Characterization of the genomic structure and function of regions influencing renin and angiogenesis in the SS rat

Timothy J. Stodola,1 Micheline M. de Resende,4 Allison B. Sarkis,1,3 Daniela N. Didier,1,2 Howard J. Jacob,1,3 Norbert Huebner,5 Oliver Hummel,5 Kathrin Saar,5 Carol Moreno,1,3 and Andrew S. Greene1,2

1Department of Physiology, 2Biotechnology and Bioengineering Center, 3Human and Molecular Genetics Center, Medical College of Wisconsin; 4Texas Heart Institute, Houston, Texas; and 5Max Delbrück Center for Molecular Medicine, Berlin, Germany

Submitted 7 September 2010; accepted in final form 19 April 2011

Stodola TJ, de Resende MM, Sarkis AB, Didier DN, Jacob HJ, Huebner N, Hummel O, Saar K, Moreno C, Greene AS. Characterization of the genomic structure and function of regions influencing renin and angiogenesis in the SS rat. Physiol Genomics 43: 808–817, 2011. First published April 26, 2011; doi:10.1152/physiolgenomics.00171.2010.—Impaired regulation of renin in Dahl salt-sensitive rats (SS/JR-HsdMcwi, SS) contributes to attenuated angiogenesis in this strain. This study examined angiogenic function and genomic structure of regions surrounding the renin gene using subcongenic strains of the SS and BN/NHsdMcwi (BN) rat to identify important genomic variations between SS and BN involved in angiogenesis. Three candidate regions on Chr 13 were studied: two congeneric strains containing 0.89 and 2.62 Mb portions of BN Chr 13 that excluded the BN renin allele and a third strain that contained a 2.02 Mb overlapping region that included the BN renin allele. Angiogenesis induced by electrical stimulation of the tibialis anterior muscle was attenuated in the SS compared with the BN. Congenics carrying the SS renin allele had impaired angiogenesis, while strains carrying the BN renin allele had angiogenesis restored. The exception was a congeneric including a region of BN genome 0.4 Mb distal to renin that restored both renin regulation and angiogenesis. This suggests that there is a distant regulatory element in the BN capable of restoring normal regulation of the SS renin allele. The importance of ANG II in the restored angiogenic response was demonstrated by blocking with losartan. Sequencing of the 4.05 Mb candidate region in SS and BN revealed a total of 8,850 SNPs and other sequence variants. An analysis of the genes and their variants in the region suggested a number of pathways that may explain the impaired regulation of renin and angiogenesis in the SS rat.

renin-angiotensin system; physiological genomics; consomic; congeneric

ANGIOGENESIS IN ADULT TISSUES is a complex process that allows vascular adaptation to an increase in metabolic demand. In skeletal muscle, the increase in capillary density involves a variety of mediators including the renin-angiotensin system (RAS). The RAS has long been known to be an essential modulator of sodium homeostasis and vascular resistance. Recently increasing evidence has implicated this system in the control of microvascular density since inhibition of the RAS pharmacologically (3, 4, 19, 27, 44), genetically (5, 14), and by high-salt diet (15, 16, 50, 51) inhibits angiogenesis. Others have described local tissue RAS in a variety of organs that act both independently and in concert with the circulating system (22, 35, 43, 47). We have shown that it is angiotensin II (ANG II), the major active product of the RAS, that is an essential regulator of skeletal muscle angiogenesis induced by exercise (4), electrical stimulation (15, 16, 51), and vasodilation (50). The Dahl salt-sensitive SS/JR-HsdMcwi (SS) rat strain is an animal model that exhibits disease traits similar to human salt-sensitive hypertension (11, 13, 20, 26, 34). In this low-renin model of hypertension, impaired regulation of the RAS results in reduced microvascular density (44) and an inhibited angiogenic response in skeletal muscle (3, 4), both of which can be ameliorated by a subpressor administration of ANG II (16, 51). In previous studies we have shown that the angiogenic phenotype in the SS rat can also be restored through introgression of a portion of chromosome (Chr) 13 from Brown Norway BN/NHsdMcwi (BN) that contains the renin gene and is as small as 4.05 Mb (14). Interestingly, sequencing studies have revealed that there are no differences in the coding region or the 5ʹ-flanking proximal promoter region of the renin gene between SS and BN rats. Although deletions and sequence variants at different sites in the intronic regions and in the distant 5ʹ- and 3ʹ-flanking regions of the SS rat have been described compared with the closely related salt-resistant rat (65), these variants or differences in alleles at a closely linked locus have not been tested for functional relevance with regard to renin expression. The lack of sequence variants in the coding region of the renin gene in the SS rat suggests the existence of a nearby mutation that interacts with the renin gene on the SS rat and that may be related to the anti-angiogenic phenotype.

The goal of the present study was to identify genomic regions on Chr 13 that may regulate renin expression and thus levels of ANG II and angiogenesis in the SS rat. Three candidate regions within the already identified 4.05 Mb region (14) on Chr 13 were studied by the development of subcongenics strains; two congeneric strains capturing a 0.89 Mb portion and a 2.62 Mb portion of Chr 13, which exclude the BN renin allele, and a third strain, which contains a 2.02 Mb overlapping region including the BN renin allele, were studied. The 4.05 Mb region including all three candidate regions was also sequenced in the SS and BN strains.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee. Animals were housed and cared for at the MCW Animal Resource Center and were given food and water ad libitum. Studies were performed in male salt-sensitive hypertensive Dahl SS/JR-Hsd/Mcwi rats, referred to here as SS, SS-13BN/Mcwi congenic rats, and...
BN-13\(^{SS}\)/Mcwi consomic rats from our colonies at the MCW. The 4.05 Mb congeneric strain SS.BN-(D13Hmgc41-D13Rat101)/Mcwi was derived from the SS-13\(^{BN}\)/Mcwi consomic by marker-assisted breeding, as described previously (14, 42). Three additional Chr 13 congeneric strains were developed by crossing SS rats with the 4.05 Mb SS.BN-(D13Hmgc41-D13Rat101)/Mcwi congeneric strain. Rats from the F\(_1\) generation were intercrossed, and the progeny were genotyped for the selection of recombinant animals. Genotyping was performed with eight simple sequence length polymorphism (SSLP) markers spanning the region of Chr 13 between 45.22 and 49.34 Mb. Recombinant rats underwent one more round of backcrossing and intercrossing to reach homozygosity in the target regions to further establish and expand the congeneric strains. In total, four generations of breeding were necessary for the development of the congeneric strains from the congeneric SS.BN-(D13Hmgc41-D13Rat101)/Mcwi. Additional genotyping with 11 SSLP markers and by direct sequencing was performed on selected congeneric strains to further define the flanking regions and to ensure the relative position with respect to the renin gene (Fig. 1).

All markers developed and/or used in these studies have been posted for marker size information and strain polymorphisms at the Rat Genome Database (http://rgd.mcw.edu/). As demonstrated in Fig. 1, we developed and studied a total of four congeneric rat strains that included or excluded the renin locus: line 13D [SS.BN-(D13Hmgc41-D13Rat101)/Mcwi], line 13D\(_A\) [SS.BN-(D13Hmgc41-D13Hmgc23)/Mcwi], line 13D\(_B\) [SS.BN-(D13Hmgc41-ss256302599)/Mcwi], and line 13D\(_C\) [SS.BN-(D13Rat124-D13Rat101)/Mcwi]. All rats were generated and housed at MCW. Lines 13D (4.05 Mb) and 13D\(_A\) (2.02 Mb) included the BN renin allele, while lines 13D\(_B\) (0.89 Mb) and 13D\(_C\) (2.62 Mb) excluded it. The BN-13\(^{SS}\)/Mcwi reverse congeneric strain, in which chromosome 13 from the SS rat is introgressed onto the BN background, was also developed by marker-assisted breeding, as described previously (58).

**Experimental protocol.** All rats underwent 7 days of hindlimb electrical stimulation and received different treatment during the entire stimulation protocol, being randomly assigned to the following groups: no drug, line 13D\(_C\) rats treated with losartan (50 mg kg\(^{-1}\)·day\(^{-1}\) in the drinking water, gifted by Merck Pharmaceuticals, SS treated with ANG II (3 ng kg\(^{-1}\)·day\(^{-1}\), venous infusion), and SS treated with ANG II and losartan. All rats received a 0.4% NaCl diet (Dyets, Bethlehem, PA) and completed experimental protocols at 8–10 wk of age.

**Electrical stimulation surgery.** Rats were anesthetized with an intramuscular injection mixture of ketamine (70 mg/kg), xylazine (4 mg/kg) and acepromazine (1 mg/kg). Under aseptic conditions, subcutaneous incisions were made over the thoracolumbar region and medial aspect of the right leg, and a miniature battery powered stimulator was implanted as previously described (38). After 24 h of recovery, the stimulator was activated and electrodes located near the common peroneal nerve in the lower leg produced square-wave impulses of 0.3 ms duration, 10-Hz frequency, and 3-V potential, causing intermittent contractions of the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles for eight consecutive hours, daily. The contralateral leg was used as a control. All animals were euthanized after 7 days of stimulation.

**ANG II infusion.** A jugular catheter was implanted during electrical stimulation surgery. SS rats fed a low-salt diet (0.4% NaCl) were continuously infused intravenously with ANG II dissolved in sterile saline or vehicle via the jugular catheter at a suppressor dose (3 ng kg\(^{-1}\)·min\(^{-1}\)) throughout the 7-day electrical stimulation period, according to the protocol previously described (51).

**Tissue harvest and morphological analysis of vessel density.** The animals were euthanized by an overdose of Beuthanasia solution, and the stimulated and contralateral unstimulated TA and EDL muscles were removed and weighed. A 100-mg section was taken from the TA muscle and immediately frozen in liquid nitrogen for mRNA analysis. The remaining tissue was fixed overnight in 0.25% formalin solution and sectioned. The sections were immersed in a solution of 30 µg/ml rhodamine labeled Grifonia simplicifolia I lectin (Sigma) for 2 h. The sections were rinsed and mounted on microscope slides as described previously (51). The sections were visualized with a video fluorescent microscope system (Nikon E-801 microscope with COHU camera, ×200). Twenty representative fields from each muscle were digitally photographed and computer analyzed by Metamorph software (Molecular Devices) based on Riede et al. (53). Vessel counts from all fields were averaged to a single vessel density, defined as the mean number of vessel-grid intersections per microscope field (0.077 mm\(^2\)) for each muscle.

**rt-PCR analysis.** Frozen tissue was homogenized in TRIzol (Invitrogen, 25 µg tissue in 1 ml) with a bead homogenizer (TissueLyserII, Qiagen). RNA was isolated as previously described (37, 60) and purified with DNase treatment per manufacturer protocol (Fermentas). Purified RNA from each muscle was quantified with NanoDrop spectrophotometer (Thermo Scientific) and run on a 7900HT real-time PCR machine (Applied Biosystems). Samples were run with the Taqman one-step kit (Applied Biosystems) per manufacturer’s instructions and the following oligos: renin forward 5'-GGTGCCCTCCACCAAGGTG, renin reverse 5'-GCTAGAG-GATTCGAGGAGTC primers, renin probe 5'-[FAM]TCCCC-TTCTACTGCCTGTGAGATTCACA[TAMARA] (Sigma), or the Taqman Ribosomal control kit. Analysis was performed as described by Knoll et al. (31).

**Renin activity assay.** Renin activity was measured as previously described (56). In brief, kidney and TA muscle homogenates from line 13D rats were incubated with renin substrate tetradecapeptide (0.3 mM, Sigma) for 10 min to 1 h at 37°C in the presence of phenylmethylsulfonyl fluoride (0.25%) and maleic acid (6.6 mM, Sigma). Each sample was also incubated in the presence of a renin inhibitor peptide (WFML, Anaspec) to determine enzyme specificity. Incubated samples were separated by HPLC (Beckman PF2D), and the ANG I peak area for each sample was measured. A standard curve was generated to quantify nmoL ANG I relative to peak area. Results

---

**Physiol Genomics · VOL 43 · www.physiolgenomics.org**

---

1. **Fig. 1.** Illustration of the 4 congeneric lines (13D, 13D\(_A\), 13D\(_B\) and 13D\(_C\)) created around the renin gene. All lines carry a Dahl SS/JrHsdMcwi (SS) rat background genome. Brown Norway (BN) rat chromosome inserts are denoted by the black bars. The renin gene is marked by the horizontal line.
were expressed as nmol ANG I per mg tissue per hour incubation for each sample.

**Immunohistochemistry for renin.** Immunohistochemistry was performed as previously described (41). Briefly, tissues were fixed in a 10% formalin solution in phosphate buffer and then paraffin embedded using an automatic tissue processor (Microm HMP 300). Embedded tissues were cut in 3 μm sections (Microm HM355S) and mounted on silanized/charged slides. Slides were deparaffinized and incubated in 0.3% hydrogen peroxide for 30 min followed by the endogenous avidin and biotin blocking kit (Vector Laboratories). Slides were blocked in horse serum, incubated against anti-renin antibody (1:100, sc-27318, Santa Cruz Biotechnology), and then incubated with a biotinylated anti-goat secondary antibody (Vector Laboratories), followed by Vectastain Elite ABC reagent per the manufacturer’s instructions (Vector Laboratories). Staining was visualized with 3,3′-diaminobenzidine and peroxidase. Tissue sections were photographs using a Nikon E-400 microscope fitted with a Spot Insight camera.

**Genomic sequencing.** Sequencing of the SS strain, which includes the 4.05 Mb region included in congenic line 13D, was performed on an Illumina Genome Analyzer II by the Max Delbrück Center for Molecular Medicine in Berlin, Germany (6).

**Sequence annotation and analysis.** The Illumina sequence reads were initially aligned against the reference using Illumina’s analysis pipeline, CASAVA. Upon receiving the Illumina read data, we assembled them into contigs with Mosaic. Variants were found using Consed (25). The identified nonsynonymous variants were analyzed using Polyphen (52) and SIFT (45) for predicted damage to the protein. Predicted microRNA target sites in the 3′-untranslated regions of known genes were identified by TargetScan (23).

To identify possible connections between renin and the genes identified with nonsynonymous variants we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING v8.3)(28). The program was seeded with nonsynonymous variants identified as being damaging to the protein as well as renin. The STRING network was generated with the following parameters: gene fusion, co-occurrence, coexpression, experiments, databases, and text-mining. Network depth was 1 and required confidence was >0.4.

**Data analysis and statistics.** Muscle, body weights, and renin activity were expressed as means ± SE and evaluated using a paired t-test.

Vessel counts from all fields were averaged to a single vessel density, defined as the mean number of vessel-grid intersections per microscope field (0.077 mm²) for each muscle. Within experimental groups, mean vessel densities of stimulated muscles were compared with contralateral unstimulated muscles, with all values presented as means ± SE and evaluated by paired t-test. Differences between groups were evaluated with one-way ANOVA, and significant differences were further investigated with Holm-Sidak or Dunn’s post hoc test.

Real-time PCR analysis was performed as described by Knoll et al. (31). Renin mRNA expression was normalized to endogenous 18S rRNA and results expressed as renin mRNA units relative to 18S rRNA. Differences between groups were evaluated by two-way repeated-measures ANOVA, and significant differences were further investigated with Holm-Sidak.

**RESULTS**

Table 1 summarizes the body weight and muscle-to-body weight ratios of the congenic animals. All animals had a similar significant increase in body weight over the course of the experiment. Several congenic rat strains showed increase in EDL or TA muscle weight with stimulation.

- As previously shown, SS rats on a low-salt diet did not increase vessel density in response to electrical stimulation (Fig. 2). Intravenous infusion of a low dose of ANG II restored normal angiogenesis, and this restoration was completely blocked by oral coadministration of the angiotensin type 1 receptor (AT₁) antagonist losartan (50 mg·kg⁻¹·day⁻¹, Fig. 2). Also in agreement with our previous results (15, 16), transfer of the entire Chr 13, containing the functioning renin gene from a BN rat, onto a SS background (SS-13⁵⁵/Mcwi) restored the angiogenic response to electrical stimulation (16 ± 2.2% and 8.6 ± 2.5% in the TA and EDL muscles, respectively, Fig. 3).

When the reverse consomic BN-13⁵⁵/Mcwi (SS Chr 13 introgressed onto the BN background) was subjected to 7 days of electrical stimulation, no angiogenesis was observed (Fig. 3).

Angiogenesis in the congenic strains is represented in Fig. 4. Congenic strains in which the genomic region containing the BN renin allele was transferred onto the SS background (lines 13D and 13DA) showed a significant increase in vessel density after stimulation in both the TA (line 13D, 21.1 ± 5.8% vessel density increase, line 13DA, 8.6 ± 4.4%) and EDL (line 13D, 17.9 ± 3.3% increase, line 13DA, 8.2 ± 2.9%) muscles. In contrast line 13Db animals, which contained

---

**Table 1. Body weight and EDL and TA muscle weight-to-body weight ratio of all experimental groups**

<table>
<thead>
<tr>
<th></th>
<th>Start Weight</th>
<th>End Weight</th>
<th>EDL/BW, mg/g</th>
<th>TA/BW, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Line 13D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>273.3 ± 6.8</td>
<td>288.0 ± 7.6*</td>
<td>0.41 ± 0.01</td>
<td>1.82 ± 0.01</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.43 ± 0.01</td>
<td>1.88 ± 0.04</td>
</tr>
<tr>
<td><strong>Line 13DA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>268.3 ± 7.5</td>
<td>288.4 ± 7.4*</td>
<td>0.42 ± 0.01</td>
<td>1.76 ± 0.02</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.46 ± 0.01*</td>
<td>1.89 ± 0.04*</td>
</tr>
<tr>
<td><strong>Line 13Db</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>262.3 ± 9.8</td>
<td>285.4 ± 11.5*</td>
<td>0.39 ± 0.02</td>
<td>1.73 ± 0.03</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.44 ± 0.01*</td>
<td>1.90 ± 0.03*</td>
</tr>
<tr>
<td><strong>Line 13Dc</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>260.7 ± 8.3</td>
<td>281.8 ± 6.7*</td>
<td>0.42 ± 0.01</td>
<td>1.83 ± 0.03</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.45 ± 0.01*</td>
<td>1.91 ± 0.03</td>
</tr>
<tr>
<td><strong>SS-13⁵⁵/Mcwi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>294.8 ± 8.3</td>
<td>316.4 ± 6.8*</td>
<td>0.42 ± 0.01</td>
<td>1.81 ± 0.02</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.44 ± 0.01</td>
<td>1.85 ± 0.04</td>
</tr>
<tr>
<td><strong>BN-13⁵⁵/Mcwi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>215.3 ± 6.9</td>
<td>227.3 ± 6.9*</td>
<td>0.52 ± 0.01</td>
<td>1.90 ± 0.03</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.52 ± 0.01</td>
<td>1.88 ± 0.05</td>
</tr>
<tr>
<td><strong>13Da+Los</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>262.6 ± 9.2</td>
<td>275.8 ± 9.6*</td>
<td>0.40 ± 0.01</td>
<td>1.74 ± 0.02</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.46 ± 0.01*</td>
<td>1.84 ± 0.05</td>
</tr>
<tr>
<td><strong>SS</strong></td>
<td>249.8 ± 14.4</td>
<td>271.8 ± 13.4*</td>
<td>0.42 ± 0.01</td>
<td>1.80 ± 0.06</td>
</tr>
<tr>
<td><strong>SS+ANG II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>259.4 ± 5.9</td>
<td>270.8 ± 7.8*</td>
<td>0.40 ± 0.02</td>
<td>1.68 ± 0.04</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.41 ± 0.02</td>
<td>1.74 ± 0.08</td>
</tr>
<tr>
<td><strong>SS+ANG II+Los</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>278.8 ± 3.0</td>
<td>282.8 ± 2.9</td>
<td>0.43 ± 0.01</td>
<td>1.77 ± 0.05</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td>0.46 ± 0.01</td>
<td>1.88 ± 0.04</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. TA, tibialis anterior; EDL, extensor digitorum longus; BW, body weight; U, unstimulated muscle; S, stimulated muscle. Groups are as follows: congenic line 13D (n = 9), congenic line 13Da (n = 12), congenic line 13Db (n = 13), congenic line 13Dc (n = 14), SS-13⁵⁵/Mcwi (n = 15), BN-13⁵⁵/Mcwi (n = 12) congenic line 13Dc; rats treated with losartan (13Da+Los, n = 10), SS/Mcwi (SS, n = 5), SS/Mcwi rats with angiotensin II (ANG II) infusion (SS+ANG II, n = 4), SS/Mcwi rats treated with losartan and ANG II infusion (SS+ANG II+Los, n = 3). *P < 0.05 vs. start weight or unstimulated leg weight.
The BN region above renin, did not exhibit restoration of angiogenesis (TA 2.8\%/H10006 3.4\% increase, EDL 5.1\%/H10006 2.8\%).

Line 13DC, in which a 2.62 Mb region below renin was introgressed, animals had a significant increase in microvessel density (TA 22.4\%/H10006 2.9\% increase, EDL 18.7\%/H10006 3.7\%).

We further investigated the restored angiogenesis in congenic line 13D and 13DC rats by examining the mode of inheritance of the response to stimulation. Heterozygote line 13D and 13DC rats had an angiogenic response that was equal to that measured in the line 13D and 13DC homozygotes (line 13D heterozygote 21.3 ± 5.0\% TA, 23.6 ± 6.7\% EDL; line 13DC heterozygote 14.9 ± 5.2\% TA, 24.2 ± 5.6\% EDL). To assess the ANG II dependence of the restoration of the angiogenic response in the line 13DC animals we administered losartan to the 13DC homozygote subcongenics. Losartan significantly attenuated the angiogenic response to electrical stimulation in the 13DC rats (Fig. 5), indicating that the restoration of angiogenesis in line 13DC was mediated through interactions with the RAS.

There were no differences in the relative level of mRNA for renin in the unstimulated TA between the different congenic strains (Fig. 6). Muscle stimulation induced a significant increase in renin mRNA levels (two-factor repeated-measures ANOVA). Congenic strains with restored angiogenesis (lines 13D P < 0.05, 13DA P = 0.117, 13DC P < 0.05) had greater increases and higher absolute renin mRNA levels in their stimulated TA than were found in the stimulated TA of animals that did not have an increase in vessel density (SS, line 13DB, BN-13SS/Mcwi). Comparison of renal tissue to stimulated muscle revealed an ~2.5-fold greater amount of renin mRNA in the kidney compared with the stimulated muscle in SS and line 13DC rats.

Renin activity in skeletal muscle also increased with stimulation (data not shown). A 47\% increase in renin activity was observed in the stimulated TA muscle of line 13D rats (40.6 ± 3.9 nmol ANG I·mg tissue\(^{-1}\)·h\(^{-1}\)) relative to unstimulated TA (29.9 ± 7.8 nmol ANG I·mg tissue\(^{-1}\)·h\(^{-1}\), P = 0.079, n = 3). Kidney expressed twofold higher renin activity (63.5 nmol ANG I·mg tissue\(^{-1}\)·h\(^{-1}\)) relative to unstimulated TA. Histological analysis of SS-13BN rats found positive staining for renin in the smooth muscle layer of large vessels in both stimulated and unstimulated TA muscle (Fig. 7, A and B).

Assembly of Illumina SS sequence reads for the 4.05 Mb region of interest resulted in 5,638 contigs, covering 3,277,840 bp (79.3\% of the region) with an average contig length of 581 bp. Compared with the BN reference, a total of 8,850 variants (0.21\% of the sequence) were identified by
two or more reads with an occurrence ≥30%. Of these variants, 5,181 were found to be intergenic and 3,562 within introns or untranslated regions. The remaining 107 variants were within known coding regions.

Of the variants within coding regions, 29 were determined to cause amino acid changes or nonsynonymous variants (Table 2). Using the software Polyphen and SIFT, we predicted 14 of the nonsynonymous variants to be damaging to the protein. Five of the variants found in coding sequence were located in the chromosomal region in common to lines 13DA and 13DC (Table 2, boldfaced variants). We also looked at the presence of predicted microRNA that could act as inhibitors of renin transcription in this 593 kb region. There were 19 conserved predicted microRNA target sites in eight genes, none of which contained sequence variations between strains. Possible connections between renin and the genes with predicted damaging variants in Table 2 were identified by STRING and are shown in Fig. 8.

DISCUSSION

Chromosome substitution is powerful tool for studying complex multigenic diseases. Through the development of consomic and congenic lines our group has been able to isolate regions of the SS rat genome that play a role in this animal’s inability to regulate renin, which in turn drives the phenotype of angiogenesis impairment in response to electrical stimulation of skeletal muscle (5, 14). In previous studies we have shown that a lack of ANG II, driven by inhibition of the RAS or an impairment in the function of renin, impacts endothelial function and survival by modulating apoptosis of endothelial cells. This effect was dramatically altered by high-salt diet, a known suppressor of renin (15). Further studies have shown that impairments in renin-mediated ANG II production is essential for normal bone marrow and endothelial progenitor cell function in our model of muscle stimulation (17).

In the present study we used marker-assisted breeding to produce three new congenic strains from the previously described renin and angiogenesis-restored SS.BN-(D13hmgc41-D13rat101)/Mcwi (line 13D) (14). These 1 to 2 Mb regions spanned the length of the line 13D insert, with one upstream of renin (line 13DB), one spanning from upstream through
the renin gene (line 13DA), and the last entirely downstream of renin (line 13DC). We found line 13DA, and unexpectedly line 13DC, to restore the angiogenesis phenotype and renin response to electrical stimulation, while line 13DB exhibited the SS phenotype. We have been further able to verify that this restored angiogenesis is ANG II dependent as treatment of line 13DC animals with the AT1 antagonist losartan significantly attenuated the vessel density increase in these animals compared with the untreated group. The angiogenic effect of line 13DC, which contains the SS renin allele combined with data on renin expression, demonstrates that there is a renin regulatory region acting in *trans* on the renin gene itself, and located >452 Kb downstream of renin. The SS allele impairs renin and angiogenesis stimulation and acts in a manner that implies that the SS allele is recessive.

The congenic strains that include the BN renin allele also restore angiogenesis, which in the past has lead us to hypothesize that renin or a nearby regulator was impaired in the SS rats. One of these strains (13DA) shares a 593 Kb stretch with line 13DC, which suggests that in these strains it is not necessarily the renin gene, but the region between 452 and 1,045 Kb downstream of renin, that contains a sequence variation participating in the impaired renin regulation observed in the SS rat. It is also possible that there could be two loci present in the region covered by congenic line 13DA that regulate renin and angiogenesis, or just the one shared with line 13DC, downstream of renin. Further narrowing the region by development of additional subcongenic strains to isolate these chromosomal segments will be required for the positional clonig of this/these loci.

Extensive research has been performed on the *cis*-acting proximal promoter and enhancers of renin in mouse, human, and rat models (8, 48). In mice, ~4 kb of the 5′-flanking sequence of renin contains the most important regulatory regions (48). The proximal promoter contains three highly conserved transcription factor-binding sites including the HOX-PBX complex and Pb site, and in mice there are four additional transcription factor-binding sites not found in human or rat (the M3 insert −564 to −80), and a mutation in this region reduces mouse renin transcription 90%, bringing renin levels near those found in human and rat (48). Six transcription factor-binding sites have been found human choriocarcin cell model system, and three of the transcription factors, HOX-PBX and cAMP-responsive element CRE, have been identified (8).

It is not known which transcription factor-binding sites are present in rat (48); however, the strong sequence conservation in the mouse, human, and rat promoter region would suggest similar regulatory mechanisms in all three species. A 242 bp region has been identified in all three models that acts as a renin enhancer, approximately −2.8 kb upstream in mouse, −11 kb in human (75% conserved from mouse enhancer), and −5.8 kb in rat (85% homologous to the mouse enhancer) (48). While transfection studies have shown the enhancer can increase renin production >50-fold in mice, the enhancer causes a limited increase in human tissue (1- to 2-fold) and no increase in rat (48). The relationship between these known regulatory elements and the candidate region in the current study remains unknown.
Variants in the human genes coding for renin, angiotensin converting enzyme (ACE), angiotensinogen, and the AT1 receptor have now been shown to be associated with a variety of complex multigenic diseases (10, 57). Although no differences in the coding sequence or the proximal promoter of the renin gene were identified in SS and BN rats, variations in the expression of renin and its downstream actions in these strains suggest that the differences in the regulation of renin depend on an intermediate mechanism.

Sequence analysis of the SS and BN strains in the 4 Mbp region covered by line 13D identified 12 nonsynonymous variants that were predicted to be damaging to the encoded protein. Genes containing these variants include Ptprv, a protein involved in p53-dependent cell cycling, Syt2, a negative regulator of chemotaxis; and Pik3c2b, a member of the phosphoinositide 3-kinase family, which collectively control multiple relevant cellular responses, including proliferation, growth, chemotaxis, and survival. None of the genes are known to directly play a role in the regulation of renin expression; however, a pathway analysis using STRING revealed a number of interesting connections between a few of the identified sequence variants and renin expression including inactivation through renin binding protein, connections to the wnk1 serine/threonine kinase pathway and modulation through the AT1, angiotensin type 2 (AT2), and MAS receptors. From the predicted damaging variants, only Chit-1 and Mybph single nucleotide polymorphisms were located in the common region between lines 13DA and 13DC. The putative connection between the Chit-1 (chitinase-1) gene (chitotriosidase-1 precursor, EC 3.2.1.14) and renin expression acting through the renin-binding protein [renbp, N-acylglucosamine 2-epimerase (EC 5.1.3.8)], which binds to renin, forming high-molecular-weight renin, is intriguing. Chit-1 is secreted by activated macrophages, and mutations have been shown to be functionally relevant in asthma (63). Renbp has a leucine zipper motif that may be important in the formation of a heterodimer with renin. Both of these proteins are involved in the formation of N-Acetyl-D-glucosamine as part of the amino sugar metabolic pathway (KEGG)(29). As for Mybph, this gene encodes for myosin binding protein H. This gene has not been directly related to the RAS but is highly expressed in skeletal muscle (59, 66) and cultured human myotubes, suggesting a possible role for this gene in angiogenesis (21). How a mutation in this gene would affect renin expression and result in an impaired angiogenic response is unknown.

Fig. 8. Possible connections between the genes identified in Table 2 and renin identified by STRING. The program was seeded with nonsynonymous variants identified as being damaging to the protein (Table 2) as well as renin. The STRING network was generated with the following parameters: gene fusion, cooccurrence, coexpression, experiments, databases, and text-mining. Network depth was 1 and required confidence was >0.4.
With ~850 kb of the 4.05 Mb region unsequenced, other variants may exist that have yet to be identified. As a more complete reference sequence emerges, these few remaining unsequenced regions will be compared and any additional variants identified. Beginning with the 10 genes we have identified that contain deleterious variants, future work will be done to analyze expression differences between strains in the mRNA and proteins.

The present study emphasizes the importance of maintaining normal renin regulation as well as ANG II levels during the angiogenesis process in adult skeletal muscle. Increased expression of renin with electrical stimulation of the hindlimb was demonstrated in the current study and verified by the measurement of renin activity and renin protein. Although the exact mechanism by which stimulation increases in renin expression is not known, it has been suggested that hypoxia may stimulate renin gene expression. This may not be a direct mechanism however, since hypoxia had no effect on renin secretion or renin gene expression in isolated renal juxtaglomerular cells (54). Alternatively, mechanical forces induced during stimulation may also play a role expression of RAS genes (36), although this is somewhat controversial since direct assessment of renin gene expression in As4.1 cells indicated decreased renin expression with cyclical stretch (55).

Studies in the literature have shown involvement of the RAS in angiogenesis of the eye (47), heart (43), kidney (22), and chicken chorio-allantoc membrane (35). In the current study physiological, genetic, and pharmacological manipulation of the RAS resulted in impaired muscle angiogenesis, which was assessed by comparison of microvessel density in the stimulated vs. the unstimulated hindlimb of the same animals. In previous studies using this model we have shown that the contralateral hindlimb does not undergo significant remodeling and provides an excellent control because it controls for interanimal variability (38). Interestingly, no direct link was observed between microvascular angiogenesis and muscle trophicity in the stimulated or unstimulated leg in these studies (Table 1 and Fig. 2), suggesting these two responses to stimulation may be independent of one another. Understanding the mechanism by which angiogenesis is impaired in the SS and restored by ANG II has major therapeutic importance since ACE inhibitors and ANG II receptor blockers continue to be prescribed for the treatment of a growing number of conditions (1, 2, 7, 18, 33, 39, 40, 49, 61, 67). While the present study does not address the specific mechanism by which impaired renin regulation alters angiogenesis, it does provide direct evidence that genetic dysregulation of the renin expression, acting through the RAS, can produce significant abnormalities in vessel growth and suggests several pathways by which this may occur. Insufficient growth of the microcirculation as well as abnormal capillary regression may be involved in a wide range of diseases including heart and brain ischemia (24, 32), neurodegeneration (12, 46), hypertension (9), nephropathies (30), atherosclerosis, and diabetes (62, 64); identification of the molecular and genetic factors that affect the RAS could have a dramatic effect on the growth and stability of the microcirculation and consequently on the treatment of these diseases.

GRANTS
This work was supported by National Institutes of Health Grants HL-29587, HL-82798, and NIH-NHLBI contract N01-HV-28182.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

Physiol Genomics • VOL. 43 • www.physiolgenomics.org
REGULATION OF RENIN AND ANGIOGENESIS


