Molecular Cloning of Human Angiotensinogen cDNA and Evidence for the Presence of Its mRNA in Rat Heart

Satya P. Kunapuli and Ashok Kumar

Human angiotensinogen cDNA clone was isolated from a liver cDNA library using a 32-nucleotide-long, synthetic oligonucleotide. The cDNA insert was 1,030 bp long and coded for the secretory and biologically active angiotensin II regions of the angiotensinogen molecule. The RNA from rat liver, brain, and heart was analyzed by the Northern hybridization procedure using nick translated angiotensinogen cDNA as a probe. In addition to liver, the angiotensinogen mRNA is present in the brain and the heart. The angiotensinogen mRNA in the heart is at least fourfold to fivefold more abundant as compared with the liver. We also provide evidence that angiotensinogen mRNA is present in the rat atria and right ventricle but not detectable in the left ventricle. The size of the angiotensinogen mRNA is the same from all three of the tissues, as judged by their electrophoretic mobilities. (Circulation Research 1987;60:786-790)

Renin-angiotensin system plays an important role in the control of blood pressure. Angiotensin II (ang II) is the most active pressor substance known. This octapeptide is synthesized by the proteolytic cleavage of its precursor molecule, angiotensinogen. Renin cleaves the angiotensinogen to produce a decapeptide angiotensin I, which is then converted by angiotensin-converting enzyme (dipeptidyl carboxypeptidase, peptidyl dipeptide hydrolase) to ang II. Angiotensinogen is normally synthesized in the liver and secreted into the blood. It has also been shown to be present in the brain by immunohistochemical methods, and the evidence has been presented that all the components of the renin-angiotensin system are present in the brain. It has been suggested that angiotensinogen in the central nervous system controls the secretion of the antidiuretic and adrenocorticotropic hormones and is also involved in causing thirst.

We have recently isolated human angiotensinogen cDNA clone from a liver cDNA library using synthetic oligonucleotide approach. In this paper, we provide evidence for the presence of angiotensinogen mRNA in the liver as well as in the heart. While this work was in progress, Ohkubo et al. also reported the presence of angiotensinogen mRNA in the brain. However, this is the first report on the presence of the angiotensinogen mRNA in the heart. We have also localized angiotensinogen mRNA in various regions of the heart and provide evidence that it is present in the atria and right ventricle but not found in the left ventricle.

Materials and Methods

Synthesis of Oligonucleotide

A partial amino acid sequence consisting of 13 amino terminal amino acids of human angiotensinogen has been described. A mixture of 32 base oligonucleotides, complementary to the putative mRNA sequence derived from the published amino acid sequence of human angiotensinogen, were chemically synthesized. Synthesis was performed on a solid support (silica gel) using manual addition of nucleoside phosphoramidites in the presence of tetrazole. After completion of the synthesis, oligonucleotide was purified by preparative gel electrophoresis using 20% acrylamide. The oligonucleotide was then phosphorylated using γ-32P-ATP, and the radiolabeled oligonucleotide was used to screen a human liver cDNA library. The amino acid sequence of angiotensinogen and the nucleotide sequence of the synthetic oligonucleotide derived from the amino acid sequence are shown in Figure 1.

Screening of Human Liver cDNA Library

The human liver cDNA library (kindly provided by Dr. S. Orkin) was plated on nitrocellulose filters at 1,000 colonies per plate. Duplicate filters were made according to the method of Hanahan and Meselson. The filters were prehybridized in 6 x SSC (standard saline citrate), 5 x Denhardt's solution, 0.1% SDS, 0.1 mg/ml sheared and denatured salmon sperm DNA, and 0.05% sodium pyrophosphate (1 x SSC is 15 mM...
1. Val - Tyr - Ile - His - Pro - Phe - His - Leu - Val - Ile - His
   U U U U U CUX U

2. 5'- GUX - UA - AUC - CA - CCX - UU - CA - G - GUU - AUC - CA
   C A C C C UUA A
   A A A A A GAX A

3. 3'- CAX - AT - TAG - GT - GGX - AA - GT - G - C - CAX - TAG - GT
   G T G G G AAT T

4. 3'- CA - ATG - TAG - GTG - GGG - AAG - GTG - GA - CA - TAG - GT

5. 3'- CAC - ATG - TAT - GTG - GGG - AAG - GTG - GAG - CAG - TAG - GT

FIGURE 1. Partial amino acid sequence of human angiotensinogen and mixture of 32-nucleotide-long, synthetic oligonucleotides used to isolate cDNA clone. 1: The amino acid sequence (3-13) of human angiotensinogen, 2: nucleotide sequence of putative mRNA corresponding to the amino acid sequence, 3: deoxyoligonucleotide sequence complementary to the mRNA sequence, 4: nucleotide sequence of a mixture of chemically synthesized 32-mers, and 5: nucleotide sequence of this region obtained by sequence determination of the cDNA clone. X represents all 4 nucleotides. Preferential human codon usage and assumption that guanosine can base pair with cytosine and/or uracil and thymine can base pair with a denosine and/or guanosine was made in deriving the synthetic sequence. Asterisk in 5 indicates the only mismatch found in the synthetic and natural sequence of cDNA clones.

Na citrate and 0.15 M NaCl, and 1 × Denhardt’s solution is 0.02% Ficoll, 0.02% polyvinyl pyrrolidine, and 0.02% bovine serum albumin) for at least 2 hours at 37°C. Hybridization with the 32P-labelled oligonucleotide probe was carried out in 6 × SSC and 5 × Denhardt’s solution containing radiolabelled probe (0.5–1.0 × 10⁶ cpm/ml) for 16–18 hours at 37°C. The filters were washed 4 times at room temperature in 6 × SSC and 0.05% sodium pyrophosphate and then once for 15 minutes at 65°C. Next, the filters were air dried and exposed to Kodak XAR-5 x-ray film for 18 hours at −70°C with an intensifier screen.

DNA Sequence Analysis
Plasmid DNA, prepared by the method of Norgard et al., was treated with restriction enzyme Pst I, and DNA fragments were separated by gel electrophoresis. DNA fragments of about 450 and 150 bp were isolated by low melting point agarose method. The fragments were subcloned in M13mp18, and its nucleotide sequence was determined by Sanger’s dideoxy chain termination technique, using α-35S-dATP. The sequencing reactions were electrophoresed and autoradiographed.

Isolation of RNA and Northern Blot Hybridization
A 10% tissue homogenate was prepared in guanidinium isothiocyanate solution, and the total RNA was isolated by cesium chloride gradient. Poly-A+ RNA was isolated by oligo-dT cellulose column chromatography. Poly-A+ RNA was separated by 1.4% agarose-formaldehyde gel electrophoresis and transferred to nitrocellulose paper. The nitrocellulose paper was prehybridized with 6 × SSC, 5 × Denhardt’s solution, 0.1% SDS, and 0.1 mg/ml sheared and denatured salmon sperm DNA for 2 hours at 65°C and then hybridized with 32P-labelled nick translated cDNA probe (a mixture of 450 bp Pst I fragments) in 6 × SSC and 5 × Denhardt’s solution at 65°C for 16–20 hours. The filter was then washed with 1 × SSC 4 times for 15 minutes at room temperature and twice for 30 minutes each at 52°C. The filters were then exposed to Kodak XAR-5 x-ray film with an intensifier screen at −70°C.

Dot Blot Analysis of Rat Liver, Brain, and Heart RNA
Total RNA isolated from the rat liver, brain, and whole heart as well as the atria, left ventricle, and right ventricle of the heart (20 μg/ml) was denatured in 20 × SSC and formaldehyde at 65°C for 15 minutes and applied at different dilutions (10 μg in the first spot, 5 μg in the second, etc.) on nitrocellulose paper using BRL hybridblot apparatus (Bethesda Research Laboratories, Gaithersburg, Md.). The filter was air dried for 1–2 hours and baked for 3 hours at 80°C in a vacuum oven. The filter was then hybridized with the nick translated cDNA probe (10⁶ cpm/ml) at 65°C, washed in 1 × SSC 4 times at room temperature and twice at 52°C, and exposed to Kodak XAR-5 x-ray film for 16 hours. The autoradiograph was scanned in a spectrophotometer at 600 nm. The dots from the nitrocellulose filter were cut and counted in a scintillation counter.

Results
Isolation of Human Angiotensinogen cDNA Clone
A human liver cDNA library prepared in pKT 218 was screened using the 32P-labelled oligonucleotide mixture described above (Figure 1). Four recombinant clones were isolated by the colony hybridization procedure from a total of 20,000 clones plated on duplicate filters. The plasmid DNA was isolated, and the cDNA inserts were released by treatment with restriction enzyme Pst I. The DNA was separated by 0.8% agarose gel electrophoresis, transferred to nitrocellu-
lose paper, and analyzed by Southern hybridization using 32P-labelled oligonucleotide. All 4 clones had Pst I fragments (about 450 bp) that hybridized with the synthetic oligonucleotide. Since the restriction pattern of all the 4 clones was identical, the first clone designated as pHAG 1 was used for further characterization.

Nucleotide Sequence Analysis of cDNA Clone

The cDNA inserts were released by restriction enzyme Pst I treatment and separated by preparative gel electrophoresis. Two fragments (450 and 150 bp) were subcloned in M13mp18 for sequence analysis. The

Figure 2. Nucleotide sequence of human angiotensinogen cDNA and the derived peptide sequence. The nucleotide sequence was determined by the Sanger dideoxy chain termination method. Restriction fragments were subcloned in M13mp9 vector for sequence determination. Universal primer and synthetic oligonucleotides were used as primers to derive the complete sequence. Angiotensin II sequence is underlined.
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sequence analysis revealed that 450 bp fragments were in a mixture of 2 components and that one of them had the oligo guanosine nucleotide tail. Similarly, 150 bp fragments had the oligo guanosine nucleotide tail and, therefore, had the cloning site. The nucleotide se-
quence of 1,030 bp human angiotensinogen cDNA insert and the derived amino acid sequence is presented in Figure 2.

Analysis of RNA From Rat Liver, Brain, and Heart

The poly-A+ RNA from rat liver, brain, and heart was analyzed by Northern hybridization using nick translated 450 bp Pst I fragments of human angiotensinogen cDNA. The cDNA probe hybridized to an RNA species of about 18s (corresponding to 2 kb) in liver, brain, and heart (Figure 3). The intensity of RNA bands suggests that angiotensinogen mRNA is less abundant in the brain and more abundant in the heart than in the liver. To quantitate the angiotensinogen messenger RNA levels in these tissues, the RNA was subjected to dot blot analysis, and the results of the experiment are shown in Figure 4 (Densitometer scanning: liver 88 ± 8, heart 370 ± 16; scintillation counter: liver 90 ± 6, heart 393 ± 12). The results clearly show that angiotensinogen mRNA is at least 4 to 5 times more abundant in heart compared with liver. The results of the dot blot analysis of the RNA isolated from the atria and left and right ventricles of the heart are shown in Table 1. These data indicate that angio-
tensinogen mRNA is present in the atria and right ventricle but not detectable in the left ventricle.

Discussion

The study presented describes the isolation of angio-
tensinogen cDNA clone from a human liver cDNA library. The cDNA clone was isolated using a 32-
nucleotide-long, synthetic oligonucleotide, the se-
quence of which was derived from the known 13 N-terminal amino acid sequence of human angioten-
sinogen. The synthetic sequence was derived by as-
suming that guanosine can base pair with cytosine and/or uracil and thymine can base pair with adenosine and/or guanosine. While this work was in progress, Kageyama et al18 also reported the isolation and nu-

![Figure 3. Northern hybridization of rat poly-A+ RNA. Poly-
A+ RNA from rat 1) liver (2 µg), 2) brain (2 µg), and 3) heart (1 µg) was electrophoresed on 1.4% agarose-formaldehyde gel, blotted to nitrocellulose paper, and hybridized with nick trans-
lated human angiotensinogen cDNA clone at 65° C for 18 hours
in 6 x SSC and 5 x Denhardt's solution. The filter was washed
at 52° C in 1 x SSC and autoradiographed. The position of
ribosomal RNA markers is indicated on the left.]

![Figure 4. Dot blot hybridiza-
tion of rat total RNA. Total RNA
(20 µg/ml) from rat 1) liver, 2) brain, and 3) heart were blotted
to nitrocellulose paper at differ-
ent dilutions (10 µg in the first spot, 5 µg in the second spot, etc.) using BRL hybrid blot appara-
ratus. The filter was hybridized with nick translated human an-
giotensinogen cDNA clone (mixture of 450 bp Pst I fragments) at
65° C for 18 hours in 6 x SSC and 5 x Denhardt's solution, washed at 52° C in 1 x SSC, and autoradiographed.]

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The nucleotide sequence of 1,030 bp fragments presented in this paper is identical with the sequence published by those workers.

This study also provides evidence that the angiotensin gene is expressed in the liver, brain, and heart. Recently, Ohkubo et al. reported that angiotensin mRNA is present in the rat brain, kidney, adrenal gland, ovary, and lung. However, our studies provide the first evidence of angiotensin mRNA in the rat heart. Ohkubo et al. have shown that at least 4 different size classifications of the angiotensin mRNA are present in both hepatic and extrahepatic tissues, and all these multiple mRNA species are encoded by a single gene in the rat genome. An analysis of the angiotensin mRNA by Northern blot hybridization (Figure 3) indicates that its size is the same in the liver, brain, and heart. Using a dot blot hybridization procedure, angiotensin mRNA is at least fourfold to fivefold more abundant in the heart compared with the liver. Our analysis of the RNA isolated from different regions of the heart indicates that angiotensin mRNA is present in the atria and the right ventricle but not found in the left ventricle.

Recently, renin was found in the mouse heart and in vascular endothelial cells in culture. Angiotensin-converting enzyme has also been localized to the endothelial cell surface, where it converts ang I to ang II. Until now, the origin of angiotensin in the vascular system was not known, and it had been postulated that angiotensin was taken up from the serum and internalized. However, the evidence presented here shows that angiotensin mRNA is present in the heart and suggests that internally synthesized angiotensin may play an important role in the vascular system. Formation of ang II by the sequential action of renin and angiotensin-converting enzyme in the vascular tissue may result in the control of vascular tone either directly or indirectly through the induction of prostaglandin synthesis. It remains to be established whether angiotensin synthesized in the heart is of the intracellular form or secretory form as in the case of liver. Experiments are now in progress to answer these questions.

Acknowledgments

We are thankful to Dr. S. Orkin for the human liver cDNA library and to S. Kumar for technical assistance.

Table 1. Distribution of Angiotensinogen mRNA in the Rat Heart

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<th>Section</th>
<th>Densitometer reading</th>
<th>Radioactivity counts</th>
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<tr>
<td>Whole heart</td>
<td>382 ± 15</td>
<td>395 ± 12</td>
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<tr>
<td>Atria</td>
<td>737 ± 21</td>
<td>768 ± 19</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>20 ± 2</td>
<td>15 ± 3</td>
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<tr>
<td>Right ventricle</td>
<td>376 ± 12</td>
<td>412 ± 16</td>
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Reference


Key Words: angiotensinogen mRNA • rat heart • cDNA • nucleotide sequence • tissue distribution
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