3cat under control of SV40 early promoter (horizontal hatched box) (positive control) as 100% in each cell line. The black boxes indicate SV40 polyadenylation signals.

Concluding Remarks

The application of recombinant DNA techniques has led to new directions for the study of the angiotensinogen gene. In order to analyze regulatory phenomena at the transcriptional level, I cloned and sequenced the gene for human angiotensinogen. Sequence analysis reveals that the gene is 12-kb long and consists of five exons and four introns, and indicates a conservation of the gene structure as compared to the rat angiotensinogen gene and the human α_1-antitrypsin gene, α_1-antichymotrypsin gene, and the presence of nucleotide sequence involved in the regulation by steroid hormones and so on in the 5' flanking region of the gene.

As a first approach to understand expression of the human angiotensinogen gene, I examined the basal regulation of the gene expression in hepatic cell line, and determined the 76-bp region from -32 to +44 containing the TATA box and first exon required for its cell-specific expression of the gene.

Although angiotensinogen is synthesized mainly in the liver, mRNA for angiotensinogen has been localized in variety of rat extrahepatic tissues such as adrenal, brain, kidney,large intestine, lung, ovary, spleen, and stomach. Thus, I examined the extrahepatic expression of human angiotensinogen gene by Northern blot hybridization with the cloned gene as a probe. The mRNA for angiotensinogen was detected in human kidney and human glioblastoma (A172) cells. Of particular, A172 may be useful as a brain model for studying the biosynthesis and gene expression of human angiotensinogen.

Furthermore, to examine the regulation of the human angiotensinogen gene *in vivo*, I have generated two lines of transgenic mice harboring the 14-kb human angiotensinogen gene, which includes 1.3 kb of 5' and 3'-flanking regions. The transgene was expressed predominantly in the liver. Notably, mRNA for the transgene was detectable in the kidney, where is normally the minor source of angiotensinogen, to levels comparable to that in liver. The

additional transfection analyses showed that transcription of the CAT gene under control of the 1.3-kb angiotensinogen gene promoter is all activated in human hepatoma HepG2 and human embryonic kidney 293 cells, demonstrating that the 1.3-kb upstream sequences play a regulatory role in liver and kidney cells.

These *in vivo* and *in vitro* experiments suggest that the 1.3-kb 5'- flanking sequences are essential for expression of the angiotensinogen gene in hepatic and renal cells and the microinjected DNA lacks key control element that normally function to repress the gene in renal cells.

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