Congenic strains provide evidence that four mapped loci in chromosomes 2, 4, and 16 influence hypertension in the SHR

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HYPERTENSION is a complex phenotype in which the cumulative effects of many genetic determinants influenced by environmental factors contribute to a variety of phenotypes. Rat models of spontaneous hypertension were developed beginning in the late 1950s by brother-sister mating the successive offspring of an initially random rat population and concomitant selection for high blood pressure (BP), presumably fixing a number of unknown alleles that contributed to the hypertensive phenotype. These animals captured features of the large spectrum of BP-associated phenotypes observed in human hypertension (19). Among them, the spontaneously hypertensive rat (SHR) has been instrumental in advancing our understanding of how BP control systems are modulated throughout development or on challenge with a variety of environmental stimuli (27). However, the genetic determinants underlying the disease in these experimental models and in humans remain largely elusive.

Quantitative trait locus (QTL) mapping has been applied to identify chromosomal regions containing putative genetic variant(s) that affect quantitative traits, such as BP (8, 9). In this regard, several studies have successfully identified a number of BP-related QTLs in the rat with a variety of crosses between normotensive and hypertensive strains and analysis of their progeny with informative panels of molecular markers covering the rat genome (3, 5, 7, 20). This powerful analytical strategy, however, requires additional validation including synthetic approaches such as the development of congenic strains in which the mapped QTL chromosomal region is exchanged among different genotypes. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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careful evaluation of the effects emerging from interactions between multiple QTLs. Such studies may facilitate our understanding of the genomic context-dependent interactions among different QTLs and shed light on the possible physiological mechanisms governing these interactions. Ultimately, such information may play a pivotal role in identifying candidate genes and designing appropriate studies to test their roles in the physiopathology of hypertension.

We previously reported (20) the identification of five BP-related QTLs with a genomewide search that explained ∼38% of the BP variance under high salt intake in an intercross between the SHR and Brown Norway (BN) rat strains. In the present study we report the development of congenic lines to assess the physiological roles of four of the five BP QTLs. Finally, the analytical predictions using the data from the mapping study (F2s) were compared with the results from the rat congenic lines to gain additional insight on the influence of the genetic background on the role of these QTLs in BP variance. Our data reveal unique physiological characteristics associated with each QTL, unforeseen by the analysis of the F2 cross.

MATERIALS AND METHODS

Animals and generation of congenic strains. Inbred colonies of SHR and BN rats were maintained at the University of São Paulo Medical School animal facility. Four congenic strains, for chromosomes 2 (SHR.BN2a and SHR.BN2c), 4 (SHR.BN4), and 16 (SHR.BN16), were established by using a backcross marker-assisted breeding schedule to fix the BN interval (donor) onto the SHR genome (recipient). This required the production of an F1 generation through the crossing of the normotensive BN rat and the hypertensive SHR. Male F1s, from the intercross between male SHR, to fix the Y chromosome, and female BN rats, were backcrossed against the SHR parental strain. Animals were selected for the presence of BN markers within the desired interval and for SHR markers in the remainder of the genome before each new round of backcrossing. After an average of 10–13 successive generations of selective backcrossing, the BN genetic background was eliminated by >99.9%, as indicated by 83 polymorphic markers (Supplemental Table S1). Once the segment of interest was fixed, selected male and female animals were crossed to obtain homozygous animals for each congenic strain. All rats were housed under controlled conditions of temperature (21°C) and light (12:12-h light-dark cycle) and maintained on normal rat chow and water ad libitum. The experimental procedures followed institutional guidelines for care and use of laboratory animals and were approved by the Institutional Review Board of the University of São Paulo Medical School (no. 019/06).

DNA extraction and genotyping. Congenic animals (4–6 wk of age) were briefly anesthetized with ether, and a 10-mm tip from the tail was surgically removed and placed into a 1.5-ml microfuge tube. Genomic DNA was isolated from tail snips by proteinase K digestion and ethanol precipitation, and samples were genotyped by PCR amplification of DNA. Primers were selected based on their map locations and on their being polymorphic between parental strains (http://rgd.mcw.edu; http://www.genome.wi.mit.edu). The markers used in the screening protocol for generating congenic strains were essentially the same as reported previously for genomic scans on mapping BP QTLs (20). The congenic status of the background was confirmed with polymorphic microsatellite markers scattered throughout the genome (Supplemental Table S1). PCR reaction was performed in a total volume of 25 μl in the presence of 100 ng of genomic DNA, 1.25 mM dNTP mix, 0.1% Triton X-100, 50 mM KCl, 2 mM MgCl2, 10 mM Tris·HCl, each primer at 10 μM, as well as 2.5 U of Taq polymerase (Invitrogen). The thermal profile used on a Peltier thermocycler (PTC-200/MJRresearch) consisted of an initial 94°C denaturation for 3 min, followed by 35 cycles of 94°C (60 s), 58°C (60 s), and 72°C (60 s), and a 10-min 72°C extension at the end. After PCR, loading buffer (50% glycerol, 0.25% bromophenol and xylene cyanol) was added to each sample, and the amplification products were checked for the predicted sizes by 4% Agarose 1000 gel electrophoresis with ethidium bromide staining. Markers that could not be resolved under these conditions were amplified and resolved on denaturing polyacrylamide gel, stained with SYBR Green, and visualized on a PhosphorImager.

Experimental design. Male 12-wk-old rats were chosen from separate litters from each of the congenic lines to minimize potential environmental influences on phenotyping accuracy. Different groups of animals from each line received tap water or high salt loading (1% NaCl solution in drinking water) over 2 wk (SHR n = 23 and 24, SHR.BN2a n = 22 and 23, SHR.BN2c n = 22 and 23, SHR.BN4 n = 22 and 23, SHR.BN16 n = 22 and 23 for normal and high salt intake, respectively), followed by BP determination as described below.

Blood pressure measurement. For mean (MBP), systolic (SBP), and diastolic (DBP) BP measurements, femoral artery polyethylene indwelling catheters were implanted into the vessel and then tunnelled to the back of the rat for BP recording (20). In brief, the surgical procedure was performed in anesthetized rats 24 h before recordings. For BP recording, the arterial cannula was connected to a pressure transducer (P23Db; Statham, Hato Rey, Puerto Rico). The pulse pressure signal from the transducer was fed to an amplifier (GPA-4 model 2; Stemtech, Wood Dale, IL) and further to a 10-bit analog-to-digital converter (DataQ Instruments, Akron, OH). Recordings of SBP and DBP were obtained in the undisturbed state in conscious, freely moving animals, always in the afternoon. Measurements were made over a period of at least 30 min, and the mean values for SBP and DBP were calculated from the whole period of recording. BP was analyzed on a beat-to-beat basis at a frequency of 2.0 kHz. Heart rate (HR) was obtained from BP pulses.

Statistical analysis. The variables of BP (SBP, DBP, MBP, HR), and body weight were analyzed by analysis of variance (ANOVA), considering a 2 × 5 factorial design. The following sources of variation were taken into account: the main effects of rat strains and salt loading as well as the effects of the interaction between these factors. The study of significant effects was conducted with multiple-comparison tests between means and with an adjusted calculation of P value. Where P < 0.05, the values were considered statistically significant. Data are expressed as means ± SE.

The results of the analysis of the systolic pressure variable (SBP) are shown in greater detail in this article in order to J) study the impact of the interaction between strains and salt loading in such a way as to identify classes of phenotype variation, defined according to the regulation pattern of the four chromosomal regions under study, and 2) conduct a comparative analysis of the adjustment of the ANOVA model, considering the data from the congenics experiment and the data from the F2 experiment for mapping BP QTLs [analytically inferred from Schork et al. (20)].

Assuming an additive regulation mode among the four regions, one can obtain from Table 1 the additive genetic model, assuming there is no interaction effect between the four chromosomal regions, i.e., taking parameters ßq<sub>k</sub>, as being null. Considering the data from the congenics experiment and the additive genetic model, the parameters in Table 1 were estimated through adjustment of the classic ANOVA model [see, for instance, Neter et al. (14)] and the results were compared with the corresponding estimates inferred from the F2 data of Schork et al. (20). This being the case, the effect of the genetic background on the estimates of genetic parameters was analyzed by comparing the results of two independent designs of experiments.

1 The online version of this article contains supplemental material.

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Table 1 lists the contrasts between the means and parameters of the additive genetic model and the model with interaction. Under the specifications of the models with interaction between the four regions, these contrasts estimate the corresponding parameters \( \beta_{ij} \), enhanced by interaction effects. The bottom row of Table 1 expresses the expected mean of systolic pressure for SHR animals with the four regions of BN animals. As shown, in the presence of interaction effects between the loci one cannot expect to find the same estimates.

**RESULTS**

**Development of congenic lines.** Five rat congenic strains were expected through introgression of BN rat BP-lowering intervals for chromosomes 2, 4, 8, and 16 onto the SHR background. Two regions from BN rat chromosome 2 resulted in SHR.BN-(D2Rat144-D2Rat123)/Jk and SHR.BN-(D2Rat226-D2Rat294)/Jk, arbitrarily abbreviated as SHR.BN2a and SHR.BN2c, respectively; one from BN chromosome 4 designated as SHR.BN-(D4Rat28-D4Rat54)/Jk, abbreviated as SHR.BN4; and one from BN chromosome 16 designated SHR.BN-(D16Rat87-D16Mgh1)/Jk, abbreviated as SHR.BN16. The fifth congenic strain was not successfully developed because of breeding difficulties. Through rounds of backcrossing and marker-assisted selection of the offspring, we fixed each BP-lowering chromosomal interval on the N10-13 F3 generation. Brother-sister mating was then performed to obtain animals harboring the homozygous chromosome intervals that gave rise to the congenic strains described here. The size and position of each transferred BN chromosome segment as defined by testing with molecular markers are shown in Fig. 1.

**Influence of individual QTLS on blood pressure-related phenotypes.** Development of congenics provided evidence that all four mapped QTLs evaluated in this study affected SBP levels under normal or high salt intake or the salt sensitivity component of the BP observed in the SHR. Under normal salt intake SBP was reduced in congenics SHR.BN2a, SHR.BN2c, and SHR.BN4, whereas under high salt all but congenic line SHR.BN4 showed smaller SBP levels and the salt sensitivity phenotype was abrogated in congenics SHR.BN2a and SHR.BN16 (Fig. 2 and Supplemental Table S2).

In congenic rats fed a normal salt diet, QTLs mapped on chromosomes 2a, 2c, and 4 were associated with significant changes in SBP (13, 20, and 15 mmHg, respectively), whereas the QTL on chromosome 16 had no measurable effect under these conditions (Table 2). Nevertheless, on feeding the animals a high-salt diet the chromosome 16 QTL had a marked impact on SBP, as did the QTLs on chromosome 2a and 2c (18,
17, and 19 mmHg, respectively) but not the QTL on chromosome 4, despite its clear role under normal salt intake (Table 2).

Thus three classes of SBP-related phenotypes were revealed: QTLs 2a and 2c alter SBP regardless of salt intake (although congenic line SHR.BN2a was not salt sensitive), QTL 4 only under normal salt intake, and QTL 16 only associated with salt loading.

**Analytical prediction vs. experimental evidence: F2 mapping data vs. congenics.** It has been difficult to assess the predictions obtained with analytical tools, such as total genome scan to map QTLs, and to later validate their quantitative contribution to the genesis of complex phenotypes with synthetic tools. To this end, the effect of each QTL on high-salt SBP estimated from the linkage study using the F2 rat progeny [inferred analytically from Schork et al. (20) considering an additive genetic mode of inheritance] was compared with the experimental findings from the congenic lines described in the present study (Table 3). The increase in BP associated with each QTL showed the same trend, that is, BN alleles decrease BP and SHR alleles increase BP. It should be noted, however, that despite similar trends the quantitative changes between the F2s, in which each animal represents a unique mixed distribution of BN and SHR genomes, versus the congenics, in which there is a fixed SHR background, are not the same.

**Table 2. Blood pressure variation for congenics and SHR at 14 wk on either normal or high salt for 2 wk**

<table>
<thead>
<tr>
<th>Contrast</th>
<th>BP Variation</th>
<th>2.5% CI</th>
<th>95% CI</th>
<th>t Value</th>
<th>SE</th>
<th>P Adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>SHRs-BN2a</td>
<td>13.23</td>
<td>4.68</td>
<td>21.78</td>
<td>3.81</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>SHRs-BN2c</td>
<td>20.19</td>
<td>11.64</td>
<td>28.73</td>
<td>5.81</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>SHRs-BN4</td>
<td>15.28</td>
<td>6.73</td>
<td>23.83</td>
<td>4.40</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>SHRs-BN16</td>
<td>6.91</td>
<td>−1.64</td>
<td>15.46</td>
<td>1.99</td>
<td>3.47</td>
</tr>
<tr>
<td>High salt</td>
<td>SHRs-BN2a</td>
<td>17.25</td>
<td>8.68</td>
<td>25.82</td>
<td>4.96</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>SHRs-BN2c</td>
<td>18.95</td>
<td>10.48</td>
<td>27.41</td>
<td>5.51</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td>SHRs-BN4</td>
<td>7.89</td>
<td>−0.48</td>
<td>16.26</td>
<td>2.32</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>SHRs-BN16</td>
<td>18.10</td>
<td>9.73</td>
<td>26.48</td>
<td>5.33</td>
<td>3.40</td>
</tr>
<tr>
<td>Difference</td>
<td>SHRs (s − c)</td>
<td>8.67</td>
<td>−0.13</td>
<td>17.47</td>
<td>2.55</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>SHRs.BN2a (s − c)</td>
<td>4.65</td>
<td>−4.55</td>
<td>13.85</td>
<td>1.31</td>
<td>3.55</td>
</tr>
<tr>
<td></td>
<td>SHRs.BN2c (s − c)</td>
<td>9.91</td>
<td>0.82</td>
<td>19.00</td>
<td>2.82</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>SHRs.BN4 (s − c)</td>
<td>16.06</td>
<td>7.06</td>
<td>25.05</td>
<td>4.63</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>SHRs.BN16 (s − c)</td>
<td>−2.52</td>
<td>−11.52</td>
<td>6.47</td>
<td>−0.73</td>
<td>3.47</td>
</tr>
</tbody>
</table>

BN, Brown Norway; BP, blood pressure; CI, confidence interval; s, high salt; c, control.

To gain additional insight into these quantitative discrepancies, we estimated the role of each QTL first considering the mixed F2 background, followed by the SHR (congenics) background (Table 4). Arbitrarily, we designated the BN genome as 0 and the SHR genome as 1. Thus the first row of data in Table 4 represents a hypothetical SHR, with respect to the QTL intervals, since it has 1s for all four QTL columns. Similarly, the second through fifth rows depict each of the congenic lines and the estimated BP-lowering QTL effect on both genetic backgrounds, the mixed F2s and the fixed SHR in the congenics. The bottom row depicts the intercept values (μ estimation) from the regression analysis, or the estimated effects of all low-BP BN QTLs in both genetic backgrounds. The effect of alleles at putative QTLs near each marker was estimated by a linear regression-based genetic model assigning simple indicator variables to the rats based on genotype information. This linear model was previously described in detail by Schork et al. (20). In the presence of all four BP-lowering BN QTLs, high-salt SBP values were remarkably similar regardless of the genetic background, mixed BN-SHR versus SHR (133 ± 4 vs. 134 ± 9 mmHg, respectively). However, when adding either one by one or all four high-BP SHR QTLs at once, the estimated means for the congenics were always greater than the F2s. This finding is particularly important considering that two of the loci, 2a and 4, influence BP in a recessive mode and...
eventual heterozygosity would not interfere with the modeling, whereas for the two co-dominant loci, 2c and 16, the model may overestimate the differences in the case that the F2s are predominantly heterozygous.

Interestingly, if one assumes that there is genetic interaction among the QTLs, the estimated BP values in the absence of the four high-BP QTLs (bottom row, Table 1) would be lower since the overall effect would be greater than the sum of the individual contributions predicted from the mapping data assuming an additive model (bottom row, Table 1). The fact that the intercept values were not different gave further support to the hypothesis that these four QTLs indeed influence BP in an additive manner, as predicted previously (20).

DISCUSSION

Three major aspects emerge from the present study. First, we provide evidence, to our knowledge for the first time, that four of five significant SHR BP QTLs mapped under the same conditions give rise to independent congenic lines in which the individual role of each QTL in the control of BP phenotypes was experimentally verified and confirmed. Second, we uncovered physiological nuances underlying each QTL, evidenced by their unique abilities to influence BP under differing salt diets. Finally, by comparing the data from the congenic study presented here (the synthetic approach) with the inferred data from the F2 animals [the analytical approach from the BP QTL mapping (20) performed by the same group with comparable methods], we find no evidence for QTL-QTL interaction but provide an indication that the final expression of the mapped BP QTLs is influenced by other SHR alleles.

In the present study, the BN BP-lowering chromosomal intervals were introgressed into the SHR background. We chose this design instead of the other way around since we reasoned that it would be less likely to detect the effect of a single SHR BP-elevating QTL on a normotensive BN background with its entire compensatory BP control capacity. Indeed, we expected that placing a single BP-lowering QTL allele from the BN into the hypertensive strain would allow some improvement in BP control capacity, which would then be translated into a lower basal and/or salt-loaded BP phenotype in the congenic lines, as was observed.

The decomposition of the complex genetic architecture into its individual components, by introgressing BN alleles corresponding to each QTL into the SHR genome, revealed previously unsuspected nuances of the physiological roles of each QTL. As expected, MBP, DBP, and basal SBP values of the congenics SHR.BN2a and SHR.BN2c were significantly lower than the values in the SHR strain. A significant BP decrease of 20 mmHg was observed in the SHR.BN2c, which confirms the existence of a QTL located in the region between D2Rat226 and D2Rat294, as previously suggested by genomic scan mapping. Since our initial work, rat chromosome 2 has been repeatedly shown to contain QTLs with BP-raising alleles originating from various hypertensive rat models (2, 4, 6, 12, 17).

Transfer of a chromosome 4 segment from the BN strain into the SHR significantly reduced basal SBP compared with the SHR, indicating that a BP QTL mapped between markers D4Rat28 and D4Rat54 (~20 cM position). Interestingly, with the high-salt diet, these congenics exhibited a great increase in BP, which left them only 8 mmHg lower than, and not different from, the values observed in the SHR \( (P = 0.071, \text{Table 2}) \). These results may appear inconsistent with the mapping data that indicated that the most significant changes occurred under the high-salt diet, but it may also reemphasize the proposed genetic background influence over the BP QTL effect on two very particular conditions [F2 mapping (mixed BN and SHR) vs. congenics (pure SHR background)]. Pravenec et al. (18) demonstrated that transfer of a segment of chromosome 4 localized between markers IL6 and Npy (~0–42 cM position including Cd36 gene) from the BN rat into the SHR background resulted in BP lowering and improvement in fructose-induced glucose intolerance, hyperinsulinemia, and hypertriglyceridemia. More recently they provided evidence that lower expression of cd36 in the kidney is associated with an increase in BP (16). This region does not overlap with our introgressed interval on chromosome 4, but it is in close proximity to our originally estimated marker, Npy2.

The transfer of segments of chromosome 16 between markers D16rat87 and D16mgh1 (0.0–12.35 cM) from the BN strain into the SHR had no effect on basal BP but prevented BP increase from increasing on salt loading. Interestingly, Moujahidine et al. (13) replaced segments of the Dahl S rats with the homologous segment from the Lewis rat between the telomere and D16Rat21 (5.4 cM position) and observed a significant decrease in BP with a 2% NaCl diet. It is therefore possible that the BP effects observed in both studies are associated with the same gene(s).

The development of congenic strains represents an important step in validation of QTL mapping, but rarely have multiple

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Table 4. Estimated blood pressure values for effect of mapped BP QTLs individually or in combination under mixed BN-SHR or SHR genetic background

<table>
<thead>
<tr>
<th></th>
<th>Chr2a (R5129)</th>
<th>Chr2c (R5159)</th>
<th>Chr4 (R5114)</th>
<th>Chr16 (R762)</th>
<th>Exp. F2*</th>
<th>Congenics†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>171.1±(3.3)</td>
<td>196.6±3.2</td>
</tr>
<tr>
<td>SHR.BN2a</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>162.5±(3.3)</td>
<td>179.3±3.5</td>
</tr>
<tr>
<td>SHR.BN2c</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>161.5±(3.3)</td>
<td>177.6±2.0</td>
</tr>
<tr>
<td>SHR.BN4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>157.1±(3.3)</td>
<td>188.7±2.4</td>
</tr>
<tr>
<td>SHR.BN16</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>165.2±(3.3)</td>
<td>178.5±2.7</td>
</tr>
<tr>
<td>BN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>133.1±(3.3)</td>
<td>134.4±8.7</td>
</tr>
</tbody>
</table>

Values are mean ± SE estimated systolic blood pressure (SBP) values for effect of mapped BP QTLs individually or in combination under a mixed BN-SHR (experimental F2s) or SHR (congenics) genetic background. 1 and 0, Origin of QTL interval in homozygosity from SHR and BN, respectively. *SEs (shown in parentheses) were calculated considering values estimated by linear regression analysis (shown in Table 3); †n = 179.

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QTLs identified in one mapping effort been validated with this approach as presented here.

Moreover, three classes of phenotypic variation were identified among the four congenic lines and salt challenge. The specific roles associated with each locus have not been carefully investigated yet, but it can be noted that loci 2c and 4 from BN lower BP and the animals are salt sensitive, while loci 2a and 16 from the BN abrogate salt sensitivity. Thus one may speculate that loci 2a and 16 preferentially influence sodium/water handling, whereas loci 2c and 4 predominantly influence changes in BP. We also were able to infer as to the importance of the genetic background for the expression of the QTLs individually or as a group. The issue of background effect is important for the understanding of complex traits, especially considering human genetic diversity and multifactorial approaches to analysis of global patterns of gene expression that may be useful to identify the genetic variants in the background that influence the effect of QTLs (10).

Collectively, our results suggest that BP effects observed in SHR derive from the composite effects of multiple and separate QTLs and the QTL effects depend on the genetic background. Because the QTLs identified contain several potential candidate genes, substrains with progressively smaller chromosomal regions are being created and the physiological significance of relevant genes involved in regulation of BP will be further investigated by a variety of approaches.

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