Structure and Regulation of Human and Rat Renin Genes

Institute of Applied Biochemistry

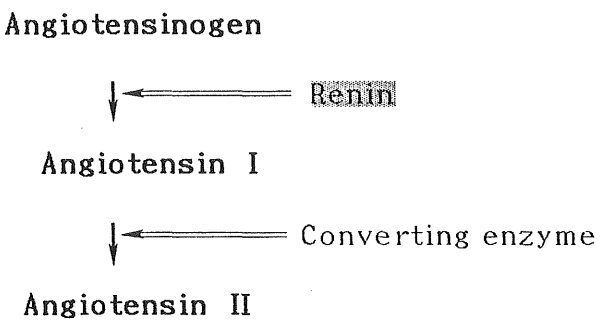
University of Tsukuba

Akiyoshi Fukamizu

Contents

Chapter I: Preface

Since the discovery of renin 90 years ago, there have been remarkable advances in our understanding of the renin-angiotensin system. The system as it is known today is summarized below. Renin controls the rate



-limiting step in the formation of the potent vasoactive peptide, angiotensin II, by catalyzing the formation of angiotensin I from angiotensinogen. Angiotensin II has several important physiological functions such as pressor action, stimulation of aldosterone or cathecolamines secretion. Through these actions, the renin-angiotensin system plays an important role in the regulation of blood pressure and of the volume and composition of the extracellular fluid.

The conventional concept that the renin-angiotensin system is a circulating endocrine system has been challenged recently by the observations that components of the system can be detected in many tissues (1). Particularly, in reproductive organs, it was suggested that renin expression is regulated by gonadotropic hormones (2,3).

Attempts to analyze the structure and function of renin have been

hampered by the difficulties involved in purification of this enzyme, which is unstable and of low abundance. However, the advent of protein purification and recombinant DNA technology has obviated some of these difficulties. Particularly, the DNA recombinant technology allowed us to isolate mouse (4), human (5), and rat (6) renin cDNAs, and to express these cDNAs in *Escherichia coli* and mammalian cells (7,8).

A central problem in eukaryotic molecular biology is to understand the mechanisms by which specific genes are expressed in a temporal or tissue -specific manner or are activated in response to extracelluar inducers. The development of methods for cloning and characterizing individual genes has provided the opportunity to study these mechanisms at the molecular level.

Recent studies (9,10) indicated that genes expressed in a particular tissue contain *cis*-acting elements which are required for their tissue-specific transcription. Three classes of such elements have been distinguished. Firstly, upstream promoter elements, which are located 5' -regions of the TATA boxes (11). Secondly, cellular enhancers increase transcription rate independently of their orientation and distance from the promoter. Finally, the steroid response elements (SRE), which bind steroid hormone receptors, have enhancer-like characteristics (12).

From these situations, the renin-angiotensin system may provide a very attractive model to analyze how these three classes of *cis*-acting elements control gene transcription in different cell types. Therefore, I have cloned and sequenced the exon-intron junctions of the human renin gene (13). However, little is known about the renin gene expression.

In this thesis, to examine more precisely the regulation of renin gene, I have cloned and characterized the 5'-flanking region (promoter and regulatory regions) of the human renin gene from a juxtaglomerular cell (JGC) tumor (14) (Chapter II), and determined the complete nucleotide sequence of rat renin gene including its 5'- and 3'-flanking regions (15) (Chapter III). Furthermore, I have characterized the expression of the rat renin mRNA in ovary and uterus (61) (Chapter IV), and of the human renin mRNA in an ileal carcinoma (an ectopic renin-secreting tumor) by Northern blot analysis (62).

Chapter II: Human Renin Gene of Renin-Secreting Tumor

Abstract

A large amount of renin mRNA was found to be expressed in the juxtaglomerular cell (JGC) tumor, as determined by Northern analysis. I have isolated the long 5'-flanking region of the human renin gene from the tumor, and characterized the promoter region with respect to DNA sequence and mRNA transcription start point. Of two sets of CAAT and TATA boxes, the TATA box at 29 base pairs (bp) upstream from the capping site is demonstrated to be a functional promoter by primer extension analysis. The 1.6 kilobase (kb) sequence, containing the 5'-flanking region, exon 1, and part of the first intron, obtained from the tumor was in complete agreement with that of the clone from fetal liver, which does not produce renin. This indicates that abnormal expression of the renin gene in the JGC tumor involves no major alteration in the primary structure within 1.2 kb of the 5'-flanking region. Within 1.2 kb of the 5'-flanking region, there are several nucleotide segments exhibiting homology with the glucocorticoid, estrogen, and progesterone receptor-binding sites and enhancers. These structures may be related to the tissue-specific expression of the renin gene.

Introduction

Renin is an aspartyl proteinase and catalyzes the initial and rate-limiting reaction, which plays an important role in the control of blood pressure and in the pathogenesis of hypertension. Renin is mainly synthesized in the JGCs of kidney, which are located in the afferent arterioles immediately proximal to the glomeruli (16). The biosynthesis of renin is considered to be under complex regulation, which rouses interest about the detailed structure of the gene. We therefore cloned and sequenced cDNA (5) and genomic clones (13).

The JGC tumor, a benign tumor, secretes very large quantities of renin into the bloodstream (17,18). This tumor provides an excellent material for studying the molecular events controlling renin gene expression. It is interesting to know the mechanism(s) through which normal controls are altered in tumor tissue.

In the present study, I have cloned and sequenced the 5'-flanking region immediately upstream from the start site for transcription of the human renin gene in the tumor tissue.

The results shows no difference in the nucleotide sequence of the 5'-flanking region between the normal and tumor renin gene. However, in this region, I found potential control elements such as enhancers, steroid hormone receptor-binding sites, and cAMP-regulatory units, which may contribute to the cell-specific expression of the human renin gene.

Materials and Methods

(a) *Materials*

All restriction endonucleases, DNA modifying enzymes, and reverse transcriptase were obtained from Takara Shuzo, Nippon gene, and Toyobo. [γ-^{32}P]ATP and [α-^{32}P]dCTP were from Amersham.

(b) *Preparation of DNA and RNA*

Human genomic DNA was isolated from different tissues (placenta, kidney, and JGC tumor) by the method as described (19).

Total RNA was isolated from a human JGC tumor and normal kidney by the guanidinium thiocyanate procedure (20). RNA was enriched for poly(A)$^+$-containing sequences by two passage over oligo(dT)-cellulose.

Charon 4A recombinant phage and plasmid DNAs were purified as described (21).

A 920-bp *Pst*I-*Hpa*II fragment was isolated from pHRn321 (5), coding for the 5'-portion of the human renin mRNA. This fragment was labeled with [α-^{32}P]dCTP by nick translation (22) and used as a hybridization probe.

(c) *Construction and screening of the juxtaglomerular cell tumor genomic library*

Human JGC genomic DNA fragments were generated by partial *Eco*RI digestion and cloned into Charon 4A by the method as described (21). The library was screened by the method of Benton and Davis (23), for sequences that hybridize with the probe as described above.

Hybridization was conducted at 65℃ for 20 hr and the filters were subsequentially washed several times in (i) 0.3 M NaCl/ 0.03 M sodium citrate/ 0.1% SDS at room temperature, (ii) the same buffer at 65℃, (iii) 15 mM NaCl/ 1.5 mM sodium citrate/ 0.1% SDS at 65℃, and (iv) (i) buffer at room temperature and exposed to Fuji X-ray film RX-50 with a DuPont Cronex Lightning Plus screen. Positive clones were picked and rescreened at low plaque density to achieve high purity.

(d) *Primer extention analysis*

pUX77 was constructed by subcloning a 770-bp *Xba*I fragment into pUC19. The 100-bp *Dde*I-*Dra*I fragment of pUX77, labeled at its *Dra*I 5'-end with [γ-^{32}P]ATP, was used as a primer. This DNA fragment was denatured at 90℃ for 5 min and then hybridized with poly(A)$^{+}$RNA from the JGC tumor and total RNA from normal kidney in 80% formamide (deionized), 0.4 M NaCl, 40 mM PIPES, pH 6.4, and 1 mM EDTA for 3 h at 55℃. After precipitation of the DNA-RNA hybrid, the pellet was dissolved in reverse transcriptase buffer containing 50 mM Tris-HCl, pH 8.3, 100 mM KCl, 10 mM $MgCl_2$, and 10 mM DTT. The primer was extended with 20 units of Rous associated virus (RAV-2) reverse transcriptase, using 1 mM each of the four dNTPs. The primer-extended cDNAs were analyzed on a 7 M urea/ 6% polyacrylamide sequencing gel. The size markars used were M13 mp18 single-stranded DNA.

(e) *Southern blot analysis*

A 20 μg of DNA from human placenta, JGC tumor, and kidney were

digested with various restriction endonucleases, electrophoresed on a 0.7% agarose gel, and transferred to a nitrocellulose filter (24). Hybridization was performed as described above.

(f) *Northern blot analysis*

The poly(A)$^+$RNAs from normal kidney and JGC tumor were denatured with glyoxal and electrophoresed on a 1.2% agarose gel. The separated RNAs were transferred to diazobenzyloxymethyl-paper according to the procedure of Alwine *et al.* (25). The paper was prehybridized at 42℃ for 12 h in 50% formamide/ 0.75 M NaCl/ 0.075 M sodium citrate/ 50 mM sodium phosphate buffer, pH 7.0/ 0.02% polyvinylpyrrolidone/ 0.02% Ficoll/ 0.02% bovine serum albumin/ 1% glycine/ 0.2% SDS/ 100 μg denatured salmon sperm DNA per ml. The paper was hybridized at 42℃ for 24 h in the same buffer solution except that glycine was omitted and that the solution contained the ^{32}P-labeled probe described above. After the hybridization, the paper was washed at 42℃ in 0.45 M NaCl/ 0.045 M sodium citrate/ 0.1% SDS and subjected to autoradiography.

(g) *DNA sequence analysis*

DNA sequencing was performed by the dideoxy-chain termination method (26) following subcloning into M13 mp10 and mp11.

Results and Discussion

(a) *Tissue-specific expression of the human renin gene*

To determine whether the primary transcript from the renin gene is correctly spliced in the JGC tumor, poly(A)$^+$RNAs were isolated from the tumor, and normal kidney, and were subjected to Northern blot analysis using a human renin cDNA as a hybridization probe. In both cases, as shown Fig.1, one major 16S RNA was detected. The amount of the 16S mRNA species was apparently larger in the tumor in the JGC tumor tissue (Fig.1, lane 2). No 16S renin RNA was found in liver or small intestine (data not shown). These results suggest that the primary transcripts are similarly spliced in both narmal and tumor tissues, and that the renin gene is expressed only in specialized cells.

(b) *Identification of recombinant phage carrying the human renin gene derived from the JGC tumor*

To characterize the 5'-flanking sequences of the human renin gene of the JGC tumor, the human genomic library of the tumor was screened with the human renin cDNA probe. 15 plaques that hybridized to the probe were detected among a total of 8 x 10^5 phages. One positive clone, designated as λHJg 52, containing an insert of approx. 20 kb, was characterized by a combination of restriction enzyme and Southern blot analysis. As displayed in Fig. 2, this DNA contains exon 1, 2, 3, and the 5'-flanking region, and shows a restriction map indistinguishable from that of clone λHR_n 88 obtained from the fetal liver, which does not produce renin. Seven other clones containing the above region from the tumor exhibit

the same restriction map as λHJg 52.

(c) *No gross rearrangement of the human renin gene in the JGC tumor genome*

Soubrier *et al.* (27) have shown that the renin gene exists in a single copy in the human genome. To establish whether the rearrangements of major sequence occur in the JGC tumor, restricted DNA was analyzed by Southern blotting. As shown in Fig. 3, the blot hybridization analysis of total cellular DNA obtained from human placenta, JGC tumor, and kidney showed that they all produced identical hybridization-positive fragments, indicating that no gross rearrangement of the renin gene occurred from non-expressing to expressing cells, and that there are no other closely related genes or pseudogenes in the human genome.

(d) *Identification of the 5'-end of the renin gene mRNA*

To define precisely the start site for transcription, I performed a primer extension experiment. For this analysis, a 100-bp *Dde*I-*Dra*I fragment of pUX77 (Fig. 4-B), labeled at the *Dra*I 5'-end, was used as a primer, hybridized with the RNAs from human JGC tumor and normal kidney, as shown in Fig. 4. A main fragment of 137 bp was observed (Fig. 4-A), indicating that transcription is initiated at 29 bp downstream from the TATA box in both tissues, coinciding with the typical range for eukaryotic genes: 34-26 downstream.

(e) *Nucleotide sequence of the 5'-flanking region of the human renin gene*

To compare DNA sequences of the 5'-flanking region between the normal and tumor renin genes, the nucleotide sequences extended up to 1200 bp upstream from the putative capping site in both fetal liver (λHR_n 88) and tumor (λHJg 52) were determined. The sequence of the tumor is in complete agreement with the clone obtained from the fetal liver, which does not produce renin.

I found a number of possible control elements in the 5'-flanking region and first intron (Fig. 5). Two potential TATA boxes (11) are located at positions -29 and -77. Associated with these TATA boxes are two CAAT boxes (28) at positions -51 and -92. The hexanucleotide sequences TGTTCT and TGTCCT have been proposed as glucocorticoid receptor-binding sites of the human renin gene (13). In the present investigation, we confirmed the above sequences at the positions (-185 to -190 and -451 to -456), and also found the hexanucleotide sequence AGTCCT (29) as a glucocorticoid receptor-binding site in the first intron at +233 to +238.

There are two DNA segments, at -981 to -999 and -300 to -318, with differing degrees of similarity to the consensus sequence of the progesterone receptor-binding site (30). Nine out of eleven nucleotides at -335 to -345 match the central sequence of the estrogen receptor complex binding reported for the chicken vitellogenin gene (31).

Moreover, another noteworthy feature is the presence of a sequence complementary to a 29-nucleotide sequence at -117 to -145, which has been proposed as a mediator of cAMP-regulated transcription (32).

It is interesting that at five nucleotide positions in the 5'-flanking region and one within the first intron, sequences are located which are

very similar to the consensus or complementary sequence for several enhancers, since the enhancers function independent of location or orientation and, in some cases, confer tissue specificity (33); the exact positions are: GTGGCAAG (-1223 to -1230), GGGGAAAG (-973 to -980), CTACCCAC (-573 to -580), GTTCCCAC (+186 to +193) similar to the SV40 enhancer (34), and TGTCCATCA (-669 to -677), TAGTGGAGA (-635 to -642) similar to the polyoma enhancer (35).

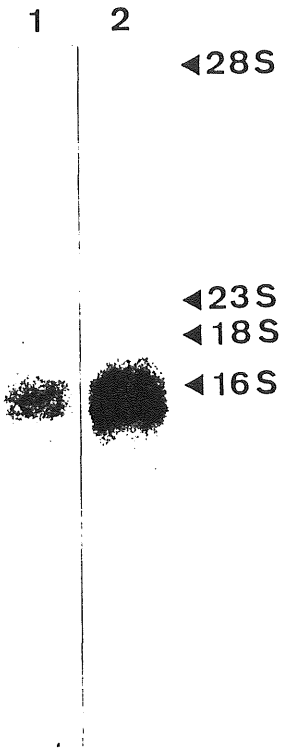


Fig. 1. Northern blot analysis. A 20-μg sample of mRNA from human normal kidney (lane 1) and 0.5 μg of mRNA from JGC tumor (lane 2) were subjected to Northern blotting as described in **Materials and Methods**. Exposure on X-ray film was done at -70℃ for 24 h (lane 1) or 2 h (lane 2) with an intensifying screen. The size markers used were calf and *E. coli* rRNA.

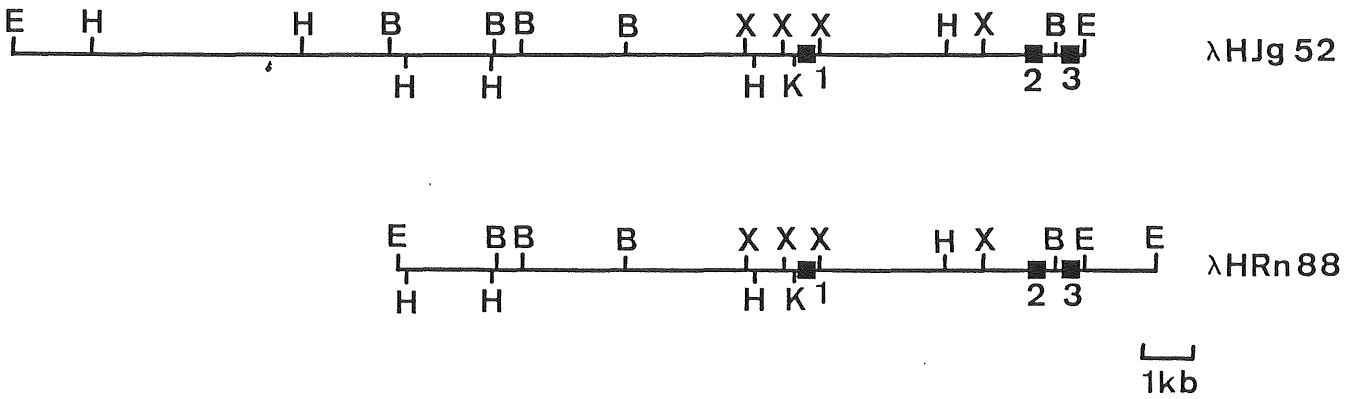


Fig. 2. Restriction maps of λHJg52 and λHRn88. Restriction sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I. Small black boxes represent exons; their numbers are given. λHJg52 was cloned from the human JGC tumor genomic library, and λHRn88 from the human fetal liver genomic library (13).

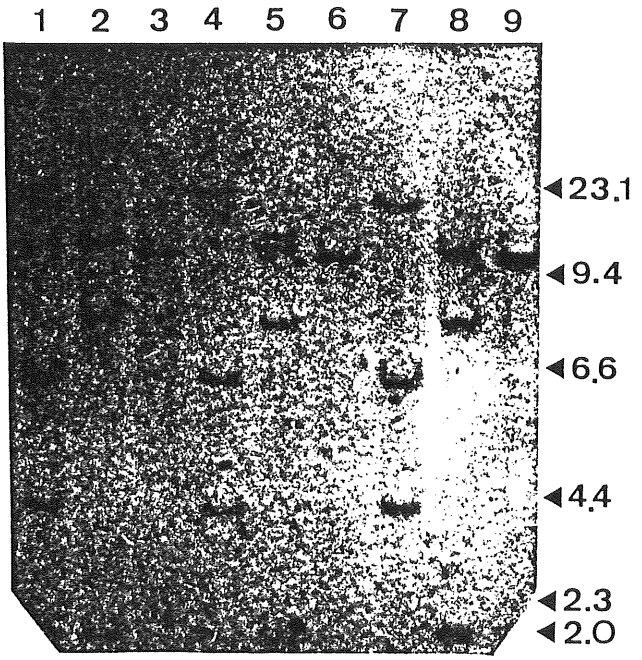


Fig. 3. Southern blot analysis of genomic human DNA. Samples of 20 μg DNA from human placenta (lanes 1-3), JGC tumor (lanes 4-6), and kidney (lanes 7-9) were digested with the restriction endonucleases, *Eco*RI (lanes 1, 4, and 7), *Bam*HI (lanes 2,5,and 8), or *Kpn*I (lanes 3,6, and 9). Exposure on X-ray film was done at -70℃ for 48 h using an intensifying screen. λDNA digested with *Hin*dIII served as a size marker (sizes in kb in right-hand margin).

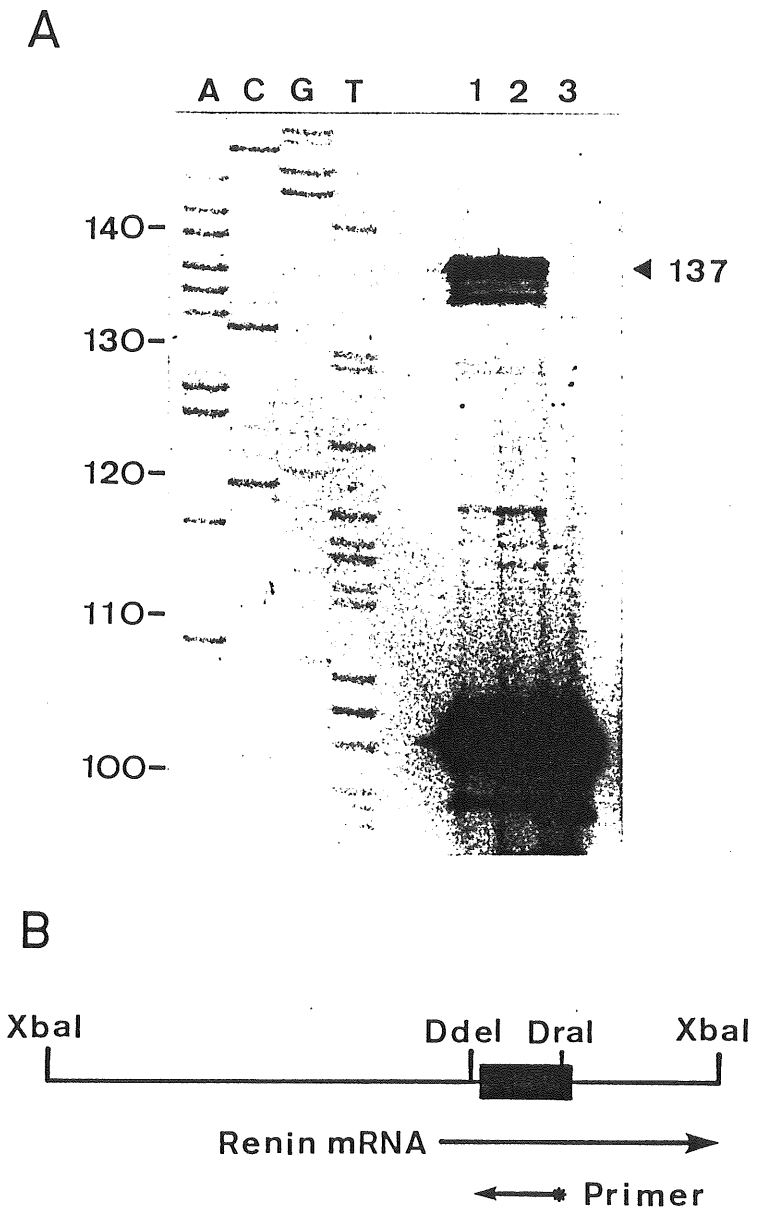


Fig. 4. Primer extension analysis of the 5'-end of renin mRNA from JGC tumor and normal kidney. (A) Autoradiograph of the primer extended cDNA transcripts. Lane 1, 0.1 μg of human JGC poly(A)$^+$RNA; lane 2, 50 μg of human normal kidney poly(A)$^+$RNA; lane 3 , 10 μg yeast tRNA. A dideoxy sequence ladder was used as size markers (lanes A, C, G, T). (B) Schematic representation of the primer. The asterisk denotes the labeling position; black box represents the first exon.

```
-1245 TCTAGACCAGGTATTGTGGCAAGGAAAGTACCTTATTCAGAGAACCAGAAAACCAAGAAG
-1185 ATGGTGGACCAGCATGATAAAGAACCATCTGAAGTCAGCATGAACGTTAGGCTCTTCTTT
-1125 ATGTTAAGGGAAGAGGAAGAAGAAGGGGATTGGGATCAAGAGGGGACTGATGACCACAGA
-1065 CACCTGGGTGCCAGCAAGGGTATACATATTTAACATAACATTGTTATTTGTCTGTATATT
-1005 TCCTTATCTCCTTGGGGGTTAGTTTGGGGAAAGGAACTGTTACCATTTTTTTTAAAGTTG
-945 AACTGCAAGCTAAACTCCTATAATTAGCTGGTCTATGTACAGAGCTAAGCAGAAGCTTTT
-885 AGCTAAAGGATAATACCCCTGGGGGTGCAGAGGCAAAATGGAGTCAGTCATGCTAAGTCT
-825 CCCTCCACTCTCTTTCTTTTTTGAGATGGGATTTCACTCTTATTGCCAGGCGCGGAGTAG
-765 TGGCATGATTCTCAGCTCACTGCAACCTCCGCCTCCTGGGCCTAAGCAATTCTCTTGCTC
-705 CAGCCTCCTGAGTAGCTGAGATTACAGGTGTCCATCACCACACCCAGCTAATTTTTGTAG
-645 TTTAGTGGAGATGGGGTTTCACCATTGTTGGTCAGGCTGGTCTGGAACTCCTGACCTCAG
-585 GTGATCTACCCACCTTGGCCTCCCAAAGTGCTGGGACAGGTGTGAGCCACCATGCCTGGC
-525 CCCTCTACTCTTATAATTAAACCAGCTGTTGCTTTTCCTGCCAAGAAACCAGTCATGAAG
-465 ATTCACCCATGTTCTAGATGGGAAAACTGGGCTGTAGCCTGGGAGAGGCCAGTCAGGGAC
-405 AAAGCCAAAGTTAATATAGAGAATGGAGCTTCCAGGGTATAGGGGTTGGGTCTGGGCTAG
-345 GGAGCTGGAAACCTAGGTTTTACGCTTGTCCCAGTTTTGATGTTAGCCCTGAGCAGTGCT
-285 GTTTCTCATCAGCCTCTGCCTGCTCCAGGGGTCACAGGGCCAAGCCAGATAGAGGGCTGC
-225 TAGCGTCACTGGACACAAGATTGCTTTCCCACAGCTGTCCTTCCTCCAGCCCCTCTGCTC
-165 CCCATCCGGAAACCTGGGTACCCTTCACCCACCTAGCTCTGTCCCGCAGTGAGATTTATT
-105 GCTGACTGCCCTGCCATCTACCCCAGGGTAATAAATCAGGGCAGAGCAGAATTGCAATCA
-45 CCCCATGCATGGAGTGTATAAAAGGGGAAGGGCTAAGGGAGCCACAGAACCTCAGTGGAT
+1
+16 CTCAGAGAGAGCCCCAGACTGAGGGAAGCATGGATGGATGGAGAAGGATGCCTCGCTGGG
M D G W R R M P R W
+76 GACTGCTGCTGCTGCTCTGGGGCTCCTGTACCTTTGGTCTCCCGACAGACACCACCACCT
G L L L L L W G S C T F G L P T D T T T
+136 TTAAACGGTAATTGGTAACTCAGGCAGAGAAGGGGTGGGAGGGGTGCAGGGTTCCCACCT
F K R
+196 TCCCAACACCCTGGCTTTTCCACATGAGGTGTCATTCAGTCCTTACGATCAGCTGGACAG
+256 GGAAGTATGGACCTGTTGACATAGGTCAAGTGACTTGCCCAATAAATGACACTAGTAGTC
+316 AGTCTAGA
```

Fig. 5. Nucleotide sequence of the 5'-flanking region, first exon, and part of the first intron. Nucleotides are numbered on the *left* with the cap site, designated +1. The predicted amino acid sequence of the protein is displayed below the nucleotide sequence. The CAAT and TATA sequences are underlined by dashed lines. The nucleotide residues homologous to the putative estrogen (□), progesterone (■), and glucocorticoid (▽) receptor-binding sites are indicated. Closed and open circles indicate the sequences homologous or complementary to SV40 and polyoma enhancer core sequences, respectively. The sequence related to cAMP-regulated genes is indicated (▲).

Chapter III: Primary Structure of Rat Renin Gene

Abstract

I have isolated the renin gene from a Sprague-Dawley rat genomic library and determined its complete nucleotide sequence. The single rat renin gene is approximately 11,000 bases in length and consists of nine exons and eight introns. The amino acid sequence predicted from the genomic sequence indicates that the rat renin precursor consists of 402 amino acid residues, and shows 85%, 82%, and 68% homology to the mouse *Ren-1* and *Ren-2*, and human renins, respectively. The canonical promoter "TATA" boxes, TATAAA and TAATAA, are found 27 and 57 base-pairs (bp) upstream from the putative cap site, respectively. Several attractive sequences analogous to glucocorticoid, estrogen receptor-binding sites, cAMP-responsive element and SV40 enhancer core sequences were noted in the 5'-flanking region of the gene. In the first intron, segments with an average size of 38 bp containing a *Nco*I site are present at 46 tandem repeats within 1710 bp. A "CA" element consisting of $(CA)_{27}$ was identified in intron 3. Furthermore, intron 8 contains a sequence that shows about 93% homology to that of the neuronal identifier sequence.

Introduction

Although renin is classically considered to be synthesized by the kidney and secreted into the circulation, a number of studies have identified renin and its mRNA in adrenal, brain, ovary, spleen, testis and uterus (1-3), as determined by immunohistochemistry and blot hybridization.

In order to understand the mechanism(s) of the regulation of renin gene expression in renal and extra-renal tissues, isolation and complete characterization of renin gene are necessary. However, only partial nucleotide sequences of renin genes were determined from mouse (36), and human (13,14). In the present study, the complete nucleotide sequence of the renin gene, including its flanking regions, was elucidated from rats that were used most commonly in the experiments on hypertension.

Materials and Methods

(a) *Enzymes and biochemical reagents*

Restriction endonucleases, DNA-modifying enzymes, Rous associated virus 2 reverse transcriptase, and M13 cloning vectors were purchased from Takara Shuzo, Toyobo, and Nippon Gene. [α-^{32}P]dCTP (>400 Ci/mmol and ~3000 Ci/mmol) and [γ-^{32}P]ATP (>5000 Ci/mmol) were obtained from Amersham. All other chemical reagents were of the highest quality available.

(b) *Screening of the rat genomic DNA library*

The rat genomic DNA library (kindly provided by Drs. H. Esumi and T. Sugimura, National Cancer Center, Research Institute, Tokyo) constructed as a partial *Eco*RI digest of rat liver DNA cloned into bacteriophage Charon 4A arms, was screened as described by Benton and Davis (23) using ^{32}P-nick-translated mouse renin cDNA (358-bp *Acc*I-*Rsa*I fragment) (4) as a probe. Hybrydization was carried out in 6 x SSC (1 x SSC, 0.15 M NaCl and 0.015 M sodium citrate), 5 x Denhardt's solution (1 x Denhardt's , 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), and 100 μg/ml sonicated salmon sperm DNA at 55℃ for 16 h. The filters were washed four times with 2 x SSC at room temperature.

(c) *Preparation of DNA and Southern hybridization*

Rat genomic DNA was isolated from liver by the method of Blin and Stafford (19). Charon 4A recombinant bacteriophage and plasmids were

purified as described (21). Genomic and purified DNAs were digested by restriction endonucleases, electrophoresed on a 0.7% agarose gel, and transferred to the nitrocellulose filter (24). Hybridization was carried out as described above and in the case of genomic Southern hybridization, the filters were hybridized with ^{32}P-nick-translated genomic DNA fragment (1-kb *Eco*RI fragment from λRRn-1) at 65℃ for 42h, and washed four times with 0.1 x SSC/0.1% SDS at 65℃.

(d) *DNA sequence analysis*

DNA sequencing was performed by the dideoxynucleotide chain termination method (26) following subcloning into M13 mp18 and mp19. In some instances, a set of overlapping deletions was generated by the procedure as described (37). 7-Deaza-2'-deoxyguanosine 5'-triphosphate was used to prevent the formation of secondary structure (38).

(e) *Primer extension analysis*

Total RNA was prepared from rat kidney and liver as described (20). Poly(A)$^+$RNA was purified by oligo(dT)-cellulose affinity chromatography. The 50-bp *Ava*II fragment from exon 1 labeled with [γ-^{32}P]ATP was used as a primer. The labeled primer coprecipitated with 10μg of poly(A)$^+$RNA was denatured at 90°C for 5 min, and then hybridized in 80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, and 1 mM EDTA for 16 h at 60℃. After precipitation of the DNA-RNA hybrid, the pellet was dissolved in reverse transcriptase buffer containing 50 mM Tris·HCl, pH 8.3, 100 mM KCl, 10 mM $MgCl_2$, and 10 mM dithiothreitol. The primer was extended with 20

units of Rous associated virus 2 reverse transcriptase, using 1 mM of each dNTP. The primer-extended cDNAs were analyzed on a 6% polyacrylamide /7 M urea sequenceing gel. An M13 mp18 dideoxy sequencing ladder was used for molecular markers.

Results and Discussion

(a) *Organization of rat renin gene*

Using mouse renin (*Ren-2*) cDNA (4) as a probe, a rat genomic DNA library was screened for clones containing the renin gene. One of the positive clones, λRRn-1, was purified and characterized. The complete nucleotide sequence of all exons and introns of the rat renin gene was determined according to the strategy outlined in Fig. 6. The entire DNA sequence for the gene is shown in Fig. 7. The rat renin gene is about 11,000 bases long and consists of nine exons interrupted by eight introns. The total Southern blot analysis suggested that there is a single gene encoding renin in the rat (Fig. 8).

The location and sizes of the exons and introns are summarized in Table I. The exons range in size from 99 to 354 bp. The average size of 159 bp is similar to that of 150 bp reported for exons in higher eukaryotes (39,40).

(b) *Amino acid sequence of rat renin*

The amino acid sequence of rat renin was deduced from the genomic sequence and compared with those of other species (5,36). Considerable similarity among four known renin amino acid sequences is shown in Fig. 9. The overall similarities among the precursors are indicated by following percentages of identical amino acids: rat/mouse *Ren-1*, 85%; rat/mouse *Ren-2*, 82%; rat/human, 68%. A study by Shinagawa *et al* (41) indicated that the amino terminus of the mature form of human renin is

identical with that deduced from the human renin cDNA (5). From this, the amino terminus of the mature form of rat renin is predicted to be Ser65 (Fig. 9), although no direct evidence exists. This postulated mature form consists of 338 amino acid residues, M*r* 36917, close to the literature report for rat kidney renin (42,43). The two catalytically important aspartyl residues, Phe-*Asp*-Thr-Gly-Ser at positions 101 to 105 and Val-*Asp*-Thr-Gly at positions 286 to 289, are completely conserved among three species of renin. These catalytically important sequences are identical with other aspartyl proteinases (44). Three potential *N*-linked glycosylation sites, Asn-X-Ser/Thr, are present in rat renin as well as mouse *Ren-1*, although there is none in mouse *Ren-2*. Their positions are at 69, 139, and 320 (Fig. 9).

(c) *5'-flanking region*

I have characterized and determined the nucleotide sequence of the 5'-flanking region and the start site for transcription (Fig. 7 and 10). In the region preceding the putative transcription initiation site, there are several possible promoter and regulator elements. Two "TATA" sequences, TATAAA and TAATAA, are present at positions -27 and -57, respectively. Nine out of eleven nucleotides, GAAGCTAAAG (at positions -228 to -237), match the central sequence of the estrogen receptor-binding site, GGAGCTGAAAG, reported for the chicken vitellogenin II gene (31). Additionally, we found, at positions -1048 to -1053 and +208 to +213, AGGACT and AGTCCT sequences that is present in the rabbit uteroglobin gene for the glucocorticoid receptor-binding site

(29). Moreover, we note the presence of two sequences, TTTCCAC (at positions -448 to -454) and GTGGAAA (at positions -1116 to -1202), identical with the SV40 enhancer core sequences (34). There is another noteworthy DNA segment, CTGATGCCAG (at positions -477 to -484), which has 80% homology to the sequence necessary for the induction by cAMP in the rat somatostatin gene (45).

(d) *Intron features*

In the process of determining the DNA sequence of the rat renin gene, I found several interesting features in the introns. In the first intron, the average size of 38-bp segment containing *Nco*I site is present as 46 tandem repeats within 1710 bp. A stretch of 41 adenine residues is located close to the 5' end of exon 2. A repeat of $(CA)_{27}$ preceded by consecutive 54 pyrimidine residues is found in the intron 3. Short repeats of $(GT)_9$ and $(CA)_{3-8}$ are also present in the intron 5 and the 3'-flanking region, respectively. Of note also is the presence of the sequence in the intron 8 (at positions 10235 to 10315) that shows about 93% homology to the complementary strand of the neuronal identifier sequence (46).

Table I

Location and size of exons and introns in the rat renin gene

exon	nucleotide positions	length(bp)	amino acids	intron	nucleotides positions	length(bp)
1	1-124	124	1-31[a]	1	125-4590	4466
2	4591-4741	151	31-81	2	4742-5251	510
3	5252-5375	124	82-123	3	5376-6032	657
4	6033-6151	119	123-162	4	6152-6902	751
5	6903-7099	197	163-228	5	7100-9017	1918
6	9018-9137	120	228-268	6	9138-9513	376
7	9514-9658	145	268-316	7	9659-9922	263
8	9923-10021	99	317-349	8	10022-10685	664
9	10686-11039	354	350-402[a]			

[a]Exon 1 contains 32 bp of 5'-noncoding sequence. Exon 9 also contains 192 bp of 3'-noncoding sequence following the TAA termination codon.

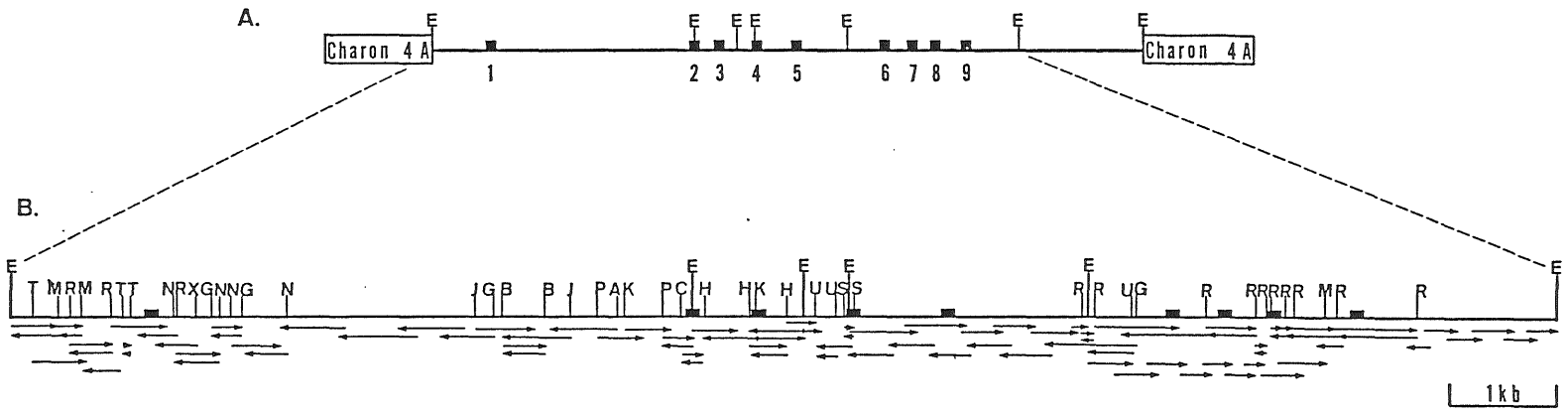


Fig. 6. Restriction endonuclease map and sequence strategy of the cloned genomic DNA fragment containing the rat renin gene. The direction of the transcription is from left to right. (A) *Eco*RI restriction map of the cloned rat renin gene, λRRn-1. The location of exons are indicated by filled boxes, and exon numbers are given. (B) Sequencing strategy. The arrows indicate the direction and extent of sequence determination. Restriction sites are indicated as follows: A, *Ava*I; B, *Ban*II; C, *Sca*I; E, *Eco*RI; G, *Bgl*II; H, *Hae*III; J, *Nhe*I; K, *Kpn*I; M, *Sma*I; N, *Nco*I; P, *Pst*I; R, *Rsa*I; S, *Sau*3AI; T, *Taq*I; U, *Alu*I.

AATTCTCAGGGGCCTCACATCCAGGAGGGTGTTGTCTCTGAGGTAAAAACACATTTCTTAACCCACAGCTAGAGACCAAGGCCTGTTTCTCTCACTGTCA -1119
TTGCTGCCCAGAGATGGTGGAAAAGCAGTCAGGACACACACTTAGAATACCGGTGCCTCTGGGTCAGGACTGCCCTTGGGGTCCACTCAGCTGTTTCACA -1019
GGGGCCGAGAGAAGGGAAGAGAGGGGTCCAGCCTCGATGTCACACATCGGTAGCACCTAGGGCTCTCCCGTTCTGGCATTTGATAAACAGCACACTCCAC - 919
TGTGGCCAAGAGGCTCAGGCACAAACATTACTTGGCTGCAAAGGGATGAAGACACACCCTGGAGGTCACACCTCTCACAGGGTGAGAGGCCACAGTAGAA - 819
GATCGTCCATGGCCCTTAGGGTCTGCCTTGTGGCATTGGTGGCCCATTTGAAGTGATCCCGGGGGAACAAACAGGGAGAGGAGCAGAGTGGGCACTTAGG - 719
GAGAGTAAGTAGTGCTCTGGGACCTAGGGTTAAGTGCATTTAGGTAGTTCTGTCAGGCTTCTGAGAGGTACAGGGAGGAAGGAATGGGGCAGAGAAATAG - 619
GTCTAGGGAGTGAAGGGCCAATAGGGGGATTGATGAACTGGAAACCAGTTATCCTGGAGACCCGGGGTTCTTTGAGAGCTACCCTTCCTGTCCACCATCA - 519
CTAACCCCAGGAAGGGACAGCCCACCCCACTCCCTGATGCCAGAGGTTAGATAGGATCTTACTTTTTCCACTAGGCAGCAGCCAAAAGGAGTCACCCATG - 419
ATCAGGATGGAAGACTGGGGCCTCGGAGGAGCCAGTCAGGGGTAATACAAAGGTTAATATAGACGATGCAGGCTCAAGGACATAGATGGTGGGGCCTGAG - 319
TTGGAAGTGCAAAGCCAGGGTCTATGCTGTCTGTACCCTGACCTTATGCTTGAACCCCACAGTTCCTCATCAGCCTCCGTCGAAGCTAAAGGTTGCAGGG - 219
CCAGGCCAAACAGGGACACCAGCGTCACTGGGTTCAGCCATGTTTCCCACACTCGATTCCTGCCACTCTGCTTGGCTTCCGGCTCCTGCTTATCCCTCAC - 119
CCACCTAGCTTGGCCTCACGGGCTAGGATTTATGAGGGCTGCCCTGCCATGGACCCTGGGGTAATAAATCAGAGCAGGGCCTGTGGGTGTGTATAAAGA - 19
AGGCTCAGGGTCTGGGCTACACGGCTCTCAGAAAGCCTTGGCTGGACCAG ATG GGC GGG AGG AGG ATG CCT CTC TGG GCA CTC TTG T 69
Met Gly Gly Arg Arg Met Pro Leu Trp Ala Leu Leu L
TG CTC TGG ACC TCT TGT AGC TTC AGT CTC CCG ACA GAC ACA GCC AGC TTT GGA CG GTAACTTGGGCAGAGAAGGGGCGGGA 150
eu Leu Trp Thr Ser Cys Ser Phe Ser Leu Pro Thr Asp Thr Ala Ser Phe Gly Ar
GGGTGCGGGAACTGGCCTCTTACTAACGCTCTCAGCTTTTGATATGTGGGGTCATCCAGTCCTTATGTCCAGTCAGCCATGGATGTATCCTCCCGCTCTG 250
ACAGTCTGAGCAACTAGCCCAAGGGTACACGGCCATAATTAATTAGGTCCGGGAGGGTTCTCTTTGGAGTCACATCCAGAGGATGGGGAACTCAGACGGT 350
TAGAGCATCCCTGGTCCTTAAGCTCCACAACGCAGTGACTGAAGGGGGCAGTGCTTGCTAACACTCCTGGGTAAGTTAGTCGCGGCCCCTCTAGAGTTTT 450
GCAGGTTAAATTCTGCTTCACTGGCAGCGGATGAAGCAAAGCAGCAAGCAGGTCTCACCAATCCTCAGCGTGCCATCTTGAAACAGGATGATAACAGAGC 550
CTGCGTCACGGGCTGCTTGGAAGATCTGATGAAATGGTGCACACATCACTGTGGATGCTGTGCCAGGAATGGTGTAAGACACCATGGCCCCAGCGTGGAT 650
TGTCACGGAGTGTAGTAAAGGAAAGGCTCCCGGCAGCCCACGCGGGGAGGATGACACATTCGCTTAGTGCAGTTCCAGGGCCATGGCAGCAAGGTTTCT 750
TGCAGACAGGAGTAGGGAAGAGGCGAGACTAGGAGAAGGCTCACCGGAAGCCCGGGTCTGGGGACTGAGGTGAGGCGGTCCTGAGATCTGTGGCAGTGAG 850
CGTTGTGACTCTGCTGGGGCCTAGCACTTGGTGGGTTGCTTTCGTCCTAAACCGAGTCTCCAGGCCTCAGCACCTATTCTGCAGATGATGGTACCAGGGG 950
GCGTCCTCAAGGAAGGTCCGAGGGCCCCCTGGTCTTTGTTTCCCAAGCCCTGCCCCCTTGCTCCCAGGTTTGCAGGGAGCAACTGCTGCAATGCGAGAAG 1050
AGGGCTGTACTTCCTTGCACCCCCCCCCCCAGGATGAAGGGCAGAAGGAAGTAAATGCGTGTGGCAGGGAGCATGGGATCAGCTAGAGCGCAGTGGGCCT 1150
CTGTGCTACCTGCTCCGTGTAAAGTATTGTGCAGGCCACTTGAGGGGCTTTCTTCTCAGGGTCAGTTGCCTTCCATACACACGATGTCCAGCCTCCGATC 1250
TGAAGGCTGAGCTGTGACTGCCCGAGGATGGAGAGGTCAGAGAGTGCTCAGCCCCACACAGCATCCTCAGCACCGGCGGGCGGCCCATTCCACTGCTACC 1350
CATGGGCTAGGCTGTGGGAGCACTAGAGGAGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAACC 1450
CATGGGTTGGCTGTGGGAGCACTCAGGGGAACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAACCCATGGGCTGGCTGTGGGAGCACTAGGGGAAATGG 1550
ACCCATGGACTGGCTGTGGGAGCACTCAGGGGAACCCATGGGCTGGCTGTGGGAGCACTAGGGGAAATGGACCCATGGACTGACTGTGGGAGCACTCAGG 1650
GGAAATGGACCCGTGGACTGACTGTGGGAGCACTAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTAGGGAAAATGGACCCATGGGCTGGCTGTG 1750
GGAGCACTCAGGGAAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTAGCTGTGGGAGCACTAGGGAAAATGGACCCA 1850
TGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTGGG 1950
GGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTAGCTGTGGGAGCACTAGGGAAAATGGACCCATGGGCTGGCTGT 2050
GGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTGGGGGAAATGGACCC 2150
ATGGGCTGGCTGTGGGAGCACTCAGGGAAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTAGCTGTGGGAGCACTAG 2250
GGAAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGTC 2350
TGTGGGAGCACTGGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGAAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGA 2450
CCCATGGGCTAGCTGTGGGAGCACTAGGGAAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACT 2550
CAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTGGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTAG 2650
CTGTGGGAGCACTAGGGAAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGG 2750
ACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCA 2850
CTGGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTAGCTGTGGGAGCACTAGGGAAAATGGACCCATGGGCTG 2950
GCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTCAGGGGAAATGGACCCATGGGCTGGCTGTGGGAGCACTGGGGGAAATG 3050
GACCCATGGACTGGCCCCCTTGCTGAGAGCATCTAGGACTAAAGATCTAGGAGAGGAACAGAAGCAGGTTTTATGTTTATCTTCTTTTGGGACAGAGCCT 3150
CCCAACGGAGCCCGGAACTCACTCTATAGACCTGGCTGTCCTCAAACTCGCAAAATCAGCCTTCCTTTGCCTCCTGAAGGCTGAAGTTAAAGGCATGTGC 3250
CACCACACCACAGAGGGGTTTTCTAAAAGAGGAAGGGTCCGTAAAGTACAGAAAAGCATGGGTCTTTTCAGGAGATGGTGGGAAATAGACCTTTTTGGAT 3350
ATATATTCCCTTCTCCTCCTTTGTAAAGTGGGGAAATAAAAAACACCCATCTGATGGAATGGCTGGGGTCAAATAAAACAGGAGTGAAAACACTCGTAAG 3450
CTGTGATGGGACTGTTTCTGCTAGAGATTGCACAGAGAGGTGTGAAATATAAGTAAAGCAGGGAGGTGAGGGCGTGGAGTGCCAGGAGGAAGGTTAGGGC 3550

TCACGTTGGAGAACAAAGAAGGCTCTCTGGGTGTCTGGGAAACCGTGACCCTGGGCTGGCGGCATCTTCCTCCAGACAAAAAACAGTCTCCATGCCTCTC 3650
TGTGCCTGGCAGCCCTGGTAGGAAAGACAGACAGACAGACAGAGCATGGACTGTCTCTGCTTGGCATTTCTGAGTCATCTTGCAGTCCTATGAAGCTGCA 3750
ATGTCTTGGCTGGAAAACAGGGAGCTAGCTGTCATGTGGTTTGTATGAGGCTCTTGCCAGGCAGCTGGTCTGCAGCCAGTAGCAGCTCAGCGGGCCCTTG 3850
TCAAAATTAAATCTCTTGAATTTCCCCAGTCAGTGATGCATTGGAGGACAACTGATTTGAGGTGAGGAGTCCTGGGGGTTCAGGCCCAAGACACATCAAT 3950
CCCCAGGCTGAGGCTCACAGGCAATCTACAGACACTCGGTCTACCCGAGGCAGGCTGGGTGCACGTGGGTGCAGCTGGGTACATACCACCTGGCTCTGTG 4050
ACACCCTAGGGGTACCAAGTCCTTGCAGCCTGCTGTGGGCATCTTCTGCCACCTACAGGCCACCCCTGGTACTGCCTCCCCTGTCAATCAGAGGGGATCA 4150
GAGAGGCAGTATAGACCTTTATGTTCCTATCCAGAACAGTGAGGCACAGAGTAGGTGTTCAACACATGATGATAATGATGGATGGGTGGGTGGGTGGTGG 4250
GTGGGTAGGTGGGTGGGTGGGTGGTGGATGTATGGATGAATGGACGGATGGACAGACGGACAGACAGACGGATGGACAAATTGCCTTATTTAACTAAAAT 4350
ACGATCTGGGAATTATTTAGTACCAGAATAAGTGACTCCAAGAAGCTCATACCTTCTCCTGCAGAAACCCCAGGGCACAAAAAGTGATTCGTAATTGCAT 4450
GGGAGAGGGTTAGTGATAGATGTAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGGCAGCCTCTGGTTTCTGCTGACATTGGAGTAC 4550
TACAGGACTCTTCTGTTTTTCTTCCTCTATCCTGGAGCAG A ATC TTG CTC AAG AAA ATG CCC TCG GTC CGG GAA ATC CTG GAG G 4634
g Ile Leu Leu Lys Lys Met Pro Ser Val Arg Glu Ile Leu Glu G
AG CGG GGA GTA GAC ATG ACC AGG ATC AGT GCT GAA TGG GGT GAA TTC ATC AAG AAG TCT TCC TTT ACC AAT GTT A 4709
lu Arg Gly Val Asp Met Thr Arg Ile Ser Ala Glu Trp Gly Glu Phe Ile Lys Lys Ser Ser Phe Thr Asn Val T
CC TCC CCC GTG GTC CTC ACC AAC TAC TTG GAT GTGAGTGCTGGTTCATCTGGTATTTCCTTCTCTCTGGGCCGAGCAGTCCCCACTCCT 4798
hr Ser Pro Val Val Leu Thr Asn Tyr Leu Asp
TCCACACATGTCTGACTGAGGCAGGGGAGCACCAGAGCGCTCTGTAGAACTCCTACGTGCAGCCGAGGAGTCTGGTGGGGAGGGGCTGTCGTGATTATTA 4898
TGCTGTTTGTTTTGTTTTGTTTTGTTCTTGAGACAGGGTCTCCCTGGGTAGCCCTGGCAAGCCTAGAACTCACTGTACAGACTAGGCTGACCTCGAACTC 4998
ACAGACTTCTACCCACCTCTTCCTCCCAGGTGCTGGGCTTAAAGGCATGTGACATCATGCCCAAGTATTTTAGCTGCTTTTAGTAGGTTATCATGCCCCA 5098
GTGAGCCTCCTGTACCCCAGGACAGCAGAGGACAGTGCAGAGTCCTCAGCTCACCAAAAAGAAAGCCAACAGCCAAGAAGGGAACCGCAAGGCCCCCCAA 5198
CCCCGAGTCCCCTGAGCTCCATGTCCCTACTTTTCTCTGCCCACCCCCCCCAG ACC CAG TAC TAT GGT GAG ATC GGC ATT GGT ACC CC 5286
Thr Gln Tyr Tyr Gly Glu Ile Gly Ile Gly Thr Pr
A TCC CAG ACC TTC AAA GTC ATC TTT GAC ACG GGT TCA GCC AAC CTC TGG GTG CCC TCC ACC AAG TGT GGT CCC CT 5361
o Ser Gln Thr Phe Lys Val Ile Phe Asp Thr Gly Ser Ala Asn Leu Trp Val Pro Ser Thr Lys Cys Gly Pro Le
C TAC ACT GCC TGT G GTAAGAATCAAGCCTCGTGCCTCTCCCCTGCTCTGCCTGCTGCTCCTATGAACTGCTTTCCCTCAGCTCCACGCCACTGA 5455
u Tyr Thr Ala Cys G
TCCAAGAGAGAGGACGTTTCTCTCATCTTCCTGAATTGGCAACCAAGGTGAATAAGCCAGCCTTGGGAAGTGCCTGGGCACCTGGAAGGCCATTTGCCAG 5555
TGTCCCAAGGATTCAGACAGGAACAAATTTGTAAAGCCATCCAAGTCTTGAAGCTTTTCCAATGATAGCCGTAGTCAATGCCCTTGTGCTGAGGACTTAC 5655
TCTGTGCCCAGAATCTTCTTTGGCAGTGTATCGGTGTTATCAATGGTTCTAGAATTCTTTGAAACAAATGTCTCAGAATAATAAGCATTTAAGGCAAAAC 5755
CAAGAAAACACTGTCCTCCACTGGTACTACATTTTATCCAAGCTAAAAAAATACACACAAACTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC 5855
TCTCTCTCTCTCTTTCACACACACACACACACACACACACACACACACACACACACACACACACACATCTAAGTCTTCTAATCATACTACCCATAGCC 5955
ATCCCTTGTCCTACCTCTGAGCCTTTTACTTAGATCAAAAACTCACGTGCTGATTGGAGCCTGTGTTCCTCTGCTAG AG ATT CAC AAC CTC TAT 6049
lu Ile His Asn Leu Tyr
GAC TCC TCG GAA TCC TCT AGC TAC ATG GAG AAT GGG ACT GAA TTC ACC ATC CAC TAT GGA TCA GGG AAG GTC AAA 6124
Asp Ser Ser Glu Ser Ser Ser Tyr Met Glu Asn Gly Thr Glu Phe Thr Ile His Tyr Gly Ser Gly Lys Val Lys
GGT TTC CTC AGC CAA GAT GTG GTA ACT GTAAGTAGGACCACCCCTATTTACCCCCATGGGACCCATGCGGTGTGGAATATATGTGCTCCAG 6215
Gly Phe Leu Ser Gln Asp Val Val Thr
CGCCCCCTGGAGCCTGGGAGGTTGAAGTGCAACCTCTTTGGCAGCTTCCAGTAGAGCAAACTGGCTGACACTGATGGACATGGACAAGGCTTTACGTTAA 6315
CGTACATGTGGGTGTACCTAAAGCCCAGTTTCCTTCCTAGTAACCTGTCAGACTTTGCTAGTTGAGAGCAGGCTTGGAGCATTGAAATTAGGAACCAGGG 6415
TTTTGTGGCAGGGCGATTTTGCTTTCTTTCAGTCAGTGCCTAAGCCTTCGCTGCTCTGGCGTCTCTCAGCCCTGCTGTGCCAGAGGTAGGAAGAGACACA 6515
TATAGGAGCCCAGAGGAAATCAGTGCAAGCCCTTCCCTCTCCCCATGCCTGTGGAATCTGGGGGAGGGGCAGAAGGGAAGCCCTGGTTATAGTGAGGAA 6615
ATCCTCAGCCTTGGCTGGTAGGGCTAGCATCAAAGTCAGGCCTCGGGGAAGGTGCAGATCACCTGTCTGGCAGGACCTCTTTCTCAGCCCTTATAGCACT 6715
AGTCACCCCCCTCTTTCTGGCCCCCAAGCATAGAGGTGTTGAGACAGTGTGTGCACATTTGGCGAGATAGTCTCACCTTCACGGCTGTACAGAACCTGTA 6815
TCCCTGGGGCCCCTGGTGTGAAAGCCAGAGCAGATCCCAGCCAAGGTTGGGCAGTAACCTCTGTCCCTTATGTTTCTTCTCTCACAG GTG GGT GGA 6911
Val Gly Gly
ATC ATT GTG ACA CAG ACC TTT GGA GAG GTC ACC GAG CTG CCC CTG ATA CCC TTC ATG CTG GCC AAG TTT GAC GGG 6986
Ile Ile Val Thr Gln Thr Phe Gly Glu Val Thr Glu Leu Pro Leu Ile Pro Phe Met Leu Ala Lys Phe Asp Gly
GTT CTG GGC ATG GGC TTC CCT GCT CAG GCT GTT GAT GGA GTC ATC CCT GTC TTC GAC CAC ATT CTC TCC CAG AGG 7061
Val Leu Gly Met Gly Phe Pro Ala Gln Ala Val Asp Gly Val Ile Pro Val Phe Asp His Ile Leu Ser Gln Arg

GTG CTA AAG GAG GAA GTG TTT TCT GTC TAC TAC AGC AG GTGGGCCTTTGACTTCAAGGTCAGGGCAGGGAGGGGAGGACAGCTGGGA 7148
Val Leu Lys Glu Glu Val Phe Ser Val Tyr Tyr Ser Ar
GGACGGCAAAAGTCCAGTCCAGGATTTACATGTCCTGATTAGGTGGTCCTCAATCCATATCTAGAACCACACTAGCCAATTAAAATGAATGCAAGTCAAA 7248
CATTCGGTCCTGAGGGACTGGCCACACAGACACAGCTGGCTTCATGTCTTTGCTGGCACCTTTATCTGTCCTCTCATTGATCCGCTCTGTGCTCTGTTTC 7348
CATGCTCTGTGGTGGGCTCCAGGGGTGCACAGTCCACAAGACAATCCCTACCCTCCATCCTCATAGATCTCACTGCCCAGCAGGGGAGACGAGAAATACA 7448
GTACAGTGAGTGTCATTCTGAAGCTGCAGATGGTTTTTCAATTCTAGGCCAGGATTCTGGGCAGTTCAGCCAGCCTTGGAGAAGCTGTGGTCAGTGCCCC 7548
AGCGGGGCTGGGATCTGCTGCCACAGGCCGGGTTTAGAAAGATGGTCTGAGCTGGAGGTAAAATCATTACTGGTAAGAATAGAGGCCAGGGGTGGCGAGA 7648
CTGGAAGGAGGAGTAGACTAGAACAATCTCAGAGGAAGCAGGAATTTCTGGATCATGGCTCCGGCAGATTTCTGTGCAAAGAGAGGCATTGAGCAAATG 7748
ACACCTCCCGGAGTCTCCTGCGGTCAGTGGTCTTGGGGTTGTGCTTCAAAGAGACCAGAAAAACAAAGTGGCTAAGAGTGAAAGTAGATGTCAGAAAGAG 7848
GTGTCGGGGGGTGGGGGAGCAAGTCCACAAAATGTGGGCTGCTCTGGAAAATTCAACAAAGAAGGAGATGTGGGGCAGGGTGTGGGGTGAGATCCAGAGA 7948
GAAGAAGGAGGGTGTTCTAAACGCTAGAGGATGCTTGAGTAATGCTGTGGGTAGAAGAGCCATGGGAAGACTAAAATGTGGGGAGCACATGCATGGGGCT 8048
CTGGAGCAGGACCACAGAAGGAATGAGGCTCCAGCACAGGTAGCAAGCAGGCCTGGTAAGCAGGGGCCCTCTTCCCTTCCCTTGGGCTGAGAAGCAGGAA 8148
GACCTAGATAAGTCACTGACTCTTGCACAGAACATGCACAACCTACCACATGTACACATGTGCACATACATGCGTGTATGTTGTGCACATACATGTGTGT 8248
GTGTGTGTGTGTTTTGAATTCTAAGAAACTCCTTCATATATACACGAGTACCTTAAAAACCAGTCCGAGGCAGGCCTGGGCTGAAGTAGCATGATGCATG 8348
TGGAGAGGAAAGTGAGGCCAGTCAGCTTTGACTGAGAATGAGAGTGTGGCCCCAGCCATCGTGTGTCCACCAGGGTCTCACCATCTGGTGACTCAATTGC 8448
CTGGAGGGCAAAAATTAGCCTCTACTTCTAAACACAGGGTTGCTGAATCTTGCCTAAGAAGAAGGTTCTCCCTTCTGTGTCCCTCTTTGCCTCAGCAGAA 8548
ATCTTAAGGAGAGAGGAGCCCCAATGGGGCCCCAAGCTCCACTATTTCCCAGACCCTTATCCCAGAATCATACCCACCCTCCCACACATCCACTCATCAG 8648
ACCCAGCTCCACTAAAGATCTGCCTTCTTCCCTCTACACACTCTAGTCATGTCTGCCTTCTCCCCAGAGTCTGCCCCACACACCCGAGAAAGGCACAATT 8748
TACCGCCATTTGCATCTACCCAGTGTCCTGTAACTAGATGGTTTTCTGGGGGGCAGTCGGACAGTCCTAGAAATACTTCGGTTACATTAAGGGAGGGGCC 8848
AGCATGGGGAGAAGCAACAGGGTCTATGTGTCTCCTGATGACAGCTTACGACCTTGCCATCTACCCACAGAACCCTTCCTCTGCCCCAAAACCCGCCTGC 8948
ACCAGACTGGACTTTCCCTCATCCCCTAGTCTCCAGTGTGTCTCTAAGTTTCGCCGCTCTTTCCTTTAG G GAG TCC CAC CTG CTG GGG GGC 9039
g Glu Ser His Leu Leu Gly Gly
GAA GTG GTG CTG GGA GGC AGT GAC CCT CAA CAT TAC CAG GGC AAC TTT CAC TAC GTG AGC ATC AGC AAG GCC GGC 9114
Glu Val Val Leu Gly Gly Ser Asp Pro Gln His Tyr Gln Gly Asn Phe His Tyr Val Ser Ile Ser Lys Ala Gly
TCC TGG CAG ATC ACC ATG AAG GG GTCAGCAGTTGTAACCCTTCCAACCATCCAAATGCATGTCACCATGTGATCGGGGCAGGATGGGGATGG 9206
Ser Trp Gln Ile Thr Met Lys Gl
GACAGGGGGGCCACCCCTTAAGGCACAGCCACCATCTGCTTTCTGTGTGCTCTGTCCAGGATCGGGGCATGAAGATTTGAGGGTGGGACTGGGATGGAGG 9306
GGACTCAGGGAAAGGAGGTGGAGTCTGGAGGCTACCGTGTTGATGGCAAGGAACAGTCAGTACCTTTCTTTAGTGCCTCAGCCCTCCTCCAGGTCACCAC 9406
CAGAACACTTGTATGGCCTCCCATCACTCCCTAAATTGGGAAAGGCATGCAGCCGGGGGTGAGGATGGAGGTGGGGTCTGTTGCCTGTCTAACTGTAGCC 9506
TCCACAG G GTC TCT GTG GGG CCT GCC ACC TTG TTG TGT GAG GAG GGC TGT ATG GCA GTG GTG GAC ACT GGC ACA TC 9582
y Val Ser Val Gly Pro Ala Thr Leu Leu Cys Glu Glu Gly Cys Met Ala Val Val Asp Thr Gly Thr Se
C TAT ATC TCG GGC CCT ACC AGC TCC CTG CAG TTG ATC ATG CAA GCC CTG GGA GTC AAA GAG AAG AGA GCA AAT AA 9657
r Tyr Ile Ser Gly Pro Thr Ser Ser Leu Gln Leu Ile Met Gln Ala Leu Gly Val Lys Glu Lys Arg Ala Asn As
T GTAAGAGAGCTGGGTGGCGTCAAAGGGCGGGCGGCGGTGGTGAGGGGTGGTGGCAACAGCACCATCTGGCTCCAGCCTGCAAACTGTCCAGACCACAC 9756
n
TGGGTCCGAAACAAAGCCTTCCCCTCCTCACCTCTCCCACTCCCTGAATGTACGGTATGTCTCCCATAGAAGGTGACCAGGGACTGTGTCCAGGACTCTC 9856
AGTTACAGGGCTCTCTAGTCTGCCTAGAGCCTTCCACATTGACAACACTTCTCTTTACTCCCCCAG TAC GTT GTG AAC TGT AGC CAG GTA C 9947
Tyr Val Val Asn Cys Ser Gln Val P
CC ACC CTC CCC GAC ATC TCC TTC TAC CTG GGA GGC AGG ACC TAC ACT CTC AGC AAC ATG GAC TAT GTG CAA AAG G 10022
ro Thr Leu Pro Asp Ile Ser Phe Tyr Leu Gly Gly Arg Thr Tyr Thr Leu Ser Asn Met Asp Tyr Val Gln Lys
TGAGGCTGTGAGAGGGGTGGACAGGGGCCACTGAAGGTGGGACAACGTGTAATGTGCAAGTCTCCTAGTACACTCGGGGCTGACGAACGCAGCTCCTGTC 10122
ATCAGGAGAGTTTGTAGCAATCCCAGTCAGTACCCAGTCCGCATCCCTCACGCAGCAGATCAGAACTTCTGCAACAGGCACCTAGGTATCGGAGTTTTGT 10222
TTTTAAACTTTTTTTTTTCCGGAGCTGGGAACCGAACCCAGGGCCTTGCACTTCCTAGGCAAGCGCTCTACCACTGAGCTAAATCCCCAACCCAGTATTG 10322
GAGTTTTAAAAACTCCCCAGCAGATTCATTATGAAGCTATGCTGTGACTCGGTGATGGTCCTCTCTCTCCATTCGTGAGCGGCCCTCTTTCTGGCCCTCT 10422
GTTCTTTTCATGCTCCCGGGCTTAGAGAAGTTCAGTGACTTGTTTTGGGTCAAGCTAAGTGAGCCTGTGAACAAGGAGTAGACACACCTTTCGCCTGATG 10522
TGTACCGCATTGCCTGTCTGCCCCAGGAAGGCTGAGATTTAAGAAAATGGCTCTGGGAGCACATAGCGGAGAGGAGCCATACACTGAATATAGGTGCTTC 10622
ACTCCGGTGGTGGGAAGGGGTCGTGTGGCCAGGCAATAGAGCAAACTTCCTGCTTCTTGCCAG AAT CCC TTC AGG AAC GAT GAC CTG TGC 10712
Asn Pro Phe Arg Asn Asp Asp Leu Cys
ATA CTG GCT CTC CAA GGC CTG GAC ATC CCA CCA CCC ACT GGG CCT GTC TGG GTC CTG GGT GCC ACC TTC ATC CCC 10787
Ile Leu Ala Leu Gln Gly Leu Asp Ile Pro Pro Pro Thr Gly Pro Val Trp Val Leu Gly Ala Thr Phe Ile Arg

AAG TTC TAT ACA GAG TTC GAC CGG CAT AAC AAT CGC ATC GGG TTC GCC TTG GCC CGC TAA GCTCCTCTGTCACCCAGTGA 10867
Lys Phe Tyr Thr Glu Phe Asp Arg His Asn Asn Arg Ile Gly Phe Ala Leu Ala Arg ***
CCCTAGTTTTAGGCCAAGCCAAAGCTGGCGTTCCTGGGGGCCATTCTGTCTGGCTCTGCCCCCAACATAGGGACACTGGACACAGAGCCCTTGATGAGTG 10967
CTTGTCCCTTCGCCTGCCTGCACTCACCCTTCCCTGCTTTGAGGAAAAACAGAATAAAGACTGCATGTTTAGAACCTGTTTCCAATGGGTTCTTTTGAGT 11067
TTGGAGAAGGAGGGACAGCTGCATGGTCACGCATGGGGAAGACTATGACAGATGAAGCCTGAGATGTAGTTTTGTTAGTAAAATGCTTGCTTTTCAGGTG 11167
TGAGGACCTGAATTTGATCCCCAGAACCCACAATTTAAAAAAAAAAGAGTCGGCATGATGGCCGATGTTTTAATCCTGGCAACCAGGGTGACAGAGACAG 11267
GTAGGTACAGACAAAAACAAATCCCTGGAGTTTGATACAATTAAGGTTGTCCTTTGGTTAACACACACGCGCGCGCACACACACACACACACTCACTCAG 11367
AGACAGAGAGACAGAGAGAGAGACAGAGAGAGAGGGAGGGGCTATACACACATACACAGAGAGAGACAGAGAGAGAGAGAGACAGAGAGAGAGACAGA 11467
GAGACAGAGAGAGACAGAGACAGAGAGAGGGAGGAGGCTATACACACACAGACACAGAGAGAGAGAGACAGAGACAGAGAGAGACAGAGACAGAGACAGA 11567
GACAGAGGGAGGGGGCTATACACACACATACACACACACACAGAGAAAGAGAGAGACAGAGACAGAGAGAGACAGAGACAGAGAGAGGGAGGGGGCTATA 11667
CACACATACAGAGAGAGAGAGAGAGGGAGGGAGGGAGACAGAGAGACAGAAAGACAGAGAGACAGACAGAGAGAGACAGAGAGACAGAGAGACAGAGACA 11767
GAGAGAGGGAGGGGGCTATATATACACACACACACACGGGGGGGGCTACACACACAGAGACAGACACAGAGACAGAGACAGAGAAAGACAGAAACAGAGA 11867
GACAAAGACAGAGGCACGGAAAAACACACAGGCAGAGACATAGAGACATAGAGAGAAAGAGACAGAGAGATGGAGAGAGGGAGAATGTGACAAAGTAGAT 11967
TCCCACACACACACACATACACTGATGTTTTTGTGTGTAAGGTTGATGGCTTCTGTCTACTGTCCACCAGATTCACATAGAAATTTTTCATACAATTTTG 12067
GCTGGAAAGTTTACTAAGAGATAGGCCCCGTGTCCCTCTAGGCCTTAGGGCTATGGCAGTAGAGTGTTAACGGTAGTTTAATGTCAATTTGACACAAGCT 12167
AAAGTCATCTGAGAGGAAGGCACCTCAATTAAGAAGGGTGCTCCTCTGCACTCTCTGTGTGCCCCATGCCTCCAGAAGATGGGCTGTAGAATTAGTGAAT 12267
CATGGGTAAGGGTCCAGCCCATTGTGGGCCAAAAAGAGCAAGCCAGGAGGCAACACTCCTCCATGGTCTCTACAGTTCCTGCCTCCAGGTTCCTGCCCTG 12367
CTGTTGAGTTCCTGTCTTGACTTCCTTTGCTGATGACCTGTGATGTGGAAGTGTAGGCCAAACAGACCCTTTCCTCGCCAAGTGGGTCATGGTGTTTCAT 12467
CCCCCAGGCTCCGTGTTGCTGAACAATCCAACCCTATGTAG 12508

Fig. 7. Nucleotide sequence of the rat renin gene and its flanking regions. The putative transcription start site (nucleotide 1) was indicated by an arrowhead. The termination codon, TAA, is marked by asterisks. The polyadenylation site deduced from the rat renin cDNA (6) is indicated by shaded box. TATA boxes and polyadenylation signal are underlined by double lines. Filled and open triangles indicate possible glucocorticoid and estrogen-receptor binding sequences, respectively. A filled circle and a filled square indicate the putative enhancer core sequence and cAMP- responsive element, respectively. The identifier sequence, "A" stretch, and $(CA)_n$ or $(TG)_n$ are underlined. The 38-bp tandem repeats are marked by broken lines.

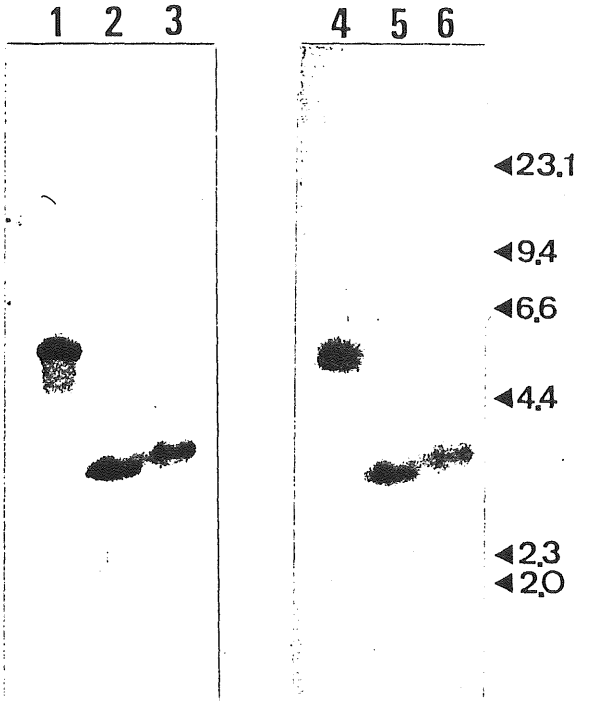


Fig. 8. Southern blot analysis of genomic DNA and phage DNA containing the rat renin gene. Genomic DNA (10 μg) (lanes 1-3) or phage DNA (0.5 μg) (lanes 4-6) was digested with the following restriction enzymes: *Xba*I (lanes 1 and 4), *Pst*I (lanes 2 and 5), and *Pvu*II (lanes 3 and 6). Phage λ DNA digested with *Hin*dIII served as a size marker (sizes in kb in right-hand margin).

```
        1                 10                20                30                40
rRen    MGG-RRMPLWALLLL-WTSCSFSLPTDTASFGRILLKKMPSVREILE
mRen-1  MDR-RRMPLWALLLL-WSPCTFSLPTRTATFERIPLKKMPSVREILE
mRen-2  MDR-RRMPLWALLLL-WSPCTFSLPTGT-TFERIPLKKMPSVREILE
hRen    MDGWRRMPRWGLLLLLWGSCTFGLPTDTTTFKRIFLKRMPSIRESLK

                50                60                70                80                90
rRen    ERGVDMTRISAEWGEFIKKSSFTNVTSPVVLTNYLDTQYYGEIGIGT
mRen-1  ERGVDMTRLSAEWGVFTKRPSLTNLTSPVVLTNYLNTQYYGEIGIGT
mRen-2  ERGVDMTRLSAEWDVFTKRSSLTDLISPVVLTNYLNSQYYGEIGIGT
hRen    ERGVDMARLGPEWSQPMKRLTLGNTTSSVILTNYMDTQYYGEIGIGT

                100               110               120               130
rRen    PSQTFKVIFDTGSANLWVPSTKCGPLYTACEIHNLYDSSESSSYMEN
mRen-1  PPQTFKVIFDTGSANLWVPSTKCSRLYLACGIHSLYESSDSSSYMEN
mRen-2  PPQTFKVIFDTGSANLWVPSTKCSRLYLACGIHSLYESSDSSSYMEN
hRen    PPQTFKVVFDTGSSNVWVPSSKCSRLYTACVYHKLFDASDSSSYKHN

        140               150               160               170               180
rRen    GTEFTIHYGSGKVKGFLSQDVVTVGGIIVTQTFGEVTELPLIPFMLA
mRen-1  GSDFTIHYGSGRVKGFLSQDSVTVGGITVTQTFGEVTELPLIPFMLA
mRen-2  GDDFTIHYGSGRVKGFLSQDSVTVGGITVTQTFGEVTELPLIPFMLA
hRen    GTELTLRYSTGTVSGFLSQDIITVGGITVTQMFGEVTEMPALPFMLA

                190               200               210               220               230
rRen    KFDGVLGMGFPAQAVDGVIPVFDHILSQRVLKEEVFSVYYSR---ES
mRen-1  KFDGVLGMGFPAQAVGGVTPVFDHILSQGVLKEEVFSVYYNR---GS
mRen-2  QFDGVLGMGFPAQAVGGVTPVFDHILSQGVLKEKVFSVYYNR---GP
hRen    EFDGVVGMGFIEQAIGRVTPIFDNIISQGVLKEDVFSFYYNRDSENS

                240               250               260               270
rRen    HLLGGEVVLGGSDPQHYQGNFHYVSISKAGSWQITMKGVSVGPATLL
mRen-1  HLLGGEVVLGGSDPQHYQGNFHYVSISKTDSWQITMKGVSVGSSTLL
mRen-2  HLLGGEVVLGGSDPEHYQGDFHYVSLSKTDSWQITMKGVSVGSSTLL
hRen    QSLGGQIVLGGSDPQHYEGNFHYINLIKTGVWQIQMKGVSVGSSTLL

        280               290               300               310               320
rRen    CEEGCMAVVDTGTSYISGPTSSL-QLIMQALGVKEKRANNYVVNCSQ
mRen-1  CEEGCAVVVDTGSSFISAPTSSL-KLIMQALGAKEKRIEEYVVNCSQ
mRen-2  CEEGCEVVVDTGSSFISAPTSSL-KLIMQALGAKEKRLHEYVVSCSQ
hRen    CEDGCLALVDTGASYISGSTSSIEKL-MEALGAK-KRLFDYVVKCNE

                330               340               350               360               370
rRen    VPTLPDISFYLGGRTYTLSNMDYVQKNPFRNDDLCILALQGLDIPPP
mRen-1  VPTLPDISFDLGGRAYTLSSTDYVLQYPNRRDKLCTLALHAMDIPPP
mRen-2  VPTLPDISFNLGGRAYTLSSTDYVLQYPNRRDKLCTVALHAMDIPPP
hRen    GPTLPDISFHLGGKEYTLTSADYVFQESYSSKKLCTLAIHAMDIPPP

                380               390               400
rRen    TGPVWVLGATFIRKFYTEFDRHNNRIGFALAR
mRen-1  TGPVWVLGATFIRKFYTEFDRHNNRIGFALAR
mRen-2  TGPVWVLGATFIRKFYTEFDRHNNRIGFALAR
hRen    TGPTWALGATFIRKFYTEFDRRNNRIGFALAR
```

Fig. 9. Comparison of rat (r*Ren*), mouse (m*Ren-1*, m*Ren-2*), and human (h*Ren*) preprorenin amino acid sequences. The sequences, indicated by standard 1-letter amino acid abbreviations, were aligned. The putative amino-terminal amino acid is marked by an arrowhead. The catalytically important aspartyl residues are indicated by asterisks. Gaps have been inserted to achieve maximum alignment.

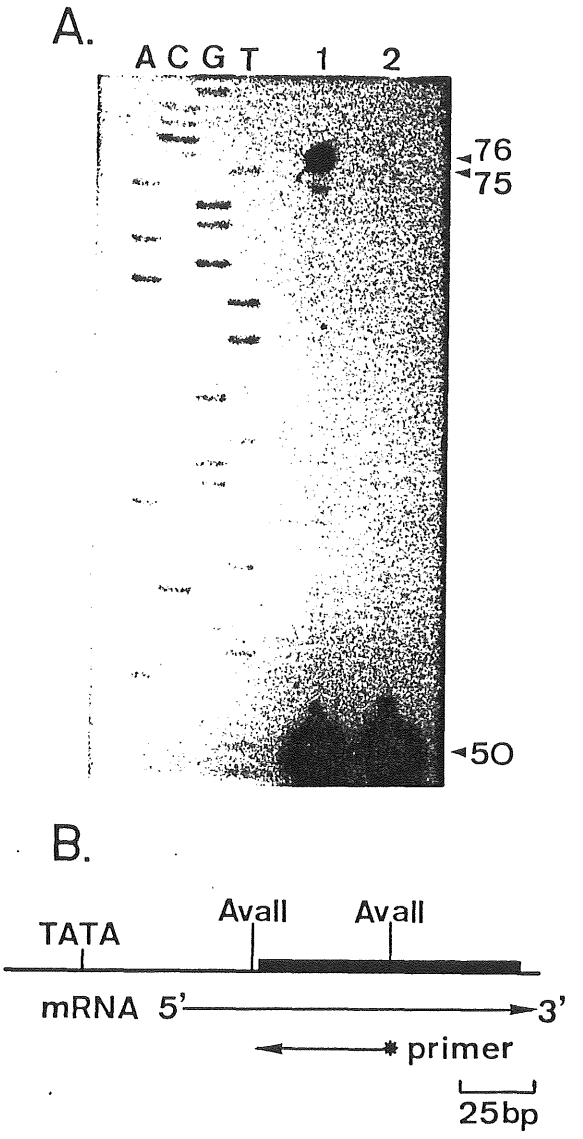


Fig. 10. Primer extension analysis of the rat renin gene. (A) A 50-bp fragment from exon 1 was kinased with [γ-^{32}P]ATP, hybridized with 10 μg of rat kidney (lane 1) and liver (lane 2) mRNA, and extended with reverse transcriptase. An M13 mp18 dideoxy sequencing ladder (lanes A,C, G,T) was used as for molecular markers. The nucleotide length of the primer (50 bp) and primer extension products (75 and 76 bp) are indicated in right-hand margin. (B) Schematic representation of the primer. The asterisk denotes the labeling position; Black box indicates the first exon.

Chapter IV: Identification of Renin and Renin mRNA in Rat Ovary and Uterus

Abstract

An increase in plasma prorenin during pregnancy suggests that prorenin is synthesized in the reproductive organs. To demonstrate the existence of renin and its mRNA in the rat ovary and uterus, I have characterized rat ovarian and uterine renin by radioimmunoassay and Northern blot analysis. These data indicate that prorenin activities in ovary and uterus were very high, and that renin mRNA was present in the reproductive organs. These findings suggest that the local renin-angiotensin system functions in the ovary and uterus.

Introduction

Maintenance of fluid and electrolyte balance are well known functions of the circulating renin-angiotensin system. In addition to its endocrine role, the renin-angiotensin system may function in a paracrine manner, since components of the system have been reported in many organs, especially those concerned with cardiovascular and reproductive organs (1). Reproductive organs in which the renin-angiotensin system components have been described include the ovary, testis, uterus, and prostate gland (2).

In the rat ovary, high levels of effector peptide angiotensin II (AII) have been measured (47). Angiotensinogen mRNA is expressed in the ovary (48), and AII receptor (47) and angiotensin-converting enzyme (49) have been localized to several rat ovarian structures. However, little information about renin is known in rat ovary and uterus. Since formation of angiotensin I (AI) by renin is considered to be the rate-limiting step in the formation of AII, the demonstration of renin and its mRNA in the reproductive organs is an important prerequisite for the demonstration of a functional tissue renin-angiotensin system. Therefore, the aim of the present study was to determine whether renin and its mRNA are present in rat ovary and uterus.

This study shows that high levels of inactive renin occur in the rat ovary and uterus and that renin mRNA is present in the reproductive organs. These findings greatly strengthen the evidence for a functional renin-angiotensin system in the rat ovary and uterus.

Materials and Methods

(a) *Determination of renin activity*

Renin activity was assayed using the partially purified hog angiotensinogen. Prorenin was determined as the increment in activity following activation with trypsin. Angiotensin I generating activity of renin from hog angiotensinogen was determined by radioimmunoassay of angiotensin I generated after incubation (50).

(b) *RNA preparation*

Female rats of Wistar-Imamichi strains (200-250g) were used for the RNA preparation from the ovary, uterus, and kidney. The total RNA was extracted from various tissues by homogenization in 5M guanidine thiocyanate and centrifugation through 5.7M cesium chloride (20). Poly(A)$^+$RNA was isolated by affinity chromatography on oligo(dT)-celluloce. In the experiments for RNA blot hybridization analysis, the total RNA (kidney) and poly(A)$^+$RNA (ovary and uterus) were used.

(c) *RNA blot hybridization analysis*

Poly(A)$^+$RNA or total RNA was denatured with 1M glyoxal/ 50% dimethyl sulfoxide, electrophoresed on a 1.5% agarose gel and transffered to diazobenzyloxymethyl paper (25). A 760 base pair fragment derived from the rat renin gene (15) was subcloned into pSP64 vector (51). The plasmid, pSPRRnRv9, was linearized with *Eco*RI and transcribed SP6 RNA polymerase in the presence of a ^{32}P-CTP (400 Ci/mmol) and used as a hybridization probe. After prehybridization for 16 h at 42℃, the blot was

hybridized with the ^{32}P-labeled rat renin cRNA for 16 h, then washed in 0.1xSSC/ 0.1% SDS (1xSSC: 0.15M sodium chloride. 0.015M sodium citrate) at 70℃. The size marker used were *E. coli* rRNAs. The filter was then subjected to autoradiography at -70℃.

Results and Discussion

(a) *Measurement of rat ovarian and uterine renin activity*

To demonstrate the existence of renin in the rat ovary and uterus, renin and prorenin activities were measured. Active renin was not present in measurable quantities in the ovary and uterus. However, prorenin activities in the ovary and uterus were very high and showed 5.1 ± 0.2 ng AI/ ml/ h, and 6.2 ± 0.3 ng AI/ ml/ h, respectively (Fig. 11). Polyclonal antibodies against rat kidney active renin completely inhibited the renin activities from the rat kidney, ovary, and uterus (data not shown). These data suggest that renin is present as an inactive form in the reproductive organs.

(b) *Northern blot hybridization analysis*

To identify the renin mRNA in the rat ovary and uterus, total RNA or poly(A)$^+$RNA derived from ovary uterus and kidney was subjected to Northern blot hybridization analysis with a rat renin cRNA probe. As shown in Fig.12, the RNA preparation from all tissues examined exhibited a single hybridization-positive band. Renin mRNAs in the ovary, uterus, and kidney of rat were indistinguishable by size according to their migration. Based on the relative hybridization signal measured in each preparation, the relative levels of renin mRNA in the ovary and uterus varied at around 1/100 of that of rat kidney.

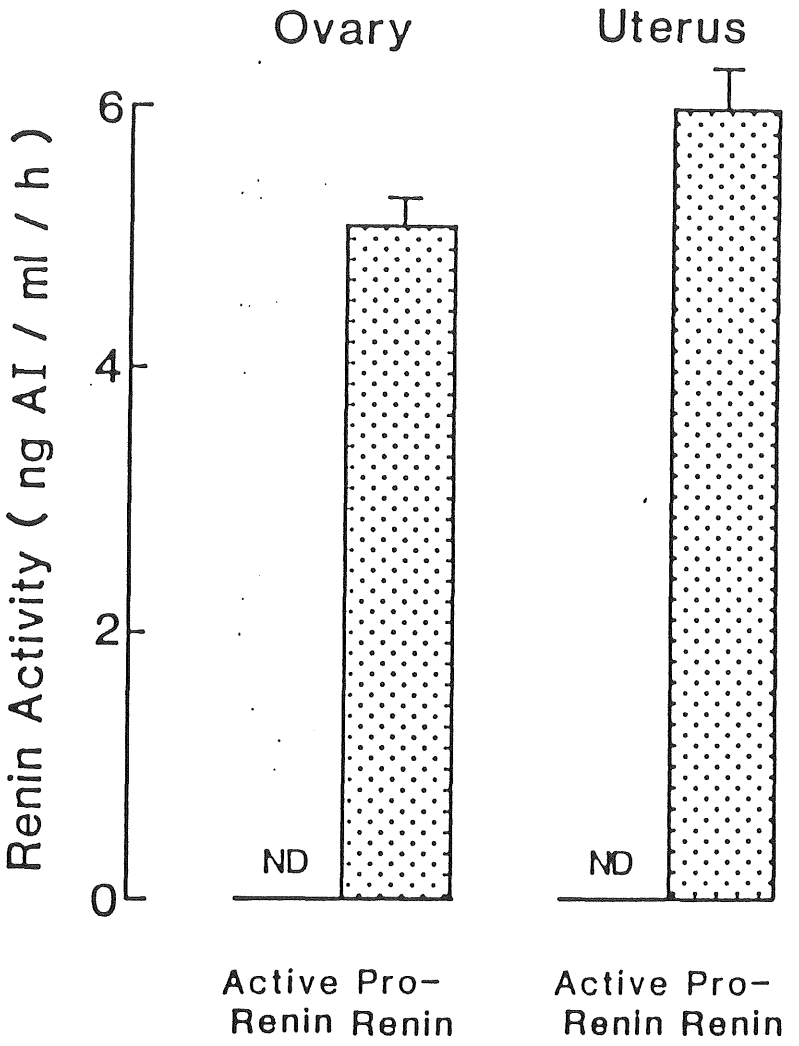


Fig. 11. Active renin and prorenin measurements in cytosol from rat ovary and uterus. Prorenin activity was calculated from total renin activity minus renin activity before trypsin activation.

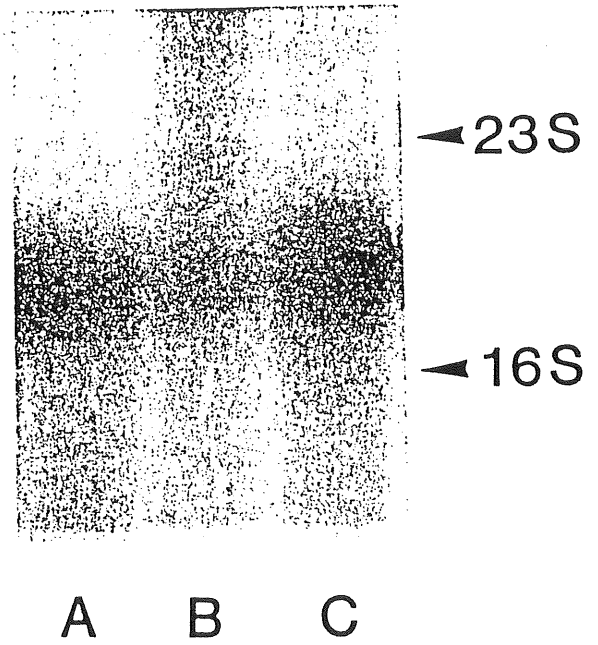


Fig. 12. Northern blot analysis of RNAs from rat ovary, uterus, and kidney. Lane A: kidney (100μg of total RNA), lane B: uterus (50μg of poly(A)$^+$RNA), and lane C; ovary (50μg of poly(A)$^+$RNA).

Chapter V. General Discussion

(i) Comparison of the 5'-flanking regions among human, mouse, and rat renin genes.

Genes harboring similar regulation in closely related species are expected to exhibit a high homology in *cis*-acting regions (DNA sequence) involved in the gene regulation.

Indeed, when 5'-flanking regions from human gene are compared with the analogous region of the corresponding genes in mouse and rat, large stretches of homology are found. The sequences of rat and human insulin genes are 75% homologous up to nucleotide -240 (52). A homology equal to 60% or more is found over 240 bp in the 5'-upstream regions of human and mouse opiomelanocortin gene (53,54). In apolipoprotein (apo) A-IV genes, a region extending from nucleotides -77 to -167 in the human gene exhibits striking sequence similarity (90% identity) to the rat gene (55). A further comparison to the promoter region of the mouse apoA-IV gene (56) reveals the same striking homology in this domain.

Renin mRNA has been identified in kidney, adrenal, testis, heart, ovary, uterus and brain of mous and rat (57-61). In human, it was also found in ileal carcinoma derived from small intestine, which does not produce renin (Fig. 13) (62). These observations suggest that *cis*-control elements of the renin genes contribute to the variety of mRNA expression. It is of interest, therefore, to compare the 5'-flanking region of the human, mouse, and rat renin genes.

Alignment of the rat, mouse, and human promoter region of renin genes,

(Fig.14) revealed a typical TATA box beginning at comparable positions in these genes. The high conservation within these regions suggested a possible biological significance.

There is another feature that the rat and mouse genes contain deletions between positions -31 and -32 (rat gene number) in comparison with the human gene. One hypothesis arising from this comparison is that these various conserved domains and/or the multiple deletions could be important in determining the observed patterns of renin tissue-specific expression in rodents and primates.

Further upstream from the promoter region, no such homology can be found among three species, although they exhibit a similar tissue-specific regulation in the kidney. Therefore, I initiated a series of *in vitro* studies to identify functionally important domains in the 5'-flanking region of the human and rat renin genes.

DNA sequences containing the 5'-flanking region of the human and rat renin genes (*i.e.* 3000 and 1200 nucleotides, respectively, upstream from the start site of transcription) were fused to the 5' end of the *E. coli* chloramphenicol acetyltransferase (CAT) gene (63). These recombinants were introduced into different cell lines (HeLa, A549, and mouse L cells), and the transient expression of the CAT gene, mediated by the promoter activity of the renin gene fragments, was measured. However, the transfection analysis can not be used to find potential *cis*-elements for their differential transcription since cell lines examined are not available.

For further investigation, it is necessary to find out appropriate cell lines which synthesize renin constitutively.

(ii) Structure of renin genes

Pepsin and chymosin, as well as renin, are the well-known members of the aspartyl proteinase group. Recently, their nucleotide sequences and the genomic structure were determined (64,65).

Since it has been proposed that the members of the aspartyl proteinase family are derived from a common ancestral gene generated by gene duplication and fusion (66), the structures of five aspartyl proteinase genes were compared (Fig. 15).

As shown in Table II, it is interesting to note that the intron positions interrupting the amino acid codons are completely conserved among these enzymes except for the exon 6 in the human renin gene. The unusual small exon consists of 9 nucleotides and codes for the extra 3 amino acids (13) not found in mouse (36) and rat (6) renins.

Other features indicated among these genes are as follows; i) These genes consists of 9 exons and 8 introns excluding the human renin gene. ii) The two catalytically important aspartyl residues are encoded by exon 3 and 7, respectively (exon 3 and 8 in the human renin gene).

The similarity in the structural organization of these genes indicates that they are evolutionally related, suggesting that the aspartyl proteinase gene family is arisen from a common origin.

(iii) **Prorenin in reproductive organs**

Extrarenal sources of renin including the placenta (67), uterus (68, 69), and brain (70) have been described previously. Recently, several groups (2,71) reported the presence of prorenin or renin-like activities in human ovarian follicular fluid. They confirmed that the follicular fluid prorenin or renin was immunochemically and biochemically identical to that of the human kidney. However, it has not been clear whether ovarian renin is synthesized endogenously or derived from the blood-borne enzyme of another origin.

The presence of renin-angiotensin system in the ovary or uterus is beginning to be understood. Biochemical characterization of the components of the system will be an important step toward understanding the mechanisms of formation and action of angiotensin II (AII), a product of renin-angiotensin cascade, at the molecular level. All of the components, except for ovarian renin, have been well characterized. Ohkubo *et al.* (48) confirmed that angiotensin mRNA is expressed in the ovary and Fernandez *et al.* (71) suggested the the presence of immunoreactive AII in human follicular fluid.

As shown in Fig.12, I employed rat renin cRNA to examine the expression of this mRNA in rat ovary and uterus. I demonstrated that renin mRNA accumulated in rat ovary and uterus. The ovary or uterus mRNA species were indistinguishable in size from that of the kidney. This provided direct evidence that renin mRNA is expressed in the ovary and uterus, thus supporting the view that renin is synthesized locally in these tissues.

In human, the presence of large proportion of prorenin in the

systematic circulation has been known (72,73). It accounts for 70-90% of the total plasma renin. The ovary and uterus also contain a large amount of prorenin which comprises well over the total renin (2). Renin or a renin-like enzyme was present in the uterus of all species examined. The concentration of renin is low in the non-pregnant uterus and increases during pregnancy. Most renin in a uterus exists as an inactive form, prorenin. The enigma of the selective secretion of prorenin in these tissues remains to be resolved. Kidney is known to secrete only a small amount of prorenin (74). Therefore, the increase of prorenin in plasma during pregnancy or the menstrual cycle might be derived from the ovary or uterus. The regional and cellular localization of all of the components of the renin-angiotensin system in the ovary and uterus suggests that these enzymes and peptides are closely involved in events related to the reproductive process.

Table II

Comparison of intron positions among the aspartyl proteinase genes, showing the interruption of codons specifyng amino acids

Proteinases	Amino acid positions interrupted by introns							
	(1)*	(2)*	(3)*	(4)*	(5)*	(6)*	(7)*	(8)*
mRen	31	81/82	123	162/163	228	268	316/317	349/350
rRen	31	81/82	123	162/163	228	268	316/317	349/350
hRen	33	83/84	125	164/165	230 (233)	273	320/321	353/354
bChy	21	71/72	111	150/151	217	255	303/304	336/337
hPep	19	73/74	113	152/153	219	258	306/307	339/340

*Intron numbers are given by 1 to 8 with the exception of the human renin gene. mRen, mouse renin; rRen, rat renin; hRen, human renin; bChy, bovine chymosin; hPep, human pepsin.

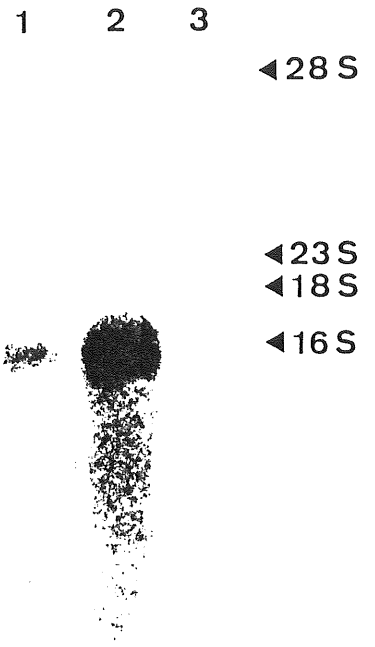


Fig. 13. Northern blot analysis. A 20-μg sample of mRNA from human normal kidney (lane 1), ileal carcinoma (lane 2), and normal small intestine (lane 3) was subjected to Northern blotting as described in **Materials and Methods** (Chapter I). Exposure on X-ray film was done at -70℃ for 24 h with an intensifying screen. The size markers used were calf and *E. coli* rRNA.

```
mRen-2                                  G     G
mRen-1                 CCCT-GGGTAATAAATCAAAGCAGATCCTGTG----------------ATACATGGTGTGTATAAAAGAAGGCTCAG
            -70        -60       -50    *     **                        -30       -20       -10
rRen   CTGCCCTGCCATGGACCCCTGGGGTAATAAATCAGAGCAGGGCCTGTG---------------------GGTGTGTATAAAAGAAGGCTCAG
                  **     *              *    *     *                         *          *    *
hRen   CTGCCCTGCCATCTACCCCA-GGGTAATAAATCAGGGCAGAGC---AGAATTGCAATCAACCCCATGCATGGAGTGTATAAAAGG-GG--AAG


mRen-1 GGTCT--GGG---CT-A
                  -1
rRen   --TCT--GGG---CT-A
                    *
hRen   ---CTAAGGGAGCCACA
```

Fig. 14. Pairwise alignment of the sequences around the promoter region of the rat, mouse, and human renin genes. *Shadded box* indicates the putative transcription start site. "TATA" boxes are displayed in the negative format. *Asterisks* indicate mismatches; *dashes* indicate gaps introduced for optimal alignment.

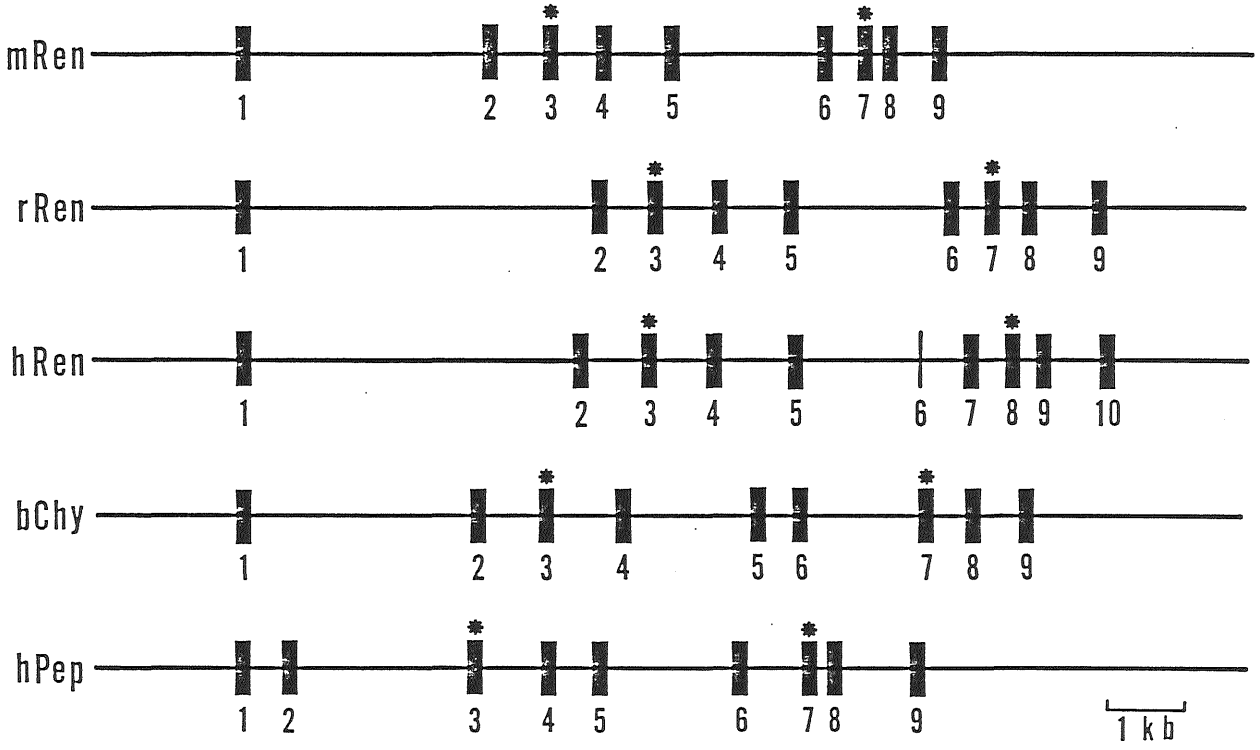


Fig. 15. Comparison of the gene structures among the aspartyl proteinases. *Black boxes* represent exons, and *solid line* indicates introns and flanking regions. *Asterisks* indicate the positions of the catalytically important aspartyl residues. Exon numbers are given. mRen, mouse renin; rRen, rat renin; hRen, human renin; bChy, bovine chymosin; hPep, human pepsin.

Chapter VI: Concluding Remarks

The application of recombinant DNA techniques to the study of eukaryotic gene regulation has led to the identification and characterization of regulatory DNA sequences (*cis*-acting elements), and significant advances are being made in the characetrization of the protein factors (*trans*-acting factors) that specifically interact with these sequences to promote or repress transcription (9).

DNA sequences involved in the initiation of transcription have been located not only in the 5'-flanking region, but also in the 3'-flanking region, or introns. These sequences may be responsible for cell-type-specific expression (75-77).

As a first approach to understanding the tissue-specific expression of renin genes, I have characterized the 5' end of the human renin gene by primer extension and sequence analyses, and determined the complete nucleotide sequence of the rat renin gene including its 5'- and 3'-flanking regions.

From these analyses, I found several possible *cis*-elements, steroid hormone receptor-binding sites or SV40 enhancer, that may confer tissue specificity of renin gene expression.

Interestingly, two groups (2,71) have reported the presence of prorenin or renin-like activities in human follicular fluid. Renin mRNA was also identified in rat ovary by blot hybridization analysis (61), and found that the ovarian renin is transcriptionally regulated by estrogen (3). Thus, the putative estrogen receptor-binding sites in

human and rat renin genes might account for a mediator of estrogen signal *via* its specific receptor on the gene regulation. However, there is no direct evidence that *trans*-acting factors bind to tentative *cis*-acting sequences in the renin genes and renin mRNA synthesis is initiated by the interaction of factors.

Since angiotensin II (AII) affects intracellular calcium and phospholipase activity, there are many potential roles for prorenin *via* AII action, ranging from an effect on pituitary luteinizing hormone release (78) to facilitation of egg extrusion (79) or alteration of ovarian steroid biosynthesis in a manner similar to the known action of angiotensin on adrenal cortical steroid biosynthesis (80). The comming years should see exciting new research in this regard. Renin has been found in the male repoductive system (57), but it remains to be determined whether prorenin is synthesized in the testis in a manner analogous to that in the ovary or uterus. Whatever the outcome, it seems likely that a new form and function of the renin-angiotensin system exists that operate independently of changes in circulating active renin, and the effect of which is regulated by changes in prorenin.

This new prorenin system seems to be involved in reproductive function. Future work to elucidate ovarian and uterine prorenin function may have clinical implications in the fields of birth control, infertility, and toxemia of pregnancy.

Acknowledgments

I would like to express my sincere gratitude to all those whose support and encouragement during the preparation of this dissertation.

First of all, I wish to express my appreciation to Professor Kazuo Murakami, University of Tsukuba, for his kind guidance and encouragement in the performance of this study.

I am grateful to Professor Shigehisa Hirose, Tokyo Institute of Technology, and Associate Professor Hiroaki Ohkubo and Professor Shigetada Nakanishi, Kyoto University, for their appropriate advice to prepare the manuscripts (Chapter II and III).

I also wish to thank Mr. Nishi, K., Mr. Cho, T., Mr. Saitoh, M., Mr. Nishimatsu, S., Mr. Tada, M., Mr. Seo, M.S., Mr. Takahashi. S., Mr. Hori, H., Mr. Hayashi, T., Dr. Imai, T., Dr. Nakayama, K., and Dr. Miyazaki, H., for their useful discussion and supports and the members of the Laboratory of Food Biochemistry in our laboratory for their friendship and companionship.

I dedicate this dissertation to my parents, sister Hiroko, my wife Chieko, and my son Yougo.

References

(1) Deschepper, C.F., Mellon, S.H., Cumin, F., Baxter, J.D., and Ganong, W.F. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 7552-7556.

(2) Glorioso, N., Atlas, S.A., Laragh, J.H., Jewelewicz, R., and Sealey, J.E. (1986) Science, 233, 1422-1424.

(3) Kim. S.-J., Shinjo, M., Tada, M., Usuki, S., Fukamizu, A., Miyazaki, H., and Murakami, K. (1987) Biochem. Biophys. Res. Commun. 146, 989-995.

(4) Masuda, T., Imai, T., Fukushi, T., Sudoh, M., Hirose, S., and Murakami, K. (1982) Biomed. Res. 3, 541-545.

(5) Imai, T., Miyazaki, H., Hirose, S., Hori, H., Hayashi, T., Kageyama, R., Ohkubo, H., Nakanishi, S., and Murakami, K. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 7405-7409.

(6) Tada, M., Fukamizu, A., Seo, M.S., Takahashi, S., and Murakami, K. (1988) Nucleic Acids Res. 16, 3576.

(7) Imai, T., Cho, T., Takamatsu, H., Hori, H., Saitoh, M., Masuda, T., Hirose, S., and Murakami, K. (1986) J. Biochem. 100, 425-432.

(8) Poorman, R.A., Palermo, D.P., Post, L.E., Murakami, K., Kinner, J.H. , Smith, C.W., Creardon, I., and Heinrikson, R.L. (1986) Proteins, 1, 139-145.

(9) Dynan, W.S., and Tjian, R. (1985) Nature, 316, 774-778.

(10) Maniatis, T., Goodbourn, S., and Fischer, J. (1987) Science, 236, 1237-1245.

(11) Breathnach, R., and Chambon, P. (1981) Ann. Rev. Biochem. 50, 349-383.

(12) Yamamoto, K.R. (1985) Ann. Rev. Genet. 19, 209-252.

(13) Miyazaki, H., Fukamizu, A., Hirose, S., Hayashi, T., Hori, H., Ohkubo, H., Nakanishi, S., and Murakami, K. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5999-6003.

(14) Fukamizu, A., Nishi, K., Nishimatsu, S., Miyazaki, H., Hirose, S., and Murakami, K. (1986) Gene, 49, 139-145.

(15) Fukamizu, A., Nishi, K., Cho, T., Saitoh, M., Nakayama, K., Ohkubo, H., Nakanishi, S., and Murakami, K. (1988) J. Mol. Biol. 201, 443-450.

(16) Oparil, S., and Haber, E. (1974) New Engl. J. Med. 291, 389-401.

(17) Robertson, P.W., Klidjian, A., Harding, L.K., Walters, G., Lee., M. R., and Robb-Smith, A.H.T. (1967) Am. J. Med. 43, 963-976.

(18) Kihara, T., Kitamura, S., Hoshino, T., Seida, H., and Watanabe, T. (1968) Acta. Pathol. Jpn. 18, 197-206.

(19) Blin, N., and Stafford, D.W. (1976) Nucleic Acids Res. 3, 2303-2308.

(20) Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Biochemistry, 18, 5294-5299.

(21) Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY.

(22) Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) J. Mol. Biol. 113, 237-251.

(23) Benton, W.D., and Davis, R.W. (1977) Science, 196, 180-182.

(24) Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.

(25) Alwine, J.C., Kemp, D.J., and Stark, G.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4835-4838.

(26) Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad.

Sci. U.S.A. 74, 5463-5467.

(27) Soubrier, F., Panthier, J.J., Houot, A.M., Rougeon, F., and Corvol, P. (1986) Gene, 41, 85-92.

(28) Benoist, C., O'Hare, K., Breathnach, R., and Chambon, P. (1980) Nucleic Acids Res. 8, 127-142.

(29) Cato, A.C.B., Geisse, S., Wenz, M., Westphal, H.M., and Beato, M. (1984) EMBO J. 3, 2771-2778.

(30) Mulvihill, E.R., Lepennec, J.P., and Chambon, P. (1982) Cell, 24, 621-632.

(31) Jost, J.P., Seldran, M., and Geiser, M. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 429-433.

(32) Nagamine, Y., and Reich, E. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4606-4610.

(33) Khoury, G., and Gruss, P. (1983) Cell, 33, 313-314.

(34) Weiher, H., Konig, M., and Gruss, P. (1983) Science, 219, 626-631.

(35) Prochownik, E.V. (1985) Nature, 316, 845-848.

(36) Holm, I., Ollo, R., Panthier, J.-J., and Rougeon, F. (1984) EMBO J. 3, 557-562.

(37) Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene, 33, 103-119.

(38) Mizusawa, S., Nishimura, S., and Seela, F. (1986) Nucleic Acids Res. 14, 1319-1324.

(39) Naora, H., and Deacon, N.J. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6196-6200.

(40) Blake, C. (1983) Nature, 306, 535-537.

(41) Shinagawa, T., Do, Y.-S., Tan, H., and Hsueh, W.A. (1986) Biochem. Biophys. Res. Commun. 139, 446-454.

(42) Matoba, T., Murakami, K., and Inagami, T. (1978) Biochim. Biophys. Acta. 526, 560-571.

(43) Figueiredo, A.F.S., Takii, Y., Tsuji, H., Kato, K., and Inagami, T. (1983) Biochemistry, 22, 5476-5483.

(44) Murakami, K., Hirose, S., Miyazaki, H., Iami, T., Hayashi, T., Hori, H., Kageyama, R., Ohkubo, H., and Nakanishi, S. (1984) Hypertension, 6 (Suppl. I) I-95-I-100.

(45) Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6682-6686.

(46) Sutcliffe, J.G., Milner, R.J., Bloom, F.E., and Lerner, R.A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4942-4946.

(47) Husain, A., Bumpus, F.M., De Silva, P., and Steph, R.C. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2489-2493.

(48) Ohkubo, H., Nakayama, K., Tanaka, T., and Nakanishi, S. (1986) J. Biol. Chem. 261, 319-323.

(49) Steph, R.C., and Husain, A. (1988) Bio. Repro. 38, 695.

(50) Goto, T., Imai, N., Hirose, S., and Murakami, K. (1984) Clin. Chim. Acta. 138, 87-98.

(51) Melton, D.A., Kreig, D.A., Rebagliati, M.R., Maniatis, T., Zinn, K. , and Greene, M.R. (1984) Nucleic Acids Res. 12, 2035-2056.

(52) Bell, G.I., Picket, R.L., Rutter, W.J., Cordell, B., Tischer, E., and Goodman, H.M. (1980) Nature, 284, 26-32.

(53) Cochet, M., Chang, A.C.Y., and Cohen, S.N. (1982) Nature, 297, 335-339.

(54) Notake, M., Tobimatsu, T., Watanabe, H., Mishina, M., and Numa, S. (1983) FEBS Lett. 156, 67-71.

(55) Elshourbagy, N.A., Walker, D.W., Paik, Y.-K., Boguski, M.S., Freeman, M., Gordon, J.I., and Taylor, J.M. (1987) J. Biol. Chem. 262, 7973-7981.

(56) Williams, S.C., Bruckheimer, S.M., Lusis, A.J., LeBoeuf, R.C., and Kinniburgh, A.J. (1986) Mol. Cell. Biol. 6, 3807-3816.

(57) Pandey, K.N., Maki, M., and Inagami, T. (1984) Biochem. Biophys. Res. Commun. 125, 662-667.

(58) Field, L.J., MacGowan, R.A., Dickinson, D.P., and Gross, K.W. (1984) Hypertension, 6, 597-603.

(59) Dzau, V.J., Ingelfinger, J., Pratt, R.E., and Ellison, K.E. (1986) Hypertension, 8, 544-548.

(60) Dzau, V.J., Brody, T., Ellison, K.E., Pratt, R.E., and Ingelfinger, J.R. (1987) Hypertension, 9, (Suppl. III) III-36-III-41.

(61) Kim, S,-J., Shinjo, M., Fukamizu, A., Miyazaki, H., Usuki, S., and Murakami, K. (1987) Biochem. Biophys. Res. Commun. 142, 169-175.

(62) Saito, T., Fukamizu, A., Okada, K., Ishikawa, S., Iwamoto, Y., Kuzuya, T., Kawai, T., Naruse, K., Hirose, S., and Murakami, K. (1989) Endocrin. Japon, 36, 41-48.

(63) Gorman, C., Merlino, G.T., Willingham, M.C., Pastan, I., and Howard, B.H. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6777-6781.

(64) Sogawa, K., Fujii-Kuriyama, Y., Mizukami, Y., Ichihara, Y., and Takahashi, K. (1983) J. Biol. Chem. 258, 5306-5311.

(65) Hidaka, M., Sasaki, K., Uozumi, T., and Beppu, T. (1986) Gene, 43, 197-203.

(66) Tang, J., James, M.N.G., Hsu, I.N., Jenkins, J.A., and Blundell, T. L. (1978) Nature, 271, 618-621.

(67) Symonds, E.M., Stanley, M.A., and Skinner, S.L. (1968) Nature, 217, 1152-1153.

(68) Skinner, S.L., Lumbers, E.R., and Symonds, E.M. (1968) Am. J. Obstet. Gynec. 101, 529-533.

(69) Hadori, A.A., Carretero, O.A., and Hodgkinson, C.P. (1969) Obstet. Gynec. 34, 358-362.

(70) Ganten, D., Minnich, J.L., Granger, P., Hayduc, K., Brecht, H.M., Barbeau, A., Boucher, R., and Genest, J. (1971) Science, 173, 64-65.

(71) Fernandez, L.A., Tarlatzis, B.C., Rzasa, P.J., Caride, V.J., Laufer, N., Negro-Vilar, A.F., DeCherney, A.H., and Naftolin, F. (1985) Fertil. Steril. 44, 219-223.

(72) Osmoand, D.H., Ross, L.J., and Sciaff, K.D. (1973) Can. J. Physio. Pharmacol. 51, 705-708.

(73) Sealey, J.E., and Laragh, J.H. (1975) Circ. Res. 36 and 37 (Suppl. I), I-10-I-16.

(74) Atlas, S.A., Laragh, J.H., Sealey, J.E., and Hesson, T.E. (1980) Clin. Sci. 59, 29s-33s.

(75) Walker, M.D., Edlu d, T., Boulet, A.M., and Rutter, W.J. (1983) Natrue, 306, 557-561.

(76) Rodgers, B.L., Sobnosky, M.G., and Saunders, G.F. (1986) Nucleic Acids Res. 14, 7647-7659.

(77) Gillies, S.D., Morrison, S.L., Oi, V.T., and Tonegawa, S. (1983) Cell, 33, 717-728.

(78) Ganong, W.F. (1984) Annu. Rev. Physiol. 46, 17-31.

(79) Virutamasen, P., Wright, K.H., and Wallach, E.E. (1972) Obstet. Gynec. 39, 225-230.

(80) Laragh, J.H., Angers, M., Kelly, W.G., Leiberman, S. (1960) JAMA, 174, 2234-240.