Transcriptional profiling with a blood pressure QTL interval-specific oligonucleotide array

Bina Joe,1 Noah E. Letwin,2,3 Michael R. Garrett,1 Seema Dhindaw,1 Bryan Frank,2 Razvan Sultana,2 Kathleen Verratti,2 John P. Rapp,1 and Norman H. Lee2,3

1Physiological Genomics Laboratory, Department of Physiology and Cardiovascular Genomics, Medical University of Ohio, Toledo, Ohio; 2Department of Functional Genomics, The Institute for Genomic Research, Rockville, Maryland; and 3Department of Pharmacology, The George Washington University, Washington, District of Columbia

Submitted 27 July 2004; accepted in final form 26 September 2005

Joe, Bina, Noah E. Letwin, Michael R. Garrett, Seema Dhindaw, Bryan Frank, Razvan Sultana, Kathleen Verratti, John P. Rapp, and Norman H. Lee. Transcriptional profiling with a blood pressure QTL interval-specific oligonucleotide array. Physiol Genomics 23: 318–326, 2005. First published October 4, 2005; doi:10.1152/physiolgenomics.00164.2004.— Although the evidence for a genetic predisposition to human essential hypertension is compelling, the genetic control of blood pressure (BP) is poorly understood. The Dahl salt-sensitive (S) rat is a model for studying the genetic component of BP. Using this model, we previously reported the identification of 16 different genomic regions that contain one or more BP quantitative trait loci (QTLs). The proximal region of rat chromosome 1 contains multiple BP QTLs. Of these, we have localized the BP QTL1b region to a 13.5-cM (20.92 Mb) region. Interestingly, five additional independent studies in rats and four independent studies in humans have reported genetic linkage for BP control by regions homologous to QTL1b. To view the overall renal transcriptional topography of the positional candidate genes for this QTL, we sought a comparative gene expression profiling between a congenic topological region of the positional candidate genes for this QTL, we sought to identify the downstream targets constituting biochemical pathways relevant to BP that are altered as a consequence of changes in gene expression of positional candidate genes. This “biological signature” is represented by differential expression of genes that are affected either directly, or indirectly, by QTL1b. The differential expression of these genes was validated with quantitative real-time RT-PCR analysis using the same RNA samples that were interrogated by microarrays, as well as from two independent

natural variation in blood pressure (BP) between the S rat and other normotensive inbred rats have been limited to linkage and substitution mapping (7, 13, 30, 36). In different genetic analyses of crosses involving the S rat, multiple loci are shown to contribute to disease (7, 30, 36). Thus far, only one locus has been subjected to high-resolution mapping to identify a single candidate gene (3, 8). Nevertheless, substitution mapping in our laboratory has generated a number of congenic strains that are genetically identical to the S rat except for introgressed regions from normotensive strains. These congenic strains serve as genetic tools to identify each of the underlying molecular determinants of BP control.

We previously constructed and characterized congenic rats by replacing the S rat alleles with corresponding Lewis (LEW) rat alleles in a BP quantitative trait locus (QTL) region on chromosome 1 (named QTL1b region) (35). This process resulted in the localization of BP QTL1b to a 13.5-cM region, which corresponds to 20.92 Mb on rat chromosome 1 containing ~231 transcripts. It is of obvious interest to identify the underlying molecular determinant(s) within BP QTL1b that predisposes S rats to hypertension.

Integration of transcriptional profiling and linkage analysis/substitution mapping is increasingly used as one of the methods to identify genes underlying disease in rat models of elevated BP (9, 12, 18, 19, 23, 25, 44). The hypothesis for this approach is that the abundance of various transcripts positioned within a BP QTL region serve as phenotypic reporters of DNA sequence variation that contribute to BP control. By using such an integrated approach in the present study, we sought to identify the genetic component of BP QTL1b that is tightly linked to the heritability of variation in gene expression of positional candidate genes. None of the commercially available rat microarrays are suitable for this purpose because they do not adequately represent all the positional transcripts within the BP QTL1b region. Therefore, we designed a custom oligonucleotide array that contained all the known transcripts within BP QTL1b to identify differences in renal transcription profiles between S and a congenic strain containing LEW alleles at QTL1b. Furthermore, using a genome-scale transcriptional profiling approach, we sought to identify the downstream targets constituting biochemical pathways relevant to BP that are altered as a consequence of changes in gene expression of the positional candidate genes. This “biological signature” is represented by differential expression of genes that are affected either directly, or indirectly, by QTL1b. The differential expression of these genes was validated with quantitative real-time RT-PCR analysis using the same RNA samples that were interrogated by microarrays, as well as from two independent

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

Address for reprint requests and other correspondence: B. Joe, Dept. of Physiology and Cardiovascular Genomics, Medical Univ. of Ohio, 3035 Arlington Ave., Toledo, OH 43614-5804 (e-mail: bjo@meduohio.edu) or N. H. Lee, The Institute for Genomic Research, 9712 Medical Center Dr., Rockville, MD 20850 (e-mail: nhlee@tigr.org).
sample sets (refer to MATERIALS AND METHODS) from rats maintained on low- and high-salt diets. The results of our study combined with the observations from comparative mapping studies justify further investigation of 17 differentially expressed positional candidate genes within BP QTL1b as potentially involved in BP control both in hypertensive rat models and in humans.

MATERIALS AND METHODS

Strains. Animal protocols were approved by the Institutional Animal Care and Use Committee, Medical University of Ohio (MUO). Inbred Dahl salt-sensitive (SS/Jr) rats (hereafter referred to as S rats) were from our colony at MUO. The congenic strain used for the present study, S.LEW(Chr1×3)×3, was generated in our laboratory and previously reported (35).

Raising animals for RNA isolation. For gene expression profiling experiments using cDNA or oligonucleotide microarrays, RNA from a maximum of eight male S rats and eight male S.LEW(Chr1×3)×3 congenic rats was used. These rats were born on the same day, weaned at 30 days, and given a high-salt (4% NaCl) diet for 2 days, on days 40 and 41. They were euthanized at 42 days of age. Whole kidneys were isolated in RNA Later (Ambion) as per the manufacturer’s procedure and stored overnight at 4°C before RNA isolation. To validate microarray results and compare specific gene expression in low- vs. high-salt-fed animals, quantitative real-time RT-PCR was employed. For these experiments, a separate group of male S (n = 16) and S.LEW(Chr1×3)×3 congenic rats (n = 16) born on the same day were weaned at 30 days of age and raised concomitantly on a low-salt diet containing 0.3% NaCl. When the rats were 40 days old, one-half of the S and S.LEW(Chr1×3)×3 congenic rats were fed a high-salt diet containing 4% NaCl, and the remaining animals were continued on a low-salt diet. All 32 rats were euthanized at 42 days for isolation of total RNA from kidneys.

RNA isolation, target labeling. Whole kidneys were processed using Trizol reagent (Invitrogen), and total RNA was extracted using RNeasy (Qiagen) according to manufacturer’s protocols. For target labeling, 15 μg of RNA from individual S and S.LEW(Chr1×3)×3 rats were reverse transcribed into cDNA and coupled with either cysteine-5 (Cy5) or Cy3 as previously described (45).

Construction of a QTL1b region-specific 70-mer oligonucleotide microarray. A combination of two approaches was used to obtain mRNA sequences for the design of 70-mer oligonucleotide probes. First, annotated rat gene sequences flanked by the genetic markers D1Rat35 and D1Rat131 (demarcating QTL1b) were downloaded from the ENSEMBL database (Release 29.3f, October 2004; http://www.ensembl.org/Rattus_norvegicus/). Second, transcribed genes represented by expressed sequence tag (EST) sequences in The Institute for Genomic Research (TIGR) Rat Gene Index (Build 13.0, October 2004; http://www.tigr.org/tdb/tgi/) were mapped onto the draft rat genome assembly (Build 3.4, December 2004; http://www.hgsc.tmc.edu), and sequences contained in QTL1b were collected. On the basis of the union of sequences identified by both approaches, a total of 231 transcribed genes could be assigned to QTL1b. The design of oligonucleotide probes for the interrogation of these transcripts was accomplished using ArrayOligoSelector (2). Oligonucleotide design considerations included uniqueness, avoidance of internal complementation, narrow melting temperature (Tm) range (70–80°C) over the entire oligonucleotide set, and masking of low-complexity sequences to minimize nonspecific cross-hybridization.

Gene expression profiling with oligonucleotide and cDNA microarrays. Pairs of Cy5- and Cy3-labeled targets were co-hybridized onto either the oligonucleotide microarray or a custom TIGR rat cDNA array consisting of 26,401 probe elements representing 20,465 unique non-QTL1b genes (http://pga.tigr.org/AnalysisTools.shtml). The fabrication of oligonucleotide and cDNA microarray chips has been described previously (20, 38). A “flip-dye” design was used as the experimental method of choice to account for potential dye-bias labeling effects (45). A total of 12 hybridizations [6 hybridizations of S vs. S.LEW(Chr1×3)×3 rats, and 6 corresponding flip-dye hybridizations] were concurrently set up for the oligonucleotide arrays to minimize experimental variation. A similar experimental design was employed for the cDNA arrays. Microarrays were washed and scanned using an Axon Genepix 4000A scanner (Axon Instruments).

Microarray data analysis. Image scanning, fluorescence intensity measurements of Cy3 and Cy5 channels, background subtraction, quality control, and experimental noise determination were performed as described previously (20, 38). An intensity-dependent locally weighted linear regression (LOWESS) procedure was implemented to normalize the microarray data (45). This method minimizes normalization errors by segmenting the entire expression range into hybridization intensity intervals and employing local weighted regression analysis within each interval (45, 46). Following the normalization process, differentially expressed genes were statistically identified using a one-sample t-test with a false discovery rate (FDR) procedure to correct for multiple testing (32). FDR was applied separately to the QTL1b and non-QTL1b genes using a nominal alpha of 5% (i.e., false positive error rate estimated at 5%).

Quantitative real-time RT-PCR. To validate microarray data, RT-PCR was performed on an ABI Prizm 7700 Sequence Detection System using SYBR Green as described previously (20). Total kidney RNA from S and S.LEW(Chr1×3)×3 congenic rats were reverse transcribed using random primers as per the manufacturer’s protocol. The resulting cDNA was diluted and used as template for RT-PCR. PCR primers were selected for specificity by the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) of the rat genome, and amplicon specificity was verified by first-derivative melting curve analysis using software provided by Perkin-Elmer/Applied Biosystems. Quantitation and normalization of relative gene expression was accomplished using the comparative threshold cycle (Ct) method or ΔΔCt (40). ΔΔCt values were converted into ratios by 2−ΔΔCt and averaged across biological replicates. The expression of the “housekeeping” genes ribosomal protein L36a (GenBank accession no. AA859783), glutamate dehydrogenase (X14049), and hydroxysteroid sulfotransferase (AA817986) was used for normalization, as these genes did not exhibit differential expression in our microarray assays.

Primers for RT-PCR of different genes are given in the Supplemental Methods (available at the Physiological Genomics web site).1

Gene network and interactome analyses. For each differentially regulated gene identified by microarray analysis, ~2,000 bases of the proximal promoter region (~2,000 to −1) were extracted based on the ENSEMBL annotated rat transcriptional start site and the mapping of EST assemblies onto the draft rat genome assembly. The average size of extracted promoters was 1,913 bases, range 500–2,000 bases. Promoter regions were used as the target database when performing multimission archive at space telescope (MAST; version 3.0) searches of cis-regulatory sites defined by matrices in the Transcription Factor (TRANSFAC) database (21). A P value <0.001 was considered a significant hit. Promoters were binned according to common shared regulatory sites, providing preliminary gene network architecture (for review see, Ref. 17). For each bin, differentially expressed genes were mapped onto an “interactome” network using the Ingenuity Pathway Analysis application (Ingenuity Systems; http://www.ingenuity.com). This application queries a comprehensive database containing over 106 known mammalian gene product events (such as protein-protein, protein-nucleic acid, and protein-small molecule interactions)

1 The Supplemental Material for this article (Supplemental Methods) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00164.2004/DC1.
for prospective associations between the query genes (i.e., differentially expressed QTL1b and non-QTL1b genes) and all other gene products to generate a set of networks. Ingenuity Pathway Analysis assigns biological themes (e.g., gene expression, renal function, endocrine system) to each network based on gene content, using a Fischer’s exact test to calculate significance (P < 0.05). Biological themes associated with these networks were cross-validated using the software application Expression Analysis Systematic Explorer (EASE), which categorizes genes using Gene Ontology (10). EASE scores were calculated by performing 10,000 iterations. Significantly (P < 0.05) overrepresented classes of genes in a particular network denote biological relevance.

RESULTS

Comparative transcriptional profiling using the QTL region-specific custom oligonucleotide array. Of the informative fluorescence intensity measurements from the 14 hybridizations performed [7 hybridizations representing biological replicates (n = 7), and their flip-dye counterparts for a total of 14 hybridizations], 21 of 231 genes were differentially expressed between the two rat strains. These differentially expressed transcripts corresponded to 18 transcripts that were upregulated and 3 transcripts that were downregulated in congenic rats compared with S rats. This means that ~9% of all the candidate genes within the QTL1b region are differentially expressed. Hybridization data and parameter information can be accessed in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The GEO platform accession number is GPL2796; the flip dye normalized files are GSM73003–GSM73009.

Expression of QTL transcripts in low- vs. high salt-fed congenic and S rats. Using quantitative real-time RT-PCR, a four-way comparison of the extent of renal expression of QTL-specific genes was carried out between S rats and congenic rats raised on a 0.3% salt-containing diet (low salt) and S and congenic rats raised on a 0.3% salt- and then fed a 4% salt-containing diet (high salt) for 2 days. These experiments served to validate our earlier results on gene expression by interrogating a different set of animals and to test whether the observed differential expression of genes is sensitive to dietary salt. Of the 21 kidney transcripts defined by microarrays as differentially expressed between congenic and S rats, 19 could be validated with real-time RT-PCR. The two exceptions were elongation factor 2 (GenBank accession no. AI111597) and EST206339 (AI011888). Both of these genes were defined as upregulated in congenic rats by microarray analysis, whereas real-time RT-PCR defined the same two genes as not being differentially expressed (data not shown). This corresponds to a 90% validation success rate, which is in good agreement with our 5% FDR considering that the real-time RT-PCR assay was performed on RNA samples distinct from those used in the microarray assay. The list of validated transcripts is given in Table 1. The 19 transcripts given in Table 1 represent a total of 17 genes (note that 2 of the genes, Nox4/Kox4 and Chd2, are each represented by 2 transcripts). Of these, 14 genes are upregulated and 3 genes are downregulated in congenic rats compared with S. The map location of each gene within QTL1b is represented in Fig. 1.

The effects of dietary salt intake on renal gene expression were studied by comparing low salt- vs. high salt-fed S and congenic rats. Of the transcripts that were upregulated in low salt-fed congenic rats compared with low salt-fed S rats (Table 1, column C-LS/S-LS), Nox4 and Kox1 (both represent the gene kidney-specific NADPH oxidase 4), Folh1 (folate hydrolase), and Hdgfrp3 (hepatoma-derived growth factor, related protein 3) were significantly upregulated in S (Table 1, column S-HS/S-LS) and downregulated in congenic rats (Table 1, column C-HS/C-LS) in response to an acute increase in salt intake. All remaining transcripts displaying upregulation in low salt-fed congenic rats compared with low salt-fed S rats likewise displayed susceptibility to acute high salt intake, but only in one or the other strain. The exception, however, is Nr2f2 (nuclear receptor subfamily 2, group F, member 2), the expression of which was elevated in the congenic rats compared with S rats regardless of the acute increase in salt intake (i.e., high salt intake did not affect the expression of Nr2f2).

ManIIx (mannosidase IIx) and an unknown transcript XM_489186 were both downregulated in low salt-fed congenic rats compared with low salt-fed S rats. Analogous to Nr2f2, these two transcripts also were not affected by the acute increase in salt intake.

Expression differences of genes outside of QTL1b in S and congenic rats. Using the same kidney RNA samples from S and S.LEW(Chr1×3)/3 rats that were interrogated with our QTL region-specific oligonucleotide microarray, we profiled 20,465 unique genes (representing 20,447 genes residing outside of QTL1b) by means of a readily available in-house rat cDNA microarray. Of the informative fluorescence intensity measurements from the 12 hybridizations performed (n = 6 biological hybridizations plus corresponding flip-dye hybridizations), over 1,542 transcripts were found to be differentially expressed at a 5% FDR between congenic and S rats. Of these, 529 genes were upregulated and 1,013 genes were downregulated in congenic rats compared with S rats. With the assumption that the QTL1b region-specific genes affect the expression of these 1,542 non-QTL1b genes (either directly or indirectly), this finding was quite remarkable considering that only 17 genes in QTL1b were differentially expressed. It is also possible that sequence polymorphisms, and not just differential expression, in QTL1b genes contribute to the expression changes in genes residing outside the QTL1b region. Among the 20,465 unique genes interrogated by the rat cDNA microarray, there were 18 genes that reside within QTL1b. In agreement with our oligonucleotide microarray results, 17 of these genes did not exhibit differential expression, while ManIIx was downregulated in congenic rats compared with S rats (data not shown). Hybridization data and parameter information for the cDNA microarrays were deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The GEO platform accession number is GPL1350; the non-flip dye-normalized files are GSM27644 and GSM27658–GSM27668.

Gene networks and interactome maps. We attempted to place differentially regulated QTL1b and non-QTL1b renal genes into a gene and interactome network to identify potentially relevant pathways responsible for BP control differences between S and S.LEW(Chr1×3)/3 congenic rats. The following steps were implemented: 1) extract promoter sequences (~2,000 to ~1) corresponding to the differentially regulated genes; 2) search the promoters for known cis-regulatory sites defined in the TRANSFAC database using MAST, and place genes into bins based on common shared sites (17); 3) utilize Ingenuity Pathway Analysis and EASE to define an interac-
formed to determine the renal expression of these and five on the microarrays, quantitative real-time RT-PCR was per-

promoters contain the regulatory sites for Hnf4a, TCF, RARA, and JUN. Moreover, 477 gene cis-transcription factors: Hnf4a, TCF1, TCF2, RARA, RXRA, PPARG, and JUN (Fig. 2). Of interest, promoters harboring the binding sites for TCF1/TCF2 and RARA. Furthermore, the renal expression of Hnf4a, NR4A1, p53, PCBD, ZFP2 transcription factors in S rats and congenic rats. In the majority of instances, transcription factor transcripts were downregulated (9 of 12) in congenic rats relative to S rats raised on a low-salt diet (Table 2, column C-LS/S-LS). Furthermore, the renal expression of Hnf4a, NR4A1, p53, PCBD, RARA, RXRA, and TCF1 transcripts in both strains was insensitive to acute high salt intake (Table 2, columns S-HS/S-LS and C-LS/S-LS). Lastly, many of the transcription factors appear to regulate the expression of one another in apparent feedback and feedforward mechanisms (17) based on promoter analysis (Fig. 2).

To view our microarray data in a more meaningful biological context, differentially regulated genes within each bin were placed into interactome maps/modules using the software applications Ingenuity Pathway Analysis and EASE (28, 37). Particularly noteworthy was the finding that modules could be assigned to cardiovascular-related functions such as renal system, lipid metabolism, endocrine system, and hematological system (Fig. 2). On average, each interactome module contained ~15 differentially regulated genes. In some extreme instances, as seen in the Hnf4a lipid metabolism and TCF renal function modules, the vast majority of genes were downregulated in the kidneys of congenic rats compared with their S rat counterpart (Fig. 2). Also of interest, Nr2f2 and the “bin-

Table 1. Real-time RT-PCR of transcripts that are detected as differentially expressed and located within the WP QTL1b region

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TC494777_236</td>
<td>Kox-1: NADPH oxidase 4</td>
<td>AB042745</td>
<td>NM_053524 (Nox4)</td>
<td>3.52 (2.37)±0.55*</td>
<td>0.99±0.17</td>
<td>2.07±0.29*</td>
<td>0.58±0.06*</td>
</tr>
<tr>
<td>TC487979_1544</td>
<td>Nox4: NADPH oxidase 4</td>
<td>NM_053524</td>
<td>NM_053524</td>
<td>2.96 (1.81)±0.49*</td>
<td>1.00±0.12</td>
<td>2.15±0.34*</td>
<td>0.51±0.09*</td>
</tr>
<tr>
<td>TC478317_383</td>
<td>Fohl1: folate hydrolase</td>
<td>NM_057185</td>
<td>NM_057185</td>
<td>3.20 (1.99)±0.75*</td>
<td>1.02±0.22</td>
<td>2.77±0.33*</td>
<td>0.61±0.11*</td>
</tr>
<tr>
<td>TC466856_2716</td>
<td>Hdgfp3: hepatoma-derived growth factor, related protein 3</td>
<td>NM_145785</td>
<td>NM_145785</td>
<td>2.07 (1.62)±0.21*</td>
<td>1.03±0.10</td>
<td>1.41±0.14*</td>
<td>0.63±0.08*</td>
</tr>
<tr>
<td>TC473474_911</td>
<td>Homer2: homolog 2</td>
<td>NM_053309</td>
<td>NM_053309</td>
<td>2.76 (1.52)±0.48*</td>
<td>1.03±0.09</td>
<td>2.45±0.35*</td>
<td>1.08±0.12</td>
</tr>
<tr>
<td>TC480795_56</td>
<td>Zfp366: similar zinc finger protein</td>
<td>XM_214980</td>
<td>XM_214980</td>
<td>2.46 (1.75)±0.43*</td>
<td>2.95±0.13*</td>
<td>1.10±0.07</td>
<td>1.77±0.16*</td>
</tr>
<tr>
<td>TC472260_559</td>
<td>Nr2f2: nuclear receptor subfamily 2, group F, member2</td>
<td>NM_009697</td>
<td>NM_080778</td>
<td>2.14 (1.55)±0.11*</td>
<td>2.27±0.06*</td>
<td>0.95±0.17</td>
<td>1.04±0.08</td>
</tr>
<tr>
<td>TC490507_216</td>
<td>Chd2: chromodomain helicase DNA binding protein 2</td>
<td>BE108294</td>
<td>AK088690</td>
<td>LOC308738</td>
<td>2.19 (2.09)±0.19*</td>
<td>1.03±0.22</td>
<td>1.14±0.12</td>
</tr>
<tr>
<td>TC496154_63</td>
<td>Chd2: chromodomain helicase DNA binding protein 2</td>
<td>X62953</td>
<td>X62953</td>
<td>1.99 (1.75)±20*</td>
<td>1.04±0.08</td>
<td>1.03±0.06</td>
<td>0.57±0.03*</td>
</tr>
<tr>
<td>TC497582_214</td>
<td>unknown</td>
<td>AK035146</td>
<td>LOC308738</td>
<td>2.00 (2.53)±0.12*</td>
<td>1.03±0.16</td>
<td>1.07±0.08</td>
<td>0.53±0.04*</td>
</tr>
<tr>
<td>TC497555_4</td>
<td>Prcl: protein regulator of cytokinesis 1</td>
<td>XM_218820</td>
<td>XM_218820</td>
<td>1.68 (1.51)±0.05*</td>
<td>0.96±0.09</td>
<td>1.09±0.12</td>
<td>0.57±0.02*</td>
</tr>
<tr>
<td>TC464394_1641</td>
<td>Bhrd1: BTB (POZ) domain containing 1</td>
<td>NM_146193</td>
<td>NM_001011932</td>
<td>1.52 (1.61)±0.04*</td>
<td>1.02±0.11</td>
<td>1.09±0.08</td>
<td>0.59±0.02*</td>
</tr>
<tr>
<td>TC490700_381</td>
<td>Chd2: chromodomain helicase DNA binding protein 2</td>
<td>NM_006871</td>
<td>LOC308738</td>
<td>1.51 (1.72)±0.09*</td>
<td>0.97±0.10</td>
<td>0.97±0.07</td>
<td>0.56±0.03*</td>
</tr>
<tr>
<td>TC467898_1602</td>
<td>Csc: cathepsin C</td>
<td>NM_017097</td>
<td>NM_017097</td>
<td>1.46 (1.73)±0.06*</td>
<td>0.94±0.06</td>
<td>0.95±0.06</td>
<td>0.69±0.03*</td>
</tr>
<tr>
<td>TC475180_92</td>
<td>Surpe: small nuclear ribonucleoprotein E</td>
<td>XM_341120</td>
<td>XM_341120</td>
<td>1.46 (1.53)±0.06*</td>
<td>0.97±0.05</td>
<td>1.00±0.02</td>
<td>0.72±0.07*</td>
</tr>
<tr>
<td>TC471485_938</td>
<td>unknown</td>
<td>XM_489186</td>
<td>XM_344916</td>
<td>0.70 (0.73)±0.02*</td>
<td>0.64±0.05*</td>
<td>0.98±0.08</td>
<td>1.03±0.07</td>
</tr>
<tr>
<td>TC478102_24</td>
<td>Midori: similar to myocytic induction/differentiation originator</td>
<td>NM_054085</td>
<td>XM_344916</td>
<td>0.66 (0.69)±0.04*</td>
<td>1.10±0.07</td>
<td>0.71±0.04*</td>
<td>0.97±0.08</td>
</tr>
<tr>
<td>TC484378_127</td>
<td>Man2a2/Mannilx: mannosidase 2, alpha 2 or Mannosidase Ix</td>
<td>NM_172903</td>
<td>LOC308757</td>
<td>0.39 (0.64)±0.03*</td>
<td>0.40±0.03*</td>
<td>0.99±0.15</td>
<td>0.92±0.14</td>
</tr>
</tbody>
</table>

Probe name listed in italics indicates that there is an alternate transcript also representing the same gene; C, congenic; S, Dahl salt sensitive; LS, low salt; HS, high salt. Ratio values are means ± SE of 6–8 independent experiments performed in duplicate. Microarray ratio values are given in parentheses. *Significant differential expression (P < 0.05).
controlling” transcription factors (Hnf4a, RARA, RXRA, PPARG, and JUN) were grouped together by Ingenuity Pathway Analysis as potential heterodimerization partners belonging to an interactome module that controls gene expression (Fig. 2).

**Discussion**

A classic method of finding disease/trait-causative genes has been to create congenic strains with progressively higher resolution chromosome substitutions, but this is a rather laborious and time-consuming process. The experimental design of overlaying this approach with applications such as global gene expression analysis is aimed at expediting causative gene identification, but this strategy can be associated with a potential risk, since the differential expression of a positional candidate gene within a given QTL does not necessarily imply causation. Notwithstanding, the approach of combining QTL and microarray analyses allows the investigator to prioritize candidate genes for future functional analysis and/or transgenic animal experiments, as has been the case of past successful studies (1, 6, 14, 34). A recent study has taken this combinatorial approach a step further by prioritizing differentially expressed genes as candidates for physiological QTLs in recombinant inbred rat strains (12).

BP QTL1b was previously localized by substitution mapping using S.LEW congenic strains (35). Other investigators have suggested that the major proportion of the BP effect of loci on RNO1 is mediated through the kidney based on transplantation experiments (4). Therefore, it is reasonable to expect the causal effect of this BP QTL to reside in the kidney. In this study, we sought to identify positional candidate genes residing in BP QTL1b that are tightly linked to heritable differential expression. Using a custom-designed saturated QTL gene chip, we have identified 17 such genes in the kidney. Moreover, we designed additional experiments to identify differentially regulated non-QTL1b genes and exploited this information to study potential interactions between QTL1b and non-QTL1b genes. In that sense, our strategy is atypical compared with earlier combinatorial studies that have exclusively studied QTL genes (1, 23, 29).

The S rat, although studied extensively for salt-induced elevation in BP, does develop hypertension, even in the absence of a high-salt diet (31). Salt as an environmental factor rapidly amplifies the elevation of BP in the S rat. Therefore, genetic factors that cause high BP in the S rat can be viewed as belonging to one of two categories, i.e., those that exert their effects either dependently or independently of salt as an environmental factor. Of the 17 genes within the QTL1b region that were differentially expressed between S and congenic strains, there are only three whose differential expression was not influenced by dietary salt (Table 1). Two of these transcripts, ManIIx (NM_172903) and an unknown transcript (XM_489186), were downregulated in the congenic strain compared with S, whereas the remaining one, Nr2f2 (NM_009697), was upregulated in the congenic strain compared with S.

The potential roles of ManIIx, unknown transcript XM_489186, and Nr2f2 in hypertension of S rats have not been previously explored. ManIIx is an enzyme that trims mannose residues with unique specificity from oligosaccharide chains attached to proteins and therefore has been suggested to be

![Fig. 1. Location of differentially expressed transcripts within the blood pressure (BP) quantitative trait locus (QTL)1b region. Map depicts the physical coordinates of the BP QTL1b region on rat chromosome 1. Locations of the differentially expressed transcripts were obtained by BLAST searching with each of the transcript sequences at http://www.ensembl.org. Gene names are indicated wherever available. GenBank rat accession nos. are given in parentheses. *Accession nos. corresponding to the mouse sequences.](http://www.physiolgenomics.org/archives/124749.png)
involved in the complex deglycosylation of proteins in the Golgi (27). Ogawa et al. (26) first identified ManIIx, which was subsequently demonstrated to exhibit enzyme activity in vitro (27). The endogenous glycoprotein substrates of ManIIx are unknown in mice, rats, and humans. Because ManIIx is differentially expressed in the kidneys of congenic and S rats, this differential expression may be linked to the differential deglycosylation of endogenous substrates involved in BP regulation.

Table 2. Real-time RT-PCR of transcription factor genes

<table>
<thead>
<tr>
<th>Name</th>
<th>GenBank Acc. No.</th>
<th>C-LS/S-LS</th>
<th>C-HS/S-HS</th>
<th>S-HS/S-LS</th>
<th>C-HS/C-LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLI</td>
<td>XM_345832</td>
<td>2.04 ± 0.23 *</td>
<td>1.87 ± 0.14 *</td>
<td>1.08 ± 0.06</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>HNF4A</td>
<td>NM_022180</td>
<td>0.54 ± 0.07 *</td>
<td>0.53 ± 0.06</td>
<td>0.98 ± 0.10</td>
<td>1.14 ± 0.09</td>
</tr>
<tr>
<td>JUN</td>
<td>NM_021835</td>
<td>0.61 ± 0.06 *</td>
<td>1.53 ± 0.09 *</td>
<td>0.59 ± 0.07 *</td>
<td>1.47 ± 0.13 *</td>
</tr>
<tr>
<td>NR4A1</td>
<td>NM_024388</td>
<td>0.58 ± 0.07 *</td>
<td>0.43 ± 0.05 *</td>
<td>0.95 ± 0.10</td>
<td>1.13 ± 0.11</td>
</tr>
<tr>
<td>p53</td>
<td>NM_030989</td>
<td>0.64 (0.62) ± 0.09 *</td>
<td>0.56 ± 0.04</td>
<td>0.96 ± 0.08</td>
<td>1.10 ± 0.06</td>
</tr>
<tr>
<td>PCBD</td>
<td>M83740</td>
<td>0.42 (0.56) ± 0.07 *</td>
<td>0.59 ± 0.06</td>
<td>1.05 ± 0.04</td>
<td>1.01 ± 0.12</td>
</tr>
<tr>
<td>PPARG</td>
<td>NM_013124</td>
<td>1.08 ± 0.06</td>
<td>1.11 ± 0.04</td>
<td>0.96 ± 0.06</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>RARA</td>
<td>NM_031528</td>
<td>0.55 ± 0.06 *</td>
<td>0.46 ± 0.10 *</td>
<td>0.94 ± 0.09</td>
<td>1.15 ± 0.09</td>
</tr>
<tr>
<td>RXRA</td>
<td>NM_021805</td>
<td>0.46 ± 0.05 *</td>
<td>0.45 ± 0.05</td>
<td>1.06 ± 0.19</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>TCF1</td>
<td>NM_012669</td>
<td>0.59 ± 0.14 *</td>
<td>0.30 ± 0.03 *</td>
<td>1.10 ± 0.11</td>
<td>1.09 ± 0.11</td>
</tr>
<tr>
<td>TCF2</td>
<td>NM_013103</td>
<td>0.58 ± 0.05 *</td>
<td>1.67 ± 0.20 *</td>
<td>0.64 ± 0.06 *</td>
<td>2.03 ± 0.23 *</td>
</tr>
<tr>
<td>ZFPM2</td>
<td>NM_235253</td>
<td>1.73 ± 0.15 *</td>
<td>1.06 ± 0.05</td>
<td>1.53 ± 0.09 *</td>
<td>0.97 ± 0.07</td>
</tr>
</tbody>
</table>

GLI, glioma-associated oncogene homolog; HNF4A, hepatocyte nuclear factor 4 alpha; NR4A1, nuclear receptor subfamily 4 group A member 1; PCBD, 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of TCF1; PPARG, peroxisome proliferative-activated receptor, gamma; RARA, retinoic acid receptor alpha; RXRA, retinoic X receptor alpha; TCF1, transcription factor 1 (hepatic nuclear factor 1); TCF2, transcription factor 2 (hepatic nuclear factor 2); ZFPM2, zinc finger protein multitype 2. Ratio values are means ± SE of 8 independent experiments performed in duplicate. Microarray ratio values are given in parentheses. *Significant differential expression (P < 0.05).
Interestingly, complex glycosylation and deglycosylation of epithelial sodium channel (ENaC) subunits are important determinants of the efficiency of maturation, assembly, and stability of ENaC at the kidney cell surface (33).

Perhaps an even more intriguing QTL1b BP candidate is Nr2f2, a transcription factor belonging to the nuclear factor superfamily (28, 37). To identify putative targets of this cis-acting candidate gene, we interactively analyzed data obtained from two hybridization experiments, i.e., hybridizations performed on the 1) QTL1b region-specific oligonucleotide array and 2) genome-scale cDNA array that interrogates non-QTL1b genes. On the basis of promoter and interactome analyses, differential expression of non-QTL1b genes appears to be a consequence of either Nr2f2 homodimerization or heterodimerization. Nr2f2 can heterodimerize with at least nine different transcription factors (28, 37), including Hnf4a, RARA, RXRA, PPARG, and JUN (Fig. 2). This phenomenon has been suggested to mechanistically interfere with the ability of transcription factors such as Hnf4a to homodimerize or heterodimerize with its “normal” partners (e.g., Hnf4a/RARA, Hnf4a/RXRA). As a consequence, Nr2f2 behaves as a repressor of genes regulated (positively or negatively) by these normal dimerization partners (28, 37). Our findings support the notion of a complex circuitry of interacting transcription factors that regulate the expression of each other and non-QTL1b genes, and residing at the center of the regulatory circuit is Nr2f2 (Fig. 2). We speculate that the observed differential expression of Nr2f2 in S rats and congenic rats has consequences in multiple processes (e.g., interactome modules) linked to BP regulation. In other words, the inherent BP differences between S and congenic rats may be explained by the differential regulation of Nr2f2 within this circuitry. For example, the K+ inwardly rectifying channel (KCNJ1) and furosemide-sensitive Na+–K+–Cl– cotransporter (SLC12A1) gene promoters have transcription factor-binding sites for Hnf4a, RARA, RXRA, and JUN (Fig. 2). In congenic animals relative to S rats, the transcripts for SLC12A1 and KCNJ1 are downregulated more than twofold, presumably because of the overexpression of Nr2f2, which facilitates the formation of heterodimers of Nr2f2/Hnf4a, Nr2f2/RARA, Nr2f2/RXRA, and Nr2f2/JUN (Fig. 2).

Overall, the evidence provided in Figs. 1 and 2 constitutes the framework for prioritizing further substitution mapping of BP QTL1b, which is necessary to validate genotype-phenotype correlations of each of the differentially expressed candidate genes and BP.

Our study has benefited by design of a rigorous transcriptional profiling strategy of a relatively small, well-defined QTL region and illustrates two important points: 1) The importance of using a custom-designed saturated QTL array, without

---

Fig. 3. Comparative mapping of rat BP QTL1b region with human BP QTLs. The physical limit of the BP QTL1b is represented by the light-gray box on the schematic representation of RNO1. Blocks of conserved homologous segments of QTL1b region on human chromosome 15 are represented by dashed lines drawn across from RNO1 to HSA15. Solid black vertical bars represent BP QTLs in independent studies, represented as follows: a, Ref. 43; b, Ref. 11; c, Ref. 15; d, Ref. 5; e, Ref. 24; f, present study; g, Ref. 16; h, Ref. 39; i, Ref. 41; j, Ref. 42.
which a thorough and comprehensive transcriptional profiling of the BP QTL1b region could not be conducted. For example, using the global cDNA rat array enabled us to track the altered expression of only one transcript, i.e., ManIlx, whereas the custom-designed QTL array allowed for the identification of 18 other positional candidate genes within the BP QTL1b region that were significantly altered between S and the congenic strain. 2) The trans effects that a few differentially expressed genes can have over the amplification of differential expression events of genes throughout the genome.

It should be noted that the elevation in BP leads to secondary physiological and pathological consequences, which makes it difficult to identify high BP-causative factors in older rats or in rats that have been administered a high salt-consuming diet for prolonged periods. A strategy of combining gene expression analysis with substitution mapping using congenic strains was utilized by McBride et al. (23), wherein they identified glutathione-S-transferase (Gstm1) as a BP candidate gene. However, for their study, 15-wk-old rats were used that were already hypertensive, and thus they could not differentiate between expression differences in Gstm1 being the “cause” or the “consequence” of BP differences. Nevertheless, a subsequent publication using young rats clarified this issue and presented a logical case for Gstm1 as a BP-causative gene (22). The ideal solution would be to look for causative genetic factors in the absence of adaptive responses caused by long-term differences in BP. The most obvious problem, however, is that it is impossible to predict and define whether and when this occurs for a particular genetic factor. Clearly, the use of young rats has a chronological advantage compared with older rats in terms of minimizing the secondary physiological consequences associated with increases in BP. In the present studies, we used 42-day-old rats. Therefore, it is not likely that the altered gene expression between the S rat and the congenic strain reported in this study is secondary to strain differences in BP.

The corollary to identifying BP-causative genes in animal models of hypertension is to understand their relevance to BP control in humans. From this perspective, it is particularly interesting to find that QTL1b, which controls BP in S rats, overlaps with BP QTLs identified by five other substitution mapping studies using rat congenic strains and four independent linkage analyses in humans (Fig. 3). Also note that BP QTL1b is localized to the smallest genomic region compared with all other BP QTLs represented in Fig. 3. With the identification of differentially expressed putative candidate genes underlying QTL1b, it would be of interest to relate this finding among rats and in human populations wherein overlapping BP QTLs are identified.

GRANTS

Grant support to B. Joe (RO1-HL-075414) and N. H. Lee (U01-HL-66619) from the National Heart, Lung, and Blood Institute (NHLBI) and support to B. Joe from the Visiting Scientist Program of the Physiological Genomics Initiative of NHLBI are gratefully acknowledged.

REFERENCES


