Locating a Blood Pressure Quantitative Trait Locus Within 117 kb on the Rat Genome
Substitution Mapping and Renal Expression Analysis

Michael R. Garrett, Haijin Meng, John P. Rapp, Bina Joe

Abstract—Previously, a blood pressure (BP) quantitative trait locus (QTL) on rat chromosome 9 (RNO9) was localized to a <2.4 cM interval using congenic strains generated by introgressing segments of RNO9 from the Dahl salt-resistant (R) rat into the background of the Dahl salt-sensitive (S) rat. Renal gene expression using Affymetrix gene chips was profiled on S and a congenic strain spanning the 2.4-cM BP QTL interval. This analysis identified 20 differentially expressed genes/expressed sequence tags. Of these, the locus with the greatest differential expression (30- to 35-fold) was regulated endocrine-specific protein 18 (Resp18), which also mapped in the 2.4-cM BP QTL interval. Additional substitution mapping located the QTL to <0.4 cM or ≈493 kb. This newly defined QTL region still included Resp18. Nucleotide variants were identified between S and R genomic DNA of Resp18 in the coding, 5’ regulatory and 3’ untranslated regions. The coding sequence variation (T/C) occurs in exon 2 and predicts an amino acid change (Ile/Val) in the protein product. Resp18 was considered a differentially expressed positional candidate for the QTL. To fine-map the BP QTL, we constructed a congenic strain with a smaller introgressed region. Compared with the S rat, this strain (1) had significantly lower BP, (2) did not contain the R form of Resp18, and (3) did not retain the rather spectacular differential expression of Resp18. Together, these results demonstrate that a BP QTL independent of Resp18 exists within the newly defined 117-kb QTL region on RNO9. (Hypertension. 2005;45:451-459.)

Key Words: hypertension, genetic \[\text{genes} \] cardiovascular diseases

In the 1960s, Lewis Dahl selected rats for sensitivity (S rats) or resistance (R rats) to the hypertensive effect of high-salt (NaCl) diet.\(^1\) Inbred strains of S and R rats were subsequently developed from Dahl’s selectively bred stocks.\(^2\) Dahl rats and other hypertensive strains have undergone extensive genetic analysis in the form of describing quantitative trait loci (QTLs) for blood pressure (BP) and the development of congenic strains around these QTLs.\(^3,4\) A compilation of BP QTL in the rat is on the Rat Genome Database (www.rgd.mcw.edu).

Identifying the genetic variation responsible for a QTL in any species is a challenge, but more than a few have been described in plants, Drosophila, and mammals.\(^5\) For Dahl rats in particular, BP QTLs have been located on almost every chromosome in crosses involving Dahl S rats with a variety of normotensive strains. These are reviewed in Joe et al.\(^4\) We identified the causative gene for 1 BP QTL in Dahl rats on rat chromosome (RNO) 7\(^6,7\) using congenic strains for fine substitution mapping around a strong candidate gene (Cyp11b1).

However, it is somewhat more of a challenge to identify the causative gene for a QTL when there is no obvious candidate in a congenic region encompassing the QTL. Gene expression analysis using microarray technology is a recently popular way for finding candidate genes and has been discussed in the context of complex physiological traits such as hypertension.\(^8–10\) It is obvious a priori that contrasting alleles at a locus causing a QTL may or may not be expressed differently. Thus, the expression technology may or may not direct attention toward the correct candidate gene for a QTL. Admittedly, the technology is useful, but it can also be misleading. This article presents a striking example of the latter.

A BP QTL with a logarithm of odds score of 5.0 on RNO9 was identified previously in our laboratory using an F\(_2\) population of 233 rats derived from Dahl S and Dahl R strains.\(^11\) Localization of this BP QTL from a 34.2-cM interval to a 2.4-cM region on RNO9 was accomplished by multiple iterations of substitution mapping using congenic strains.\(^12\) In the present work, we used microarray technology to find a differentially expressed candidate gene in this 2.4-cM region. Further substitution mapping was applied to evaluate the candidate.

Methods

A brief summary of the methods used is given below. An expanded version of the Methods section is available online at http://www.hypertensionaha.org.
Animals
Inbred Dahl salt-sensitive (S) and salt-resistant (R) rats were maintained in our animal facility. The construction of the progenitor congenic strain and the methods for construction of subsequent congenic substrains were described previously. Other inbred rats used in this study, namely Lewis (LEW), spontaneously hypertensive rats (SHR), and Wistar Kyoto rats (WKY), are all from our colony, the original sources of which are as described previously.

GeneChip Microarray Experiment and Data Analysis
Three male S control and 3 male congenic S.R(9)x3A rats born on the same day were selected, weaned at 30 days of age, and caged with 1 congenic and 1 S rat per cage. They were raised on a low-salt (0.3%) diet (Harlan Teklad diet TD 94217) for 24 days. Starting from 39 to 42 days of age, rats were fed a 2% NaCl diet (Harlan Teklad diet TD 7034; Harlan–Sprague-Dawley) and killed at 51 days of age for RNA isolation. cRNA was prepared for each rat, fragmented as suggested by Affymetrix technical manual, and simultaneously hybridized (15 hours). RNA from animals listed as set 1 under the Quantitative Real-Time PCR section of the expanded Methods (see online supplement) was reverse transcribed and subjected to real-time PCR analysis. The data shown used primers directed at exon 6 (forward-GAATTTGCCCCTTGGAG, reverse-GTTTCTTTGGGCC-ATTCC) and normalized to the β-actin control. Similar data were obtained when data were normalized to GAPDH or using primers directed at exon 2 to 3 (forward-CTTACACGTTCTATTCCACCACA, reverse-ATGGAGTCTGCTGAGGTGCT). Error bars are SEM.

Quantitative Real-Time Polymerase Chain Reaction
Real-time polymerase chain reaction (PCR) was performed using the My q single-color real-time PCR detection system using the BioRad iQ SYBR green method. Two different controls were used: β-actin and GAPDH. A melt-curve analysis was performed for each sample to demonstrate a single PCR target was amplified. Statistical analysis of expression data was performed using an independent sample t test (Figure 1) or ANOVA with multiple comparisons (Figure 2) using SPSS software (SPSS).

Results

Genome-Wide Expression Profiling With GeneChip Microarray
To determine the renal gene expression of positional candidates within the BP QTL region on RNO9, cRNA from S and a congenic strain S.R(9)x3A were compared using Affymetrix gene chips. Based on 3 statistical analyses (ie, Student t test, change call count test, and Mann–Whitney test), 20 annotations were consistent as differentially expressed between S and S.R(9)x3A. The Table lists the annotations/descriptions of each of the differentially expressed genes and expressed sequence tags (ESTs), along with their chromosome location and fold change. Two of these differentially expressed genes (regulated endocrine-specific protein 18 [Resp18] and glutathione transferase 1 [Gsta1]) were located on RNO9. However, Resp18 but not Gsta1 mapped to the BP QTL region. Resp18 was expressed 7.3-fold lower in the congenic rat compared with the S.

Confirmation of Resp18 Renal Differential Expression
Renal expression of Resp18 was compared between S rats and the congenic strain S.R(9)x3x2C by quantitative real-
Differentially Expressed Genes and Expressed Sequence Tags in the Kidneys of S and S.R(9)×3A Congenic Rats

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<th>Congenic S</th>
<th>Fold Change</th>
<th>Chr.</th>
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<th>Gene Description (GenBank Accession No.)</th>
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Affymetrix U34 microarray chips were used. The table gives the average signals (in arbitrary units) of 3 replicates for the probes listed (SD is in parentheses), fold change of average signals, and chromosomal locations and gene descriptions. For the purpose of comparison, S array sets were arbitrarily assigned as the baseline and the congenic array sets as the experimental arrays. Thus, a negative number for fold change indicates that the congenic was lower than the S; a positive number indicates that the congenic was higher than the S; Un, indicates unknown; Chr., chromosome.

time PCR. The congenic strain, which contains the R form of Resp18, had 30- to 50-fold lower expression compared with the S rat (P<0.0001; Figure 1). The expression difference (30- to 50-fold) determined using real-time PCR was greater than the difference as determined using the Affymetrix gene chips (7-fold). The differential expression of Resp18 remained unchanged between the S rats and the congenic rats in response to acute changes of NaCl in their diet (Figure 1). Although Resp18 was detected in the kidney, it is mainly expressed in the brain, especially the pituitary and the hypothalamus. However, there was no expression difference (the fold change is equal to 1) detected in the pituitary between the S and congenic rats, either for animals on low salt or in response to acute changes in dietary NaCl (Figure 1).

Inbred Strain Survey for Resp18 Renal Differential Expression

Real-time PCR was performed on kidney samples from 4 inbred strains (R, LEW, WKY, and SHR) and compared with the expression of Resp18 in the S rat (Figure 2). Figure 2 shows that there is no significant change in Resp18 expression between the S and either the LEW or SHR (ie, the fold change is equal to 1). However, there was...
a significant 2-fold (P<0.0001) expression difference between S and WKY, with WKY less than S. The 50-fold difference (P<0.0001) seen in Figure 1, with the R allele less than the S allele, is also seen in the comparison of S and R and in the comparison of S and congenic strain S.R(9)x3x2C in Figure 2. These data demonstrate that the R form of \( \text{Resp18} \) is expressed at much lower levels when compared with the S and, therefore, also at much lower levels than LEW, WKY, and SHR.

**Single Nucleotide Polymorphisms**

Sequencing of \( \text{Resp18} \) from S and R genomic DNA revealed several nucleotide variants \( S' \) to the gene and also a coding sequence variant in exon 2 (Figure 3). Using a panel of genomic DNA from 6 inbred strains, 6 single nucleotide polymorphisms (SNPs) were identified in the 1.5-kb region upstream of the \( \text{Resp18} \) gene. Four of the SNPs (at positions \(-218, -422, -637, \) and \(-1063 \)) were unique to the R strain, whereas the SNP at position \(-537 \) was unique to the WKY.
A coding sequence variant was found in exon 2 at position 272, where it is a thymine in the S and a cytosine in the R. This nucleotide substitution results in an amino acid change of the predicted protein from Ile in S rats to Val in R rats. The SNP at exon 2 is unique to the R strain. All other inbred strains sequenced carried the S-allelic variant (ie, thymine at nucleotide position 272 of exon 2).

Fine-Mapping the BP QTL to a 493-kb Region
Concurrent with the renal expression profiling using Affymetrix gene chips, substitution mapping was used to further localize the BP QTL region. Figure 4 depicts the congenic strains used to further localize the previously described BP QTL on RNO9. In Figure 4, the congenic strains S.R(9)x3A (used for renal expression profiling) and S.R(9)x3F were reported previously. The other 2 (ie, S.R(9)x3x3A and S.R(9)x3x4A) are newly constructed congenic strains (Figure 4). The BP of congenic strains S.R(9)x3x3A and S.R(9)x3F was not significantly different from their S controls. Therefore, these 2 strains are referred to as the negative strains. However, the S.R(9)x3A congenic strain, which has an introgressed region that spans the interval between the 2 negative strains, significantly lowered BP (−19 mm Hg; \( P<0.0001 \)) compared with the concomitantly studied S rats. Comparing these 3 strains, the BP QTL can be localized to a 493-kb region, as indicated in Figure 4.

The congenic strain S.R(9)x4A (Figure 4) also confirmed the BP effect and the localization of the QTL. BP of this strain was measured by 2 methods: tail-cuff and telemetry. By the tail-cuff method, S.R(9)x4A had significantly lower BP (−21 mm Hg; \( P<0.0001 \)) compared with S, which was consistent with previous experiments. For BP measurements by telemetry, a group of 8 S and 8 S.R(9)x4A were selected randomly from those animals that had their BP taken by the tail-cuff method. Telemetry probes were implanted. One week later, BP data were collected for a period of 8 days. S.R(9)x4A had lower BP ranging from −23 mm Hg (postoperative day 7) to −35 mm Hg (postoperative day 14) compared with S (Figure 5, left panel), thus confirming BP measurements by the tail-cuff method.

The introgressed region of the congenic strain used to conduct the renal expression analysis [ie, S.R(9)x3A] spans roughly 6 Mb on RNO9 and contains 78 known and predicted genes. Substitution mapping was successful in refining the position of the QTL to 493 kb containing 23 known and predicted genes. Substitution mapping was successful in refining the position of the QTL to 493 kb containing 23 known and predicted genes, thereby eliminating the need to further characterize the other 55 genes contained in the congenic strain used in the expression analysis study. Thus, this further substitution mapping eliminated >70% of the genes within the congenic interval “trapped” in S.R(9)x3A, but the differentially expressed gene (Resp18) still was within the newly reduced QTL region of <493 kb (Figure 4).

High-Resolution Mapping of the BP QTL to a 117-kb Region
Figure 6 shows additional substitution mapping that localized the BP QTL to a small genomic interval. Portions of congenic strains S.R(9)x3F and S.R(9)x3x2C from Figure 4 are shown again in Figure 6 because they are important in delineating the QTL region. The other 2 congenic strains (ie, S.R(9)x3x2C and S.R(9)x3x2Bx1) are new congenic strains. S.R(9)x3x2C had significantly lower BP (−27 mm Hg; \( P<0.0001 \)) compared with S as measured by the tail-cuff method. BP of this congenic strain was also measured by telemetry. BP of S.R(9)x3x2C by telemetry was lower than S control rats, and the difference in BP from day 7 to day 14 (postoperative) ranged from −25 to −34 mm Hg (Figure 5, right panel). Thus, the magnitude of change in BP between S and S.R(9)x3x2C was independent of the method of BP measurement. Although data obtained from S.R(9)x3x2C did not improve the mapping of the QTL, it provided additional BP data and confirmation for the location of the BP QTL.

BP of the congenic strain S.R(9)x3x2Bx1 and concomitantly raised control S rats was measured by telemetry only. S.R(9)x3x2Bx1 had significantly lower systolic BP, diastolic BP, and pulse pressure compared with the S control (Figure 4).
For the most part, there was no detectable difference in heart rate between the strains (Figure 7). A major portion of the introgressed region of S.R(9)x3x2Bx1 overlaps with the S.R(9)x3F congenic strain, which does not have a significant effect on BP (Figure 6). This means that the BP QTL is located within the interval that is the differential segment between the lower ends of S.R(9)x3x2Bx1 and S.R(9)x3F. This region is <117 kb in size (Figure 6).

Renal mRNA levels of Resp18 in the congenic strain S.R(9)x3x2Bx1 were compared with that of S rats by real-time PCR. Data presented at the bottom of Figure 6 indicate that unlike the differential expression of Resp18 observed between S and the congenic strain S.R(9)x3x2C, there was no differential expression of Resp18 between S and the congenic strain S.R(9)x3x2Bx1. Because the congenic strain S.R(9)x3x2Bx1 retained a BP-lowering effect even in the absence of differential expression of Resp18 compared with S rats, these data provide clear evidence for the existence of a BP QTL that is independent of the differences in transcriptional regulation of Resp18 between S and S.R(9)x3x2C congenic rats. In other words, the observed differential expression of Resp18 was uncoupled from the BP-lowering effect of the QTL (Figure 6).

An interesting feature about the congenic strain S.R(9)x3x2Bx1 is the region where the lower recombination occurred (ie, between the markers D9Mco43 and Resp18–intron 2 shown in Figure 6). This region is expanded in Figure 8 alongside the schematic of the Resp18 gene structure. Note that Resp18 is oriented on the chromosome with the 5′-end toward the distal end of the chromosome (Figure 8). This means that recombination occurred within, or very close to, the Resp18 gene. To fine-map the site of recombination and determine which allele of Resp18 is present in S.R(9)x3x2Bx1, the region between D9Mco43 and Resp18–exon2 was sequenced using S and R genomic DNA. The S and R genomes in this region are identical except for 3 blocks.
of sequence variations, represented by a deletion of 13 bases (Figure 8, deletion 1) and 2 insertions in the R genome (insertion 1 of 3 bases and insertion 2 of 1 base; Figure 8). The congenic strain S.R(9)x3x2Bx1 contains the R allele at deletion 1 and insertion 1, both of which are located away from the 3'-untranslated region (UTR) of Resp18 and the S allele at insertion 2, which is in intron 2 of Resp18. Because the portion of the genomic DNA sequence of Resp18 from deletion 1 and insertion 1, both of which are located away from the 3'-untranslated region (UTR) of Resp18 and the S allele at insertion 2, which is in intron 2 of Resp18. Because the portion of the genomic DNA sequence of Resp18 from
insertion 2 to the 3′-UTR (Figure 8) is identical between S and S.R(9)x3x2Bx1, Resp18 was eliminated as the underlying cause of the BP QTL trapped within the S.R(9)x3x2Bx1 congenic strain. The interval between insertion 1 and the 3′-UTR of Resp18 defines the lower end of S.R(9)x3x2Bx1.

Discussion
In this study, we sought to identify positional candidate genes for a BP QTL on RNO9, the levels of renal mRNA of which were different between S and S.R congenic rats. At first, evidence from the renal gene expression profiling approach pointed at Resp18 as a differentially expressed candidate gene within a small region of <493 kb containing the BP QTL. The R allele of Resp18 was expressed at much lower levels compared with the S or any other inbred strain tested. The R rat, among all the strains tested, also contained the most sequence variants in the putative 5′ promoter region (Figure 3). The function of Resp18 is unknown, but evidence from the literature indicated that it could be involved in the action of dopamine.\(^1^4\) Dopamine in the kidney is involved in regulating sodium balance, and therefore, BP.\(^1^5\)–\(^1^8\) Thus, Resp18 remained an important positional and physiological candidate gene for the BP QTL on RNO9. Further substitution mapping indicated that neither the Resp18 gene nor its differential expression is a requirement for the BP effect of the QTL residing within the newly defined region spanning <117 kb. This is a major improvement over our previously published localization of this BP QTL.\(^1^2\)

The experimental design of our study has enabled us to detect a BP QTL within 117 kb on RNO9. R alleles of this QTL lower BP in the congenic rat irrespective of whether there are S or R alleles at a nearby locus, Resp18. One might ask whether the converse is true (ie, whatever alleles of Resp18 have a role in controlling BP), which is independent of S or R alleles at the BP QTL within the 117-kb region. This issue is not addressed in our report. Note that the BP effects of congenic strains with either R alleles at Resp18 (Figure 6; S.R(9)x3x2C) or S alleles at Resp18 (Figure 6; S.R(9)x3x2Bx1) were comparable (Figure 6). This indicates that the BP effect of Resp18, if at all present, is not additive with the BP effect of the QTL within the 117-kb region. On the other hand, the genetic association of Resp18 expression differences with the QTL could be an example of genetic drag that occurred during the selective breeding of S and R rats. That is, the actual locus causing the BP QTL on RNO9 is so closely linked to Resp18 that selection for the S or R alleles at the causative locus necessarily also selected for S or R alleles at Resp18.

There is at least 1 other example in which the differential expression of a gene was thought to account for a BP QTL, but it was subsequently eliminated by substitution mapping. The SA gene on RNO1 was initially discovered by its differential expression in the kidney between SHR and WKY.\(^1^9\) It was contained in a BP QTL when the QTL was only localized to a broad region in congenic strains.\(^2^0–^2^3\) However, the SA gene was eliminated as causative for a BP QTL on RNO1 by subsequent finer substitution mapping using congenic strains\(^2^4–^2^6\) and using an SA knockout mouse.\(^2^7\)

The 117-kb BP QTL region on RNO9 represents 0.0043% of the rat genome, the total size of which is estimated at 2.75 Gb.\(^2^8\) To date, this is the smallest region within which a BP QTL has been mapped. The only other rat BP QTL mapped to a similarly small region is the BP QTL on RNO7, which we localized to a <177-kb segment.\(^7\) In this case, the causative gene was shown to be steroid 11β-hydroxylase (Cyp11b1).

Perspectives
The use of expression profiling to identify causative gene(s) underlying QTL, although proven to be very useful in some cases of QTL gene identification,\(^2^9–^3^2\) should be interpreted with caution. The fact that Resp18 remained a positional candidate for the BP QTL, even as the QTL was narrowed to a relatively small region of <493 kb, gives insight into how the detection of a positional candidate gene can be masked by other genes, such as Resp18, that are closely linked, markedly differentially expressed, and contain sequence variants. The results provided in this study indicate that substitution mapping to localize QTL, although considered a time-consuming procedure,\(^3^3\) remains a required exercise.

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References


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