Molecular Cloning of cDNA for Human Renin and Its Expression in *Escherichia coli*

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CONTENTS

Chapter I. Introduction

Renin (EC 3.4.99.19) is a key enzyme in the regulation of blood pressure and electrolyte balance(1,2). The renin is a highly specific aspartyl proteinase whose only known function is to generate angiotensin I from its macromolecular substrate, angiotensinogen(Fig. 1). Its reaction is apparently, unusually restricted to hydrolytic cleavage of the unique site in the substrate molecules. The decapeptide angiotensin I is subsequently converted to angiotensin II, an octapeptide that causes marked vasoconstriction of the arteriolar smooth muscle.

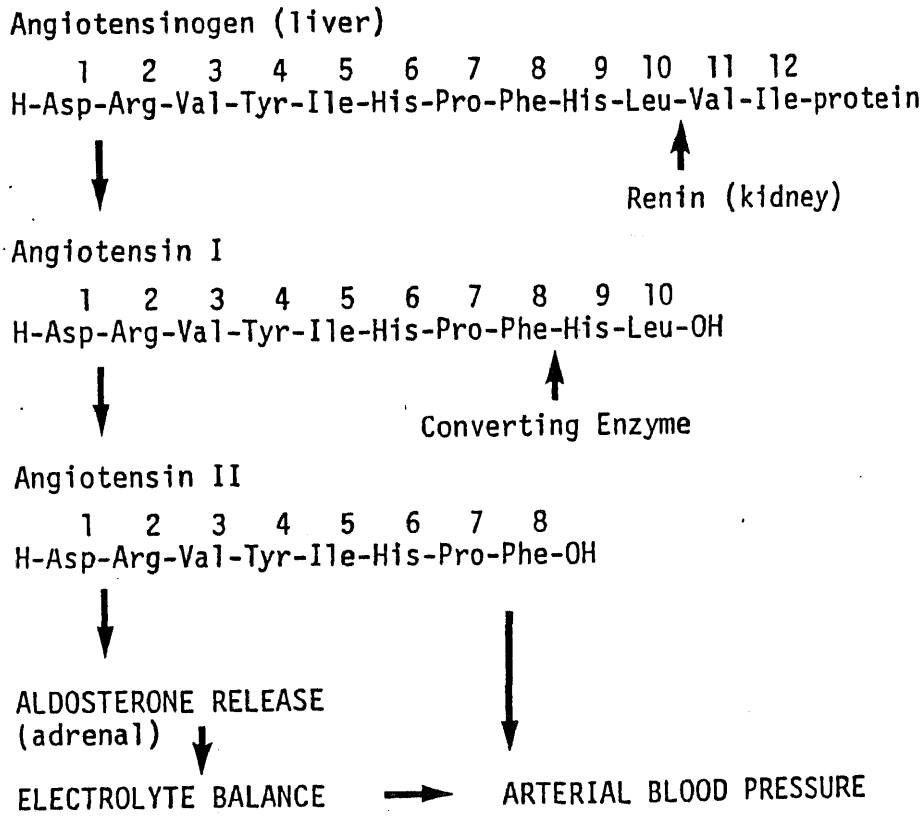

Figure 1. **Renin - Angiotensin System.**

Angiotensin II is also a potent stimulator of aldosterone secretion from the adrenal cortex. Renin is stored in the juxtaglomerular cells of the kidney and is released into the bloodstream by the renin-angiotensin system into the complex process of circulatory homeostasis.

Since the renin is the initial and the rate-limiting component of the system, there has been considerable interest in the study of this enzyme yet, progress in elucidating the biochemical properties of renin, especially human renin, has been slow because of the limited supply of human kidney and minute quantities of renin present in the kidney. Although the use of sophisticated methods of protein purification and characterization has circumvented these potential problems to some extent and improved the knowledge on renin significantly, precise identification of the structure of the renin molecules has not been achieved yet. For example, there are conflicting reports on the molecular weight and the amino acid composition of human renin(3-6).

Comparative biochemical studies have revealed that human renin is unique among other animal renins in its molecular properties such as molecular weight, isoelectric pH, and antigenicity(3). The renin-angiotensinogen reaction is also species dependent(7): renin isolated from the kidney of monkeys and humans reacts with the substrate from all mammals, whereas renin

from other mammals does not release angiotensin I from angiotensinogen in monkyes or humans.

The approach by recombinant DNA techniques has permitted analysis of eukaryotic gene structure, its organization, expression, and evolution. Using genetic engineering of microorganism, these techniques have also allowed the production of exogenous or heterologous proteins of artifically introduced genes.

Because an extremely low concentration of renal renin prevented purification in amounts sufficient to determine the complete amino acid sequence and tertiary structure of the protein, the complementary DNA(cDNA) for human renin was cloned and the enzyme was produced in Escherichia coli using the recombinant DNA techniques. The present investigation in this thesis is concerned with: i) the molecular cloning and sequence analysis of complementary DNA for human renin precursor (Chapter II); ii) the complete amino acid sequence of human preprorenin deduced from its cDNA(Chapter II); iii) the expression of renin gene in Escherichia coli (Chapter III); and iv) the characterization of renin produced in Escherichia coli (Chapter III).

Chapter II. Molecular Cloning and Sequence Analysis of cDNA for Human Renin Precursor

SUMMARY

The primary structure of human renin precursor has been deduced from its complementary DNA(cDNA) sequence. A library of cDNA clones was constructed from human kidney poly(A)$^+$RNA by applying the vector/primer method of Okayama and Berg[Okayama, H. and Berg, P.(1982) Mol. Cell. Biol. 2, 161-170]. The library was screened for human renin sequences by hybridization with the previously cloned mouse renin cDNA. Of the 240,000 colonies screened, 35 colonies that were positive for hybridization were isolated. Two recombinant plasmids containing long inserts of about 1,300 and 1,600 base pairs were selected for sequence analysis. The amino acid sequence predicted from the cDNA sequence shows that human renin precursor consists of 406 amino acids (Mr = 45,057) with a pre- and prosegment carrying 20 and 46 amino acids, respectively. A high degree of sequence homology, especially in the catalytically important region, was found upon comparison of mouse and human renins. An overall homology, including presequence between the two renins, is 68.7 %. Close similarities were also observed in the primary structure of renins and other aspartyl proteinases with defined three-dimentional structures, suggesting a tertiary structure for renin

that is similar to the other enzymes. These results indicate that human kidney renin is homologous with mouse submandibular renin in primary and tertiary structures, proteolytic processing, and catalytic apparatus with small differences. The major structural difference distinguishing the two renins was the presence of the two possible glycosylation sites in human kidney renin, which was not observed in mouse submandibular gland renin.

INTRODUCTION

The structure of renin has been the subject of intense interest for many years due to its important physiological role in the regulation of blood pressure and its stringent substrate specificity.

Renin from mouse submandibular gland is an excellent alternative for the study of renin structure since it represents as much as 2 - 5 % of its total protein content. In 1982, a complete amino acid sequence of renin and its precursor from the mouse submandibular gland was determined by the techniques of conventional amino acid sequence(8) and the nucleotide sequence of the complementary DNA(cDNA) coding renin precursor(9). However, the renin from mouse submandibular gland is different from renal renin, especially human renin, in their physiological function(10), and molecular and genetic properties(11-13). Because of renin's very low concentration in its primary tissue source, the kidney (3-5), it has been difficult to obtain adequate quantities to determine its amino acid sequence.

Therefore, the attempts were made to obtain full-length cDNA clones from poly(A)$^+$RNA extracted from a surgically removed ischemic kidney in which the renin content was markedly increased due to the stenosis of the renal artery. Although a variety of procedures had been reported for synthesis of complementary DNA library(14-17), the vector/primer method of Okayama and Berg(18) was

used to construct the cDNA library from the mRNA preparation of human kidney because it was a very effective method for cloning of full-length cDNA. In order to screen the renin cDNA clone from the library a mouse submandibular gland renin cDNA, which had been cloned and characterized in our laboratory(19), was used as a hybridization probe. The important evidence that the mouse renin cDNA cross-hybridizes with human renin gene had already been obtained(19).

The results presented here describe the cloning and sequence analysis of human renin cDNA. Determined were the entire 3' non-coding sequence preceding the poly(A) tail, 1,218 nucleotides coding for renin precursor, and of the 5' non-coding region, 42 nucleotides.

MATERIALS AND MRTHODS

Materials

Materials were obtained from the following sources: restriction enzymes from Takara Shuzo(Kyoto, Japan) and New England BioLabs (Beverly, MA); terminal deoxynucleotidyl transferase, T4 DNA ligase, and T4 polynucleotide kinase from Takara Shuzo; Escherichia coli DNA polymerase I and E. coli DNA ligase from New England BioLabs; E. coli RNase H and protein-A sepharose from Pharmacia (Uppsala, Sweden); oligo(dT)-cellulose from Collaborative Research (Lexington, MA); nitrocellulose filters from Millipore (Bedford, MA); aminobenzyloxymethyl papers from Schleicher and Schuell(Posfach, West Germany); rabbit reticulocyte lysate, L-[^{35}S] methionine (>800 Ci/mmol, 1 Ci = 3.7 x 10^{10} Bq), [γ-^{32}P]ATP (>5,000 Ci/mmol), and [α-^{32}P]dCTP ($\approx$ 3,000Ci/mmol) from Amersham (Buckinghamshire, England).

Preparation of mRNA

Total RNA, which originated from a kidney removed from a patient with severe renovascular hypertension causing renal ischemia, was extracted in 4 M guanidine thiocyanate buffer as described by Chirgwin et al.(20). The poly(A)$^+$RNA was separated from rRNA by chromatography on oligo(dT)-cellulose and the bound poly(A)$^+$RNA was further purified by a second passage on the oligo(dT)-cellulose column(21).

Translation of mRNA in a Rabbit Reticulocyte Lysate System

Translations of mRNA were carried out using a rabbit reticulocyte lysate system containing [^{35}S] methionine (22). The translation assay system contained per 34 µl: 25 µl lysate, 75 µCi of [^{35}S] methionine, and 2.5 µg mRNA. Translations of RNA were performed for 30 min at 30 °C and aliquots were taken to determine the extent of radioactive incorporation into trichloroacetic acid(TCA)-insoluble materials. The remainder was diluted 40-fold with NET buffer(150 mM NaCl, 5 mM sodium ethylenediamine-tetraacetic acid (EDTA), 0.02 % sodium azide(NaN_3),20 mM Tris-HCl, pH7.4, 0.05 % Nonidet P-40, 1 mM methionine) (23), and products were precipitated using anti-human renin antibody(24).

Immunoprecipitation and Analysis of Translation Products

The translation products, diluted with NET buffer, were incubated overnight at 4 °C with undiluted rabbit antiserum against purified human kidney renin(6); final dilutions of the antiserum were approximately 1:1,000. The antigen-antibody complexes were precipitated by adsorption to protein A-Sepharose 4B and washed four times with NET buffer. The complexes were disrupted by boiling in sodium laurylsulfate(SDS) sample buffer(25) and protein A-gel was removed centrifugation. The samples were subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) by the

method of Laemmli(25) using 10 % slab gels. After electrophoresis, the gels were treated with 1 M sodium salicylate and autoradiograms were prepared by exposing the vacuum-dried gels to Kodak X-Omat R films(26).

Construction of a Human Kidney cDNA Library

A cDNA library was constructed by applying the method of Okayama and Berg(18) as follows.

Synthesis of first-strand cDNA was carried out in a reaction volume of 80 μl containing 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 0.3 mM dithiothreitol(DTT), 2 mM each dATP, dTTP ,dGTP and dCTP, 80 μCi of [α-^{32}P] dCTP, 22 μg of poly(A)$^+$RNA, 5.6 μg of vector/primer DNA(18), and 160 U of reverse transcriptase. cDNA synthesis was initiated by the addition of reverse transcriptase and continued at 37 °C for 30 min. The reaction was stopped by adding EDTA to 25 mM. The products were extrtacted with 0.25 % SDS and phenol-chloroform, and precipitated with ethanol out of 2 M ammonium acetae twice. The amounts of first strands synthesized were estimated by assaying TCA-insoluble radioactivity.

The pellet containing the plasmid-cDNA:mRNA was dissolved in 80 μl of 130 mM sodium cacodylate-30 mM Tris-HCl, pH6.8 buffer containing 1 mM $CoCl_2$, 0.1 mM DTT, 0.1 mM dCTP, 40 μCi [α-^{32}]dCTP, 0.64 μg of poly(A), and 20 U of terminal deoxynucleotidyl transferase. The reaction was carried out at 37 °C for 10 min to permit

the addition of approximately 10 residues of dCMP per end and then terminated with 4 µl of 0.5 M EDTA. The products were extracted with phenol-chloroform and then precipitated out of 2 M ammonium acetate as described above.

The pellet was dissolved in 80 µl of buffer containing 20 mM Tris-HCl,pH 7.4, 7 mM $MgCl_2$, 60 mM NaCl, and 0.1 mg of bovine serum albumin(BSA) per ml and then digested with 24 U of Hin dIII endonuclease for 80 min at 37 °C. The reaction was terminated with EDTA and, after extraction with phenol-chloroform followed by the addition of ammonium acetate, the DNA was precipitated with ethanol.

To check the sizes of first strands synthesized by reverse transcriptase and the digestion of the vector with Hin dIII endonuclease, these samples were subjected to alkaline agarose gel electrophoresis by the method of McDonnell et al.(27).

A 0.8 pmol sample of the Hin dIII-digested oligo(dC)-tailed cDNA:mRNA plasmid was incubated in a mixture (200 µl) containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl and 1.6 pmol of oligo(dG)-tailed linker DNA (18) at 65 °C for 2 min, shifted to 42 °C for 30 min, and then cooled to 0 °C. The mixture was adjusted to a volume of 2 ml containing 20 mM Tris-HCl, pH7.4, 4 mM $MgCl_2$, 10 mM ammonium sulfate, 100 mM KCl, 50 µg of BSA per ml, and 0.1 mM β-NAD. After 4 U of E. coli ligase

was added, the solution was incubated overnight at 12 °C.

To replace the RNA strand of the insert, the ligation mixture was adjusted to contain 40 μM of each of the four deoxynucleotide triphosphates, 0.15 mM β-NAD, 6 U of additional *E. coli* DNA ligase, 1 U of *E. coli* RNase H, and 30 U of *E. coli* DNA polymerase I. The mixture was incubated at 12 °C for 1 hr, and shifted to 25 °C for 1 hr. Transformation of *E. coli* HB101(28) was carried out using the procedure described by Morrison(29). After transformation of *E. coli* HB101, the cells were plated on nitrocellulose filters on ampicillin-containing agar plates.

Identification of Human Renin cDNA Clones

The human kidney cDNA library was screened by colony hybridization using a nick-translated *Acc* I/*Rsa* I fragment(358 base pairs) from renin cDNA of a mouse submandibular gland(19).

Prehybridization was performed at 55 °C in 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1 M NaCl, 0.2 % polyvinylpyrrolidone, 0.2 % Ficoll, 0.2 % BSA, 60 μg of denatured *E. coli* DNA per ml. Hybridization was carried out at 55 °C for 18 hr in the same buffer containing a ^{32}P-labeled probe(1 x 10^8 cpm/μg). Filters were washed six times with an excess of 0.3 M NaCl, 0.03 M sodium citrate, 0.1 % SDS at 37 °C and subjected to autoradiography.

The plasmid DNAs detected using the colony

hybridization were extracted using the method of Birnboim and Doly(30). These plasmids were cleaved with several restriction enzymes using the buffers described by Davis et al.(31). The cleavage products were separated by polyacrylamide gel electrophoresis(5 % and 8 %)(32) and/or agarose gel electrophoresis(1 %, 1.2 %, 1.5 %, and 1.8 %)(33). The clones with renin cDNA insert were selected for DNA sequence analysis by the method of Maxam and Gilbert(32).

Comparisons of the nucleotide and the amino acid sequences for human renin with those of mouse submandibular gland renin were used a dot matrix technique(34) and maximum match method(35), respectively.

Blot Hybridization Analysis

The poly(A)$^+$RNAs from human liver and infarcted kidney were denatured with glyoxal(36) and electrophoresed on 1.5 % agarose gel. The separated RNAs were transferred to diazobenzyloxymethyl-paper according to the procedure of Alwine et al.(37). The diazo paper was prehybridized at 42 °C for 24 hr in 50 % (vol/vol) 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 % BSA, 1 % glycine, 0.2 % SDS, 100 µg denatured salmon sperm DNA per ml. The paper was hybridized at 42 °C for 24 hr in the same solution except that glycine was omitted and that the solution contained a ^{32}P-labeled probe (nick-translated Ava II fragment

containing nucleotides 170-1,127 from clone pHRn321). After the hybridization, the paper was washed at 42 °C in 0.45 M NaCl, 0.045 M sodium citrate, 0.1 % SDS, and subjected to autoradiography.

All of the cloning procedures were conducted in accordance with the guide lines for research involving recombinant DNA molecules issued by the Ministry of Education, Science and Culture of Japan.

RESULTS

Cell-Free Translation of Human Renin mRNA

Concentration of human renin mRNA was at a very low level in normnal kidney. Therefore, a kidney that had been removed from a patient with severe renovascular hypertension caused by renal ischemia was used for the source of poly(A)$^+$RNA. Initial extraction of total RNA was carried out by the guanidine thiocyanate method of Chirgwin et al.(20) to isolate intact RNA. From 26 g of an infarcted human kidney, approximately 66 mg of RNA was obtained. Passage of total RNA over on oligo(dT)-cellulose column gave a 5 % yield of poly(A)$^+$RNA. The presence of intact renin mRNA in this preparation was confirmed by cell-free translation of the poly(A)$^+$RNA in the presence of [^{35}S] methionine. Immunoprecipitation of translation products with anti-renin antibody and analysis of the radioactive precipitates by SDS-polyacrylamide gel electrophoresis and fluorography revealed one major specific band with a Mr of 45,000(Fig. 2).

Construction, Selection, and Characterization of Human Renin cDNA Clones

A library of cDNA clones was constructed from the human renal poly(A)$^+$RNA by using the vector/primer method of Okayama and Berg(18), which provides a highly efficient means for obtaining full-length cDNAs. Initially, the

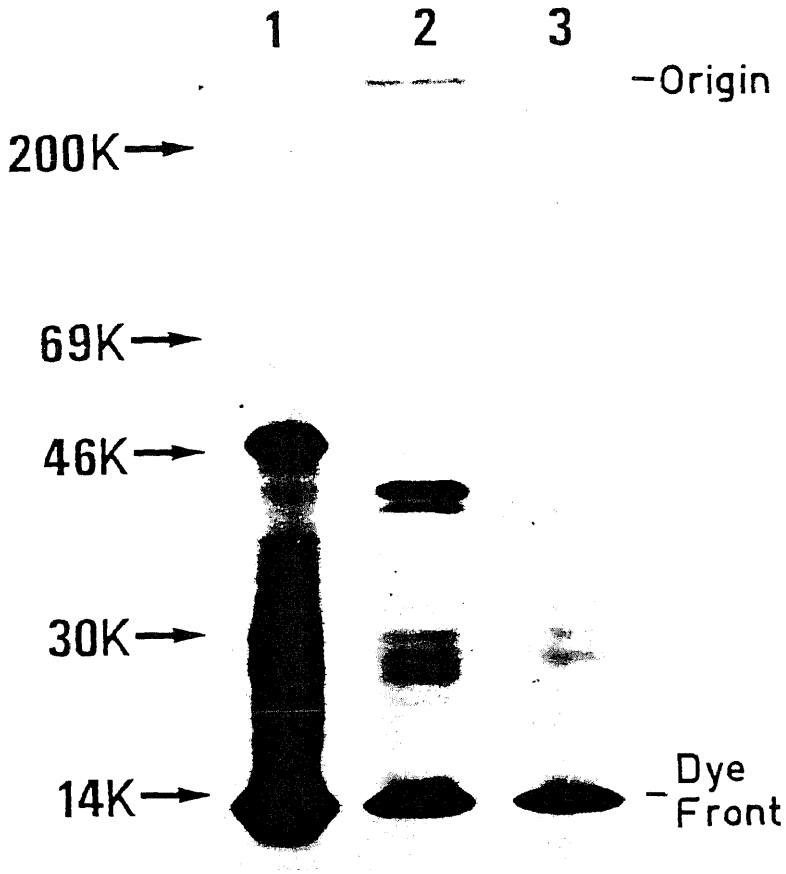

Figure 2. **Autoradiogram of SDS-polyacrylamide gel of human renin synthesized in a cell-free system containing** [^{35}S]methionine. The translation products were immunoprecipitated by use of anti-human renin antibody and analyzed by electrophoresis on a 10 % SDS-polyacrylamide gel. Lane 1: total translation products (2 µl of reaction mixture). Lane 2: proteins immunoprecipitated by an anti-renin antibody in 34 µl of reaction mixture. Lane 3: proteins immunoprecipitated using preimmune rabbit serum in 34 µl of reaction mixture. Molecular weight markers were [^{14}C]methylated myosin (200,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

library was screened for human renin sequences by hybridization with a 358-base-pairs Acc I/Rsa I restriction fragment obtaind from the previously cloned mouse renin cDNA(19) (Fig. 3). Of the 50,000 colonies screened, 6 colonies positive for hybridization were isolated. Recombinant plasmids from the selected colonies were prepared, digested with various restriction enzymes, and electrophoresed in agarose gels to determine the size. The insert, which contained 800-1,300 base pairs, seemed unable to cover the entire coding sequence since mRNA coding for a Mr 45,000 protein is expected to have at least 1,400 nucleotides, including 5' and 3' noncoding sequence; in fact, this was proved to be the case by later sequence analysis(Fig. 4) and blot hybridization analysis(Fig. 6). To isolate longer cDNAs, therefore, 190,000 colonies were further screened by using the Taq I (390)/Taq I (630) restriction fragment (Fig. 4) excised from the longest human renin cDNA insert (pHRn011) cloned above, and 29 clones positive for hybridization were recovered. Size analysis of their inserts indicated that one recombinant plasmid, pHRn321, contained an insert with about 1,600 base pairs. This clone and the pHRn011 cDNA clone were subjected to sequence analysis.

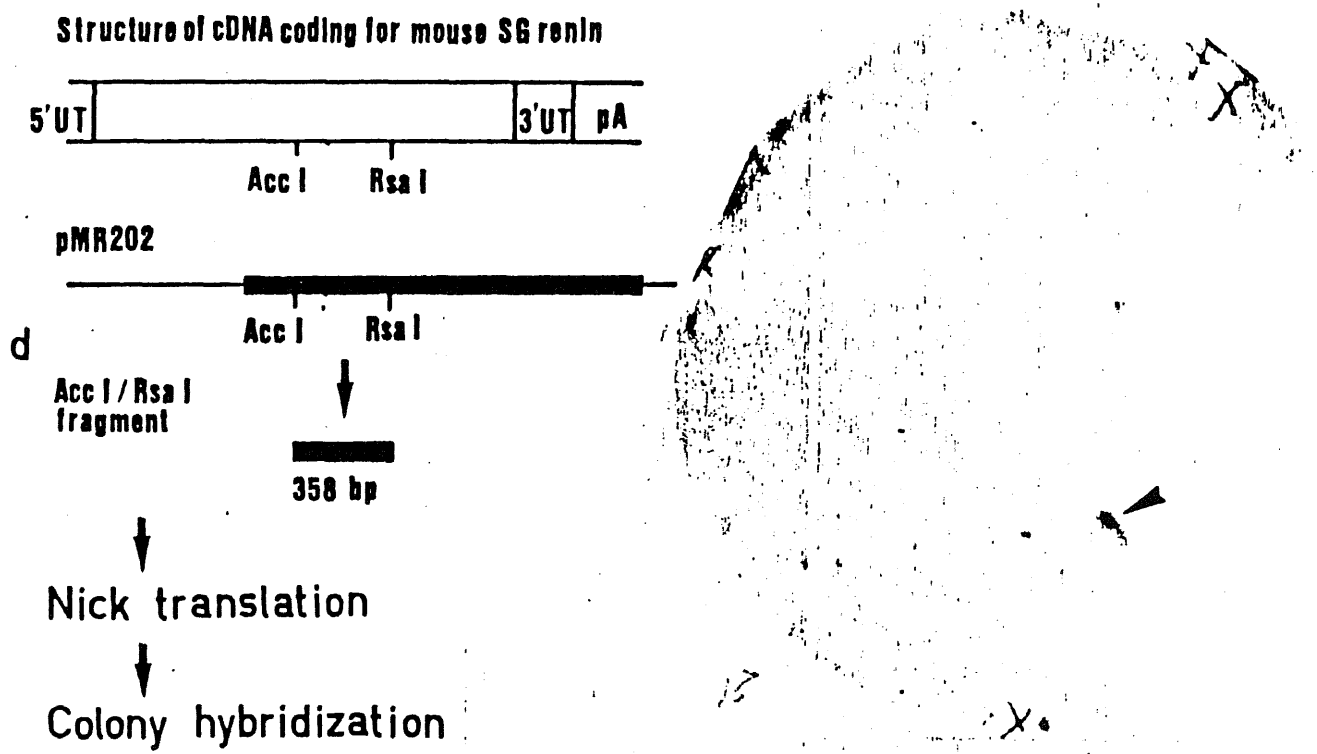

Figure 3. Screening of Escherichia coli containing human renin cDNA. SG, submandibular gland. bp, base pairs.

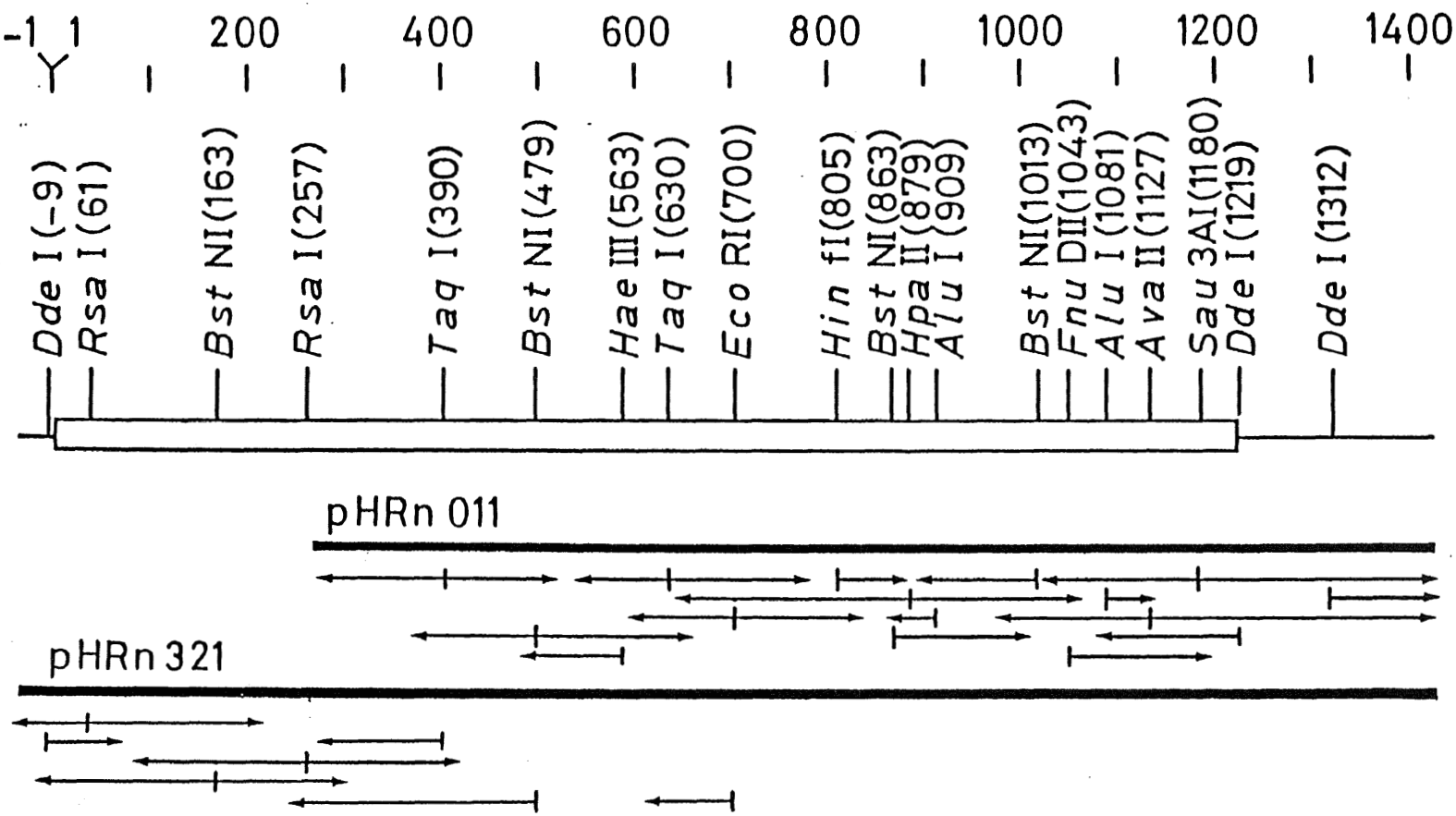

Figure 4. Sequence analysis strategy of the human renin cDNA clones. For the isolation of clone pHRn011, see the text. For the isolation of clone pHRn321, cDNA library was rescreened by hybridization at 68 °C with nick-translated Taq I fragment containing nucleotides 390-630 from clone pHRn011. The sequence corresponding to the coding region is indicated by the open box. Only the positions of the relevant restriction sites are indicated (for the nucleotide numbers, see Figure 5). The poly(dA)-poly(dT) and poly(dG)-poly(dC) tails are not included in the map. The horizontal arrows indicate the direction and extent of sequence determination. The sites of 5'-end-labeling are indicated by short vertical lines on the arrows.

Nucleotide Sequence Analysis

The sequences of two different clones were determined to verify the absolute fidelity of the cDNA sequence as a copy of the mRNA. The inserts from pHRn011 and pHRn321 were analyzed with a series of restriction enzymes. Figure 4 shows the restriction map and sequence analysis strategy. The complete nucleotide sequence of the longer clone (pHRn321) determined by the chemical degradation method is shown in Figure 5. The entire sequence was obtained from both the message and complementary strands. There was complete agreement in the nucleotide sequences obtained from the overlapping areas. The insert contained 1,459 nucleotides and the length of the poly(A) tail was not included in this determination. The DNA sequence of the shorter insert, pHRn011, was found to be identical with that of the corresponding region of pHRn321. The cDNA insert of pHRn321 contained 42 nucleotides in the 5' noncoding region, 1,218 nucleotides in the coding region, and 199 nucleotides in the 3' untranslated region preceding the poly(A) tail. From this information it was possible to analyze most of the structure of human renin mRNA. The 5' and 3' untranslated regions of the mRNA share many of the features already noted in order eukaryotic mRNA sequences; for example, the nucleotide sequence around the initiation codon is consistent with the sequence A/G-N-N-A-U-G-G, which has been shown to be most favored for

Figure 5. **Nucleotide and corresponding amino acid sequence of plasmid pHRn321 and pHRn011 encoding human renal preprorenin.** Nucleotide are numbered in the 5'-to-3' direction, beginning with the first residue of the ATG triplet encoding the initiator methionine, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The deduced amino acid residues are indicated below the nucleotied triplets. The mature polypeptide begins at the Leu residue labeled {1}. The numbers in the braces refer to amino acid positions within the mature renin. The single and double arrowheads indicate the probable ends of leader sequence and prosequence, respectively, as predicted by comparison with mouse renin precursor(9) and human prepepsinogen(47). The two active-site aspartic acid residues, potential N-glycosylation sites, and the A-A-T-A-A-A sequence within the 3' untranslated region are underlined.

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                                                                -42 AACCTCAGTGGATCTCAGAGAGAGCCCCAGACTGAGGGAAGC   -1

  1 ATG GAT GGA TGG AGA AGG ATG CCT CGC TGG GGA CTG CTG CTG CTG CTC TGG GGC TCC TGT   60
    Met Asp Gly Trp Arg Arg Met Pro Arg Trp Gly Leu Leu Leu Leu Leu Trp Gly Ser Cys
      1                    {-60}          10                    {-50}          20

 61 ACC TTT GGT CTC CCG ACA GAC ACC ACC ACC TTT AAA CGG ATC TTC GTC AAG AGA ATG CCC  120
    Thr Phe Gly Leu Pro Thr Asp Thr Thr Thr Phe Lys Arg Ile Phe Leu Lys Arg Met Pro
                       {-40}          30                    {-30}          40

121 TCA ATC CGA GAA AGC CTG AAG GAA CGA GGT GTG GAC ATG GCC AGG CTT GGT CCC GAG TGG  180
    Ser Ile Arg Glu Ser Leu Lys Glu Arg Gly Val Asp Met Ala Arg Leu Gly Pro Glu Trp
                       {-20}          50                    {-10}          60

181 AGC CAA CCC ATG AAG AGG CTG ACA CTT GGC AAC ACC ACC TCC TCC GTG ATC CTC ACC AAC  240
    Ser Gln Pro Met Lys Arg Leu Thr Leu Gly Asn Thr Thr Ser Ser Val Ile Leu Thr Asn
                       {-1}{1}        70                    {10}           80

241 TAC ATG GAC ACC CAG TAC TAT GGC GAG ATT GGC ATC GGC ACC CCA CCC CAG ACC TTC AAA  300
    Tyr Met Asp Thr Gln Tyr Tyr Gly Glu Ile Gly Ile Gly Thr Pro Pro Gln Thr Phe Lys
                       {20}           90                    {30}           100

301 GTC GTC TTT GAC ACT GGT TCG TCC AAT GTT TGG GTG CCC TCC TCC AAG TGC AGC CGT CTC  360
    Val Val Phe Asp Thr Gly Ser Ser Asn Val Trp Val Pro Ser Ser Lys Cys Ser Arg Leu
                       {40}           110                   {50}           120

361 TAC ACT GCC TGT GTG TAT CAC AAG CTC TTC GAT GCT TCG GAT TCC TCC AGC TAC AAG CAC  420
    Tyr Thr Ala Cys Val Tyr His Lys Leu Phe Asp Ala Ser Asp Ser Ser Ser Tyr Lys His
                       {60}           130                   {70}           140

421 AAT GGA ACA GAA CTC ACC CTC CGC TAT TCA ACA GGG ACA GTC AGT GGC TTT CTC AGC CAG  480
    Asn Gly Thr Glu Leu Thr Leu Arg Tyr Ser Thr Gly Thr Val Ser Gly Phe Leu Ser Gln
                       {80}           150                   {90}           160

481 GAC ATC ATC ACC GTG GGT GGA ATC ACG GTG ACA CAG ATG TTT GGA GAG GTC ACG GAG ATG  540
    Asp Ile Ile Thr Val Gly Gly Ile Thr Val Thr Gln Met Phe Gly Glu Val Thr Glu Met
                       {100}          170                   {110}          180

541 CCC GCC TTA CCC TTC ATG CTG GCC GAG TTT GAT GGG GTT GTG GGC ATG GGC TTC ATT GAA  600
    Pro Ala Leu Pro Phe Met Leu Ala Glu Phe Asp Gly Val Val Gly Met Gly Phe Ile Glu
                       {120}          190                   {130}          200

601 CAG GCC ATT GGC AGG GTC ACC CCT ATC TTC GAC AAC ATC ATC TCC CAA GGG GTG CTA AAA  660
    Gln Ala Ile Gly Arg Val Thr Pro Ile Phe Asp Asn Ile Ile Ser Gln Gly Val Leu Lys
                       {140}          210                   {150}          220

661 GAG GAC GTC TTC TCT TTC TAC TAC AAC AGA GAT TCC GAG AAT TCC CAA TCG CTG GGA GGA  720
    Glu Asp Val Phe Ser Phe Tyr Tyr Asn Arg Asp Ser Glu Asn Ser Gln Ser Leu Gly Gly
                       {160}          230                   {170}          240

721 CAG ATT GTG CTG GGA GGC AGC GAC CCC CAG CAT TAC GAA GGG AAT TTC CAC TAT ATC AAC  780
    Gln Ile Val Leu Gly Gly Ser Asp Pro Gln His Tyr Glu Gly Asn Phe His Tyr Ile Asn
                       {180}          250                   {190}          260

781 CTC ATC AAG ACT GGT GTC TGG CAG ATT CAA ATG AAG GGG GTG TCT GTG GGG TCA TCC ACC  840
    Leu Ile Lys Thr Gly Val Trp Gln Ile Gln Met Lys Gly Val Ser Val Gly Ser Ser Thr
                       {200}          270                   {210}          280

841 TTG CTC TGT GAA GAC GGC TGC CTG GCA TTG GTA GAC ACC GGT GCA TCC TAC ATC TCA GGT  900
    Leu Leu Cys Glu Asp Gly Cys Leu Ala Leu Val Asp Thr Gly Ala Ser Tyr Ile Ser Gly
                       {220}          290                   {230}          300

901 TCT ACC AGC TCC ATA GAG AAG CTC ATG GAG GCC TTG GGA GCC AAG AAG AGG CTG TTT GAT  960
    Ser Thr Ser Ser Ile Glu Lys Leu Met Glu Ala Leu Gly Ala Lys Lys Arg Leu Phe Asp
                       {240}          310                   {250}          320

961 TAT GTC GTG AAG TGT AAC GAG GGC CCT ACA CTC CCC GAC ATC TCT TTC CAC CTG GGA GGC 1020
    Tyr Val Val Lys Cys Asn Glu Gly Pro Thr Leu Pro Asp Ile Ser Phe His Leu Gly Gly
                       {260}          330                   {270}          340

1021 AAA GAA TAC ACG CTC ACC AGC GCG GAC TAT GTA TTT CAG GAA TCC TAC AGT AGT AAA AAG 1080
     Lys Glu Tyr Thr Leu Thr Ser Ala Asp Tyr Val Phe Gln Glu Ser Tyr Ser Ser Lys Lys
                        {280}          350                   {290}          360

1081 CTG TGC ACA CTG GCC ATC CAC GCC ATG GAT ATC CCG CCA CCC ACT GGA CCC ACC TGG GCC 1140
     Leu Cys Thr Leu Ala Ile His Ala Met Asp Ile Pro Pro Pro Thr Gly Pro Thr Trp Ala
                        {300}          370                   {310}          380

1141 CTG GGG GCC ACC TTC ATC CGA AAG TTC TAC ACA GAG TTT GAT CGG CGT AAC AAC CGC ATT 1200
     Leu Gly Ala Thr Phe Ile Arg Lys Phe Tyr Thr Glu Phe Asp Arg Arg Asn Asn Arg Ile
                        {320}          390                   {330}          400

1201 GGC TTC GCC TTG GCC CGC TGAGGCCCTCTGCCACCCAGGCAGGCCCTGCCTTCAGCCCTGGCCCAGAGCTGGA 1273
     Gly Phe Ala Leu Ala Arg
                        {340}406

1274 ACACTCTCTGAGATGCCCCTCTGCCTGGGCTTATGCCCTCAGATGGAGACATTGGATGTGGAGCTCCTGCTGGATGCGT 1352

1353 GCCCTGACCCCTGCACCAGCCCTTCCCTGCTTTGAGGACAAAGAGAATAAAGACTTCATGTTCAC
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eukaryotic initiation sites(38). The 3' untranslated region contains, in the expected position (20 nucleotides upstream from the poly(A) tail), the hexanucleotide 5'-A-A-U-A-A-A-3' which could function as a signal for poly(A) addition or termination of transcription(39).

Predicted Amino Acid Sequence of Human Renin

The amino acid sequence deduced from the nucleotide sequence analysis is shown in Figure 5. At the NH_2 terminus is a sequence of hydrophobic amino acids characteristic of the signal peptides found in the precursors of many secreted proteins. The two adjacent basic residues, Lys-Arg at position 65-66, may represent the site of proteolytic cleavage upon the conversion of prorenin to mature renin; the presence of prorenin was suggested by the finding that human kidney contains inactive renin that can be activated by trypsin. The

Table 1. Amino acid composition of human renin

Ala	16	Leu	28
Arg	10	Lys	15
Asp	17	Met	8
Asn	12	Phe	19
Cys	6	Pro	13
Glu	16	Ser	33
Gln	12	Thr	30
Gly	34	Trp	3
His	6	Tyr	17
Ile	22	Val	23

Total 340 amino acids

molecular weight (Mr = 37,200, sugar residues not included) calculated from the predicted sequence is in agreement with that determined by Yokodswa et al.(3) for the purified protein. The amino acid composition of the mature protein which consists of 340 amino acid residues, is indicated in Table 1.

Codon utilization for human preprorenin is not random and exhibits a marked preference for codons ending with G or C(Table 2). For example, while 20 codons for valine end in G or C, only four end in A or T; similarly for leucine, the codons with G or C in the third position occur 8 times more frequently than the codons ending in A or T. Such a nonrandom codon usage has been observed in other animal genes (40).

Identification and Size Determination of Renin mRNA by Blot Hybridization Analysis

Takahashi and Tang(41) have recently shown that bovine cathepsin D and renin from the mouse submandibular gland are unexpectedly similar in their amino acid sequences. Therefore, to confirm that the cloned pHRn321 is complementary to renin mRNA and not to cathepsin D and to determine the size of human renin mRNA, cloned cDNA was used as a probe to hybridize to mRNA isolated from human kidney, a major source of renin, and from the liver, an abundant source of cathepsin D. As anticipated, the probe hybridized only to kidney mRNA (Fig. 6).

Table 2. Codon usage in human preprorenin mRNA

UUU	Phe	9	2.2%	UCU	Ser	4	1.0%	UAU	Tyr	6	1.5%	UGU	Cys	4	1.0%
UUC	Phe	13	3.2%	UCC	Ser	15	3.7%	UAC	Tyr	11	2.7%	UGC	Cys	3	0.7%
UUA	Leu	1	0.2%	UCA	Ser	4	1.0%	UAA	***	0	0.0%	UGA	***	0	0.0%
UUG	Leu	4	1.0%	UCG	Ser	3	0.7%	UAG	***	0	0.0%	UGG	Trp	7	1.7%
CUU	Leu	2	0.5%	CCU	Pro	3	0.7%	CAU	His	1	0.2%	CGU	Arg	2	0.5%
CUC	Leu	14	3.4%	CCC	Pro	11	2.7%	CAC	His	5	1.2%	CGC	Arg	4	1.0%
CUA	Leu	1	0.2%	CCA	Pro	2	0.5%	CAA	Gln	4	1.0%	CGA	Arg	3	0.7%
CUG	Leu	15	3.7%	CCG	Pro	2	0.5%	CAG	Gln	9	2.2%	CGG	Arg	2	0.5%
AUU	Ile	6	1.5%	ACU	Thr	4	1.0%	AAU	Asn	4	1.0%	AGU	Ser	3	0.7%
AUC	Ile	17	4.2%	ACC	Thr	19	4.7%	AAC	Asn	8	2.0%	AGC	Ser	8	2.0%
AUA	Ile	1	0.2%	ACA	Thr	9	2.2%	AAA	Lys	5	1.2%	AGA	Arg	3	0.7%
AUG	Met	13	3.2%	ACG	Thr	3	0.7%	AAG	Lys	14	3.4%	AGG	Arg	5	1.2%
GUU	Val	2	0.5%	GCU	Ala	1	0.2%	GAU	Asp	8	2.0%	GGU	Gly	8	2.0%
GUC	Val	8	2.0%	GCC	Ala	13	3.2%	GAC	Asp	12	3.0%	GGC	GLY	14	3.4%
GUA	Val	2	0.5%	GCA	Ala	2	0.5%	GAA	Glu	8	2.0%	GGA	Gly	11	2.7%
GUG	Val	12	3.0%	GCG	Ala	1	0.2%	GAG	Glu	11	2.7%	GGG	Gly	7	1.7%

On the basis of its migration in a denaturring gel system, it was estimated that the sequence of mature human renin mRNA is 1,600 nucleotides long. A protein with 406 amino acid residues requires 1,218 bases for its coding sequence. The renin mRNA must, therefore, have about 380 noncoding bases, including the 3'-poly(A) tail.

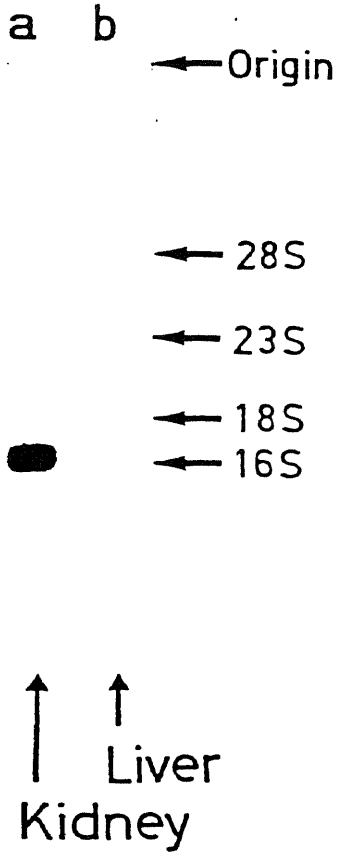


Figure 6. Blot hybridization analysis of liver and kidney mRNA. Lane (a) was obtained with 10 µg of poly(A)$^+$RNA from human kidney, whereas lane (b) had corresponding quantities of human liver poly(A)$^+$RNA. The positions of the size markers (E. coli and human ribosomal RNAs) are indicated.

DISCUSSION

Emphasis was placed on the study of human renin, which is involved in the pathogenesis of several forms of hypertension. Human kidney renin is a key enzyme in the control of blood pressure as well as water and electrolyte balance. Its nucleotide and amino acid sequences had not been analyzed previously. The selection of transformants containing sequences corresponding to human renin was greatly facilitated by the availability of the defined cDNA of the mouse submandibular renin, which has previously been shown to cross-hybridize with human genomic DNA(19). In recent years the primary structure of the renin of the mouse submandibular gland has been determined independently by Misono *et al.*(8), and Panthier *et al.*(9) through chemical sequence analysis of purified protein and sequencing of a cDNA clone, respectively. Since the primary sequence of human renin was unknown, the final confirmation of the identity of the clones was obtained by determining the sequences of the cDNA inserts and comparing the nucleotide and the derived amino acid sequences with published sequences of the renin cDNA of the mouse submandibular gland(9). A considerable degree of homology was thus found between the nucleotide and amino acid sequences of the two enzymes, as discussed below.

Comparison of Nucleotide Sequences Between Human Kidney and Mouse Submandibular Gland Renin cDNAs

A complete nucleotides sequence of the cDNA coding for human preprorenin was consisted of 1,218 bases. Comparison of the nucleotide sequence of human renin cDNA with that of mouse submandibular gland renin by a dot matrix technique denotes that the human and mouse sequences are remarkably homologous(Fig.7). The overall homology between the two sequences is 76 %.

Amino Acid Sequences of Human and Mouse Preprorenins and Comparison with Those of Other Aspartyl Proteinases

The amino acid sequence of human preprorenin was deduced from the nucleotide sequence analysis of its cDNA. This sequence(Fig. 8) is aligned to maximize homology with that of mouse submandibular gland renin using the method of maximum match. A sequence of hydrophobic amino acids characteristic of the signal peptide (such as Leu-Leu-Leu-Leu) was present at the NH_2-terminus: this presequence of human renin contains amino acids through 1 to 20 in Figure 8. The two adjacent basic residues, Lys-65 and Arg-66 in Figure 8, may represent the site of proteolytic cleavage upon the conversion of prorenin to mature renin. The presence of prorenin was suggested by the finding that human kidney contains inactive renin that can be activated by trypsin(12).

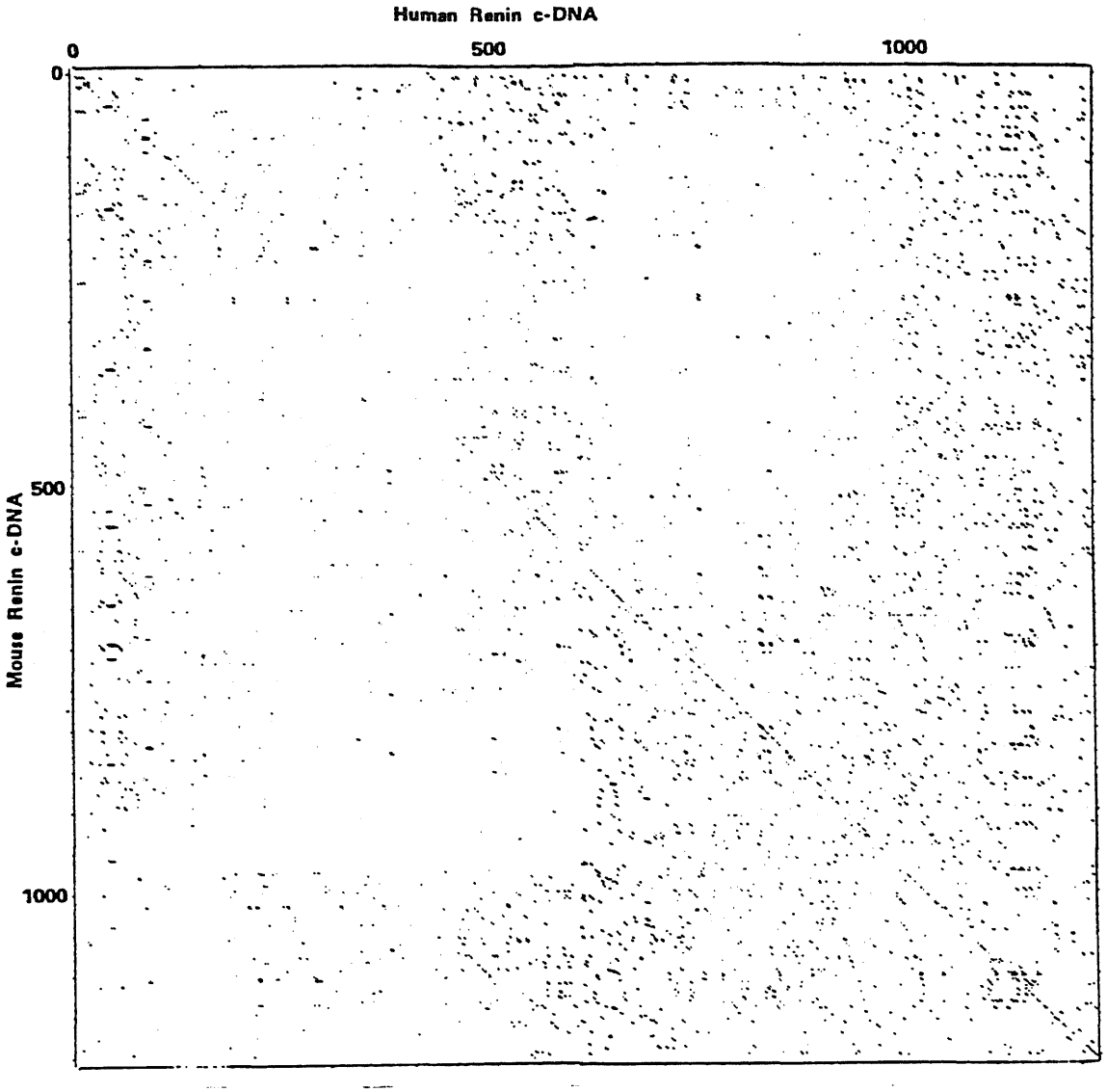
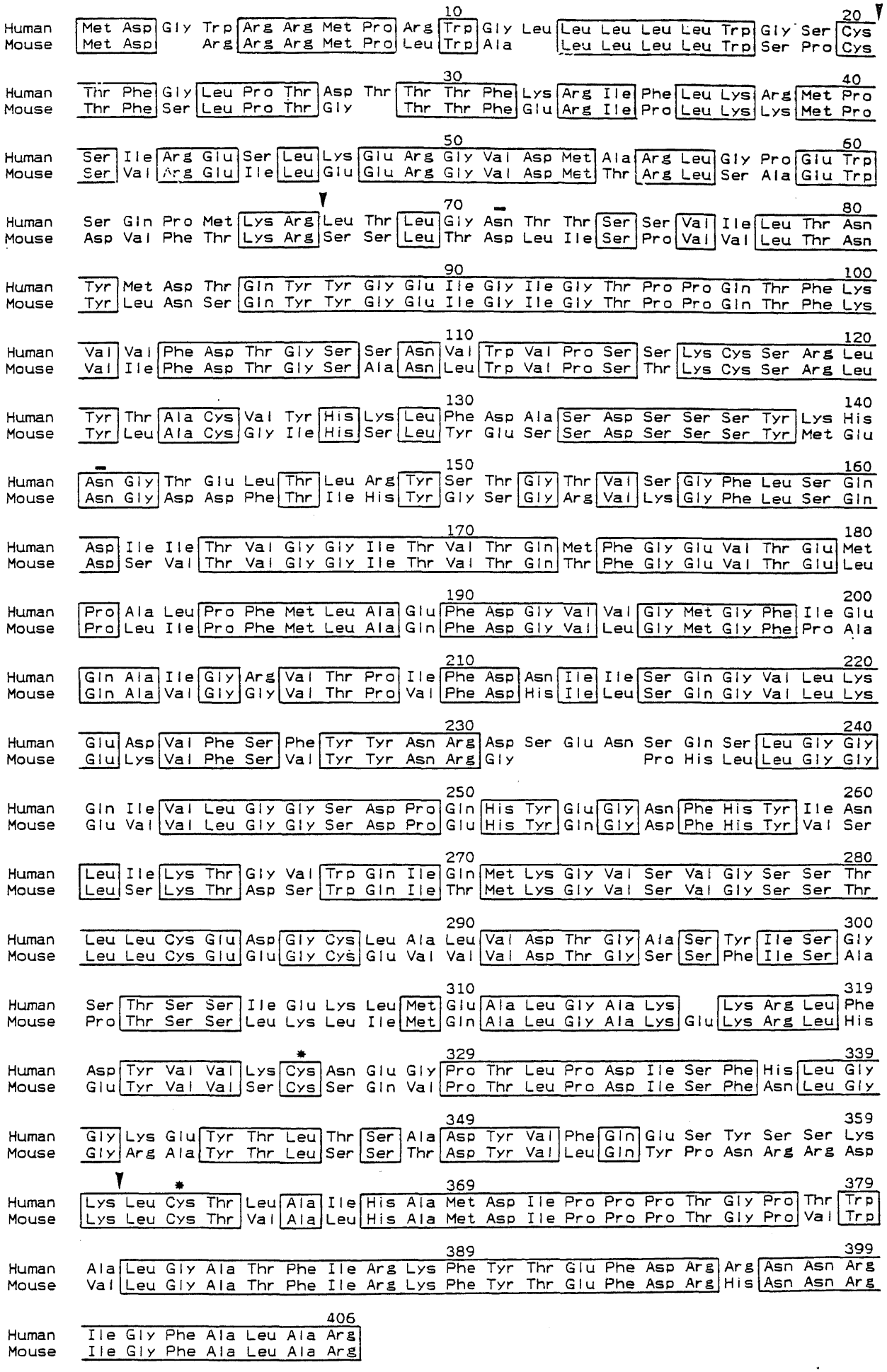


Figure 7. Homology of the nucleotide sequence between two preprorenin cDNAs from human kidney and mouse submandibular gland by a dot matrix technique. On the horizontal and vertical axes, respectively, are 1,218 nucleotide bases of human cDNA and 1,203 bases of mouse cDNA, encoding the complete amino acid sequence of preprorenin. More than four consecutive dots on which each dot presents a base that coincided in the two sequences are recorded.

Figure 8. Homology of amino acid sequence between two preprorenins from human kidney and mouse submandibular gland. Identical residues are enclosed in solid lines. The homology search was carried out by a computer-aided maximum match(15). [20-21] and [66-67] indicate the proteolytic cleavage sites for prorenin and mature renin, respectively. The arrow (▼) indicates possible sites attacked by trypsin-like enzyme(s).


Human Met Asp Gly Trp Arg Arg Met Pro Arg Trp Gly Leu Leu Leu Leu Leu Trp Gly Ser Cys
Mouse Met Asp Arg Arg Arg Met Pro Leu Trp Ala Leu Leu Leu Leu Leu Trp Ser Pro Cys
Human Thr Phe Gly Leu Pro Thr Asp Thr Thr Thr Phe Lys Arg Ile Phe Leu Lys Arg Met Pro
Mouse Thr Phe Ser Leu Pro Thr Gly Thr Thr Phe Glu Arg Ile Pro Leu Lys Lys Met Pro
Human Ser Ile Arg Glu Ser Leu Lys Glu Arg Gly Val Asp Met Ala Arg Leu Gly Pro Glu Trp
Mouse Ser Val Arg Glu Ile Leu Glu Glu Arg Gly Val Asp Met Thr Arg Leu Ser Ala Glu Trp
Human Ser Gln Pro Met Lys Arg Leu Thr Leu Gly Asn Thr Thr Ser Ser Val Ile Leu Thr Asn
Mouse Asp Val Phe Thr Lys Arg Ser Ser Leu Thr Asp Leu Ile Ser Pro Val Val Leu Thr Asn
Human Tyr Met Asp Thr Gln Tyr Tyr Gly Glu Ile Gly Ile Gly Thr Pro Pro Gln Thr Phe Lys
Mouse Tyr Leu Asn Ser Gln Tyr Tyr Gly Glu Ile Gly Ile Gly Thr Pro Pro Gln Thr Phe Lys
Human Val Val Phe Asp Thr Gly Ser Ser Asn Val Trp Val Pro Ser Ser Lys Cys Ser Arg Leu
Mouse Val Ile Phe Asp Thr Gly Ser Ala Asn Leu Trp Val Pro Ser Thr Lys Cys Ser Arg Leu
Human Tyr Thr Ala Cys Val Tyr His Lys Leu Phe Asp Ala Ser Asp Ser Ser Ser Tyr Lys His
Mouse Tyr Leu Ala Cys Gly Ile His Ser Leu Tyr Glu Ser Ser Asp Ser Ser Ser Tyr Met Glu
Human Asn Gly Thr Glu Leu Thr Leu Arg Tyr Ser Thr Gly Thr Val Ser Gly Phe Leu Ser Gln
Mouse Asn Gly Asp Asp Phe Thr Ile His Tyr Gly Ser Gly Arg Val Lys Gly Phe Leu Ser Gln
Human Asp Ile Ile Thr Val Gly Gly Ile Thr Val Thr Gln Met Phe Gly Glu Val Thr Glu Met
Mouse Asp Ser Val Thr Val Gly Gly Ile Thr Val Thr Gln Thr Phe Gly Glu Val Thr Glu Leu
Human Pro Ala Leu Pro Phe Met Leu Ala Glu Phe Asp Gly Val Val Gly Met Gly Phe Ile Glu
Mouse Pro Leu Ile Pro Phe Met Leu Ala Gln Phe Asp Gly Val Leu Gly Met Gly Phe Pro Ala
Human Gln Ala Ile Gly Arg Val Thr Pro Ile Phe Asp Asn Ile Ile Ser Gln Gly Val Leu Lys
Mouse Gln Ala Val Gly Gly Val Thr Pro Val Phe Asp His Ile Leu Ser Gln Gly Val Leu Lys
Human Glu Asp Val Phe Ser Phe Tyr Tyr Asn Arg Asp Ser Glu Asn Ser Gln Ser Leu Gly Gly
Mouse Glu Lys Val Phe Ser Val Tyr Tyr Asn Arg Gly Pro His Leu Leu Gly Gly
Human Gln Ile Val Leu Gly Gly Ser Asp Pro Gln His Tyr Glu Gly Asn Phe His Tyr Ile Asn
Mouse Glu Val Val Leu Gly Gly Ser Asp Pro Glu His Tyr Gln Gly Asp Phe His Tyr Val Ser
Human Leu Ile Lys Thr Gly Val Trp Gln Ile Gln Met Lys Gly Val Ser Val Gly Ser Ser Thr
Mouse Leu Ser Lys Thr Asp Ser Trp Gln Ile Thr Met Lys Gly Val Ser Val Gly Ser Ser Thr
Human Leu Leu Cys Glu Asp Gly Cys Leu Ala Leu Val Asp Thr Gly Ala Ser Tyr Ile Ser Gly
Mouse Leu Leu Cys Glu Glu Gly Cys Glu Val Val Val Asp Thr Gly Ser Ser Phe Ile Ser Ala
Human Ser Thr Ser Ser Ile Glu Lys Leu Met Glu Ala Leu Gly Ala Lys Lys Arg Leu Phe
Mouse Pro Thr Ser Ser Leu Lys Leu Ile Met Gln Ala Leu Gly Ala Lys Glu Lys Arg Leu His
Human Asp Tyr Val Val Lys Cys Asn Glu Gly Pro Thr Leu Pro Asp Ile Ser Phe His Leu Gly
Mouse Glu Tyr Val Val Ser Cys Ser Gln Val Pro Thr Leu Pro Asp Ile Ser Phe Asn Leu Gly
Human Gly Lys Glu Tyr Thr Leu Thr Ser Ala Asp Tyr Val Phe Gln Glu Ser Tyr Ser Ser Lys
Mouse Gly Arg Ala Tyr Thr Leu Ser Ser Thr Asp Tyr Val Leu Gln Tyr Pro Asn Arg Arg Asp
Human Lys Leu Cys Thr Leu Ala Ile His Ala Met Asp Ile Pro Pro Pro Thr Gly Pro Thr Trp
Mouse Lys Leu Cys Thr Val Ala Leu His Ala Met Asp Ile Pro Pro Pro Thr Gly Pro Val Trp
Human Ala Leu Gly Ala Thr Phe Ile Arg Lys Phe Tyr Thr Glu Phe Asp Arg Arg Asn Asn Arg
Mouse Val Leu Gly Ala Thr Phe Ile Arg Lys Phe Tyr Thr Glu Phe Asp Arg His Asn Asn Arg
Human Ile Gly Phe Ala Leu Ala Arg
Mouse Ile Gly Phe Ala Leu Ala Arg

The molecular weight of mature human renin (Mr = 37,236) calculated from the amino acid sequence is in agreement with that determined by Inagami(12) for the purified protein. The molecular weight of human preprorenin (Mr = 45,057) is consistent with a Mr of 45,000, which was obtained by the methods of the cell free translation of renin mRNA and SDS-polyacrylamide gel electrophoresis(Fig. 2). The molecular weights of the prepro-, pro-, and mature forms of human renin are 500 to 800 greater than those of mouse submandibular gland renin. These differences are, however, smaller than those anticipated before(13). Since human kidney renin is glycoprotein, its molecular weight is larger than that calculated by amino acid sequence. The difference in the molecular weight of human renin with and without carbohydrate residues is under investigation.

Comparison of the amino acid sequence of human renin with that of mouse submandibular renin indicates a high degree of homology between the two enzymes. An overall homology, including presequence between the two renins is 68.7 %. Moreover, certain regions of renin are remarkably conserved between the two species, particularly the regions corresponding to the two catalytically important aspartyl residues Phe-Asp-Thr-Gly-Ser at amino acid positions 103 through 107 in Figure 8, (porcine pepsin No. 31-35 in Table 3) and Val-Asp-Thr-Gly at position 291 through 294 in Figure 8(porcine

pepsin No. 214-217 in Table 3). These catalytically important sequences are also homologous with all other aspartyl proteinases(Table 3).

Table 3. Active site residues of aspartyl proteinases

Proteinase	Amino acid sequence of residues									
	30	31	32	33	34	35	36	37	39	75
Renin (human)	-Val -	Phe	- Asp	- Thr	- Gly	- Ser	- Ser	- Asn-	Trp	Tyr
(mouse)	-Ile -	Phr	- Asp	- Thr	- Gly	- Ser	- Ala	- Asn-	Trp	Tyr
Pepsin (human)	-Val -	Phe	- Asp	- Thr	- Gly	- Ser	- Ser	- Asn-	Trp	Tyr
(pig)	-Ile -	Phe	- Asp	- Thr	- Gly	- Ser	- Ser	- Asp-	Trp	Tyr
Chymosin (bovine)	-Leu -	Phe	- Asp	- Thr	- Gly	- Ser	- Ser	- Asp-	Trp	Tyr
Penicillopepsin	-Asn -	Phe	- Asp	- Thr	- Gly	- Ser	- Ala	- Asp-	Trp	Tyr

	213	214	215	216	217	218	219	220	222	308
Renin (human)	-Leu -	Val -	Asp	- Thr	- Gly	- Ala	- Ser	- Tyr-	Ser	Arg
(mouse)	-Val -	Val -	Asp	- Thr	- Gly	- Ser	- Ser	- Phr-	Ser	Arg
Pepsin (human)	-Ile -	Val -	Asp	- Thr	- Gly	- Thr	- Ser	- Leu	Thr	Arg
(pig)	-Ile -	Val -	Asp	- Thr	- Gly	- Thr	- Ser	- Leu	Thr	Arg
Chymosin (bovine)	-Ile -	Leu -	Asp	- Thr	- Gly	- Thr	- Ser	- Lys	Val	Arg
Penicillopepsin	-Ile -	Ala -	Asp	- Thr	- Gly	- Thr	- Thr	- Leu	Leu	Lys

Amino acid residues are numbered according to porcine pepsin.

In addition to the two active site aspartyl residues (Asp-32 and Asp-215 in Table 3), the other catalytically important residues in aspartyl proteinase are also found in human renin. For example, Ser-35 in human renin (Table 3) may be hydrogen-bonded to the carboxylate oxygen of Asp-32, as suggested in mouse submandibular gland renin(13). The sequence Thr-Gly(216-217 in Table 3), whose peptide moiety has been implicated in the catalytic mechanism of penicillopepsin(13), again is found in human renin in the present investigation and in mouse renin(13). Both of these structures, Ser-35 and

Thr-Gly(216-217), are within close proximity of the two active site aspartyl residues Asp-32 and Asp-215, respectively. In addition, Tyr-75 and Arg-308 in porcine pepsin or Tyr-75 and Lys-308 in penicillopepsin implicated in the catalytic mechanism are also identical or functionally conserved in human and mouse renins(Table 3).

These findings strongly support the argument that, in spite of the marked difference in the pH optimum of the catalysis, the catalytic mechanism of renin is very similar to that of other aspartyl proteinases. The sequence identity of human prorenin with human pepsinogen(47) was 34 %. Moreover, the amino acid residues in human renin identical to those in pepsin or penicillopepsin are distributed throughout the length of the molecule, which suggests that the tertiary structure of renin should be similar to those of pepsin and penicillopepsin.

Potential Glycosylation Sites

As discussed above, human kidney renin is homologous to the renin from mouse submandibular gland. This homology includes the primary and most likely the tertiary structure and catalytic apparatus. The major structural difference distinguishing the two enzymes is the presence of two glycosylation sites in human kidney renin. Human renin has two glycosylation sites Asn-X-Thr in residues

71 through 73 and 141 through 143 in Figure 8 which are not observed in mouse submandibular gland renin(8,9). The threonyl residue two away from the glycosylated Asn residue is the normal glycosylation signal for N-linked oligosaccharides(42). The result that human kidney renin is glycoprotein is consistent with other biochemical data obtained previously(12). There is another aspartyl proteinase, porcine spleen cathepsin D, which has two glycosylation sites(Asn-67 and Asn-183, based on the residue number of porcine pepsin)(42,43). It is interesting to note that the two glycosylation positions in cathepsin D are located one in each half of the molecule, whereas those in human kidney renin are located only in one-half of the molecule. It is not clear that this difference between the two enzymes implies any physiological significance, although the function of these oligosaccharides, at least in cathepsin D, is to serve as makers of packaging of these enzymes.

Model for Cellular Processing of Renin

A model for the processing of human and mouse preprorenin to prorenin and mature renin is shown in Figure 9. The signal peptide attached to the beginning of the proteins helps to direct the newly synthesized preprorenin to its destination and is clipped off when it passes through the membrane of endoplasmic reticulum. The signal peptides (presequences) consist of 20 (human)

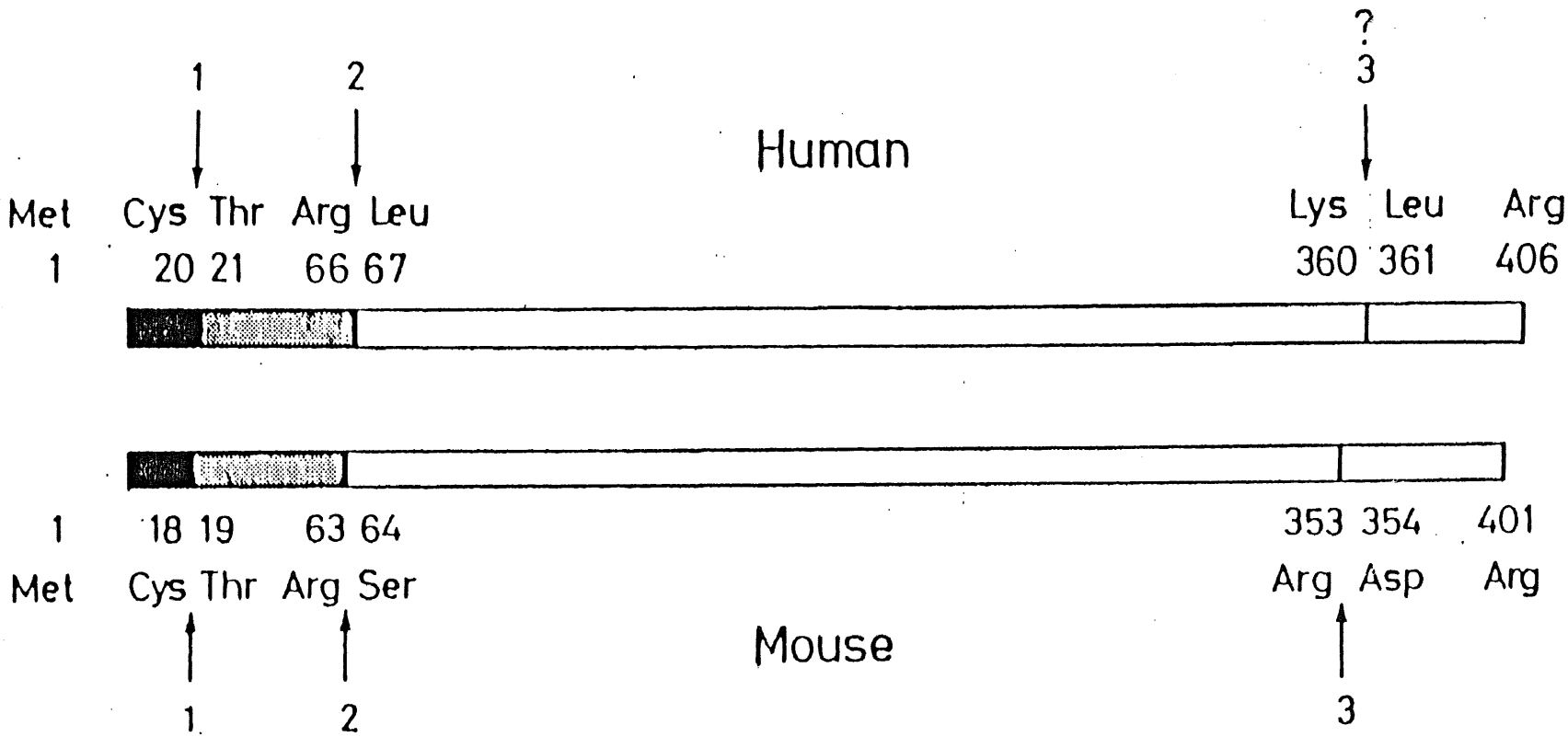


Figure 9. Processing of preprorenin to prorenin and mature renin in human kidney and mouse submandibular gland. Black bar = presequence; hatched bar = pro-sequence; white bar = mature renin. Arrows 1, 2, and 3 indicate the cleavage sites attacked by processing enzymes.

and 18 (mouse) amino acids and are cleaved by a proteolytic enzyme in the membrane to form prorenins. Prosequences consisting of 46 (human) and 45(mouse) amino acids are cleaved by an activating enzyme to produce mature renins. The mature renins are composed of 340 (human) and 338(9) or 333(8) (mouse) amino acids. The mouse renin consists of a heavy chain(64-353) and a light chain(354-401), as shown in Figure 9. Consequently, human renin may be composed of a heavy chain(67-360) and a light chain(361-406).

Knowledge of renin has recently become very detailed but confusing. Multiple forms of active renin have been extracted from kidneys of several species(48). The probability that these renins are the products of separate renin genes seems to be very low because all the human renin cDNAs cloned in this study yield identical patterns of restriction fragments when digested with Ava II, Rsa I, or Bst NI and analyzed by electrophoresis. Therefore, the different forms of renin may result from posttranslational modifications or more likely represent a mere experimental artifact induced by limited proteolysis during the extraction and purification procedures. In support of this view, four cleavage sites with two basic amino acids(Lys-Arg(250-251), Lys-Lys(293-294), Arg-Lys(321-322), and Arg-Arg(329-330)) (Fig. 9)

are known to occur near the COOH terminus of mature renin. In addition to the active renins, inactive renins that can be activated by trypsin have been demonstrated in human kidneys and plasma. Biochemical characterizations have suggested that inactive renin in the kidney corresponds to prorenin, a biosynthetic precursor of renin. However, the relationship between the renal prorenin and the inactive material in plasma, which accounts for more than 80 % of the total plasma renin, is not clear(for reviews see refs. 49-51). Attempts to show that inactive renin in plasma is a precursor of the circulating active renin have produced conflicting results, and currently available data do not allow one to determine whether the plasma inactive renin represents the prorenin secreted from the kidney or the previously active renin covalently combined with an inactivating protein. It is anticipated that the size and the amino acid sequence of the pro segment reported here may contribute to a more precise analysis of the exact nature of plasma inactive renin.

Recent studies that have demonstrated the presence of renin in several organs other than the kidney have modified the classic assumption that the renin-angiotensin system is a system of renal origin that regulates blood pressure. Especially, renin from the brain has drawn a great deal of attention because it is implicated in the regulation of numerous activities of

the central nervous system(52,53). Full-length renin cDNA clones here could serve as useful tools for the cloning of extrarenal renin cDNAs as well as for the analysis of the organization of human renin genes and for the production of human renin in heterologous cells such as Escherichia coli.

Chapter III. Expression of Human Prorenin Gene in *Escherichia coli*

SUMMARY

DNA sequences encoding Ile-Glu-Gly-Arg and human prorenin were joined and placed under the transcription control of the *Escherichia coli trp* promoter-operator in the expression plasmid pTR501. *E. coli* cells transformed with pTR501 expressed high levels (30 % of total cell protein) of prorenin as part of a hybrid protein with the *trp E* gene product. The chimeric protein, accumulated in a sedimentabale form, was dissolved in 6 M guanidine hydrochloride, purified to near homogeneity, and renatured by dialysis. The complete prorenin sequence was then excised from the renatured hybrid protein using blood coagulation factor Xa, a proteinase which is highly specific for the tetrapeptide insert Ile-Glu-Gly-Arg introduced between the 9 amino terminal residues of the *trp E* gene product and the first amino acid (Thr 1) of prorenin. Human prorenin thus obtained was readily activatable with trypsin and showed close similarities to naturally occurring prorenin in its biochemical and immunochemical properties.

INTRODUCTION

Plasma inactive renin, which comprises 70-90 % of total circulating renin and exhibits a unique reversible activation when exposed to acid, has been a focal point of much investigation(49-51, 54, 55). In recent years, plasma inactive renin was identified with prorenin, a biosynthetic precursor(56,57). Although its complete amino acid sequence(Chapter II)(58) and gene structure (59,60) have been determined by nucleotide sequencing of cDNA and cloned chromosomal DNA, human renin still presents experimental difficulties in its characterization and elucidation of the mechanisms of activation mainly because of its low concentration.

Therefore it was attempted to produce human prorenin in quantities sufficient for its biochemical characterization and clarification of the activation mechanisms, using the expression system for foreign genes in Escherichia coli.

Many kinds of expression vectors have been developed to produce proteins of biochemical interest(61,62). The expression vector contains sequences of DNA that are required for the transcription of desired gene and the translation of its mRNA in E. coli. The major requirements for expression of a cloned gene are an E. coli promoter sequence and a ribosome-binding site (Fig. 10).

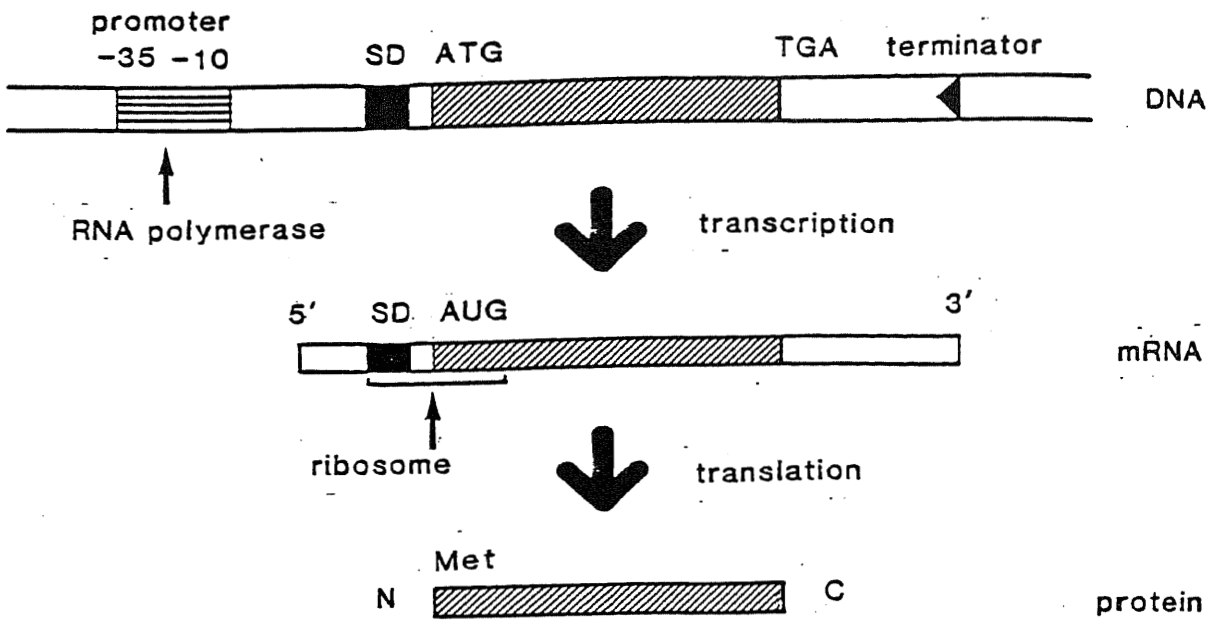


Figure 10. **Gene structure and flow of genetic information in Escherichia coli.** SD, Shine-Dalgarno sequence which is complementary to the 3' end of E. coli 16S rRNA; N, amino terminus; C, carboxyl terminus.

If the desired gene is of E. coli origin, its promoter must function properly in E. coli. Therefore, the gene coding for the protein, along with its promoter, can be inserted directly into a high-copy-number E. coli plasmid present in about 30 - 50 copies per cell. If the gene functions as well when located on the plasmid as it did when chromosomally located, roughly a 30- to 50-fold increase in the amount of the desired protein per transformed cell occurs. For instance, the E. coli lac Z gene product produced up to 30 % of the total soluble protein of the cell.

However, for expression of the foreign gene in E. coli, the gene coding for the protein must be placed under the control of an E. coli promoter such as the lac promoter and trp promoter which are efficiently recognized by E.

coli RNA polymerase. Such recombinants can direct the synthesis of large amounts of protein when present in E. coli hosts(64,65).

To achieve high levels of gene expression in E. coli, it is necessary to use not only strong promoters to generate large quantities of mRNA, but also ribosome binding sites to ensure that the mRNA is efficiently translated.

The efficiency of translation of an mRNA could be affected by several factors: 1)the degree of complementarity between the Shine-Dalgano(SD) sequence (66,67) and the 3' end of the 16 S rRNA; 2)the spacing and possibly the RNA sequence lying between the SD sequence and the initiation codon(AUG)(68-70); and 3)the nucleotide sequences following the AUG(71).

These factors must be optimized to obtain efficient production of unfused eukaryotic proteins(72-74)(Fig. 11-A). For example, the optimization of the distance between the bacterial SD sequence and the ATG of the eukaryotic gene may be achieved by the methods described in Roberts et al.(72). And if the DNA sequence to be expressed, lacks the ATG codon, then one must be provided by chemical DNA synthesis(72,75). And the direct expression often results in production of a protein bearing an additional methionine residue at its amino terminus. It seemed to be a very critical and complicated procedure.

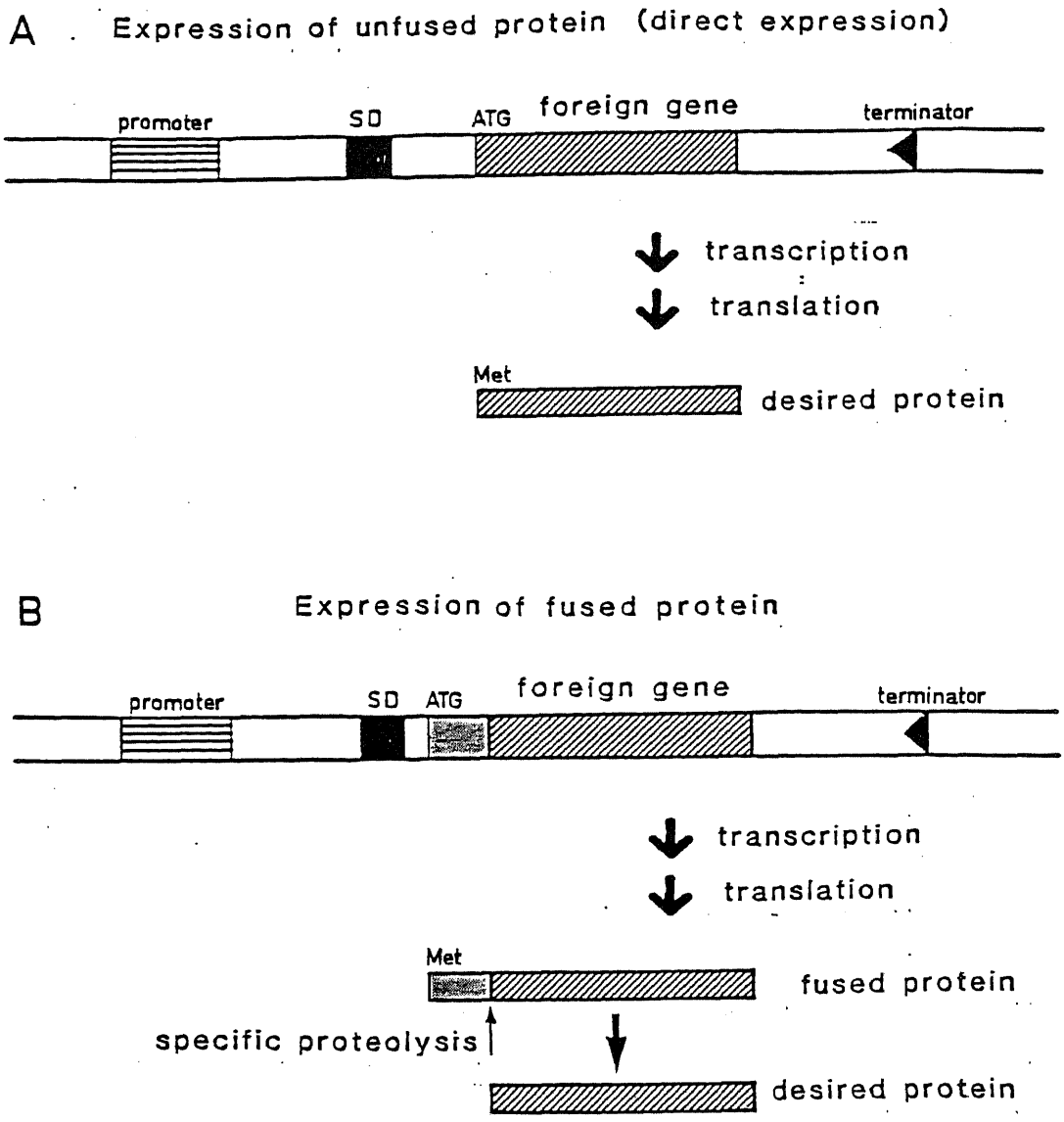


Figure 11. **Expression of foreign genes in E. coli.** ▒▒, prokaryotic sequence; ▨, eukaryotic sequence; SD, Shine-Dalgarno sequence.

To avoid these problems, another expression system that the proteins are synthesized as a hybrid proteins has been developed(Fig. 11-B). A strong promoter, the

ribosome binding site and the part of coding sequence from highly expressed E. coli genes were placed in front of the foreign DNA sequences, and the expected fusion proteins were produced at high levels. Such a hybrid protein is not suitable for functional studies or clinical use. Recently, however, Nagai and Thøgersen have divised a novel method of producing a hybrid protein from which the desired authentic sequence can be released by sequence-specific proteolysis(76).

The author used similar strategies to construct the expression plasmid that directs the synthesis of prorenin as a hybrid polypeptide fused to a NH_2-terminal portion of the trp E product by inserting the cDNA version of the prorenin gene in down stream of the trp promoter-operator through the linker which specifies the unique recognition sequence of blood coagulation factor Xa(FXa), i.e. Ile-Glu-Gly-Arg(76). The essential feature of this construction is that the complete prorenin sequence can be excised from the fusion product by using FXa. The expected fusion protein was obtained, purified, and cleaved with FXa. The liberated prorenin can be readily activated by limited proteolysis. The availability of large quantities of bacterially produced trypsin-activatable prorenin will open the way to a number of biochemical experiments aimed at defining the properties, roles, and activation mechanisms of plasma and renal prorenin(77).

MATERIALS AND METHODS

Materials

Some of the reagents are described in the preceding chapter(Chapter II). Other materials were obtained as follows: trypsin-TPCK from Worthington Biochemicals (Freehold, NJ); soybean trypsin inhibitor from Miles Laboratories (Elkhart, IN); Cellulofine GCL-2000 and egg white lysozyme from Seikagaku Kogyo (Tokyo, Japan). Pure preparation of blood coagulation factor Xa was kindly supplied by Dr. Sadaaki Iwanaga (Kyushu University, Japan)(78).

Construction of Expression Plasmid for Human Prorenin

See Figure 12. The plasmid, pKN205(79), carrying the E. coli trp promoter was kind gifts from Dr. Tsutomu Masuda (Kikkoman Corporation, Japan).

Two complementary oligodeoxyribonucleotides, 5'-dAGCTA-TCGAAGGCCGC and 5'-dGCGGCCTTCGAT, coding for the amino acid sequence Ile-Glu-Gly-Arg which is cleavable by blood coagulation factor Xa(76), were prepared on an automated DNA synthesizer (Applied Biosystems, model 380 A) according to the procedure recommended by the manufacturer.

The plasmid containing the promoter of tryptophan operon of E. coli, pKN205, was cleaved with Hin dIII and Pst I, and the fragment containing the promoter was recovered. This fragment also contains trp L and the 5' region of trp E that codes the N-terminal 7 amino acids

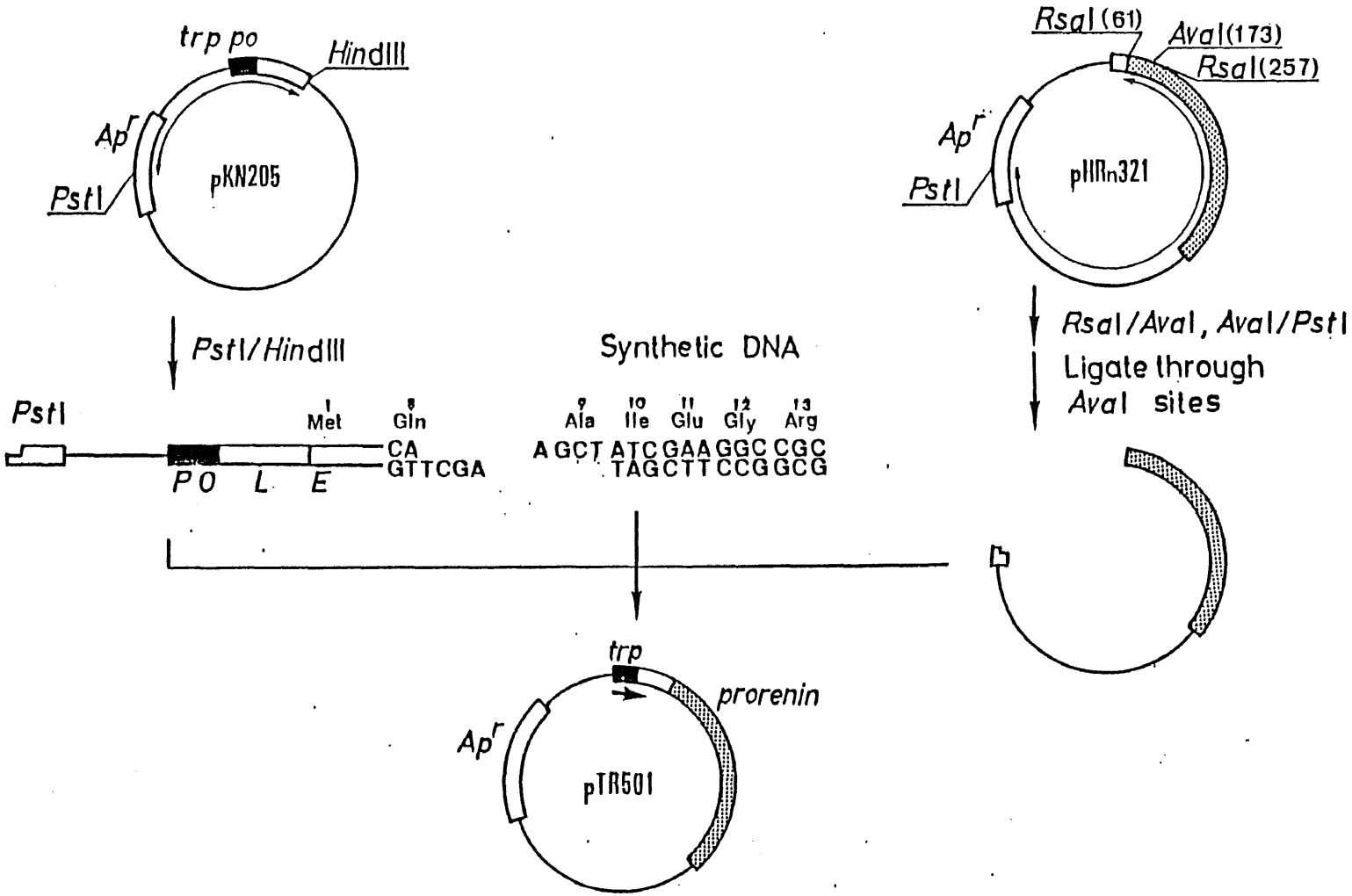


Figure 12. **Construction of expression plasmid.** Prorenin coding sequences are represented by the shaded box. The solid thick line represents the trp promoter region. The thin single line represents pBR322 sequences. Ap^r indicates the β-lactamase gene. Only the positions of the relevant restriction sites are shown (for the nucleotide numbers in renin gene, see Figure 5). Double arrows indicate fragment isolated.

of anthranilate synthetase plus Gln and Ala. A 5' fragment of the prorenin gene, containing nucleotides 61-173, was excised from pHRn321 by treating with Rsa I/Ava I. The nucleotides 1-60 which is supposed to encode the preregion was removed. Similarly, a 3' fragment of the gene was obtained as a Ava I(nucleotide number 171)/Pst I (in the β-lactamase gene, Ap^r) fragment. The 5'- and 3'-fragments were recombined through the Ava I cohesive ends and ligated by using T4 DNA ligase. The expression plasmid was constructed by ligation of the reconstituted prorenin gene to the promoter fragment through the synthetic oligonucleotides.

Escherichia coli HB101 was transformed and ampicillin-resistant clones were selected by the method of Morrison (29).

Recombinant plasmids from the selected colonies were prepared, digested with various restriction enzymes, and electorophoresed in agarose and polyacrylamide gels to confirm the structure. These procedures are described in Chapter II. The DNA sequence of the junction between trp promoter and renin cDNA of pHRn321 was determined by the method of Sanger et al.(80,81).

Media and Growth Condition

Bacterial transformants were grown in Luria-Bertani Medium (1 % Bacto-trypton, 0.5 % Bacto-yeast extract, 0.5 % NaCl, 0.1 % glucose) with 100 μg ampicillin per ml. A 1:100 dilution of the overnight culture was made into M9-

GC medium lacking tryptophan and containing per liter: 5.9 g Na_2HPO_4, 1.2 g KH_2PO_4, 2 g NaCl, 0.4 g NH_4Cl, 4 g glucose, 10 mg gelatin, 14.7 mg $CaCl_2$-$2H_2O$, 95.2 mg $MgCl_2$, 0.27 mg $FeCl_3$-$6H_2O$, 5 g Casamino acids (Difco Laboratories, Detroit MI), and 1 mg apmicillin. Cells were grown at 37 °C to OD 600 = 0.3. Expression from the trp promoter was induced by adding 3-β-indole acrylic acid (IAA) to 20 μg/ml and incubation continued for an additional 20 hr.

Analysis of Protein Expressed in Escherichia coli

The bacterial cells were suspended in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and lysed 0.5 mg of egg white lysozyme per ml. After successive incubation at 25 °C for 30 min and at 0 °C for 90 min(82), samples were sonicated (200 W for 10 min) and centrifuged for 10 min at 4,200 x g to precipitate inclusion body. Aliquots of each supernatants and pellets were analyzed on a pair of 12.5 % SDS-polyacrylamide gels(25). After electrophoresis, one of the gels was stained with Coomassie brilliant blue R-250, and the other was subjected to electroblotting onto a nitrocellulose filter. An anti-human renin antibody (24) and IMMUN-BLOT ASSAY kits(Bio-Rad) were used for the immunological detection of prorenin by protein blotting (83).

Renaturation of the Hybrid Protein Produced in an Insoluble Form in Escherichia coli

The inclusion body pelleted by low speed centrifugation

was dissolved in 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, and fractionated by gel filtration chromatography over a Cellulofine GCL-2000 column (1.5 x 90 cm) in the same buffer. The fractions (1 ml each) were dialyzed against 8 M urea, 20 mM Tris-HCl, pH 8.0, and aliquots of each fraction were analyzed on SDS-polyacrylamide gels. The fraction containing fusion protein were pooled and used as the starting material for renaturation of prorenin.

Conditions for renaturation were determined according to the procedure of Kawaguchi et al.(84). The fractions containing fusion protein were diluted to about 120 μg/ml with the urea buffer after adjusting 0-2 M NaCl concentration. The samples were dialyzed against 20 mM Tris-HCl, pH 8.0, at room temperature. The same samples used above in 8 M urea, 20 mM Tris-HCl, pH8.0, 1 M NaCl were adjusted to pH 6-12 with 1 N HCl or 1 N NaOH and dialyzed against the same pH buffer for 6 hr and then against 20 mM Tris-HCl, pH 8.0, overnight at room temperature.

Excision of Authentic Prorenin from the Hybrid Protein

Prorenin was cleaved out from the solubilized fusion protein using blood coagulation factor Xa(FXa) as follows. A 200 μg of the hybrid protein was incubated in a mixture(1 ml) containing 50 mM Tris-HCl,pH7.5, 10 mM $CaCl_2$, and 10 μg of FXa at 37 °C for 2 hr. The reaction was terminated by heating at 100 °C for 5 min in a sample

buffer(25). Gel electrophoresis was carried out using the 20 cm long model. After electrophoresis, gel was stained with Coomassie brilliant blue R-250.

Activation of the Prorenin Produced in Escherichia coli

Trypsin activation was carried out by adding 10 µl of appropriate concentration of trypsin solution into a tube containing 90 µl of sample in 20 mM Tris-HCl, pH 8.0 and 1 mg bovine serum albumin per ml. After incubation for 10 min at 4 °C, trypsin was neutralized with 10 µl of 20 mg/ml soybean trypsin inhibitor solution. Renin activity was determined by the radioimmunoassay of angiotensin I generated during the incubation of trypsin treated samples with hog substrate for 1 hr at 37 °C(85).

True renin activity was estimated by the extent of suppression of renin activity by pepstatin and anti-renin antibody. Inhibition of renin activity by 10 µM pepstatin and rabbit anti-human renin antiserum diluted to 1:100 was measured according to the standard methods(86-88).

Inhibition of trypsin activation with the prosequence-specific anti-Pro 3 antibody (56) was carried out as follows. The reaction mixture was adjusted to volume of 175 µl containing 20 mM Tris-HCl, pH8.0, 0.5 M NaCl, 0.3% BSA, and 6 µg of prorenin. After 1.6 µl of rabbit anti-Pro-3 antiserum was added, the solution was incubated overnight at 4 °C. The trypsin treatment and the measurment of renin activity was performed as described

above.

Protein concentration was estimated from sample absorbances assuming that 1 absorbance unit at 280 nm is 1 mg protein / ml. For insoluble samples, the buiret method(89) was used with bovine serum albumin as the standard.

RESULTS

Construction of a Plasmid for Expression of Human Prorenin in *Escherichia coli*

In order to produce human prorenin in *Escherichia coli*, an expression plasmid, named pTR501, was constructed (Fig. 12). pTR501 was designed to allow synthesis of a chimeric protein comprising the NH_2-terminal region of anthranilate synthetase, *trp E* gene product, and prorenin. To release intact prorenin from the fused protein, a pair of complementary oligonucleotides that codes Ile-Glu-Gly-Arg for which factor Xa is specific was synthesized and inserted between the *trp E* and prorenin genes according to the procedure of Nagai and Thøgersen (76). Expression of the hybrid gene carried on plasmid pTR501 is under control of the *trp* promoter and stimulated by an inducer such as 3-β-indole acrylic acid.

The plasmid pTR501 was introduced into *Escherichia coli* HB101 by transformation. Transformants were selected for ampicillin resistance. Clones expressing human prorenin were identified by immunoblot analysis of their extracts. The plasmid DNAs were isolated from these bacterial cells, their restriction maps and DNA sequence of the junction area between the promoter and renin cDNA were verified to have the exact nature of the construction. A representative clone was selected for further study.

Expression of the Prorenin in *Escherichia coli* and its Identification

Cells containing pTR501 expressed a new protein with an apparent Mr of 43,500 which was drastically induced with IAA as the induction time increased. This protein was not present in control cells carrying pHRn321 and pTR501 without induction, and specifically recognized with an anti-human renin antibody (Fig. 13). To facilitate large scale purification of the newly synthesized protein, optimal conditions for its production were examined. Optimal inductions were observed when IAA was added after a 3-hr preincubation (OD 600 = 0.3, early logarithmic phase) at concentration of 20 μg/ml. The relative amount of the desired product (a hybrid of the *trp E* gene product and human prorenin which are connected through the FXa recognition sequence) continued to increase for at least several hours and maximum yields was obtained by overnight culture. Under these optimized conditions, levels of expression, as determined by densitometry of the Coomassie blue-stained SDS-polyacrylamide gel electrophoresis profiles, amounted to as much as 30 % of total *E. coli* protein. After cell lysis and fractionation by centrifugation, almost all of the fusion product was recovered as inclusion body in the insoluble membrane fraction which could be clearly observed by an electron microscopy(Fig. 14).

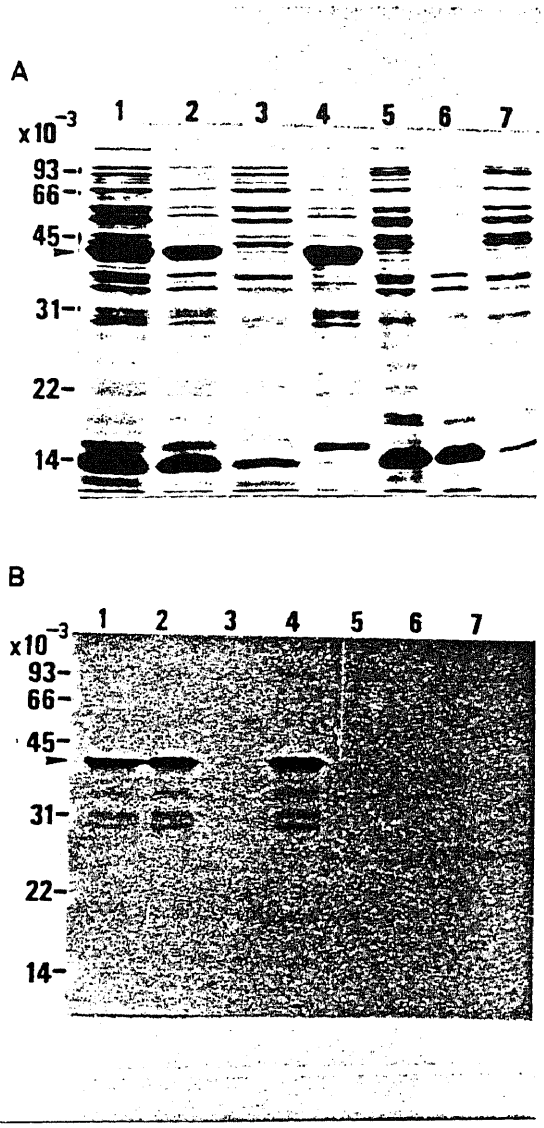


Figure 13. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of the hybrid protein synthesized in E. coli. E. coli HB101 containing pTR501 (lanes 1-4) or pHRn321 (lanes 5-7) were grown under inducing condition (20 µg/ml IAA). These bacterial cells were lysed by lysozyme treatment followed by sonication. The samples were analyzed on a pair of 12.5 % SDS-polyacrylamide gels. After electrophoresis, one of the gels was stained with Coomassie brilliant blue R-250 (A), and the other was subjected to electroblotting onto a nitrocellulose filter and treated by anti-renin antibody (B). Lane 1 and 5 show total proteins synthesized in E. coli. The total proteins were divided into soluble fraction and insoluble fraction by centrifugation at 40,000 x g for 30 min. Lane 2 and 6 show insoluble proteins. Lane 3 and 7 show soluble proteins. Lane 4 shows insoluble proteins sedimented by low speed centrifugation (4,200 x g for 10 min). The arrow marks the human prorenin as the fusion product. Molecular weight markers were phosphorylase B (Mr = 92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

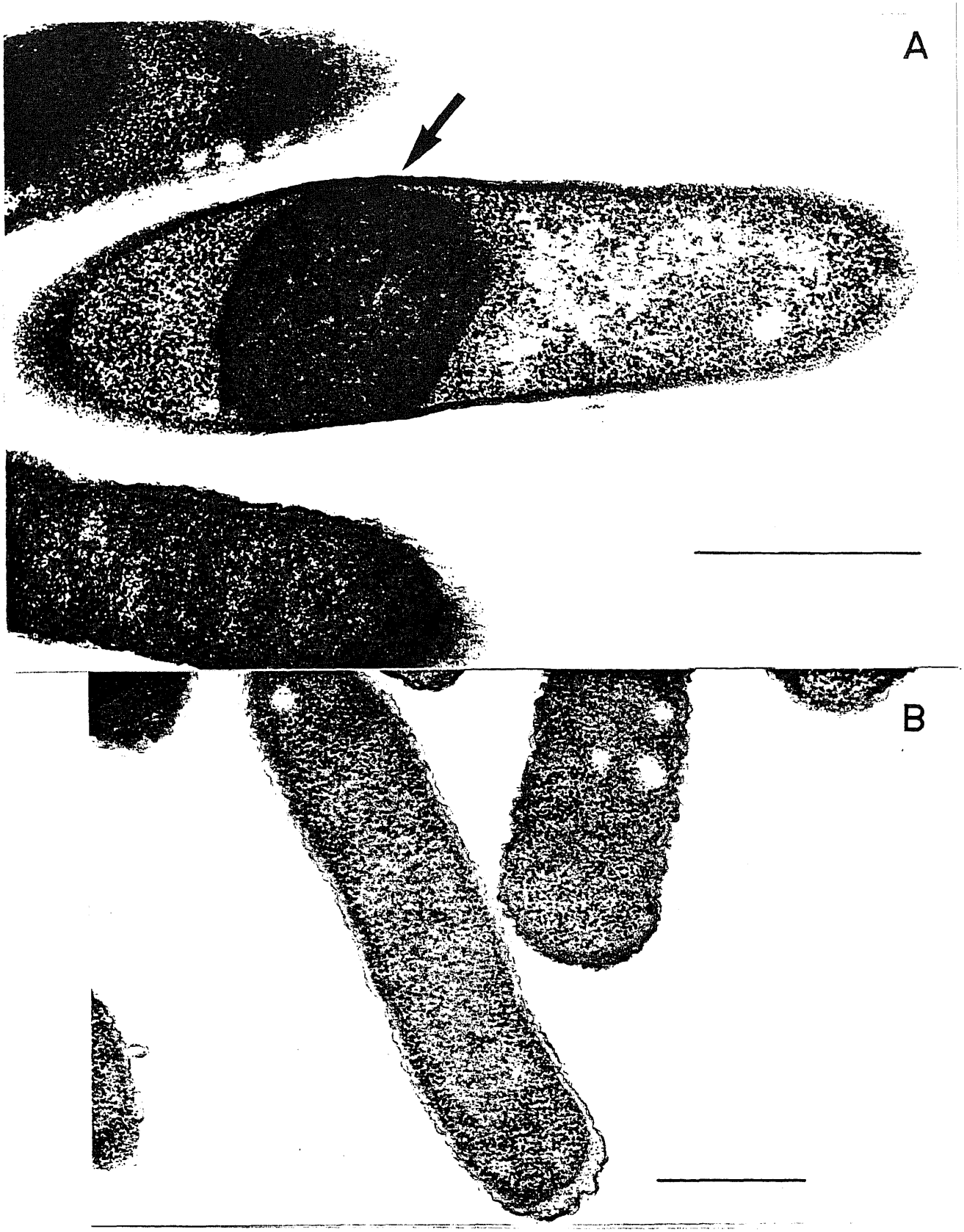


Figure 14. **Electron micrographs of Escherichia coli**
(A) Cells cantaining expression plasmid pTR501. Arrow indicates inclusion body of trp E-prorenin. Original magnification x 5,300; bar, 1 μm. (B) Cells containing pHRn321. The photographs were kindly provided by Dr. Yasuo Uchiyama, Institute of Basic Medical Sciences, University of Tsukuba.

Excision of Authentic Prorenin from the Hybrid Protein

The hybrid human prorenin synthesized in E. coli HB101 transformed with pTR501 was purified by gel filtration for further characterization. Inclusion bodies were first isolated by low-speed centrifugation and dissolved in 6 M guanidine-HCl. The solubilized proteins were then chromatographed on Cellulofine GCL-2000 in the presence of 6 M guanidine-HCl and the fractions were dialyzed against 8 M urea, 20 mM Tris-HCl, pH 8.0, and those containing the chimeric protein were pooled. SDS-polyacrylamide gel electrophresis analysis of the Cellulofine eluate indicated that a high degree of purity (>95 %) was already obtained(Fig. 15).

The next step was to renature the unfolded prorenin by gradually removing urea by dialysis. The efficiency of this renaturation process may be governed by such factors as pH and ionic strength(84). Therefore, the effects of pH and salt concentration on the refolding of prorenin polypeptide chain were investigated. As shown in Figure 16, the renaturation of prorenin was highly dependent on ionic strength and pH: the maximal renaturation, as monitored by its trypsin activatability, occurred between 0.8 and 1.0 M NaCl; the restoration of conformation or conformations close to native structure occurred optimally between pH 9.5 and 10.0 in the presence of 1.0M NaCl. Accordingly, the purified material in 8 M urea, 20 mM Tris-HCl, pH 8.0, including 1.0 M NaCl was adjusted

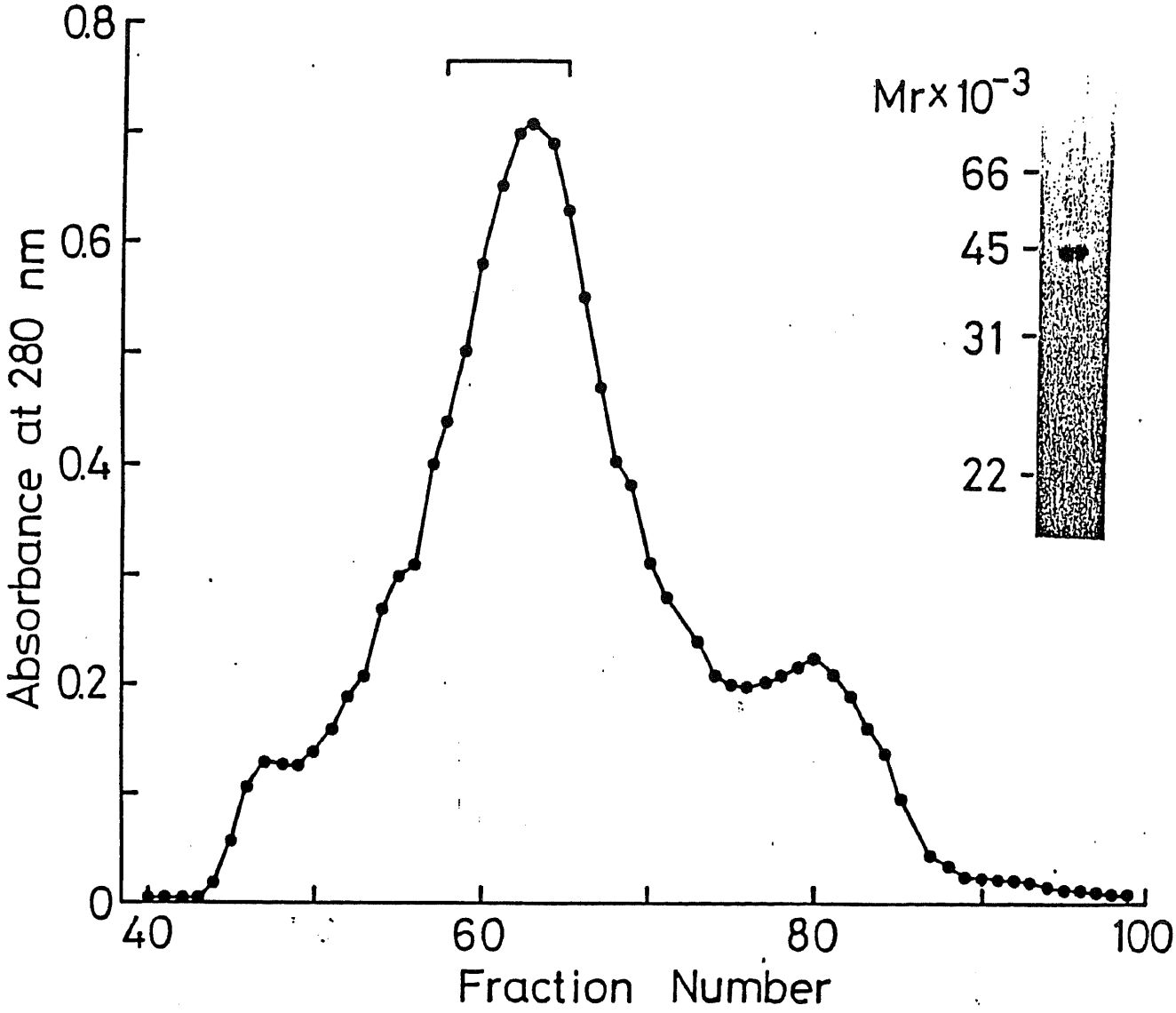


Figure 15. **Purification of the hybrid protein by gel filtration in 6 M guanidine-HCl on Cellulofine GCL-2000.** The samples of each fraction were dialyzed against 8 M urea, 20 mM Tris-HCl, pH 8.0 and aliquots of each fraction were analyzed on a 12.5 % SDS-polyacrylamide gel without reduction. Fraction indicated by bracket were pooled for renaturation step. Insert, SDS-polyacrylamide gel electrophoresis of the hybrid protein (fraction number 61).

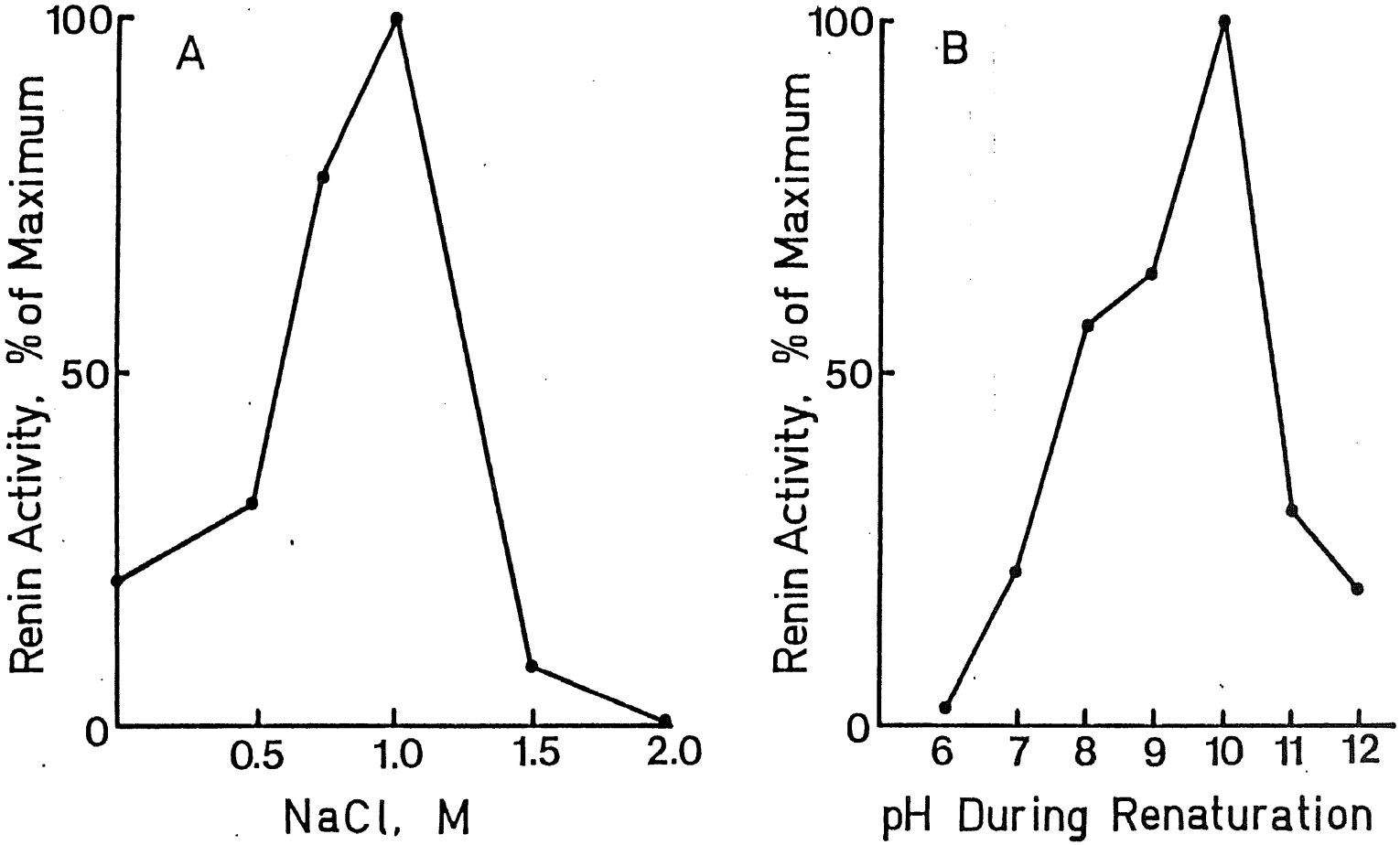


Figure 16. **Renaturation of prorenin.** The fraction containing fusion protein (Fig. 15) were dialyzed overnight against 8 M urea, 20 mM Tris-HCl, pH 8.0 and diluted to about 120 µg/ml with the same buffer after adjusting 0-2 M NaCl concentration. The samples were dialyzed against 20 mM Tris-HCl, pH 8.0 at room temperature (A). The same samples used above in 8 M urea, 20 mM Tris-HCl, pH 8.0, 1 M NaCl were adjusted to pH 6-10 with 1 N HCl or 1 N NaOH and dialyzed against the same pH buffer for 6 hr (84) and then against 20 mM Tris-HCl, pH 8.0 overnight at room temperature (B). Activities are expressed as percentages of the maximum value obtaind.

to pH 10.0 and then dialyzed against 20 mM Tris-HCl, pH 10.0 buffer.

From the dialyzed hybrid protein, authentic prorenin was liberated using the highly specific proteinase FXa(90) (Fig, 17). The size of this final product was exactly that expected for nonglycosylated human prorenin (Chapter II).

Activation of Prorenin

The prorenin produced in E. coli was examined for renin activity after trypsin treatment. These proteins were inactive without trypsin treatment but converted into active form when these were treated with the enzyme. Maximum activation was achieved with 0.9 μg of trypsin per 1 μg of the proteins at 4 °C for 10 min in the presence of 0.1 % of BSA(Fig. 18). At higher concentrations of trypsin, however, renin activity gradually declined probably because of further proteolytic degradation of the activated renin.

The activity of trypsin-treated renin, measured by its ability to generate angiotensin I from the protein substrate angiotensinogen, was copmletely inhibited by 10 μM of pepstatin or 1:100 diluted anti-human renin antiserum(Fig. 18).

Activation of prorenin with trypsin was completely protected by prosequence-specific anti-Pro 3 antibody. This property of the renin precursor was very similar to that of plasma inactive renin(56).

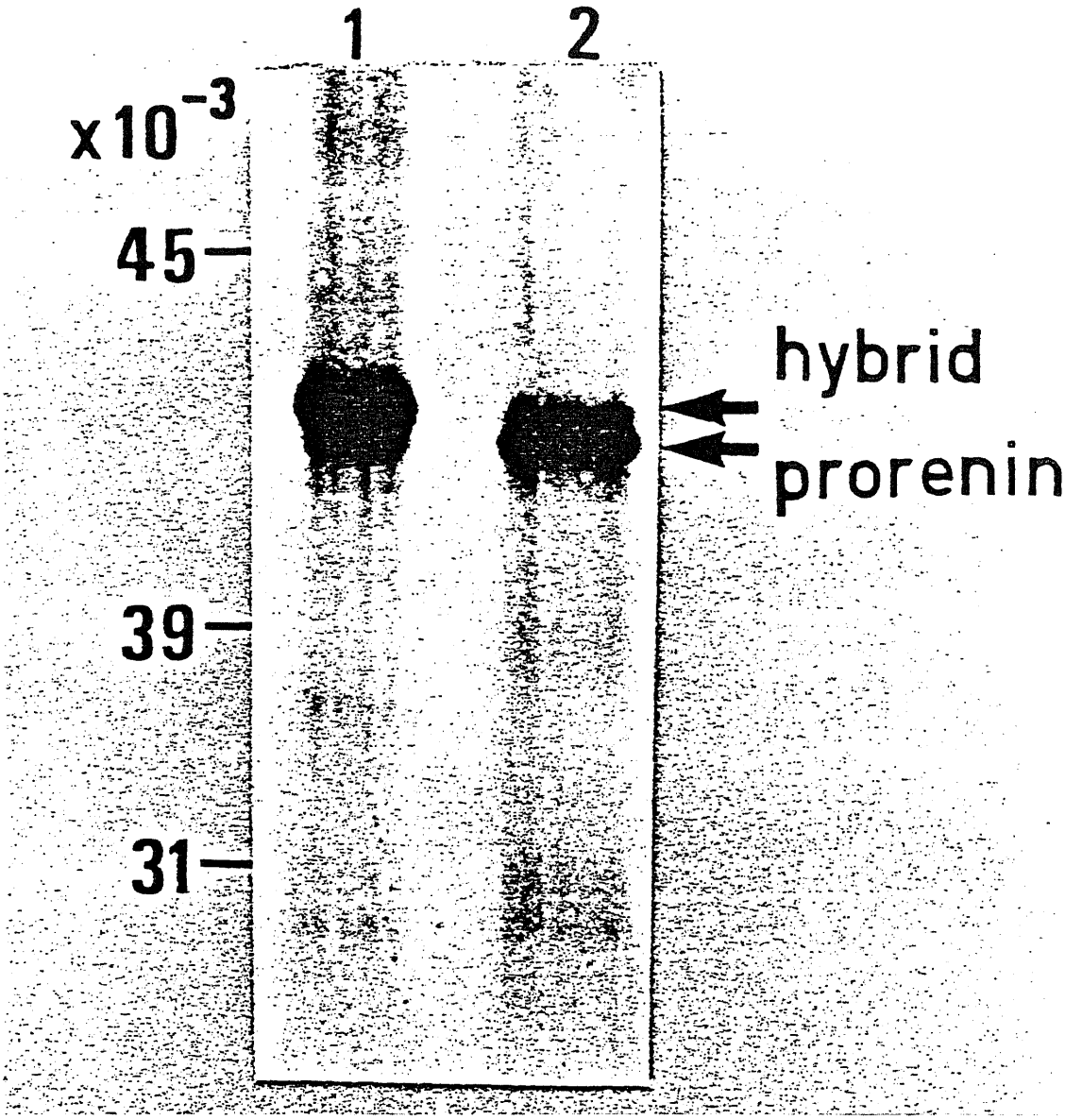


Figure 17. **Cleavage of hybrid protein synthesized in E. coli with factor Xa.** The hybrid protein renatured at the opotimal condition as described in Figure 16 was cleaved with blood coagulation factor Xa. The product was analyzed by electrophoresis on a 10 % SDS-polyacylamide gel. Lane 1, hybrid protein (Mr = 43,500); lane 2, cleaved product (Mr = 42,500). Molecular weight markers were ovalbumin (45,000), mature renin synthesized in E. coli (37,200), and carbonic anhydrase (31,000).

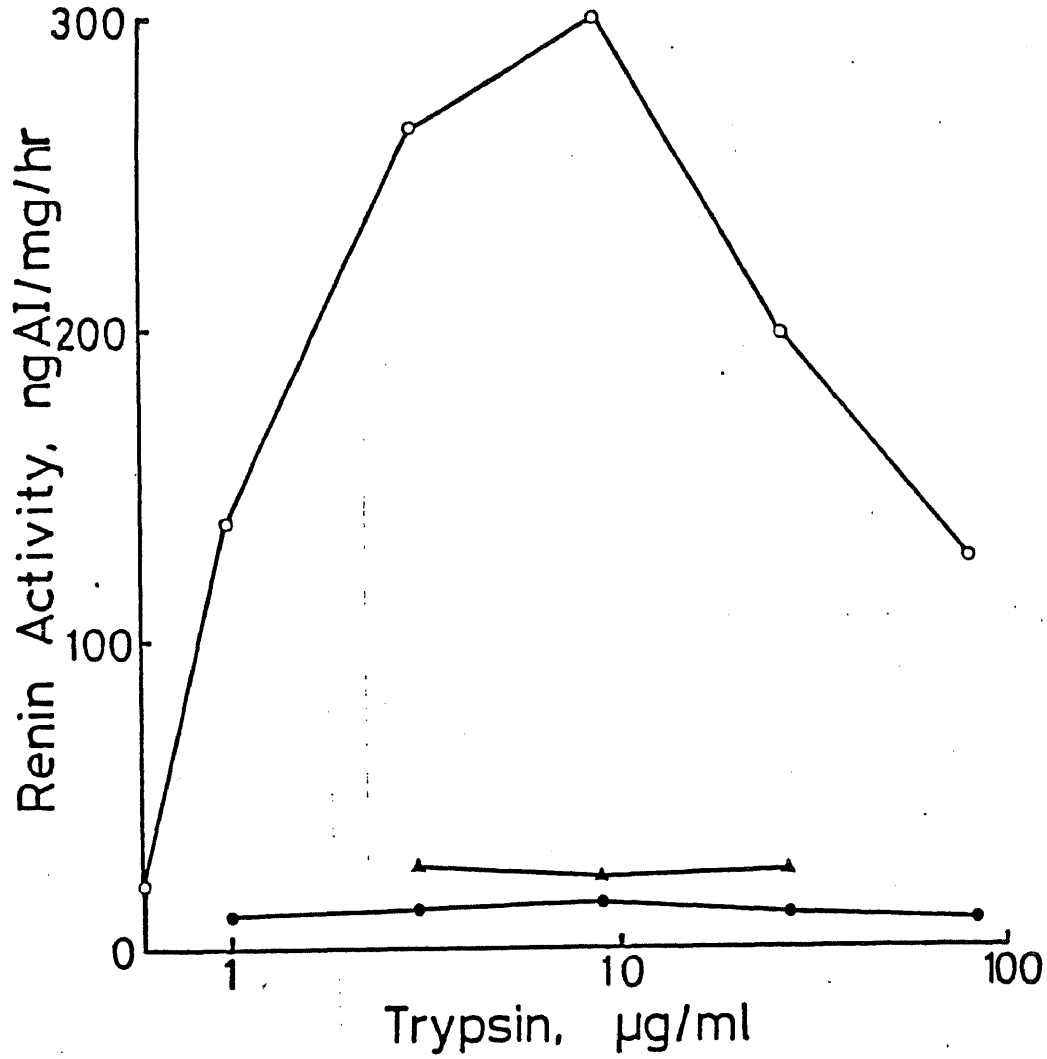


Figure 18. **Enzyme activity of bacterially produced prorenin after trypsin treatment and its inhibition by anti-renin antibody and pepstatin.** A 1-µg sample of the hybrid protein renatured at the optimal condition (Fig. 16), was digested at 4 °C with appropriate quantity of trypsin in a reaction mixture (100 µl) containing 20 mM Tris-HCl, pH 8.0, and 0.1 % BSA. After 10 min, the digestion was terminated with 10 µl of soybean trypsin inhibitor (20 mg/ml). Renin activity (o—o) was determined as previously described(85). Inhibition of trypsin-activated renin activity by rabbit anti-human renin antiserum diluted to 1:100 (●—●) and 10 µM pepstatin (▲—▲) was measured according to the standard methods(86-88).

DISCUSSION

Human prorenin was synthesized with 11 extra amino acids in its amino terminus in Escherichia coli carrying the pTR501. The level of expression amounted to as much as 30 % of cellular protein.

However, one problem remainded; the product was not soluble without strong denaturing agents such as urea and guanidine hydrochloride. Recently, it has been clarified that a large quantity of heterologous proteins synthesized in E. coli frequently precipitated inside the cell as inclusion bodies. The inclusion body, also called "crystalline protein" or "protein encased in stainless steel balls", was observed in many E. coli cells in which a large amount of mammalian proteins was produced by genetic engineering(91). The mechanisms of formation of such large aggregates are unknown; cross-linking by inappropriate disulfide bonds is suspected in some cases, but, at least in the present case, it does not seem responsible since SDS-polyacrylamide gel electrophoresis under nonreducing conditions revealed no oligomeric forms of prorenin. Although an advantage is that the aggregated proteins are protected from rapid proteolytic degradation in E. coli, accumulation of the plasmid-encoded protein in a sedimentary form poses solubilization and renaturation problems.

The insoluble protein was dissolved in 6 M guanidine-

HCl, and then dialyzed against 8 M urea, a more weakly denaturing medium. To renature the unfolded prorenin, urea was removed under conditions for protein folding. However, it is very difficult to estimate the refolding efficiency of the denatured prorenin because there is neither pure standard preparations of prorenin nor the human renin without carbohydrates residues which may effect its specific activity. Moreover, the amino terminal of authentic human prorenin has not been experimentally determined(58,60), and the E. coli-produced prorenin might differ slightly from authentic prorenin(60).

So, if it assumed that the bacterially produced-prorenin was properly activated by trypsin and its maximum specific activity was 20 mg Angiotensin I/ mg protein/hr(6), the highest specific activity of pure human kidney renin containing carbohydrate residue, the procedure employed here resulted in only a small percentage(<1.0 %) of recovery of renin activity. The 1% recovery of renin activity means only 1 % of the molecule is 100 % active or that 100 % of the molecule has only 1 % activity. To clarify this point, the activated prorenin preparation was applied on pepstatin-aminohexylagarose (92) which could only absorb active renin. Most proteins of the activated prorenin preparation passed through the pepstatin column and the very small percentage of the protein containing most of

renin activity was absorbed and eluted from the column by 0.15M Tris buffer, pH 7.5 as observed in authentic human kidney renin(6). This result indicates that the very small portion of the renatured prorenin molecule has the same tertiary structure as authentic prorenin, but the most of the other molecule is a denatured product although it has the same primary structure as authentic prorenin. This is most probably due to the difficult process in refolding of the denatured prorenin. This problem is common in almost all genetically engineered mammalian protein in E. coli(91). Therefore alternative expression system, such as extracellular secretion systems, may be developed for production of human renin in the future.

Although the renaturation problem remains to be solved in future, the E. coli-produced, guanidine-HCl-solubilized and renatured prorenin can be used: 1) as a powerful tool for studying the detailed mechanisms of activation; 2) as substrate for searching as-yet-unidentified activator(s) of prorenin; 3) to examine whether prorenin can be activated by kallikrein(49,50,54) or renin itself(93,94) as already suggested; and 4) to raise prorenin-specific monoclonal antibodies for diagnostic purposes. Because its properties closely resemble those reported for naturally occurring prorenin(49,55,95) in: i) molecular weight(Fig. 17); ii) activatability with trypsin(Fig. 18); iii) cross-

reactivities with an anti-human renin antibody (Fig. 13); iv) inhibition of their trypsin action with the prosequence-specific anti-Pro 3 antibody; and v) inhibition of trypsin-activated renin activity with pepstatin or anti-mature human renin antibody(Fig. 18).

Most of the previous studies concerning prorenin have been carried out with whole plasma or crude kidney extracts. Such complex systems render the interpretations of the experimental results tentative. It is expected that high-level expression of human prorenin in E. coli achieved in the present study will bring a breakthrough in the studies of prorenin which have been hampered by its extremely low concentration both in the kidney and in the plasma, and unravel the physiological and pathological significance of circulating prorenin.

Chapter IV. Conclusion

The renin-angiotensin system plays an important role in the regulation of blood pressure and electrolyte balance. Activation of the system is initiated by the release of the enzyme renin from the kidney into the bloodstream, where it acts on its substrate to produce angiotensin I. Although renal renins were isolated from many sources including human kidney, their extremely low concentration in kidneys prevented their purification in amounts to determine the complete amino acid sequence and to clarify the nature of their catalytic mechanisms and processing. To circumvent these problem, attempts were made (i)to obtain full-length cDNA clones for human renin from poly $(A)^+$RNA extracted from kidney, and (ii)to produce human prorenin in Escherichia coli in quantities sufficient for its biochemical characterization and clarification of the activation mechanism.

Molecular Cloning and Sequence Analysis of cDNA for Human Renin Precursor

The primary structure of human renin precursor was deduced from its cDNA sequence. The predicted sequence consists of 406 amino acids (Mr = 45,057) with a pre- and a prosegment carrying 20 and 46 amino acids, respectively. The molecular weight of mature human renin was calculated at a 37,236 without carbohydrate residues. A high degree of sequence homology, especially in the catalytically important region such as Phe-Asp-Thr-Gly-

Ser (residues 37-41) and Val-Asp-Thr-Gly (residues 225-228), was found upon comparison of the mouse and human renins. An overall homology, including presequence between the two renins, is 68.7 %. The major structural difference distinguishing the two renins was the presence of the two possible glycosylation sites in human kidney renin, which was not observed in mouse submandibular gland renin. Close similarities were also observed in the primary structure of renins and other aspartyl proteinases with defined three-dimensional structure, suggesting a tertiary structure for renin that is similar to the other enzymes.

Expression of Human Prorenin Gene in Escherichia coli

The DNA sequence encoding human prorenin was placed under the control of the Escherichia coli trp promoter-operator in the expression plasmid pTR501. E. coli cells containing pTR501 synthesized high levels (30 % of total cell protein) of prorenin as part of a hybrid protein with the trp E gene product and the unique recognition sequence of blood coagulation factor Xa, Ile-Glu-Gly-Arg. The essential feature of this construction is that the complete prorenin sequence can be excised from the fusion product by using factor Xa. The expected fusion protein was obtained, purified, and cleaved with the factor Xa. Human prorenin thus obtained showed close similarities to naturally occurring prorenin in its biochemical and immunochemical properties such as: i) molecular

weight without carbohydrate residues; ii) activatability with trypsin; iii) cross-reactivities with an anti-human renin antibody; iv) inhibition of their trypsin action with the prosequence-specific anti-Pro 3 antibody; and v) inhibition of trypsin activated renin activity with pepstatin or anti-mature human renin antibody.

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REFERENCES

1. Oparil, S. and Haber, E. (1974) N. Engl. J. Med. 291, 389 - 401.
2. Peach, M. J. (1977) Physiol. Rev. 57, 313 - 370.
3. Yokosawa, H., Holladay, L.A., Inagami, T., Hass, E. and Murakami, K. (1980) J. Biol. Chem. 255, 3498 - 3502.
4. Galen, F.-X., Devaux, C., Guyenne, T., Menard, J. and Corvol, P. (1979) J. Biol. Chem. 254, 4848 - 4855.
5. Slater, E. E. and Strout, V. Jr. (1981) J. Biol. Chem. 256, 8164 - 8171.
6. Higaki, J., Hirose, S., Ogihara, T., Imai, N., Kisaragi, M., Murakami, K. and Kumahara, Y. (1983) Life Sci. 32, 1591 - 1598.
7. Fasciolo, J. C., Leloir, L. F., Munoz, J. M. and Braun-Menendez, E. (1940) Science 92, 554 - 555.
8. Misono, K. S., Chag, J.-J. and Inagami, T. (1982) Proc. Natl. Acad. Sci. USA 79, 4858 - 4862.
9. Panthier, J. J., Foote, S., Chambreud, B., Strosberg, A. D., Corvol, P. and Rougeon, F. (1982) Nature 298, 90 - 92.
10. Bing, J., Poulsen, K. Hackenthal, E., Rix, E. and Taugner, R. (1980) J. Histochem. Cytochem. 28, 874 - 880.
11. Panthier, J. J. and Rougeon, F. (1983) EMBO J. 2, 675 - 678.

12. Inagami, T. (1981) In Soffer, R. L. ed., Biochemical regulation of blood pressure John Wiley and Sons, New York.

13. Inagami, T., Chang, J.-J., Dykes, C. W., Takii, Y., Kisaragi, M. and Misono, K.S. (1983) Fed. Proc. 42, 2729 - 2734.

14. Efstratiadis, A. and Villa-Komaroff, K. (1979) In Stelow, J. K. and Hollaender, A. eds., Genetic engineering, vol. 1. Plenum Press, New York.

15. Robbitts, T. H. (1976) Nature 260, 221 - 225.

16. Rougeon, F., Kourilsky, P. and Mach, B. (1975) Nucleic Acids Res. 2, 2365 - 2378.

17. Wood, K. O. and Lee, J. C. (1976) Nucleic Acids Res. 3, 1961 - 1971.

18. Okayama, H. and Berg, P. (1982) Mol. Cell. Biol. 2, 161 - 170.

19. Masuda, T., Imai, T., Fukushi, T., Sudoh, M., Hirose, S. and Murakami, K. (1982) Biomed, Res. 6, 541 - 545.

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

21. Aviv, H. and Leder, P. (1982) Proc. Natl. Acad. Sci. USA 69, 1408 - 1412.

22. Fukushi, T., Masuda, T., Imai, T., Susoh, M., Kimura, S., Hirose, S. and Murakami, K. (1982) Biomed. Res. 3, 534 - 540.

23. Martial, J. A., Baxter, J. D., Goodman, H. M. and

Seeburg, P. H. (1977) Proc. Natl. Acad. Sci. USA 74, 1816 - 1820.

24. Mukai, M., Torikata, C., Hirose, S., Murakami, K. and Kageyama, K. (1984) Lab. Invest. 51, 425 - 428.
25. Lammli, V. K. (1970) Nature 227, 680 - 685.
26. Chamberlain, J. P. (1979) Anal. Biochem. 98, 132 - 135.
27. McDonnell, M. W., Simon, M. N. and Studier, F. W. (1977) J. Mol. Biol. 110, 119 - 146.
28. Boyer, H. W. and Roulland-dussoix, D. (1969) J. Mol. Biol. 41, 459 - 472.
29. Morrison, D. A. (1979) Methods Enzymol. 68, 326 - 331.
30. Birnboim, H. C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513 - 1523.
31. Davis, R. W., Botstein, D. and Roth, J. R. eds. (1980) In Advanced bacterial genetics. Cold Spring Harbor Laboratory, New York.
32. Maxam, A. M. and Gilbert, W. (1980) Methods Enzymol. 65, 499 - 560.
33. Maniatis, T., Fritsh, E. F. and Sambrook, J. eds. (1982) In Molecular cloning p. 149 - 186 Cold Spring Harbor Laboratory, New York.
34. Maizel, J. R. and Lenk, R. P. (1981) Proc. Natl. Acad. Sci USA 78, 7665 - 7669.
35. Needleman, S.-B. and Wnnsch, C. D. (1970) J. Mol. Biol. 48, 443 - 453.
36. McMaster, G. K. and Carmichael, G. G. (1977) Proc.

Natl. Acad. Sci. USA 74, 4835 - 4838.

37. Alwine, J. C., Kemp, D. J. and Stark, G. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5350 - 5354.

38. Kozac, M. (1981) Nucleic Acids Res. 9, 5233 - 5252.

39. Proudfoot, N. J. and Brownlee, G. G. (1976) Nature 263, 211 - 214.

40. Wain-Hobson, S., Nussinov, R., Brown, R. J. and Sussman, J. L. (1981) Gene 13, 355 - 364.

41. Takahashi, T. and Tang, J. (1983) J. Biol. Chem. 258, 6435 - 6443.

42. Takahashi, T., Schmidt, P. G. and Tang, J. (1983) J. Biol. Chem. 258, 2819 - 2830.

43. Takahashi, T., Schmidt, P. G. and Tang, J. (1983) In Liu, M. ed., Frontiers in biochemical studies of proteins. Elsvier Publishing Company, New York.

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

45. Andreeva, N. S. and Gustchina, A. E. (1979) Biochem. Biophys. Res. Commun. 87, 32 - 42.

46. James, M.N.G., Sielecki, A., Salituro, F., Rich, D. H. and Hoffman, T. (1982) Proc. Natl. Acad. Sci USA 79, 6137 - 6141.

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

48. Overturf, M. L., Druilhet, R. E. and Kirkendall, W. M. (1979) Life Sci. 24, 1913 - 1924.

49. Sealey, J. E., Atlas, S. A. and Laragh, J. H. (1980)

Endocrine Rev. 1, 365 - 391.

50. Inagami, T. and Murakami, K. (1980) Biomed. Res. 1, 456 - 475.

51. Leckie, B. J. (1981) Clin. Sci. 60, 119 - 130.

52. Hirose, S., Ohsawa, T., Murakami, K. and Inagami, T. (1982) Exp. Brain Res. Suppl. 4, 53 - 63.

53. Inagami, T. (1982) Neuroendocrinology 35, 475 - 482.

54. Sealey, J. E., Atlas, S. A. and Laragh, J. H. (1983) Fed. Proc. 42, 2681 - 2689.

55. Hsueh, W. A. (1984) Am. J. Physiol. 247, F205 - F212.

56. Kim, S. J., Hirose, S., Miyazaki, H., Ueno, N., Higashimori, K., Morinaga, S., Kimura, T., Sakakibara, S. and Murakami, K. (1985) Biochem. Biopys. Res. Commun. 126, 641 - 645.

57. Bouhnik, J., Fehrentz, J. A., Galen, F. X., Seyer, R., Evin, G., Castron, B., Menard, J. and Corvol, P. (1985) J. Clin. Endocrinol. Metab. 60, 399 - 401.

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

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

60. Hobart, P. M., Fogliano, M., O'Connor, B. A., Schaefer, J. M. and Chirgwin, J. M. (1984) Proc. Natl. Acad. Sci. USA 81, 5026 - 5030.

61. Maniatis, T., Fritsh, E. F. and Sambrook, J. eds. (1982) In Molecular cloning p.403 - 433. Cold Spring harbor Laboratory, New York.

62. Ikeda, Y. and Beppu, T. eds (1983) In Genetics of industrial microorganisms. Kodansha, Tokyo.

63. Roberts, T. M. and Lauer, G.D. (1979) Methods Enzymol. 68, 473 - 482.

64. Bakman, K. (1976) Proc. Natl. Acad. Sci. USA 73, 4174 - 4178.

65. Bakman, K. (1978) Cell 13, 65 - 71.

66. Shine, J. and Dalgano, L. (1975) Nature 254, 34 - 38.

67. Steitz, J. A. (1979) In Goldberger, R. F. ed., Biological regulation and development, vol. 1, p.349 Plenum Press, New York.

68. Roberts, T. M., Kacich, R. and Ptashe, M. (1979) Proc. Natl. Acad. Sci. USA 76, 760 - 764.

69. Roberts, T. M., Bikel, I., Yocum, R. R., Livingston, D. M. and Ptashne, M. (1979) Proc. Natl. Acad. Sci. USA 76, 5596 - 5600.

70. Guarente, L., Lauer, G., Roberts, T. M. and Ptashne, M. (1980) Cell 20, 543 - 553.

71. Taniguchi, T. and Weissman, C. (1978) J. Mol. Biol. 118, 533 - 565.

72. Goeddel, D. V., Heyneker, H. L., Hozumi, T., Arentzen, R.,Itakura, K., Yansura, D. G., Ross, M. J., Miozzari, G., Crea, R. and Seeburg, P. H. (1979) Nature 281, 544 - 549.

73. Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K. and Riggs, A. (1979) Proc. Natl. Acad. Sci. USA 76, 106 - 110.

74. Edman, J. C., Hallewell, R. A., Valenzuela, P., Goodman, H. M. and Rutter, W. J. (1981) Nature, 291, 503 - 506.

75. Davis, A. R., Nayak, D. P., Ueda, M., Hiti, A. 1., Dowbenko, D. and Kleid, D. G. (1981) Proc. Natl. Acad. Sci. USA 78, 5376 - 5380.

76. Nagai, K. and Thogersen, H. C. (1984) Nature 309, 810 - 812.

77. Murakami, K., Imai, T. and Hirose, S. (1985) Hypertension 7, 837.

78. Hashimoto, N., Morita, T. and Iwanaga, S. (1985) J. Biochem. 97, 1347 - 1355.

79. Masuda, T. (1984) Cloning of cDNA for mouse submandibular gland renin and its expression in E. coli. Ph. D. thesis, Tokyo University, Japan.

80. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5763 - 5467.

81. Messing, J. (1983) Methods Enzymol. 101, 20 - 78.

82. Clarke, L., Hitzeman, R. and Carbon, J. (1979) Methods Enzymol. 68, 436 - 442.

83. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350 - 4354.

84. Kawaguchi, Y., Shimizu, N., Nishimori, K., Uozumi, T. and Beppu, T. (1984) J. Biotech. 1, 307 - 315.

85. Murakami, K., Takahashi, S., Suzuki, F., Hirose, S. and Inagami, T. (1980) Biomed. Res. 1, 392 - 399.

86. Hirose, S., Yokosawa, H., Inagami, T. and Workman, R. J. (1980) Brain Res. 191, 489 - 499.

87. Mukai, M., Iri, H., Nakajima, T., Hirose, S., Torikata, C., Kageyama, K., Ueno, N. and Murakami, K. (1983) Amer. J. Surg. Pathol. 7, 679 - 690.

88. Hirose, S., Naruse, M., Ohtsuki, K. and Inagami, T. (1981) J. Biol. Chem. 256, 5572 - 5576.

89. Cornall, A. G., Bardawill, C. S. and David, M. M. (1948) J. Biol. Chem. 177, 751 - 766.

90. Magnusson, S., Petersen, T. E., Sottrup-Jensen, L. and Claeys, H. (1975) In Reich, E., Rifkin, D. and Shaw, E. eds., Protease and biological control. p, 123 - 149. Cold Spring Harbor Laboratory, New York.

91. Uren, J. (1985) Intl. Biotech. Lab. April, 26 - 31.

92. Inagami, T. and Murakami, K. (1977) J. Biol. Chem. 252, 2978 - 2983.

93. Inagaki, T., Ohtsuli, K. and Inagami, T. (1983) J. Biol. Chem. 258, 7476 - 7480.

94. Hiwada, K., Imamura, Y., Sogo, Y., Kokubu, T. and Murakami, K. (1982) Biomed. Res. 3, 693 - 694.

95. Atlas, S. A., Hesson, T. E., Sealey, J. E., Dharmgrongartama, B., Laragh, J. H., Ruddy, M. C. and Aurell, M.(1984) J. Clin. Invest. 73, 437 - 447.