 Human Molecular Genetics, 1997, Vol. 6, No. 9 **1527–1534**

Human renin binding protein: complete genomic sequence and association of an intronic T/C polymorphism with the prorenin level in males

Antje Knöll[1,3], Heribert Schunkert[2], Kathrin Reichwald[3], A.H. Jan Danser[4], David Bauer[3], Matthias Platzer[3], Günter Stein[5] and André Rosenthal[3,*]

Departments of [1]Pathology and [2]Internal Medicine, University of Regensburg, D-93042 Regensburg, Germany, [3]Department of Genome Analysis, Institute of Molecular Biotechnology, Beutenbergstrasse 11, D-07745 Jena, Germany, [4]Department of Pharmacology, Erasmus University, 3015 GE Rotterdam, The Netherlands and [5]Department of Internal Medicine, University of Jena, D-07740 Jena, Germany

Recieved April 7, 1997; Revised and Accepted June 3, 1997

The role of renin binding protein (RnBP) in human (patho)physiology, despite its biochemical characterization, is as yet unclear. RnBP has been shown to bind and inactivate renin, a key player of the blood pressure regulating renin–angiotensin system. This renders the *RnBP* gene a promising candidate gene in human hypertension. Herein, a molecular genetic approach was employed to investigate if RnBP might affect renin, prorenin and/or blood pressure levels. Sequencing of the human Xq28 chromosomal region provided the precise chromosomal location and full genomic sequence of the *RnBP* gene. All 11 exons, adjacent intronic splice sites and the promoter region were sequenced in 20 patients with essential hypertension of early onset and possible X-linked inheritance and in four normotensive individuals. The only variant found was a single base exchange polymorphism 61 base pairs upstream of the intron 6/exon 7 boundary (T61C). Several cardiovascular parameters, the renin, and prorenin levels and the T61C allele status were determined in 505 Caucasian individuals. Male individuals without medication who were hemizygous for the C allele were characterized by lower prorenin levels (196 ± 15 versus 256 ± 12 mU/l, *P* = 0.05) and a significantly higher renin/prorenin ratio (10.7 ± 1.5 versus 7.7 ± 0.3%, *P* = 0.002), whereas no variations in circulating renin, blood pressure, heart rate and left ventricular mass index were associated with the C allele. No significant association was observed in women. The data do not exclude a role of RnBP in essential hypertension. The complete genomic structure of the *RnBP* gene, including the identified repetitive sequence elements, provides an essential tool for further studies of the *RnBP* gene in hypertensive patients with a different genetic background.

INTRODUCTION

Hypertension is one of the most common multifactorial diseases, affecting 15–20% of the human population (1), and represents a complex trait which may encompass multiple syndromes with both hereditary and environmental determinants (reviewed in 2,3). Based on the results of twin studies, adoption studies and statistical analyses of blood pressure in various pedigrees, it has been estimated that 20–60% of the population variability in blood pressure is genetically determined (4). Blood pressure values are distributed unimodally in the general population, suggesting that several genes are involved in the development of essential hypertension, i.e. hypertension without obvious cause. The identification of these genes is a challenge that is met by the advances of molecular genetics. One approach is the identification and testing of candidate genes which, based on their physiological actions, may contribute to a specific phenotype. Investigation of the genes that constitute the renin–angiotensin system appeared to be particularly promising, because of the central role of this system in the regulation of blood pressure. An association between molecular variants of the angiotensinogen gene and hypertension was reported initially in 1992 (5) and subsequently has been corroborated by a number of investigations (6,7). In addition, a variant of the angiotensin converting enzyme gene was found to be over-represented in patients with myocardial infarction (8–10) and to increase the risk of premature death in families with hypertension (11), albeit a direct effect on blood pressure was not observed (12–14). With regard to renin, most studies reported a lack of genetic linkage between intragenic restriction length polymorphisms (RFLPs) and arterial hypertension (15–17).

Renin binding protein (RnBP) might be an important regulator within the renin–angiotensin system (18). RnBP was first isolated and subsequently cloned by Takahashi *et al*. (19). Recently, RnBP has been characterized in pigs as an *N*-acyl-D-glucosamine

*To whom correspondence should be addressed. Tel: +49 3641 656 241; Fax: +49 3641 656 255; Email: arosenth@imb-jena.de

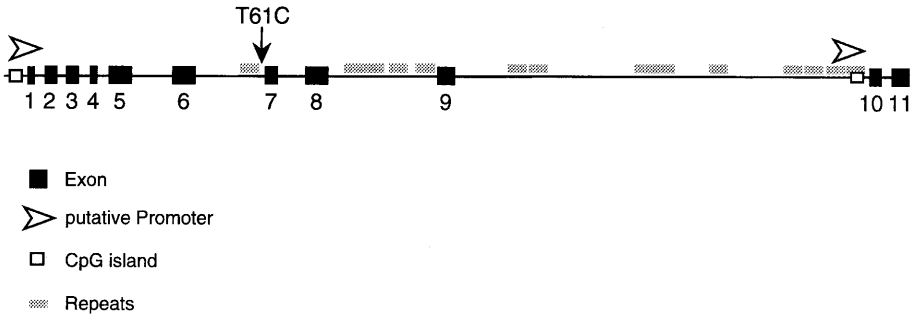

Figure 1. Genomic structure of the *RnBP* gene.

2-epimerase, an enzyme involved in the synthesis of *N*-acetylneuraminic acid (20). Recombinant RnBP injected into the circulation inactivates renin, a crucial component of the renin–angiotensin system. Sequestration of circulating renin by exogenous RnBP also results in a substantial drop in blood pressure. However, RnBP is an intracellular protein and is not detectable in plasma *in vivo*. Thus, in contrast to its documented biochemical effects, at present it is unclear whether RnBP is involved in *in vivo* regulation of renin levels. Such an interaction, either within the tissue or in the circulation, might have the potential to affect blood pressure levels and thus the manifestation of arterial hypertension. Therefore, RnBP is a promising candidate gene for hypertension.

Table 1. Genomic structure of the *RnBP* gene

Exon/intron no.	Exon length (bp)	Exon position[a] From	To	Intron length (bp)
1	23	–191	–169	165
2	110	–3	107	153
3	76	261	336	109
4	76	446	521	176
5	173	698	870	466
6	225	1337	1561	823
7	82	2385	2466	296
8	176	2763	2938	1244
9	132	4183	4314	4525
10	88	8840	8927	84
11	135	9012	9146	

[a]Position 1 refers to the ATG start codon of the *RnBP* gene (corresponding to position 97 351 in database entry U52112).

Although the *RnBP* cDNA and the exon/intron boundaries have been published before (19), the complete sequences have not yet been established for all introns. The *RnBP* gene is located in the q28 region of the human X chromosome proximal to the host cell factor 1 (HCF1) gene (21–25). Given this location, we hypothesized that the *RnBP* gene might be associated with arterial hypertension, since epidemiological surveys revealed that in males hypertension occurs more frequently and at an earlier age (26,27). While sequencing the Xq28 region on a large scale basis, we determined the complete sequence of the gene and its genomic structure. Based on the intronic sequences we designed primers and sequenced all *RnBP* exons and flanking intronic regions in 20 patients selected for early onset essential hypertension and possible X-linked inheritance and in four normotensive individuals. We identified one common polymorphism in intron 6 of the *RnBP* gene. This variant was tested for association with blood pressure and renin and prorenin levels, as well as other cardiovascular parameters, in 505 individuals.

RESULTS

Sequence of the human *RnBP* gene

During the course of a large scale sequencing project in the human Xq28 chromosomal region (Platzer *et al*., unpublished data), the complete genomic sequence of the *RnBP* gene was determined (accession no. U52112, positions 87 938–100 489). The *RnBP* gene is located on cosmid 12B2 between the genes *TE2* and *HCF1* (21–25). All three genes are transcribed in the same direction from telomer to centromer, with the *HCF1* gene ending 2948 bp telomeric and the TE2 gene starting 270 bp centromeric of the *RnBP* gene.

Table 2. Human repetitive sequence elements within the chromosomal region of the *RnBP* gene, identified by CENSOR (28)

No.	Repeat	Position From	To	Strand	Intron
1	Alu–Sg	–1320	–1017	+	5′-UTR[a]
2	Alu–Jb	–933	–664	+	5′-UTR
3	Alu–Sx	1880	2170	+	6
4	MER41	3072	3681	+	8
5	Alu–Sx	3269	3557	–	8
6	MER41	3685	3926	+	8
7	Alu–J	5031	5320	+	9
8	Alu–Sx	5323	5613	+	9
9	Alu–Sq	6386	6968	+	9
10	Alu–Jo	7213	7501	–	9
11	Alu–Y	7903	8110	–	9
12	Alu–S	8111	8190	–	9
13	Alu–Sx	8277	8568	–	9
14	MIR	8714	8778	+	9

[a]5′-UTR, 5′-untranslated region.

Table 3. Short tandem repeats within the *RnBP* gene

No.	Sequence	Position		Repeats (no.)	Identity (%)	Localisation
		From	To			
1	GGAAAAGGGCATGGCACCCAGT	5818	5880	3	100	Intron 9
2	TTTA	7172	7219	11	100	Intron 9
3	TTTG	8249	8280	9	100	Intron 9
4	CCCCGC	9076	9111	6	94	Exon 11
5	CGCCCCGCGCATTGGCC	9249	9316	4	81	3′-UTR[a]

[a]3′-UTR, 3′-untranslated region.

Table 4. Primers used for PCR, sequencing and RT-PCR

Region	Position (5′→3′)	Sequence	Application
Promotor	–640→–618	5′-ACA GAA CTT GCC TCC TGT CTA CC-3′	PCR, sequencing
	–338→–359	5′-TTG TTC CTG CCG CGA CCC TCC A-3′	PCR, sequencing
Exon 1	–442→–421	5′-GGC CAT CCC AGC TTT TCC CAC C-3′	PCR, sequencing
	–98→–119	5′-ACG GGG CTT CAG ACG TCA CCA T-3′	PCR, sequencing
Exon 2	–123→–102	5′-GGG GAT GGT GAC GTC TGA AGC C-3′	PCR, sequencing
	196→174	5′-GCC AGC AAA TCT GTG AGG TGC CT-3′	PCR, sequencing
Exon 3	162→184	5′-CTT CTC ACT CTC AGG CAC CTC AC-3′	PCR, sequencing
	433→411	5′-GGA GGG AAA GTG GCT TAA GTG GC-3′	PCR, sequencing
Exon 4	350→371	5′-AGC CTG GAA GCT CAG GCA CAC G-3′	PCR, sequencing
	619→596	5′-TTC GCT GCT TCT AGA TCC CTC TAG-3′	PCR, sequencing
Exon 5	616→637	5′-CGA AGG ACT TGG GGA GGC TCA A-3′	PCR, sequencing
	955→933	5′-GAC TTG CGT AGT GAG TGG GAA TG-3′	PCR, sequencing
Exon 6	1217→1240	5′-GGA TGA AGG TGT CTC TGG GTT CAG-3′	PCR
	1864→1841	5′-GTA ATC CTC ACT TCT GGC CTG ATC-3′	PCR
Exon 6	1265→1285	5′-AGG GCC GTG TCC AGG GGG ATG-3′	Sequencing
	1649→1630	5′-CTC TCC AGG TGT GTC GAG CC-3′	Sequencing
Exon 7	2196→2218	5′-CTA GGC CCA AGG GAG TGA ATC AG-3′	PCR
	2630→2608	5′-TGG AAG CCT GCT CCT CTG CAG AA-3′	PCR
Exon 7	2275→2297	5′-TTG GGT AAG AGG GAG GGA GGA AG-3′	Sequencing
	2583→2562	5′-CTC GCT CCT CTG CCT CCT CAA G-3′	Sequencing
Exon 8	2638→2660	5′-GGA GCT CAG AGC CTC GCT CAT TG-3′	PCR
	3051→3029	5′-AGG CCT CTA GGT TCC AAT CCA GC-3′	PCR
Exon 8	2687→2708	5′-GGG CTT TTG CTC CCT CTG TTC C-3′	Sequencing
	3021→3000	5′-TAC CCT CTA GAG AAG CAA CCA C-3′	Sequencing
Exon 9	4080→4102	5′-GCA ATT CAC CTC CAG TAG CTT GG-3′	PCR, sequencing
	4415→4394	5′-GCT GAG CGA CTG TGC TCA GCG A-3′	PCR, sequencing
Exon 10	8727→8748	5′-GGT TCT GGA GCC ACG TCA CCT G-3′	PCR, sequencing
	9024→9003	5′-ACG TGG AAA CAG CCT CCG GGT A-3′	PCR, sequencing
Exon 11	8889→8911	5′-GCA AGG TGG CCC TCT CCA TCA AG-3′	PCR
	9487→9465	5′-CAG CGG ATC GTG AAG GTG CAG TC-3′	PCR
Exon 11	8913→8935	5′-GAG GTC CTT TCA AAG GTG AGT GG-3′	Sequencing
Introns 6–7	2294→2316	5′-GAA GGG CTT TCC GGA GA[a]T GGG TC-3′	Polymorphism screening
	2529→2507	5′-CCA GAA CAC AAG GCT CAG CGC TC-3′	Polymorphism screening
Exons 5–8	831→853	5′-GAA CGA GCT GTG GAG AGC CAC AG-3′	RT-PCR
	2801→2779	5′-AAT GAC GGA GCA GAA ACC AGC CG-3′	RT-PCR

[a]At the seventh position from the 3′-end of this primer, a mismatch (A for C) has been introduced to abolish an *Alu*I restriction recognition sequence.

The RnBP gene is 9340 bp long and consists of 11 exons (Fig. 1, Table 1). The putative translation initiation site is at position 4 of the second exon. This position is referred to as +1 in the present publication.

Computer analysis of the 5′-untranslated region of the *RnBP* gene reveals a CpG island from position –512 to –232 (score 0.63, 70.5% GC, 17 CpG). One putative TATA-less promotor region was predicted with a transcription start site at position –298 (TSSW) or at position –285 (TSSG). The transcription start site has been experimentally determined before by Takahashi *et al*. (19) with a major start site at position –364 and two minor start sites at positions –319 and –312.

Table 5. Distribution of the T61C allele (intron 6 of the *RnBP* gene) in 505 Caucasians

	Males (n = 293)	Females (n = 212)
T	0.82	
C	0.18	
TT		0.66
TC		0.33
CC		0.01

There is one additional CpG island near the end of the long intron 9, extending from bp 8703 to 8858 (score 0.86, 60.9% GC, 12 CpG), and a putative promotor at position 8163 with a TATA box at position 8135 (TSSW and TSSG). This could represent the regulatory region of the *TE2* gene, which starts 559 bp 3′ of this CpG island.

We screened for common human repetitive sequence elements using the program CENSOR (28). The results are summarized in Table 2. Most of the repetitive elements are located within the large introns 8 and 9. There are two pairs of nearly perfectly inverted sequences, formed by Alu repeats 3 and 5 (85%) identity) and Alu repeats 5 and 8 (90% identity) respectively. In addition, we identified five short tandem repeats within the *RnBP* gene, as shown in Table 3.

Search for *RnBP* gene mutations by direct sequencing

We designed primers to amplify and sequence the promotor region, all 11 exons and flanking intronic sequences of the *RnBP* gene (Table 4). We selected 14 male and six female patients with familiar and/or early onset hypertension and four normotensive individuals (two males and two females) and sequenced these *RnBP* gene regions of putative functional significance.

In five patients (three males and two females) we identified one T→C sequence variation 61 bp 5′ of the intron 6/exon 7 boundary. This intronic polymorphism is referred to as T61C. Except for this variant, all patients and all normotensive control individuals revealed a sequence identical to the genomic sequence derived from cosmid 12B2.

Population screening for the T61C polymorphism and association study

The T61C base change in intron 6 of the *RnBP* gene creates a new *Alu*I restriction site which we used to determine the allele distribution in the general population. PCR-based *Alu*I restriction analysis (Fig. 2) was performed on 293 male and 212 female randomly selected Caucasian individuals (Table 5). The allele distribution was found to be in Hardy–Weinberg equilibrium (P = 0.2 for females, χ^2 test).

Anthropometric characteristics of this population have been reported before in detail (29). Table 6 displays cardiovascular and biochemical data according to the T61C allele status after exclusion of subjects treated with antihypertensive drugs. The number of subjects on antihypertensive medication was similar in the respective genotype groups (in men: 61C allele, 29.4%, 61T allele, 23.3%; in women: 61TT allele, 18.9%, 61CT allele, 14.7%; P not significant).

In men, no significant association was observed between the 61C allele and age, body mass index, blood pressure, heart rate or left ventricular mass index. Likewise, on average there was little difference between genotype groups with regard to circulating active renin (Table 6). However, prorenin levels were found to be higher in those men carrying the 61T allele (256 ± 12 versus 196 ± 15, $P < 0.05$), whereas the renin to prorenin ratio was higher in males with the 61C allele (10.7 ± 1.5 versus 7.7 ± 0.3%, P = 0.002). In women, before and after exclusion of those taking oestrogen replacement therapy, no significant differences were observed between respective genotype groups (data not shown).

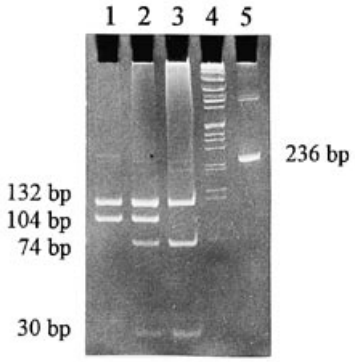

Figure 2. Polymorphism screening: *Alu*I restriction endonuclease digestion patterns after electrophoresis on 12% polyacrylamide and ethidium bromide staining. Lane 1, male individual hemizygous for the T allele; lane 2, female individual heterozygous for the C and T alleles; lane 3, male individual hemizygous for the C allele; lane 4, DNA size standard X (Boehringer); lane 5, undigested PCR product.

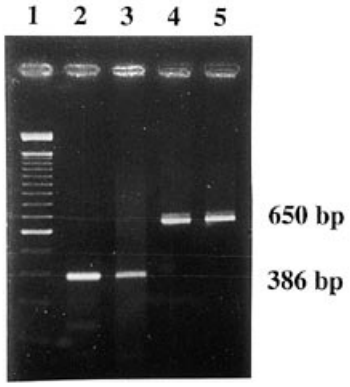

Figure 3. RT-PCR amplification of kidney *RnBP* RNA and β-actin RNA. Lane 1, DNA size standard; lane 2, RnBP amplification for male individual hemizygous for the T allele; lane 3, RnBP amplification for male individual hemizygous for the C allele; lane 4, β-actin amplification for male individual hemizygous for the T allele; lane 5, β-actin amplification for male individual hemizygous for the C allele.

RT-PCR

To investigate if the T61C polymorphism influences RNA splicing, RT-PCR was performed concurrently in two male subjects carrying the 61T and the 61C allele respectively (Fig. 3). Primers were designed to anneal to exon 5 and exon 8 sequences. An *RnBP* RNA transcript of the expected size (386 bp) was amplified from both individuals, indicating that the T61C polymorphism does not interfere with correct RNA splicing. RT-PCR for a β-actin gene transcript served as an internal control to indicate successful reverse transcription and amplification.

Table 6. Anthropometric and biochemical data of participants according to the T61C genotype, after exclusion of individuals taking antihypertensive drugs (n = 410)

Men	T (n = 182)	C (n = 35)	
Age (years)	57.5 ± 0.2	56.8 ± 0.5	
BMI (kg/m^2)	27.3 ± 0.2	26.7 ± 0.7	
Heart rate (per min)	70.4 ± 1.0	72.3 ± 3	
Systolic BP (mmHg)	146 ± 1	146 ± 3	
Diastolic BP (mmHg)	91 ± 0.7	89 ± 1.7	
LVMI (g/m^2)	107 ± 2	105 ± 6	
Renin (mU/L)	18.5 ± 0.9	19.7 ± 2.6	
Prorenin (mU/L)	256 ± 12	196 ± 15[a]	
Renin/prorenin (%)	7.7 ± 0.3	10.7 ± 1.5[b]	
Women	TT (n = 127)	TC (n = 65)	CC (n = 1)
Age (years)	58,5 ± 0.5	57.1 ± 0.5	55
BMI (kg/m^2)	26.8 ± 0.5	26.7 ± 0.6	23
Heart rate (per min)	71 ± 1.5	75 ± 1	69
Systolic BP (mmHg)	143 ± 2.4	143 ± 3.6	166
Diastolic BP (mmHg)	90 ± 1.5	87 ± 1.8	110
Renin (mU/L)	15.1 ± 1.1	15.5 ± 1.5	7.6
Prorenin (mU/L)	171 ± 11	181 ± 10	82
Renin/prorenin (%)	9.2± 0.5	8.5 ± .0.7	9.3

Values are expressed as mean ± SEM; BMI, body mass index; systolic and diastolic BP, systolic and diastolic blood pressure respectively; LVMI, left ventricular mass index.
[a]$P < 0.05$.
[b]$P = 0.002$.

DISCUSSION

Large scale genomic sequencing is an efficient tool to identify human genes and to determine their genomic structure, as well as their chromosomal location with respect to other genes. The complete genomic sequence is the ideal starting point for mutation screening of candidate genes and for the search for associations with human disease.

Direct genomic sequencing of the promoter region, the 11 exons and adjacent intronic regions of the *RnBP* gene in 20 hypertensive individuals disclosed no sequence changes except for one intronic single base exchange polymorphism well outside the known splice consensus sites. The lack of mutations within functionally important regions of the RnBP gene suggests that structural changes in RnBP are uncommon in hypertensive patients. The search for mutations was limited to 20 Caucasian individuals with essential hypertension. The selection for this screening focussed on those individuals with a positive family history compatible with X chromosomal inheritance and/or early onset of hypertension. Thus, we concentrated our search on patients with a fair chance of displaying alterations in an X chromosomal gene involved in blood pressure regulation. Nevertheless, we may have missed mutations that may occur only in a subset of patients (e.g. mild hypertension in older patients) or in other ethnic groups.

Although the detection of heterozygous sequence signals in female individuals can be complicated with fluorescent dye terminator sequencing, it seems highly unlikely that sequence changes have been missed because both strands were sequenced at least once in both directions. However, sequence variants within 5′ or 3′ regulatory regions cannot be excluded by our studies.

The identification of a common polymorphism within intron 6 provided the basis for a large population-based study to further investigate the role of the *RnBP* gene in hypertension and to possibly detect other phenotypes associated with this gene locus. In contrast to studies investigating dichotomous traits (i.e. hypertensive versus normotensive populations), this approach covers a wide spectrum of blood pressure levels and allows inclusion of other intermediate phenotypes, i.e. enzyme activities. Thus, this protocol may be much more sensitive in uncovering potential effects of RnBP, provided that the genetic marker is associated with a structural or quantitative alteration of a protein that affects these phenotypes.

Interestingly, we observed that men carrying the 61T allele displayed higher circulating prorenin levels, with no changes in active renin or blood pressure levels. An association study such as this falls short of elucidating the mechanism of this observation. However, given the known biochemical effects of the protein one may propose a hypothesis that may explain the lower level of prorenin in men carrying the 61C allele. First, circulating renin levels are known to be under tight control, including a negative feedback loop that down-regulates expression of the renin gene via angiotensin II. Second, RnBP has not yet been detected in serum, so that the protein may not alter circulating renin levels. However, men with the 61C allele may

express less RnBP and therefore display less renin sequestration within the renin-producing juxtaglomerular cells. Via a negative feedback loop, this may relate to a reduction in renin gene expression and constitutively secreted prorenin levels. These data are in good agreement with the proposed biochemical action of RnBP. The level of significance for the observed association between the renin/prorenin ratio and the 61C/T allele status in men ($P = 0.002$) makes a type 1 mistake unlikely. Taken together, RnBP may indeed participate in renin processing within juxtaglomerular cells. We were, however, unable to obtain direct evidence that RnBP may be involved in the regulation of circulating renin and, thus, the pathophysiology of essential hypertension.

From a molecular genetic point of view, one possible explanation for the observed association is that the intron 6 polymorphism is in linkage disequilibrium with functionally important sequence variants adjacent to the investigated *RnPB* gene regions. Differential splicing related to the intronic polymorphism as a potential mechanism for altered levels of RnBP was excluded in this study. Therefore, the association between T61C allele status of the *RnBP* gene and the renin/prorenin ratio in men should be corroborated by analyses of renal *RnBP* mRNA levels and replicated in other populations before definitive conclusions can be derived.

Since only one woman was found to be homozygous for the 61C allele, no statement can be made about an association between the *RnBP* gene and cardiovascular parameters in women.

To our knowledge, the present investigation is the first on the role of the *RnBP* gene in hypertension. Although we observed no association between the *RnBP* gene and blood pressure, we point out that these results are not definitive and additional data should be obtained in populations with a different genetic background. The complete sequence and precise localization of the *RnBP* gene, provided in this study, as well as the identification of an intronic polymorphism and short tandem repeats which might prove to be polymorphic, will facilitate further investigations on the molecular genetics and pathophysiological significance of the *RnBP* gene.

MATERIAL AND METHODS

Cosmid sequencing

The cosmid 12B2 was prepared and sequenced as previously described by Craxton (30). In brief, after sonication of the DNA, a 0.8–1.4 kb fraction was subcloned into the *Sma*I site of M13mp18. M13 templates were prepared with magnetic bead technology and sequenced using dye primer chemistry. The raw data were collected using ABI 373 A automated sequencers and assembled with the XBAP program (31). Gaps were closed with custom primers on M13 templates, PCR products or cosmid DNA in combination with Taq dye terminator chemistry (Perkin Elmer) or internal labelling (Pharmacia).

Computer analysis

Homology searches against the EMBL database were performed with BLAST v.1.4 (32), and FASTA v.2.0 (33). The gene prediction programs GRAIL (34) and XPOUND (35) were utilized and sequence alignments used the Global Alignment Program (GAP) (36). For promotor prediction the computer program Transcription Start Site, using both the Ghosh/Prestridge (TSSG) and Wingender (TSSW) motif databases (V.V.Solovyev, A.A.Salamov and C.B.Lawrence, personal communication), was applied. Human repetitive elements were identified with CENSOR (28).

Search for RnBP mutations by genomic sequencing

DNA was isolated from EDTA/blood using the Quiamp Kit (Qiagen).

PCR. Intronic PCR primers were designed (Table 4) to amplify the promoter region and each exon, including the adjacent intronic sequences. PCR amplification was performed using the following conditions (except exons 8 and 11): 300 ng DNA, 250 μM each dNTP, 0.5 μM each primer, 1.5 (promoter and exons 2, 5, 6 and 7), 2.125 (exons 1 and 3), 2.25 (exon 10) or 2.75 mM (exons 4 and 9) $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, and 1.25 U Taq polymerase in a total volume of 50 μl. To increase effectivness, promoter + exons 1 + 2 and exons 3 + 4 + 5 could also be amplified as continguous segments, using $MgCl_2$ concentrations of 3.375 and 4 mM respectively. After an initial denaturation step of 1 min at 95°C, each of 35 cycles consisted of 1 min at 95°C, 2 min at 65°C and 3 min at 72°C.

Exons 8 and 11 were amplified using 300 ng DNA, 350 μM dATP, 350 μM dCTP, 350 μM dTTP, 250 μM dGTP, 300 μM dITP, 0.5 mM each primer, 4 mM $MgCl_2$, 0.02% NP-40, 0.02% Tween 20, 42 μM 2-mercaptoethanol, 16.2 mM Tris–HCl, pH 9.5, and 16 U Thermosequenase (Amersham Life Science Inc.) in a total volume of 50 μl and the following cycling conditions: 1 min at 98°C, 2 min at 68°C and 3 min at 72°C for 35 cycles.

Precipitation with PEG. Polyethylene glycol (52.4 g PEG 8000, 40 ml 3 M NaOAc, pH 5.2, 1.32 ml 1 M $MgCl_2$ in a total volume of 200 ml) was used to remove unincorporated dNTPs and primers from the PCR product. The concentration of the recovered PCR product was estimated by ethidium bromide staining after agarose gel electrophoresis.

Cycle sequencing. This was performed for the promoter and each exon in both directions with either the PCR primers or internal primers (Table 4) using 30 ng PCR product and the Taq Dye Terminator protocol (ABI, Perkin-Elmer) on an ABI 373 A automated sequencer. The sequences obtained from each patient were compared with the genomic sequence derived from cosmid 12B2 using DNAstar software (DNAstar, Wisconsin).

Screening by *Alu*I restriction

PCR primers were designed to amplify a 236 bp fragment spanning the polymorphic site, which creates a novel *Alu*I recognition sequence (AG<u>T</u>T→AG<u>C</u>T). An *Alu*I recognition site only 15 bp away from the polymorphism was abolished by the introduction of a mismatch into the forward primer at the seventh position from its 3′-end. An additional *Alu*I recognition site within the PCR fragment served as an internal control for successful digestion into 132 and 104 bp fragments. PCR was performed with 300 ng DNA isolated from blood leukocytes, 250 μM each dNTP, 0.5 μM each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, and 0.5 U Taq polymerase in a total volume of 20 μl. After an initial denaturation step of 4 min at 94°C, 35 cycles consisted of 1 min at 95°C, 2 min at 68°C and 3 min at 72°C.

Aliquots of 10 µl PCR product were then incubated with 2 U *Alu*I restriction enzyme (Boehringer Mannheim) in the buffer recommended by the manufacturer at 37°C for 2 h. The restriction products were subjected to electrophoresis on a 12% polyacrylamide minigel and stained with ethidium bromide. The T allele was detected by the presence of 132 and 104 bp fragments, the C allele by the presence of 132, 74 and 30 bp fragments (Fig. 2).

RT-PCR

DNA and RNA were isolated from frozen surgical kidney specimens using the Quiamp Tissue Kit and the Rneasy Kit respectively (Qiagen). After screening the DNA for the T61C polymorphism, RT-PCR was performed on RNA from two male individuals hemizygous for each allele . Primers were synthesized to anneal to exon 5 and exon 8 sequences (Table 4). Aliquots of 500 ng total RNA were incubated with 1 µg reverse primer, 10 mmol each dNTP, 2 µl 0.1 M DTT, 4 ml 5× first strand buffer and 100 U Superscript™ II RT (Gibco-BRL) in a total volume of 20 µl at 50°C for 45 min. After RNA digestion with 1 µl RNase A for 15 min at 37°C, PCR was performed by adding 70 µl H_2O, 8 µl 10× PCR buffer, 1 µg forward primer and 2.5 U Taq polymerase (Boehringer), with 25 cycles of 1 min at 95°C, 2 min at 62°C and 3 min at 72°C. As an internal control RT-PCR was performed for β-actin, with 25 cycles of 45 s at 94°C, 30 s at 55°C and 1 min at 72°C (Fig. 3).

Biochemical measurements

Blood was drawn from non-fasting subjects who were in a supine resting position for at least 30 min. Immunoreactive renin was quantified in 200 µl plasma using an immunoradiometric assay kit (Nichols Institute, Wychen, The Netherlands), following the methods proposed by Derkx *et al*. (37). Cross-reactivity of the monoclonal renin antibody with prorenin is ~1%. The concentration of prorenin was calculated by subtracting the results obtained before activation of prorenin (i.e. active renin) from those obtained after activation (i.e. total renin). Prorenin was activated non-proteolytically, using the renin inhibitor remikiren. Results are expressed as mU/l using the international reference preparation 68/356 as standard (37).

Subjects screened for mutations in the *RnBP* gene

After giving informed consent, four normotensive Caucasian individuals (two males) as well as 20 Caucasian patients with essential hypertension (14 males) were screened for mutations within the *RnBP* gene. Hypertension was defined as a blood pressure ≥160/95 mmHg on more than three occasions or chronic intake of antihypertensive medication. The absence of secondary hypertension was excluded by a thorough clinical work-up. The onset of hypertension was before the age of 50 in all 20 patients. No patient reported excessive alcohol intake, oral contraception, diabetes mellitus or renal failure. There was a positive family history of hypertension compatible with X chromosomal inheritance in 14 of the patients (nine males, five females).

Population screened for the intronic T61C polymorphism

The subjects of this protocol had initially participated in the MONICA (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease), Augsburg, baseline survey of 1984/85 and its follow-up examination in 1987/88 (38). Subjects originate from a sex/age-stratified random sample of all German residents of the Augsburg study area. In 1994 a second follow-up examination, including echocardiographic, biochemical and anthropometric measurements, was offered to a total of 1010 men and women aged 52–65 years, of whom 646 (64%) attended.

All subjects responded to a questionnaire on medical history, physical activities, medication and personal habits. Height and weight were recorded in light clothing and body mass index was computed as weight divided by height (kg/m^2). Resting blood pressure was measured after subjects maintained a sitting position for a minimum of 30 min. Using a mercury sphygmomanometer, blood pressure was read three times on the right arm by two investigators. The mean of three measurements was used for this study. Two-dimensional guided M-mode echocardiograms were recorded on strip chart paper at 50 mm/s (Sonos 1500; Hewlett Packard Inc.). Only tracings that demonstrated optimal visualization of left ventricular interference were used, a requirement that resulted in exclusion of 17% of potential subjects. All M-mode tracings were analysed by a single cardiologist who was blind as to clinical and biochemical data. Measurements for M-mode guided calculation of left ventricular mass were taken just below the tip of the mitral valve. Left ventricular internal end-diastolic (EDD) and end-systolic dimensions (ESD) and septal (sWth) and posterior wall thickness (pWth) were measured according to the guidelines of the American Society of Echocardiography (39). Left ventricular mass (LVM) was calculated from M-mode echocardiograms according to the formula

$$\text{LVM M-mode (g)} = 0.8\,(1.04\,[\text{EDD} + \text{sWth} + \text{pWth}]^3 - \text{EDD}^3) + 0.6\ \text{g}$$

(40). LVM was indexed to body surface area as LVMI (g/m^2 body surface area).

Statistical analyses

Anthropometric and biochemical data were compared between subjects with the 61T and 61C alleles by ANOVA for the comparison of independent samples. In the case of comparisons between classified values, e.g. comparisons between T61C allele status and drug intake, χ^2 tests were performed. Subjects taking antihypertensive drugs were excluded from the analyses of potential associations between *RnBP* allele status and renin, prorenin or other cardiovascular parameters because these drugs either decrease (β-blockers) or increase (ACE inhibitors, diuretics, calcium channel blockers) renin levels. *P* values are reported for each test and statistical model.

ACKNOWLEDGEMENTS

We thank Drs A.Bosserhoff, A.Hartmann, H.-W.Hense and S.S.Sommer for many helpful discussions and suggestions regarding this manuscript and Drs F.Hofstädter and R.Büttner for generously supporting this work.

ABBREVIATION

RnBP, renin binding protein.

REFERENCES

1. James,G.D. and Baker,P.T. (1990) In Laragh,J.H. and Brenner,B.M. (eds), *Hypertension: Pathophysiology, Diagnosis, and Management.* Raven Press, New York, NY, pp. 137–145.
2. Kurtz,T.W. and Spence,M.A. (1993) Genetics of essential hypertension. *Am. J. Med*., **94**, 77–84.
3. Lifton,R.P. (1995) Genetic determinants of human hypertension. *Proc. Natl. Acad. Sci. USA*, **92**, 8545–8551.
4. Ward,R. (1990) In Laragh,J.H. and Brenner,B.M. (eds), *Hypertension: Pathophysiology, Diagnosis, and Management.* Raven Press, New York, NY, pp. 81–100.
5. Jeunemaitre,X., Soubrier,F., Kotelevtsev,Y.V., Lifton,R.P., Williams,C.S., Charru,A., Hunt,S.C., Hopkins,P.N., Williams,R.R., Lalouel,J.M. and Corvol,P. (1992) Molecular basis of human hypertension: role of angiotensinogen. *Cell*, **71**, 169–180.
6. Iwai,N., Shimoike,H., Ohmichi,N. and Kinoshita,M. (1995) Angiotensinogen gene and blood pressure in the Japanese population. *Hypertension*, **25**, 688–693.
7. Ward,K., Hata,A., Jeunemaitre,X., Helin,C., Nelson,L., Namikawa,C., Farrington,P.F., Ogasawara,M., Suzumori,K., Tomoda,S., Berrebi,S., Sasaki,M., Corvol,P. and Lifton,R.P. (1993) A molecular variant of angiotensinogen associated with preeclampsia. *Nature Genet*., **4**, 59–61.
8. Cambien,F., Poirier,O., Lecerf,L., Evans,A., Cambou,J.P., Arveiler,D., Luc,G., Bard,J.M., Bara,L., Ricard,S., Tiret,L., Amoyel,P., Alhenc-Gelas,F. and Soubrier,F. (1992) Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature*, **359**, 641–644.
9. Leatham,E., Barley,J., Redwood,S., Hussein,W., Carter,N., Jeffery,S., Bath,P.M. and Camm,A. (1994) Angiotensin-1 converting enzyme (ACE) polymorphism in patients presenting with myocardial infarction or unstable angina. *J. Hum. Hypertension*, **8**, 635–638.
10. Tiret,L., Bonnardeaux,A., Poirier,O., Ricard,S., Marques-Vidal,P., Evans,A., Arveiler,D., Luc,G., Kee,F., Ducimetiere,P., Soubrier,F. and Cambien,F. (1994) Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. *Lancet*, **344**, 910–913.
11. Morris,B.J., Zee,R.Y. and Schrader,A.P. (1994) Different frequencies of angiotensin-converting enzyme genotypes in older hypertensive individuals. *J. Clin. Invest*., **94**, 1085–1089.
12. Harrap,S.B., Davidson,H.R., Connor,J.M., Soubrier,F., Corvol,P., Fraser,R., Foy,C.J. and Watt,G.C. (1993) The angiotensin I converting enzyme gene and predisposition to high blood pressure. *Hypertension*, **21**, 455–460.
13. Tiret,L., Rigat,B., Visvikis,S., Breda,C., Corvol,P., Cambien,F. and Soubrier,F. (1992) Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am. J. Hum. Genet*., **51**, 197–205.
14. Schmidt,S., van Hooft,I.M., Grobbee,D.E., Ganten,D. and Ritz, E. (1993) Polymorphism of the angiotensin I converting enzyme gene is apparently not related to high blood pressure: Dutch Hypertension and Offspring Study. *J. Hypertension*, **11**, 345–348.
15. Naftilan,A.J., Williams,R., Burt,D., Paul,M., Pratt,R.E., , Chirgwin,J. and Dzau,V.D. (1989) A lack of genetic linkage of renin gene restriction fragment length polymorphisms with human hypertension. *Hypertension*, **14**, 614–618.
16. Soubrier,F., Jeunemaitre,X., Rigat,B., Houot,A., Cambien,F. and Corvol, P. (1990) Similar frequencies of renin gene restriction fragment length polymorphisms in hypertensive and normotensive subjects. *Hypertension*, **16**, 712–717.
17. Zee,R.Y., Ying,L.H., Morris,B.J. and Griffiths, L.R. (1991) Association and linkage analyses of restriction fragment length polymorphisms for the human renin and antithrombin III genes in essential hypertension. *J. Hypertension*, **9**, 825–830.
18. Takahashi,S., Inoue,H., Fukui,K. and Miyake,Y. (1994) Structure and function of renin binding protein. *Kidney Internatl*, **46**, 1525–1527.
19. Takahashi,S., Inoue,H. and Miyake,Y. (1992) The human gene for renin-binding protein. *J. Biol. Chem*., **267**, 13007–13013.
20. Maru, I.,Ohta,Y., Murata,K. and Tsukada,Y. (1996) Molecular cloning and identification of *N*-acyl-D-glucosamine 2-epimerase from porcine kidney as a renin-binding protein. *J. Biol. Chem*., **271**, 16294–16299.
21. Tribioli,C., Mancini,M., Plassart,E., Bione,S., Rivella,S., Sala,C., Torri,G. and Toniolo,D. (1994) Isolation of new genes in distal Xq28: transcriptional map and identification of a human homologue of the ARD1 N-acetyl transferase of *Saccharomyces cerevisiae*. *Hum. Mol. Genet*., **3**, 1061–1067.
22. Faranda,S., Frattini,A. and Vezzoni,P. (1995) The human genes encoding renin-binding protein and host cell factor are closely linked in Xq28 and transcribed in the same direction. *Gene*, **155**, 237–239.
23. Frattini,A., Faranda,S., Redolfi,E., Zucchi,I., Villa,A., Patrosso,M.C., Strina D., Susani,L. and Vezzoni, P. (1994) Genomic organization of the human VP16 accessory protein, a housekeeping gene (HCFC1) mapping to Xq28. *Genomics*, **23**, 30–35.
24. van den Ouweland,A.M.W., Verdijk,M., Kiochis,P., Poustka,A. and van Oost,B.A. (1994) The human renin-binding protein (RENBP) maps in Xq28. *Genomics*, **21**, 279–281.
25. Wilson,A.C., Parrish,J.E., Massa,H.F., Nelson,D.L., Trask,B.J. and Herr,W. (1995) The gene encoding the VP16-accessory protein HCF (HCFC1) resides in human Xq28 and is highly expressed in fetal tissues and the adult kidney. *Genomics*, **25**, 462–468.
26. National Center for Health Statistics. (1982) In Rowland,M. and Roberts,G. (eds), *The National Health and Nutrition Examination Survey II* (*NHANES II)*. Public Health Service, Rockville, MD, pp. 1–84.
27. Whelton,P.K. (1994) Epidemiology of hypertension. *Lancet*, **344**, 101–106.
28. Jurka,J., Klonowski,P., Dagman,V. and Pelton,P. (1996) CENSOR—a program for identification and elimination of repetitive elements from DNA sequences. *Comput. Chem*., **20**, 119–121.
29. Schunkert,H., Hense,H.W., Muscholl,M., Luchner,A. and Riegger,G.A.J. (1996) Association of angiotensin converting enzyme activity and arterial blood pressure in a population based sample. *J. Hypertension*, **14**, 571–575.
30. Craxton,M. (1993) In Griffin,H. and Griffin,A. (eds), *DNA Sequencing Protocols*. Humana Press, pp. 149–167.
31. Dear,S. and Staden,R. (1991) A sequence assembly and editing program for efficient management of large projects. *Nucleic Acids Res*., **19**, 3907–3911.
32. Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J. Mol. Biol*., **215**, 403–410.
33. Pearson,W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. USA*, **85**, 2444–2448.
34. Uberbacher,E.C. and Mural, R.J. (1991) Locating protein-coding regions in human DNA sequences by a multiple sensor–neural network approach. *Proc. Natl. Acad. Sci. USA*, **88**, 11261–11265.
35. Thomas,A., Skolnick,M.H. and Lewis,C.M. (1994) Genomic mismatch scanning in pedigrees. *IMA J. Math. Appl. Med. Biol*., **11**, 1–16.
36. Huang,X. (1994) On global sequence alignment. *Comput. Appl. Biosci*., **10**, 227–235.
37. Derkx,F.H., de Bruin,R.J., van Gool,J.M., van den Hoek,M.J., Beerendonk,C.C., Rosmalen,F., Haima,P. and Schalekamp, M.A. (1996) Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. *Clin. Chem*., **42**, 1051–1063.
38. Keil,U., Stieber,J., Döring,A., Härtel,U., Filipatrik,B. and Hense,H.W. (1988) The cardiovascular risk profile in the study area Augsburg: results from the first MONICA survey 1984–1985. *Acta Med. Scand*., **728** (suppl.), 119–128.
39. Sahn,D., DeMaria,A., Kisslo,J. and Weymann,A. (1978) The committee on M-mode standardization of the American Society of Echocardiography: results on a survy of echocardiographic measurements. *Circulation*, **58**, 1072–1083.
40. Devereux,R.B., Koren,M.J., deSimone,P., Okin,N. and Klingfield,P. (1993) Methods for detection of left ventricular hypertrophy: application to hypertensive heart disease. *Eur. Heart J*., **14** (suppl. D), 8–15.