Renal disease susceptibility and hypertension are under independent genetic control in the fawn-hooded rat

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Hypertension, diabetes and hyperlipidemia are risk factors for life-threatening complications such as end-stage renal disease, coronary artery disease and stroke. Why some patients develop complications is unclear, but only susceptibility genes may be involved. To test this notion, we studied crosses involving the fawn-hooded rat, an animal model of hypertension that develops chronic renal failure. Here, we report the localization of two genes, *Rf-1* and *Rf-2*, responsible for about half of the genetic variation in key indices of renal impairment. In addition, we localize a gene, *Bpfh-1*, responsible for about 26% of the genetic variation in blood pressure. *Rf-1* strongly affects the risk of renal impairment, but has no significant effect on blood pressure. Our results show that susceptibility to a complication of hypertension is under at least partially independent genetic control from susceptibility to hypertension itself.

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Hypertension, hyperlipidemia and diabetes mellitus affect hundreds of millions of people worldwide and are responsible for increased morbidity and mortality. Interestingly, these conditions do not directly impact health, but rather cause harm by increasing the risk for development of debilitating and life-threatening complications — such as end-stage renal disease (ESRD), coronary heart disease (CHD) and stroke. Curiously, patients with the same condition may develop very different complications or no complications whatsoever; the reason for this variability is unknown. One plausible hypothesis is that there are genetic factors (unrelated to the causes of hypertension, hyperlipidemia and diabetes *per se*) that determine or influence the pattern of complications that a patient will likely develop. In principle, knowledge of such factors could have important implications for preventing the morbidity and mortality of these conditions. At present, however, little is known about the genetic basis of developing particular complications.

ESRD is a major health problem in the United States, with an incidence that has been increasing steadily over the past decade[1] and more than 70% of ESRD is associated with hypertension and/or diabetes[2]. The reasons for these associations with hypertension are not known, but three possibilities exist: (i) hypertension is necessary and sufficient for producing the renal complications seen in hypertensive patients; (ii) hypertension is necessary but not sufficient, with other risk factors being required for renal complications; (iii) hypertension is neither necessary nor sufficient, but simply increases or accelerates the risk in individuals who are otherwise predisposed. Renal complications will be referred to as hypertension-*induced* in the first situation and hypertension-*associated* in the latter two situations.

Although it is difficult to distinguish between these hypotheses in humans with primary hypertension, it has been widely supposed that renal complications are directly induced by the hypertension. Recently, the alternative hypothesis — that hypertension contributes but is not sufficient to cause renal complications — has begun to gain ground. Freedman *et al.*[3] recently challenged the notion of hypertension-induced renal failure, noting that there is little evidence in support of it outside the condition of malignant hypertension and that hypertension has been shown to accelerate virtually all forms of renal failure[4,5]. Moreover, several lines of evidence suggest that genetic factors may play an important role in the risk that a hypertensive patient will develop ESRD. Only a minority (0.3% per year) of hypertensive patients develop ESRD[6], with the proportion of hypertensives that develop renal failure varying with ethnicity. African-Americans, for example, have an 8- to 25-fold higher risk than Caucasians, which cannot be explained by the severity of or treatment for hypertension[7–9]. Additionally, the presence of ESRD in an African-American individual resulted in a nine-fold increased risk of ESRD to a first-degree relative, even after controlling for hypertension in the relative[10].

Because so little is known about the genetic basis of susceptibility to renal complications, we decided to investigate renal impairment in an animal model, the fawn-hooded rat (FHR). This strain is genetically hypertensive and develops early and chronic renal disease. FHR was originally studied because of a mild bleeding disorder resulting from a platelet storage-pool disease,

which appears to be caused by a single-gene defect that maps to the region of rat chromosome 1 containing the red-eye dilution gene (*r*)[11,12]. The renal pathophysiology of FHR is characterized by systemic and glomerular hypertension, proteinuria, reduced urinary kallikrein activity levels, and glomerular sclerosis resulting in early death[13–16]. In particular, FHR have an average proteinuria level of about 69 mg/day at 12 months of age, as compared to ~8.0 mg/day in control ACI rats[18]. FHR show early expansion of the mesangial matrix and non-selective accumulation of IgG, IgM, IgA and C3, concommitant with podocyte injury and increasing proteinuria[16,17]. The frequency and severity of renal impairment is higher in FHR males than females, a feature that is shared with the human disease[13,16].

Although it is difficult to prove that hypertension directly induces the renal impairment in FHR, several lines of evidence suggest that hypertension at least plays an important role in the progression of this end-organ complication. (i) Pharmacological treatments that decrease blood pressure reduce or prevent renal damage and vascular lesions over the time course studied[19–21]. (Of course, such treatments could conceivably affect other risk factors for renal impairment in addition to blood pressure itself.) (ii) Within a closed but not inbred FHR colony, rats with the highest blood pressure have a considerably increased risk of histologically-assayed renal damage; blood pressure accounted for 42% of the variation in animals at 40 weeks of age[14]. This strong correlation suggests that blood pressure itself is an important risk factor, but also leaves open the possibility of additional factors affecting risk through an independent mechanism. (iii) The time course for development of hypertension and proteinuria in FHR parallel one another. Taken together, these observations suggest that FHR is a good model of hypertension-associated renal disease.

To study the genetic control of both hypertension and renal impairment in this animal model, we constructed a backcross between the fawn-hooded hypertensive rat (FHH) (an inbred strain of FHR developed by A.P.P.) and the normotensive ACI rat. Progeny were phenotyped both for measures of renal impairment and for blood pressure, and were genotyped for genetic markers across the rat genome.

Here, we report the genetic localization of two genes, *Rf-1* and *Rf-2*, responsible for about 50% of the genetic variation in renal impairment in the cross and one gene, *Bpfh-1*, responsible for about 26% of the genetic variation in blood pressure. Importantly, *Rf-1* exerts strong effects on the risk of renal impairment, but has no significant effect on systemic blood pressure; it appears to act through an independent mechanism. Our results demonstrate that susceptibility to hypertension and renal impairment are under at least partially independent genetic control.

Inheritance pattern of renal impairment

To determine the mode of inheritance of renal impairment, we examined F_1 progeny between FHH and ACI rats. In preliminary analyses, we (A.P.P.) found that (FHH × ACI)F_1 showed normal urinary protein levels — indicating that renal impairment, as estimated by proteinuria, is inherited as a recessive trait. Accordingly, we decided to study the inheritance of the disease in a (FHH × ACI) × FHH backcross (BC1). Progression of renal impairment was followed by a number of physiological indicators (Table 1). The principal measure was proteinuria (UPV), an early indicator of renal impairment. Other biochemical measures of the severity of impairment included: plasma creatinine (PCr), an estimate of glomerular filtration rate; plasma albumin (PAlb); urinary osmolality (UOs); urinary kallikrein-like activity (UKV); and plasma urea levels (PUr) (see Methods). In the case of UOs, no significant results were seen and this phenotype is not discussed further. The severity of renal impairment was also estimated by macroscopic examination of the kidneys recorded as the macroscopic renal index score (MRIS), based on the proportion of the kidney surface covered with visible lesions (see Methods). Finally, systolic blood pressure (SBP) was measured at 6 months.

By comparing the genetic variance for blood pressure and proteinuria in F_0, F_1 and BC1 progeny, it is possible to determine the proportion of genetic variance in the backcross and to estimate the effective number of genetic factors controlling the traits[22]. Average blood pressure was 148 ± 2.9 (mean ± SEM) in the FHR parents (n = 12), 119 ± 5.8 in the ACI parents (n = 3), 143 ± 2.0 in the F_1 progeny (n = 12), and 140 ± 1.0 in the backcross progeny (n = 128). Most of the backcross progeny showed some degree of hypertension, with only 18 animals with SBP below 130. Average proteinuria level at 6 months was 26.0 ± 1.6 in the FHR parents, 5.9 ± 0.9 in the ACI parents, 8.0 ± 0.9 in the F_1 progeny, and 19.6 ± 1.5 in the backcross progeny.

For blood pressure, roughly 60% of the backcross variance appears to be genetic and the effective number n of genetic factors is in the range 1–3, depending upon the age sampled. For proteinuria, an even larger proportion (85%) of the backcross variance appears to be genetic and n is estimated to be about 3.

The backcross progeny showed a weak, but statistically significant correlation between blood pressure and proteinuria (10% of variance, P = 0.0002). The fact that blood pressure explains a much higher percentage of the variance within a closed but outbred colony (see above) than in the backcross is consistent with the presence of major factors affecting renal impairment (independent of hypertension) segregating in the backcross.

Linkage analysis

To search for genetic factors affecting renal impairment and blood pressure, we genotyped male F_1 backcross progeny with 130 genetic markers, covering approximately 85% of the genome. Although the majority of the genetic markers were randomly-chosen simple sequence length polymorphisms (SSLPs)[23], we specifically included genetic markers in certain genes known to have a role in blood pressure regulation (including angiotensin converting enzyme, atrial natriuretic factor, 11-β-hydroxylase, and renin) and in certain regions reported to show linkage to hypertension in other crosses (such as carboxypeptidase 8 (*Carb08*) and tumour necrosis factor, and markers flanking the S_A locus (S_A) and guanyl cyclase associated with the atrial natriuretic factor receptor (*GCA*))[24–33].

Using linkage analysis, we searched for quantitative trait loci (QTLs) affecting each of the renal impairment phenotypes, as well as QTLs affecting systolic blood

Table 1 Comparison of means for animals at various genetic loci

a, *Rf-1* locus[a]

Phenotype	Mean (± SEM) Animals homozygous at *Rf-1* (*n* = 48)	Mean (± SEM) Animals heterozygous at *Rf-1* (*n* = 48)	Max lod score at *Rf-1*
SBP (6mo)	141 ± 1.43	138 ± 1.57	0.3
UKV (9mo)	44.4 ± 5.28	94.6 ± 14.7*	2.4
UPV(6mo)	24.2 ± 2.10	11.2 ± 0.68*	8.9
UPV (9mo)	39.8 ± 3.46	19.1 ± 1.39*	7.7
PAlb	23.7 ± 0.30	25.4 ± 0.30*	3.0
PCr	79.3 ± 2.78	65.7 ± 1.68*	3.7
PUr	8.52 ± 0.64	6.60 ± 0.14*	3.0
MRIS	2.43 ± 0.17	0.79 ± 0.15*	8.4

b, *Rf-2* locus[b]

Phenotype	Mean (± SEM) Animals homozygous at *Rf-2* (*n* = 56)	Mean (± SEM) Animals heterozygous at *Rf-2* (*n* = 63)	Max lod score at *Rf-2*
SBP (6mo)	144 ± 1.26	136 ± 1.37*	3.1
UKV (9mo)	47.7 ± 5.89	84.1 ± 12.7*	1.6
UPV(6mo)	20.6 ± 1.65	15.9 ± 1.66*	1.6
UPV (9mo)	35.3 ± 2.95	24.7 ± 2.30*	1.9
PAlb	23.7 ± 0.25	25.5 ± 0.31*	4.9
PCr	78.3 ± 2.3	67.7 ± 1.61*	3.2
PUr	8.19 ± .0.50	6.86 ± 0.20*	2.0
MRIS	2.10 ± 0.18	1.17 ± 0.17*	3.1

c, *Rf-1* and *Rf-2*

Phenotype	Mean (± SEM) Homozygous at *Rf-1* Homozygous at *Rf-2* *n* = 29	Mean (± SEM) Homozygous at *Rf-1* Heterozygous at *Rf-2* *n* = 16	Mean (± SEM) Heterozygous at *Rf-1* Homozygous at *Rf-2* *n* = 18	Mean (± SEM) Heterozygous at *Rf-1* Heterozygous at *Rf-2* *n* = 27
SBP (6mo)	144 ± 1.94‡	136 ± 1.83	143 ± 2.34‡	135 ± 1.97
UKV (9mo)	34.2 ± 5.31‡	55.1 ± 9.96	59.9 ± 14.6	117 ± 23.0
UPV(6mo)	28.0 ± 2.69‡	19.2 ± 3.40†	11.8 ± 0.98†	10.7 ± 0.98
UPV (9mo)	48.2 ± 4.96‡	26.6 ± 2.59†	21.6 ± 2.09†	17.8 ± 1.95
PAlb	23.0 ± 0.33‡	24.7 ± 0.48‡	24.7 ± 0.55†‡	25.8 ± 0.40
PCr	85.9 ± 3.73‡	70.4 ± 3.26	67.6 ± 2.87†	65.4 ± 2.23
PUr	9.6 ± 1.01‡	7.0 ± 0.20†	7.1 ± 1.03†	6.38 ± 0.17
MRIS	2.7 ± 0.23‡	1.9 ± 0.22	1.4 ± 0.26	0.44 ± 0.16

d, *Bpfh-1* locus[c]

Phenotype	Mean (± SEM) Animals homozygous at *Bpfh-1* (*n* = 63)	Mean (± SEM) Animals heterozygous at *Bpfh-1* (*n* = 64)	Max lod score at *Bpfh-1*
SBP (6mo)	144 ± 1.31	136 ± 1.19*	4.2
UKV (9mo)	48.8 ± 6.70	83.2 ± 11.0*	1.9
UPV(6mo)	22.1 ± 2.27	15.6 ± 1.31*	1.2
UPV (9mo)	36.3 ± 3.32	25.4 ± 2.04*	1.1
PAlb	24.1 ± 0.29	25.1 ± 0.30*	1.3
PCr	85.1 ± 8.91	68.7 ± 1.66	1.8
PUr	9.72 ± 1.77	6.99 ± 0.19	0.9
MRIS	2.08 ± 0.19	1.23 ± 0.16*	2.2

[a]According to genotype at *Rf-1* inferred from flanking markers *D1Mit6* and *D1Mgh12*.
[b]According to genotype at *Rf-2* inferred from flanking markers *r* and *D1Mit2*.
[c]According to genotype at *Bpfh-1* inferred from the adjacent marker *Mt1pa*.
SBP = systolic blood pressure (mmHg); UPV = urinary protein (mg/day per 100 g body weight); UKV = urinary kallikrein-like activity (mU/day per 100g body weight); PAlb = plasma albumin (mg/ml); PCr = plasma creatinine (μM); PUr = plasma urea (μM); MRIS = macroscopic renal index score.
* significant at (P = 0.05)
† significant difference (P = 0.05) with first column, using ANOVA with post hoc Bonferoni analyses.
‡ significant difference (P = 0.05) with first column, using ANOVA with post hoc Bonferoni analyses.

pressure. Markers were initially tested in an arbitrarily-chosen subset of 46 progeny and those regions with lod scores exceeding 1 were subsequently tested in DNA from the entire set of male F_1 progeny (see Methods). In this backcross study, there is approximately 50% power to detect QTLs responsible for at least 10% of the total phenotypic variance.

Rf-1, a locus involved in renal impairment

We found striking evidence for a gene involved in renal impairment, mapping between the markers *D1Mit6* and *D1Mgh12* on rat chromosome 1 (Fig. 1). Linkage is clear for six of the seven phenotypic measures related to renal impairment, while the lod score for the seventh (urinary kallikrein-like activity) is also high although somewhat below the genome-wide significance threshold. We refer to this locus as *Rf-1* (for *r*enal-*f*ailure-1).

Focusing on proteinuria (UPV) as the best early indicator of renal impairment, there was a lod score peak of 8.9 for proteinuria at 6 months and 7.7 for proteinuria at 9 months. The *Rf-1* locus appeared to explain about 37% of the total variance in proteinuria, corresponding to 44% of the genetic component of the variance in the cross. The mean value of the proteinuria phenotype at

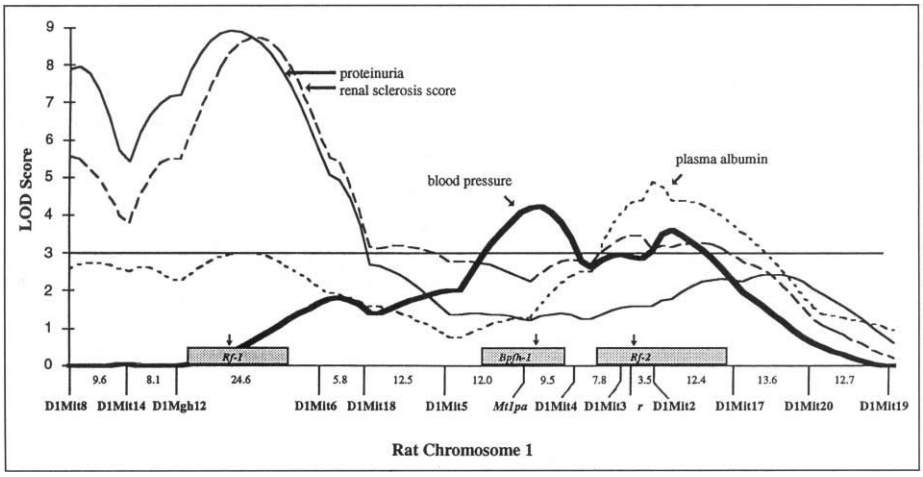

Fig. 1 Lod plot of proteinuria at 6 months (solid line), macroscopic renal index score (dashed line), plasma albumin (dotted line), and systolic blood pressure (heavy line) for rat chromosome 1. A horizontal line indicates the threshold for significance of the lod score (3.0). Maximum likelihood position and lod 1-support intervals for each QTL are indicated by tick marks and shaded interval along the horizontal axis. *Rf-1* is localized between *D1Mit4* and *D1Mit14*, *Bpfh-1* between *D1Mgh4* and *D1Mit20* and *Rf-2* between markers *D1Mit20* and *Mt1pa* using a lod-3 confidence interval. Map distances are given in centiMorgans, determined with the Kosambi map function. Distance between *D1Mit3* and *r* is 0.8 cM.

either time differed significantly ($P = 0.001$) between animals inferred to be homozygous vs. heterozygous (based on genotypes at flanking markers) at *Rf-1* (Table 1*a*). The strength of the evidence for linkage can be seen from the correlation of genotypes with UPV: *Rf-1* homozygotes have more than twice the level of proteinuria as *Rf-1* heterozygotes.

Using the macroscopic index of renal impairment, MRIS, the *Rf-1* locus showed a lod score of 8.4 and similarly accounted for about 39% of the total variance. The peak lod score for MRIS occurs in essentially the same location as for UPV (Fig. 1).

Plasma albumin also provides information about renal impairment, since albumin levels are expected to decrease at the more severe stages of kidney disease, when the liver can no longer produce enough albumin to keep pace with loss at the filtration barrier. Using plasma albumin as a phenotype, a lod score of 3.0 is found in region of the *Rf-1* locus. Three other intermediate measures of renal function, plasma creatinine, plasma urea levels and urinary kallikrein-like activity yielded lod scores of 3.7, 3.0 and 2.4, respectively. These are all detailed in Table 1*a*, along with the comparison of the means for homozygotes and heterozygotes.

Although the *Rf-1* locus showed an effect on these measures of renal impairment, it had no significant effect on blood pressure. At the maximum likelihood position for *Rf-1*, the lod score for blood pressure was very low (lod = 0.3). There was also no significant difference in mean blood pressure between *Rf-1* homozygotes and heterozygotes, even if one uses the less stringent statistical threshold appropriate for a single point comparison rather than a genome-wide search (means = 141 vs. 138, $P = 0.08$). These results indicate that *Rf-1* contributes strongly to renal impairment through a physiological mechanism other than increasing blood pressure.

To test the hypothesis that *Rf-1* causes renal impairment through a mechanism other than increasing blood pressure, we adjusted the UPV phenotype by eliminating the effect of blood pressure (SBP). Specifically, we derived a new phenotype Res(UPV;SBP) defined to be the residual when UPV is linearly regressed on SBP; the residual eliminates all linear effects attributable to blood pressure. A locus that causes proteinuria *only* through its effect on blood pressure would thus show no correlation with the phenotype Res(UPV;SBP). In fact, the *Rf-1* locus is a strong QTL for Res(UPV;SBP), with a lod score exceeding 6.5 and explaining about 25% of the variance. This confirms that *Rf-1* causes renal impairment through an independent mechanism and not simply through increasing systolic blood pressure.

Additional loci, *Rf-2* and *Bpfh-1*

Since *Rf-1* explains only about 44% of the genetic component of renal impairment and no significant proportion of blood pressure, we searched the genome for additional loci responsible for renal impairment and for loci responsible for hypertension. We found evidence for two additional loci. Both loci are also on chromosome 1, but are at a considerable distance from *Rf-1* (Fig. 1). The first has a significant effect on blood pressure, but less on renal impairment (Table 1*b*); we will refer to it as *Bpfh-1* (for *b*lood *p*ressure in *f*awn-*h*ooded-1). *Bpfh-1* is identified by the significant peak in the lod score (lod = 4.2) for systolic blood pressure near *D1Mit13* and *Mt1pa*. The inheritance pattern of *Bpfh-1* explains 16% of the total variance (or about 26% of the genetic component of the variance) in blood pressure in this cross. The second locus, *Rf-2* (for *r*enal *f*ailure-2) has a strong and significant effect on one measure of renal impairment (plasma albumin level), and significant, albeit weaker, effects on PCr and MRIS (Table 1*c*). This region containing *Rf-2* also shows some linkage to blood pressure (Table 1*c*).

When multiple QTLs appear to lie on the same chromosome, it is necessary to consider whether they might represent a single gene. It is clear that *Rf-1* cannot be identical with either *Bpfh-1* or *Rf-2*. *Rf-1* is separated by 45–60 cM, which is sufficiently large that there is no significant correlation in genotype over such distances. (We verified this by demonstrating that allowing for the presence of a QTL at *Rf-1* had no effect on the analyses of *Bpfh-1* and *Rf-2*, in a suitable multiple QTL model.)

The relationship between *Bpfh-1* and *Rf-2* is less clear. The most likely positions for the two loci are separated by about 17 cM, and the lod 1 support intervals, the traditional measure of location for genetic loci, do not overlap (Fig. 1). Moreover, the proximity of *Bpfh-1* to an important candidate locus, the S_A gene, a gene of unknown function that shows higher expression in kidney of SHR rats than WKY[24,32] and cosegregates with blood pressure in a number of crosses (including SHRSP × WKY, SHR × WKY and SS/JR × LEW)[29–33] gives us additional confidence in the localization of *Bpfh-1* to the

Table 2 Comparison of means for animals according to genotypes at both *Rf-1* and *Rf-2*.

Phenotype	Mean (± SEM) Homozygous at *Rf-1* Homozygous at *Rf-2* *n* = 29	Mean (± SEM) Homozygous at *Rf-1* Heterozygous at *Rf-2* *n* = 16	Mean (± SEM) Heterozygous at *Rf-1* Homozygous at *Rf-2* *n* = 18	Mean (± SEM) Heterozygous at *Rf-1* Heterozygous at *Rf-2* *n* = 27
SBP (6mo)	144 ± 1.9	136 ± 1.8	143 ± 2.3	135 ± 2.0
UKV (9mo)	34.2 ± 5.3	55.1 ± 10	59.9 ± 14	117 ± 23
UPV(6mo)	28.0 ± 2.7	19.2 ± 3.4	11.8 ± 1.0	10.7 ± 1.0
UPV (9mo)	48.2 ± 5.0	26.6 ± 2.6	21.6 ± 2.1	17.8 ± 2.0
PAlb	23.0 ± 0.33	24.7 ± 0.48	24.7 ± 0.55	25.8 ± 0.40
PCr	85.9 ± 3.7	70.4 ± 3.3	67.6 ± 2.9	65.4 ± 2.2
PUr	9.6 ± 1.0	7.0 ± 0.20	7.1 ± 1.0	6.38 ± 0.17
MRIS	2.7 ± 0.23	1.9 ± 0.22	1.4 ± 0.26	0.44 ± 0.16

	P value Column 1 vs. Column 2	*P* value Column 1 vs. Column 3	*P* value Column 1 vs. Column 4	*P* value Column 2 vs. Column 3	*P* value Column 2 vs. Column 4	*P* value Column 3 vs. Column 4
SBP (6mo)	NS	NS	≤0.01	NS	NS	0.05
UKV (9mo)	NS	NS	≤0.01	NS	NS	NS
UPV(6mo)	0.05	≤0.01	≤0.01	NS	NS	NS
UPV (9mo)	≤0.01	≤0.01	≤0.01	NS	NS	NS
PAlb	NS	0.04	≤0.01	NS	NS	NS
PCr	≤0.01	≤0.01	≤0.01	NS	NS	NS
PUr	NS	NS	≤0.01	NS	NS	NS
MRIS	NS	≤0.01	≤0.01	NS	≤0.01	≤0.01

Analysis of variance (ANOVA) with *post hoc* modified t test using a Bonferoni adjustment was performed. *P* values are shown for all significant interactions; non significant differences are noted (NS). For abbreviations, see Table 1.

immediate vicinity of *Mt1pa*. We therefore tentatively conclude that *Bpfh-1* and *Rf-2* represent distinct loci. However, only 7 animals are clearly recombinant between these loci, providing very little power to distinguish if their effects on blood pressure and renal impairment are independent (Tables 1*b, c*). Additional studies with more animals recombinant between *Bpfh-1* and *Rf-2* are required.

Together, *Rf-1* and *Rf-2* explain about 39% of the total variance — or 46% of the genetic variance — in proteinuria, in a two-QTL model. They also explain about 46% of the total variance in the MRIS, about 24% of total variance in PCr, and about 26% of the total variance in plasma albumin. Animals homozygous at both *Rf-1* and *Rf-2* show substantially higher indices of renal impairment than animals heterozygous at either or both loci, indicating that both loci contribute cumulatively to the development of renal impairment (Table 2). *Rf-1* and *Rf-2* contrast in that the former showed the strong effects on proteinuria, but weak effects on plasma albumin, while the latter showed the opposite pattern. These differences suggest that *Rf-1* and *Rf-2* may act through different mechanisms.

Discussion

We have identified two loci, *Rf-1* and *Rf-2*, having substantial effects on the risk of renal impairment in a (FHH × ACI) × FHH backcross. *Rf-1* is particularly interesting because it explains 44% of the genetic variance in proteinuria and strongly affects other measures of renal function, while having no apparent effect on blood pressure.

Although it is clear that *Rf-1* causes renal failure, is it fair to conclude that this renal failure is hypertension-associated? The strongest evidence for this interpretation is that renal failure in FHR can be prevented by antihypertensive treatment with ACE inhibitors (although this protection could conceivably be independent of the drug's effects on systemic blood pressure). Nonetheless, this does not exclude the possibility that *Rf-1* may cause renal impairment in both hypertensive and normotensive animals — that is, that *Rf-1* is a general locus affecting renal function. As all forms of renal disease are exacerbated by increases in blood pressure, it is possible that the effects of *Rf-1* on renal physiology only become readily apparent (at the endpoint of 9 months of age examined in this study) in the presence of an increase in blood pressure. We attempted to investigate this question by examining whether the correlation between proteinuria and blood pressure is stronger in *Rf-1* homozygotes than in *Rf-1* heterozygotes. In fact, such an effect is seen but the differences fall short of statistical significance owing to the fact that the number of *Rf-1* homozygotes is limited and *Rf-1* is linked to the independent locus *Bpfh-1* which affects blood pressure (and thereby limits the range of variation in *Rf-1* homozygotes). It would be instructive to pursue this analysis by constructing a congenic strain carrying the *Rf-1* region in an otherwise normotensive background and manipulating the blood pressure in this strain.

Rf-2 is interesting because of its differential effect on plasma albumin levels. *Rf-2* explains 16% of the total variation in plasma albumin, which is substantially higher than for *Rf-1* and suggests that this index of severe renal impairment may be independent of proteinuria. Although *Rf-2*'s effect on plasma albumin alone could be explained by a gene affecting albumin synthesis rather than renal impairment, the fact that *Rf-2* also affects the macroscopic renal index score suggests that it acts more directly. Because *Rf-2* maps near another factor affecting blood pressure, it is difficult to assess the gene's own effect on blood pressure, and it might act through a mechanism other than a direct effect on blood pressure. *Rf-2* maps to the same region as the platelet storage-pool bleeding disorder in the FHR and could be identical with the gene causing this bleeding disorder. There are several reports of patients with renal failure and various platelet disorders including a platelet storage-pool disease[35–39]. In addition, we note that the region containing *Rf-2* is conserved with a region of mouse chromosome 7 that

contains loci involved in renal failure associated with systemic lupus erythematosus[40].

In addition, we have identified a locus *Bpfh-1* explaining about 26% of the genetic variance in blood pressure. *Bpfh-1* lies in the region of the S_A gene, which has been reported to be linked to blood pressure in several rat crosses[29–33], and may be identical to it. The fact that a susceptibility locus for hypertension has been identified in this region in three different rat models suggests that it may be an important site of genetic variation in rodents and, perhaps, in humans[41].

Independent genetic control of renal impairment. The identification of genes that appear to influence whether a hypertensive rat will go on to develop renal complications may help us understand why some hypertensive patients develop renal disease. Many investigators have subscribed to the theory that hypertension *causes* renal failure. Determining causality — both clinically and experimentally — is problematic[3], because hypertension is known to accelerate renal disease[4,5].

Previous studies[20,21,42] have demonstrated that hypertension is associated with renal impairment in the FHR, as shown by the ability to reduce or prevent renal impairment through the use of pharmacological agents that reduce blood pressure; our results, however, demonstrate that hypertension alone is not sufficient to cause renal impairment. That the major loci explaining renal impairment in the FHH × ACI backcross are not those causing high blood pressure simply indicates that the majority of animals in the backcross have blood pressure levels *permissive* for the development of renal complications. In such a setting, the development of renal impairment appears to depend on the inheritance of downstream susceptibility factors, such as *Rf-1*. The fact that systemic blood pressure alone does not determine subsequent renal impairment is consistent with indirect observations from other rat strains. For example, the Milan normotensive rat develops renal sclerosis, while the Milan hypertensive rat does not, despite having higher blood pressure[43].

Genetic mapping alone does not reveal the biological role of *Rf-1* or *Rf-2* in hypertension-associated renal impairment. One possibility is that renal impairment may depend primarily on glomerular capillary pressure. High glomerular capillary pressure has been proposed as a precursor and initiator of renal damage[15,44,45]. Consistent with this notion, Simons *et al.*[15] have shown that FHH shows increased glomerular capillary pressure as compared to the normotensive WAG rat as early as 8 weeks. By contrast, the SHR rat, despite having high systemic blood pressure, shows normal glomerular filtration rate and does not develop renal impairment up through age 58 weeks[46]. Genes such as *Rf-1* may control the extent to which increased systemic blood pressure is translated into increased glomerular capillary pressure. This hypothesis can be tested by directly measuring glomerular capillary pressure in a subsequent FHH × ACI backcross; one could then study whether glomerular pressure adequately explains renal impairment and whether it genetically maps to *Rf-1* and/or *Rf-2*.

A complete understanding of these genes will clearly require their isolation. Based on the current rat genetic map[23], there are no obvious candidate genes in the region. Cloning of the rat genes would allow isolation of the human homologues and direct examination of variation in the genes in patients and families. At present, the regions of synteny conservation between rat and human for both *Rf-1* and *Rf-2* are not known.

Implications for human disease. The finding that distinct genes control the development of renal disease in a hypertensive animal model, raises the possibility that the same may be true for humans with hypertension who progress to ESRD. Clinical data and epidemiological data support this hypothesis, since improved blood pressure control has reduced the incidence of stroke and coronary artery disease, but not renal disease[1]. Moreover, ESRD occurs in only a subset of human patients with hypertension. Familial clustering of hypertension-associated complications has not been extensively studied, although a few reports based on a small number of patients have suggested familial correlation[10,34]. The genetic basis of complications in humans deserves more focused attention. It would be useful to ascertain a large collection of hypertensive affected-relative pairs to study whether risk of renal failure correlates between affected-relative pairs and, if so, to map human susceptibility factors. The regions of the human genome homologous to those containing *Rf-1* and *Rf-2* would provide initial candidate regions to test for such linkage.

In conclusion, our study provides evidence of genetic factors affecting the risk of hypertension-associated complications, independent of their effect on blood pressure. Although our studies are confined to a specific end-organ complication in a specific animal model, they suggest that complications of various common diseases may depend on inherited susceptibility factors. Understanding such genes may have important implications for the prevention of such complications and their associated morbidity and mortality.

Methods

Animals. The inbred strain of FHR, called FHH/EUR, is maintained at Erasmus University Medical School. It was derived from an outbred strain of FHR that was introduced to Europe by Tschopp in the early 1970s[47] and maintained as a closed randomly bred colony. The colony was transferred to Unilever Research Laboratories at Vlaardingen, The Netherlands, where it was maintained as a closed outbred strain until the mid-1980s, when an inbreeding program of strict brother–sister mating was initiated. Inbreeding was continued (by A.P.P.) and two inbred strains, FHH and FHL, differing in hypertension and proteinuria were selected. The colony was then transferred to Erasmus University. The FHH strain has higher levels of blood pressure and proteinuria and develops renal impairment at a younger age than FHL[48]. The FHH strain is homozygous for three recessive coat colour markers: red-eyed dilution (*r*), non-agouti (*a*), and hooded (*h*). All FHH rats used were from the 15th or 16th generation of inbreeding. ACI rats (AxC9935 Irish) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and are characterized by low UPV levels. They carry the wild-type allele at the *r*, *a*, and *h* loci.

Crosses. (FHH × ACI)F_1 × FHH backcrosses were arranged at Erasmus University. Each of the four possible arrangements of parental sexes was used, but phenotypes showed no correlation with parental sex and thus all backcross progeny were pooled for the purpose of analysis. A total of 134 male and 96 female backcross progeny were produced, of which only males were studied here. Rats were given access to food and water *ad libitum* and maintained on a 12-h diurnal period.

Phenotypes. Various phenotypes associated with hypertension and renal impairment were recorded (Table 1). SBP was mea-

Table 3 Genetic markers for rat chromosome 1

Locus[a]	Forward Primers (5'–3')	Reverse Primers (5'–3')
D1Mgh12	GCTTGCTGTACAAACCTCAGG	CAGCACGGAAGATACAAGCA
D1Mit2	ACTCAAATTCAGCTCAAATCTGC	TAGAACAACATTCACCCGACC
D1Mit3	ACTTGGTGAAGAAGAGTCAGGG	GATTTACTGTGCCTGTGGTTTT
D1Mit4	CAGTCAGAACAATGGTGCTCA	AAAGAAAGATGAAGTGCACGC
D1Mit5	AAAGTGGGGTACTCTCTATGGG	TAGGGTGATACAAGGCAGGG
D1Mit6	AGAGCAACTTCCAAACATATAGG	TGTCAATTGACCCACAGGAA
D1Mit8	ATGTTTTCCTCTGTAAGAGTTGCC	CTGTGTGCATGTGTGTGTATACG
D1Mit14	TTCCATCTACTGCTGTTTAGGG	TCTGCCTTCTCACATGAACA
D1Mit17	GTGTATGTATGGGTGCGTGC	TGGAAAGGTGGAGACAAATG
D1Mit18	GTTAGTAGCTATGAAATCATGTGGG	TAGCAAGAGAAAAGAGAGTCAACC
D1Mit20	CTGTCCCCTCTTCCCTTGTC	AGGGAGGGAGGAAAGAAGAG
Mt1pa	TGTAATGGAATCTGATGCCC	GGGCTCTATAGATAGGAGGTTTTAT

[a]Genetic marker designation, gene names when primers were taken from published sequences[51].

sured at 6 months by indirect tail-cuff method in awake, restrained animals, using a sphygmomanometer (Narco Biosystems, Houston). Direct measurements were not used because FHH rats have a bleeding disorder. Animals were trained by exposing them to restraint five times prior to blood pressure measurements. Measurements were performed on three consecutive days and the mean of these three values was used.

To collect urine for assessment of UOs, UPV and UKV levels, the animals were kept in metabolic cages. Excretion was measured for two consecutive 24-h urine samples. Urinary protein values were determined colorimetrically using a pyrogallol red molybdate complex. Kallikrein-like activity in the urine was determined by an amidolytic assay, with chromogenic substrate *D*-Val-Leu-Arg-pNa (S-2266, Kabi, Greenwich, CT) and, as expected, UKV had a significant lod score at the genetic marker for renal kallikrein (Kalb2; lod score = 3.0). Urinary measurements were made at 6 months and repeated just prior to sacrifice at 9 months, with the exception of UKV which was measured only at 9 months. Because the FHH have a reduced lifespan due to renal impairment, animals were studied at 9 months to ensure there would be sufficient animals for the study.

After completion of metabolic measurements, rats were sacrificed by decapitation and kidneys were removed and weighed. Plasma samples were obtained at autopsy and albumin, creatinine and urea levels were assessed by standard clinical chemistry techniques[49,50]. The extent of renal deterioration (MRIS) was assessed based on the macroscopic appearance of the kidneys. MRIS were assigned according to the following qualitative scale: MRIS 0 = normal kidney, no visible surface changes; MRIS 1, mild damage — isolated lesion(s) seen on < 10% of the kidney surface; MRIS,2, moderate damage — clear damage present covering < 50% of the kidney surface; MRIS3 , marked damage — damage covering > 50% of the kidney surface; MRIS4 , severe damage — damage covers >90% of the kidney surface, and MRIS5, very severe damage — kidney is completely scarred but the animal is still alive, MRIS6, terminal damage — as in MRIS5 but the animal had died prematurely. MRIS scoring was performed by a single technician blind to genotype. Of the 126 animals studied, the numbers with scores 0, 1, 2, 3, 4, and 5 were 46, 0, 47, 22, 9 and 2 respectively. Because the phenotypic distribution was skewed by some severely affected animals, scores were also regrouped to obtain a normalized MRIS consisting of three categories: 0 for animals with MRIS = 0 (n = 46); 1 for animals with MRIS = 2 (n = 47); and 2 for animals with MRIS = 3, 4 or 5 (n = 33). Analyses were performed with both scores. When a normalized MRIS is used, the result appears essentially the same: lod 8.1, 39% variance. The result of this additional analysis ensures that the linkage finding is not caused by a few severely affected animals.

Complete phenotypic data was obtained and analysed for 126 of the 134 male backcross progeny. Five rats died before phenotyping was begun and one died of renal failure before phenotyping was completed. One rat was terminally ill at the time of phenotyping and gave phenotypic values for UPV far outside (> 3 s.d.s) the range for the remaining progeny. Inclusion of this animal in the analysis had no significant effect. One animal did not have UPV/9 or UKV measured.

Genetic markers and genotypes. We have previously identified a large number of rat SSLPs in the course of our efforts and efforts by other groups to construct a genetic map of the rat[23,25,51]. Allele sizes for 200 genetic markers were initially determined for FHH and ACI, of which 130 proved to be polymorphic between these two parental strains; this subset of markers is estimated to cover about 85% of the rat genome. PCR primers for genetic markers used in the linkage map of rat chromosome 1 are shown in Fig. 1 and listed in Table 3. Additional information on genomic markers for the rat[23] may be accessed through the WWW at the address "hhtp://www-genome.wi.mit.edu". A list of markers used is available on request. The chromosomal location of 7 markers (*R261, R382, R734, R818, R1091, R1124* and *R1686*) is unknown but because there were no significant lod scores associated with them, further experiments to determine their location were not persued.

Of the 126 male backcross progeny for which complete phenotypes were available, a randomly chosen subset of 46 progeny was initially genotyped with all 130 informative SSLPs, as well as the three coat-colour markers (*r, a, h*) segregating in the cross. For genomic regions showing a lod score >1.0, the remaining 80 animals were genotyped. We calculate that our study design (requiring a lod score ≥1.0 in the initial 46 progeny, followed by a lod score ≥ 3.0 in the full cross) has 50% power to detect loci accounting for 10% of the total phenotypic variance in the backcross.

Genomic DNA sample was prepared from either rat tails or spleens of the backcross progeny[52] and was diluted to 4 ng/μl stocks in sterile, distilled water. Genotyping with SSLPs was performed essentially as previously described[23].

Somatic cell hybrids. Chromosomal assignment for markers linked to a QTL were confirmed by PCR testing of DNAs from a rat-on-mouse somatic cell hybrid panel, generously provided by C. Szpirier[53]. Because somatic cell hybrid mapping involves scoring presence or absence of a band, each PCR reaction for a rat locus also included a PCR primer pair yielding a band in the mouse host genome (*D8Mit16*), to serve as an internal positive control for amplification.

Genetic linkage analysis. Genetic markers were mapped relative to each other by using the MAPMAKER computer package[54], using an error detection procedure[55]. QTLs affecting phenotypes were mapped relative to genetic markers by using the MAPMAKER-QTL computer package[54]. Briefly, the program calculates the most likely phenotypic effect of having genotypes FHH/ACI or FHH/FHH at a putative QTL and then calculates a lod score reflecting the strength of evidence for the existence of the QTL and the proportion of the phenotypic variance explained[56]. To correct for the effect of multiple hypothesis testing, a threshold lod score of approximately 3.0 is required to establish significant linkage and is roughly equivalent to a genome-wide significance level of $P = 0.05$ [56]. The distribution of phenotypes for the traits of UPV, PAlb and PCr were normalized with a log transformation. MRIS values were evaluated as gathered and also regrouped as described above. Phenotypic comparisons for different genotypic groups were performed by using a Student's t-test or an

analysis of variance with a *post hoc* test using a modified t-test with a Bonferoni adjustment (DataDesk).

Multiple QTL analyses were conducted within the Mapmaker/QTL software. Briefly, identified QTLs are 'fixed', removing that portion of the variance that is explained by that locus from the subsequent analysis. The genome is then rescanned to identify additional QTLs. In this same manner, phenotypes can be 'fixed' and the genome rescanned to test the effects of one phenotypic trait on another[54]. In addition, residual analyses for PAlb, UPV, and MRIS regressions vs. SBP were performed and the results analysed in Mapmaker/QTL.

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