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# Transcriptional Regulation of the Novel Monoamine Oxidase Renalase: Crucial Roles of Transcription Factors Sp1, STAT3, and ZBP89

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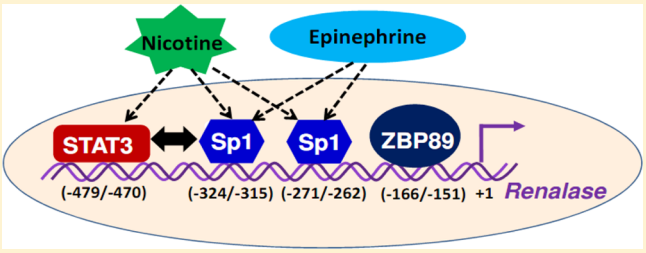
Supporting Information

**ABSTRACT:** Renalase, a novel monoamine oxidase, is emerging as an important regulator of cardiovascular, metabolic, and renal diseases. However, the mechanism of transcriptional regulation of this enzyme remains largely unknown. We undertook a systematic analysis of the renalase gene to identify regulatory promoter elements and transcription factors. Computational analysis coupled with transfection of human renalase promoter/luciferase reporter plasmids (5′-promoter-deletion constructs) into various cell types (HEK-293, IMR32, and HepG2) identified two crucial promoter domains at base pairs −485 to −399 and −252 to −150. Electrophoretic mobility shift assays using renalase promoter oligonucleotides with and without potential binding sites for transcription factors Sp1, STAT3, and ZBP89 displayed formation of specific complexes with HEK-293 nuclear proteins. Consistently, overexpression of Sp1, STAT3, and ZBP89 augmented renalase promoter activity; additionally, siRNA-mediated downregulation of Sp1, STAT3, and ZBP89 reduced the level of endogenous renalase transcription as well as the transfected renalase promoter activity. In addition, chromatin immunoprecipitation assays showed *in vivo* interactions of these transcription factors with renalase promoter. Interestingly, renalase promoter activity was augmented by nicotine and catecholamines; while Sp1 and STAT3 synergistically activated the nicotine-induced effect, Sp1 appeared to enhance epinephrine-evoked renalase transcription. Moreover, renalase transcript levels in mouse models of human essential hypertension were concomitantly associated with endogenous STAT3 and ZBP89 levels, suggesting crucial roles for these transcription factors in regulating renalase gene expression in cardiovascular pathological conditions.




Renalase is a recently discovered secretory protein that is emerging as a crucial player in the regulation of hypertension, left ventricular hypertrophy, inducible ischemia, end-stage renal disease, and diabetes.[1−5] While the major amount of renalase in the circulation is derived from kidneys (as is evident from the very small amount of this protein in subnephrectomized rats and end-stage renal disease patients), renalase is also expressed in several other tissues, including heart, liver, intestine, and various parts of the nervous system.[6−8] Renalase is considered a new member of the monoamine oxidase family and is designated as monoamine oxidase C in view of its catecholamine-metabolizing activity.[1,4,6,9] Consistently, renalase knockout mice have increased plasma levels of catecholamines (norepinephrine, epinephrine, and dopamine) and display elevated blood pressure and susceptibility to cardiac ischemia.[10] Likewise, rat models of heart failure show accumulated plasma norepinephrine levels along with a reduced level of expression of renalase.[5] Very recently, it has been shown that renalase exerts a protective effect against acute kidney injury by functioning as a signaling molecule (via activation of AKT and MAP kinase and downregulation of c-Jun N-terminal kinase through interaction with a hitherto unknown cell surface receptor) in a manner independent of its amine oxidase activity.[11]

The renalase gene is positioned in chromosome 10 in human, chromosome 19 in mouse, and chromosome 1 in rat.[1,7] The human renalase gene contains 10 exons spanning an ∼310 kb region of the genome and gives rise to seven isoforms by alternative splicing (hRenalase1−7 yielding proteins of 342, 315, 232, 200, 181, 163, and 139 amino acid residues, respectively).[11] The isoforms hRenalase1 and hRenalase2 are prominently expressed; hRenalase1, the relatively well-known isoform of renalase, has a signal peptide (amino acids 1−17), a FAD binding domain (amino acids 1−42, 109−189, and 294−341), and the putative substrate-binding domain (amino acids 43−108 and 190−294).[12,13]

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Table 1. Putative Transcription Factor Binding Sites in the Proximal Human Renalase Promoter[a]

| location | transcription factor | recognition sequence (5′ to 3′) | prediction program |
|---|---|---|---|
| base pairs −479 to −470 | STAT3 | TTAGAGGAAA | MatInspector |
| base pairs −456 to −440 | HoxB9 | ATCGCTGGTAAATTCTT | MatInspector |
| base pairs −419 to −403 | ILF1 | AATAGTAAAACAGCAAA | MatInspector |
| base pairs −409 to −400 | Pax1 | CAGCAAAAAT | Consite |
| base pairs −324 to −315 | Sp1#2 | GAGGCGGGTT | Consite/MatInspector |
| base pairs −271 to −262 | Sp1#1 | GGGGCAGGGC | Consite/MatInspector |
| base pairs −212 to −207 | SNAIL | CAGGTT | Consite |
| base pairs −212 to −207 | HNF-3$\beta$ | CAGGTT | Consite |
| base pairs −203 to −189 | FTF-2 | TCCTCAAGGACGGAG | MatInspector |
| base pairs −166 to −151 | ZBP89 | TCCTTTCCGCCCCCTG | MatInspector |

[a]The locations of the transcription factor binding sites are numbered with respect to the transcription start site (as base pair +1). Two binding sites for Sp1 were predicted. Those are shown as Sp1#1 and Sp1#2.

Although an altered expression of renalase may lead to several cardiovascular, metabolic, and kidney disorders (as mentioned above), the mechanism of regulation of renalase remains unknown except for the observations that dopamine enhances the abundance of renalase transcript in H9c2 (rat cardiomyoblast) cells and epinephrine induces renalase expression in HK2 (human renal proximal tubular epithelial) cells.[6,14] Indeed, nothing is known about the important promoter domains and transcription factors that may govern basal or inducible renalase gene expression.

In this study, we undertook a systematic analysis of the human renalase proximal promoter region (~1.3 kb upstream of the transcription initiation site) and identified two major promoter domains (base pairs −485 to −399 and −252 to −150) that are crucial for renalase expression. Computational analysis coupled with *in vitro* (exogenous expression of transcription factors with wild-type or mutated promoter reporter constructs, siRNA-mediated downregulation of transcription factors, and electrophoretic mobility shift assays) and *in vivo* (chromatin immunoprecipitation) experiments revealed regulatory roles of Sp1, STAT3, and ZBP89 in the basal and nicotine/epinephrine-induced transcription of the renalase gene. Moreover, concomitantly altered levels of renalase and STAT3 and ZBP89 in mouse models of essential hypertension provided evidence of crucial roles of these transcription factors in a pathophysiological context.

## EXPERIMENTAL PROCEDURES

**Cell Culture.** HEK-293 (derived from human embryonic kidney cells), HepG2 (a human hepatocellular carcinoma cell line), and IMR32 (a human neuroblastoma cell line) cell lines were obtained from the National Center for Cell Sciences (Pune, India). Cells were cultured in Dulbecco's modified Eagle's medium with high glucose and GlutaMAX-I (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), penicillin G (100 units/mL), and streptomycin sulfate (100 mg/mL) (Invitrogen) in 25 $cm^2$ tissue culture flasks (Nalgene-Nunc International) at 37 °C with 5% $CO_2$.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Estimation of Endogenous Renalase Expression in Cell Lines.** Total RNA samples from cultured human cell lines (viz. HEK-293, HepG2, and IMR32) were isolated using the TRIZOL reagent (Invitrogen) by following manufacturer's instructions. Estimation of RNA concentrations, assessment of the integrity of RNA molecules, and synthesis of total cDNA were conducted as described previously.[15] RT-PCRs using cDNA preparations were performed using the following human *renalase*-specific primers to amplify a 201 bp region of exon 7: forward, 5′-CAGCCTTCCAAACCAGCTAC-3′; and reverse, 5′-GTTCTGCCTGTGCCTGTGTA-3′. For normalization of human renalase expression, $\beta$-actin abundance was measured using the following primers to amplify a 174 bp DNA segment: forward, 5′-CTGGTGCCTGGGGCG-3′; and reverse, 5′-AGCCTCGCCTTTGCCGA-3′. A modified version of the touchdown PCR program described previously[16] was used to amplify the cDNA segments: the PCR protocol began with a 94 °C denaturation step for 5 min, followed by a touchdown program (94 °C denaturing step for 30 s followed by an initial annealing temperature of 70 °C, subsequently run down to 54 °C at −1 °C/cycle and a 72 °C extension step for 30 s), followed by a uniform three-step amplification profile (94 °C denaturing step for 30 s, 54 °C annealing step for 30 s, and 72 °C extension step for 30 s) for another 17 cycles, and a 72 °C for 10 min extension step and then finally held at 4 °C. The PCR products (5 $\mu$L) were loaded on a 1.5% agarose gel and stained with ethidium bromide to visualize the bands. The authenticity of the PCR products was confirmed by DNA sequencing.

**Generation of Renalase Promoter Reporter Constructs.** The human renalase promoter sequence was obtained from the UCSC genome browser (Entrez Gene ID 55328). The first nucleotide of exon 1 of the renalase gene was marked as +1. Human genomic DNA extracted from the blood sample of a healthy volunteer using Quiagen Flexigene DNA kit[17] was used to amplify various promoter regions via PCR. A series of forward primers (5′-CGGGGTACC[-1285bp]CCTTTTGGCTTTTGTCATTTGTGTC[-1261bp]-3′, 5′-CGGGGTACC[-485bp]CAAGCATTAGAGGAAAGGTTGCC[-463bp]-3′, 5′-CGGGGTACC[-393bp]CCAACAGGCATGAGAGTTATCG[-377bp]-3′, 5′-CGGGGTACC[-339bp]CGTCAGCTCTGTTGCGAGGC[-320bp]-3′, 5′-CGGGGTACC[-252bp]CCAAAGCGAGAAGACAGACAGC[-231bp]-3′; the underlined sequence indicates the added *Kpn*I site) and a common reverse primer (5′-CCGCTCGAG[-5bp]AGAGGGAGCAGCGATCCG[-22bp]-3′; the underlined sequence indicates the added *Xho*I site) were used to amplify ~1.3 kb, 480 bp, 393 bp, 334 bp, and 247 bp promoter regions (that include 130 bp from the 5′-UTR of the gene). Amplified DNA fragments were cloned between the *Kpn*I and *Xho*I sites of the pGL3-basic vector (Promega), and the resulting plasmids were named hRenPro-1285 (that contained base pairs −1285 to −5 of the renalase promoter), hRenPro-485 (containing base pairs −485 to −5), hRenPro-398 (containing base pairs −398 to −5), hRenPro-339 (containing base pairs −339 to −5), and hRenPro-252


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(containing base pairs −252 to −5). Additional promoter reporter constructs were generated by making use of restriction sites for *Pst*I (at base pairs −153 to −148), *Apa*I (at base pairs −127 to −122), and *Sma*I (at base pairs −57 to −52) in the renalase promoter. The hRenPro-1285 plasmid was digested with *Kpn*I and either *Pst*I, *Apa*I, or *Sma*I, followed by purification of the larger fragments, blunting the overhangs by Mung Bean nucleases, and reclosure by T4 DNA ligase. The resulting plasmids were named hRenPro-149, hRenPro-123, and hRenPro-54. These promoter constructs contained the putative binding sites for various transcription factors (Table 1). The authenticity of the promoter reporter constructs was confirmed by DNA sequencing.

The promoter reporter plasmid with a mutated STAT3 binding domain was generated by site-directed mutagenesis using hRenPro-485 as the template, 5′-TCAAGCATTAGAG-**CCG**AGGTTGCCCGTGGTA-3′ as the forward primer, and 5′-TACCACGGGCAACCT**CGG**CTCTAATGCTTGA-3′ as the reverse primer (mutated nucleotides are shown in bold). The resulting plasmid was named mut-STAT3-hRen-485. Two Sp1 binding domains in renalase promoter were designated as Sp1#1 and Sp1#2. The promoter reporter plasmid for mutated Sp1#1 was obtained by using hRenPro-485 as a template and the following mutagenic primers: forward, 5′-TCTGGGCGA-GG**AA**CAGGGCGCCCGGCCC-3′; and reverse, 5′-GGGCC-GGGCGCCCTGT**T**CCTCGCCCAGA-3′ (mutated nucleotides are shown in bold). The resulting plasmid was named mut-1-Sp1-hRen-485. Using this plasmid as a template, the Sp1#2 domain was mutated using 5′-CTCTGTTGCGA**AA**CGGGT-TCTCCCAA-3′ as the forward primer and 5′-TTGGGAGA-ACCCG**TT**TCGCAACAGAG-3′ as the reverse primer (mutated nucleotides are shown in bold); the resulting plasmid harboring mutations at both the Sp1 sites was named mut-1/2-Sp1-hRen-485. Similarly, a promoter reporter plasmid with a mutated ZBP89 binding domain was obtained by using hRenPro-252 as a template and the following mutagenic primers: forward, 5′-GGCCGCCTCCTTTCCGC**TT**CCTG-CAGGCCGG-3′; and reverse, 5′-CCGGCCTGCAGG**AA**GC-GGAAAGGAGGCGGCC-3′ (mutated nucleotides are shown in bold); the resulting plasmid was named mut-ZBP89-hRen-252.

**Transient Transfections of Cell Lines, Reporter Assays, and Western Blot Analysis.** HEK-293 and HepG2 cells (grown at 50−60% confluence in 12-well plates) were transfected with 1 μg of promoter reporter plasmid per well by the calcium phosphate method.[18] Similarly grown IMR32 cells were transfected with 1 μg of plasmids per well with Targetfect F2 transfection reagents (Targeting Systems). As an internal control for transfection efficiency, cells were cotransfected with 0.25−0.5 μg of a β-galactosidase expression plasmid per well, wherever possible. Cells were lysed after being transfected for 24−30 h, and luciferase and β-galactosidase assays were conducted by previously described methods.[15,19] The results were expressed as firefly luciferase/β-galactosidase activity.

In one set of experiments, a renalase wild-type or mutated promoter reporter construct or pGL3-basic plasmid (0.5 μg/well) was cotransfected with different concentrations (0.25, 0.5, or 1.0 μg/well) of a transcription factor expression plasmid (viz. pCMV-STAT3 expressing mouse STAT3, pCMV-flag-ZBP89 expressing rat ZBP89,[20] or pcDNA3-Sp1 expressing mouse Sp1)[21,22] into HEK-293 cells, followed by a luciferase assay 24−30 h after transfection. In these cotransfection experiments, the insert-free vectors pcDNA3 (in the case of Sp1 and ZBP89) and pRCMV (in the case of STAT3) were used to make equal amounts of total DNA in the transfection mixtures. As a control for varying the cell number within individual wells, total protein contents were measured in cell lysates using Bradford's assay reagent (Sigma-Aldrich) and the luciferase activities in cell lysates were normalized to total protein. Promoter activities for the wild-type or mutated constructs were then expressed as fold over the control plasmid pGL3-basic values.

To test the effect of overexpression of Sp1, STAT3, and ZBP89 on renalase protein level, HEK-293 cells were transfected with an empty vector or an expression plasmid (1.0 or 2.0 μg/well) in six-well plates, and total proteins were isolated in RIPA buffer with protease inhibitor cocktail (Sigma). Approximately 80 μg of total protein samples were resolved via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), transferred onto activated PVDF membranes, and incubated with a renalase antibody (abcam catalog no. ab178700, 1:500 dilution). The blots were reprobed with antibodies for STAT3 (Santa Cruz Biotechnology catalog no. sc-482X, 1:2500 dilution), ZBP89 (Santa Cruz Biotechnology catalog no. sc-137169X, 1:5000 dilution), and Sp1 (abcam catalog no. ab13370, 1:2000 dilution). The blots were then stripped again and probed with β-actin (Sigma catalog no. A5441, 1:7500 dilution) or GAPDH (Neo Bio Lab catalog no. AC001, 1:7000 dilution) antibodies. The bands were detected using an HRP-linked secondary conjugate specific for either mouse (Jackson Immunoresearch catalog no. 115-035-003, 1:5000−10000 dilution; for β-actin and ZBP89) or rabbit (Bio-Rad catalog no. 170-6515, 1:3000 dilution; for renalase, GAPDH, STAT3, and Sp1). A protein-specific luminescence signal was detected using the VersaDoc Chemiluminescence Detection system (Bio-Rad). Relative intensities of protein expression were quantified using NIH ImageJ.

In an additional set of experiments, HEK-293 cells were transfected with the renalase promoter construct and treated with various concentrations of nicotine (0, 0.25, 0.5, and 1.0 mM) after being transfected for 12 h. Luciferase assays were performed 30 h after transfection. For nicotine-mediated STAT3 and Sp1 activation experiments, HEK-293 cells were cotransfected with 0.5 μg of hRenPro-485 reporter plasmid per well and 0.5 μg of either pRCMV-STAT3 or pcDNA3-Sp1 per well individually and in combination. Total DNA amounts among the different conditions were made equal by using pcDNA3. In these experiments, results were represented as luciferase activity normalized to total protein.

In another set of experiments, HEK-293 cells transfected with the hRenPro-1285 construct were treated with various concentrations of epinephrine (0, 1, 5, 25, 100, and 200 μM) or dopamine (0, 5, 10, 50, and 100 μM) 8 h after transfection. The culture medium was replaced with fresh medium with the same doses of catecholamines after 10−12 h. In some experiments, the hRenPro-1285 plasmid was cotransfected with the Sp1, STAT3, or ZBP89 expression plasmid individually into HEK-293 cells and treated with epinephrine (25 μM) or dopamine (50 μM). Epinephrine and dopamine treatments were performed as described above. Luciferase and Bradford assays were performed ∼30 h after transfection.

In some experiments, the effect of serum on the renalase promoter activity was tested. Briefly, the hRenPro-485 promoter reporter construct (0.5 μg/well) was cotransfected with 500 ng of the Sp1, STAT3, or ZBP89 expression plasmid into HEK-293 cells; the total amount of DNA across the wells was made equal by using pcDNA3/pRCMV. After being

Table 2. Oligonucleotides Used in the Electrophoretic Mobility Shift Assays

| transcription factor | oligo type | sequence (5′ to 3′)[b] |
|---|---|---|
| STAT3 | wild-type | F- TCAAGCATTAGAGGAAAGGTTGCCCGTGGTA |
| | | R- TACCACGGGCAACCTTTCCTCTAATGCTTGA |
| | mutant[a] | F- TCAAGCATTAGAGccgAGGTTGCCCGTGGTA |
| | | R- TACCACGGGCAACCTcggCTCTAATGCTTGA |
| Pax1/ILF-1 | wild-type | F- AATAGTAAAACAGCAAAAATCCCAACAGGC |
| | | R- GCCTGTTGGGATTTTTGCTGTTTTACTATT |
| | mutant[a] | F- AATAGTAAAACAGCAtcAATCCCAACAGGC |
| | | R- GCCTGTTGGGATTgaTGCTGTTTTACTATT |
| HoxB9 | wild-type | F- GTGGTATCGCTGGTAAATTCTTCTTCC |
| | | R- GGAAGAAGAATTTACCAGCGATACCAC |
| | mutant[a] | F- GTGGTATCGCTGGgAccTTCTTCTTCC |
| | | R- GGAAGAAGAAggTcCCAGCGATACCAC |
| SNAIL/HNF-3$\beta$ | wild-type | F- CCGCAGCCCCAGGTTTGCTCCTCA |
| | | R- TGAGGAGCAAACCTGGGGCTGCGG |
| | mutant[a] | F- CCGCAGCCCtAGGcTTGCTCCTCA |
| | | R- TGAGGAGCAAGCCTAGGGCTGCGG |
| FTF-2 | wild-type | F- TTTGCTCCTCAAGGACGGAGGGGCC |
| | | R- GGCCCCTCCGTCCTTGAGGAGCAAA |
| | mutant[a] | F- TTTGCTCCTCAAaaACGGAGGGGCC |
| | | R- GGCCCCTCCGTttTTGAGGAGCAAA |
| ZBP89 | wild-type | F- GGCCGCCTCCTTTCCGCCCCCTGCAGGCCGG |
| | | R-CCGGCCTGCAGGGGGCGGAAAGGAGGCGGCC |
| | mutant[a] | F- GGCCGCCTCCTTTCCGCttCCTGCAGGCCGG |
| | | R- CCGGCCTGCAGGaaGCGGAAAGGAGGCGGCC |
| Sp1#1 | wild-type | F- TCTGGGCGAGGGGCAGGGCGCCCGGCCC |
| | | R- GGGCCGGGCGCCCTGCCCCTCGCCCAGA |
| | mutant[a] | F- TCTGGGCGAGGaaCAGGGCGCCCGGCCC |
| | | R- GGGCCGGGCGCCCTGttCCTCGCCCAGA |
| Sp1#2 | wild-type | F- CTCTGTTGCGAGGCGGGTTCTCCCAA |
| | | R- TTGGGAGAACCCGCCTCGCAACAGAG |
| | mutant[a] | F- CTCTGTTGCGAaaCGGGTTCTCCCAA |
| | | R- TTGGGAGAACCCGttTCGCAACAGAG |

[a]The mutated nucleotides at the transcription factor binding sites are indicated in lowercase. [b]F, forward primer; R, reverse primer.

transfected for 7−8 h, cells were subjected to serum deprivation by replacing the medium with serum-free medium. In the control wells, the culture medium was replaced with fresh medium containing 10% FBS. After transfection for 24 h, luciferase and Bradford assays were conducted, and results were expressed as luciferase activity per microgram of protein.

**Electrophoretic Mobility Shift Assays (EMSAs).** Nuclear protein extracts from HEK-293 cells were prepared using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas Life Sciences) and stored in aliquots at −80 °C until further use. The total protein amounts in these extracts were estimated using Bradford's assay reagents (Sigma-Aldrich).

The oligonucleotides used for EMSAs (Table 2) were obtained from Ocimum Biosolutions (Hyderabad, India). Single-stranded DNA oligomers (at a concentration of 5 $\mu$M) were biotinylated using 10 units of deoxynucleotidyl transferase (NEB) and Biotin-11-UTP (500 nM, Fermentas Life Sciences) following the manufacturer's protocol. The labeling reactions were terminated with 10 $\mu$M EDTA, and oligonucleotides were purified by extraction with equal volumes of a chloroform/iso-amyl alcohol (24:1) mixture. Equal amounts of each oligomer and its complementary strand were annealed to a final oligomer concentration of 50 nM. The efficiency of biotin labeling of the primers was tested by dot blot of labeled oligomers and densitometric analysis.

EMSAs were conducted by minor modifications of our previously described method.[15] In brief, 5−6 $\mu$g of HEK-293 nuclear protein extract was incubated with binding buffer [10 mM Tris, 50 mM KCl, and 1 mM dithiothreitol (pH 7.5)], 50 ng/$\mu$L poly dI-dC, and 25 fmol of the double-stranded biotinylated oligomer for 20 min at room temperature. A set of reactions that included labeled oligomers without nuclear protein or nuclear protein without any labeled oligos were used as reaction controls. The reaction mixtures were resolved on 5% nondenaturing polyacrylamide gels and transferred to nylon-66 membranes (Amersham), followed by UV cross-linking of the DNA oligomers to the membrane. The biotinylated probes were detected by chemiluminescence using the LightShift Chemiluminescent EMSA kit (Pierce). In some EMSA experiments, the nuclear extracts were incubated with excess amounts (5−100-fold molar excess) of the unlabeled oligomers having either identical or mutated sequences (Table 2) in the binding buffer prior to addition of biotin-labeled probes.

**Chromatin Immunoprecipitation (ChIP) Assays.** ChIP assays were performed using the Simple ChIP kit (Cell Signaling Technology) and ChIP-grade STAT3, Sp1, and ZBP89 antibodies from Santa Cruz Biotechnology. Anti-histone antibody (Cell Signaling) was used as a positive control, and anti-rabbit IgG antibody (Sigma) was used as a negative control. The following pair of primers was used for the


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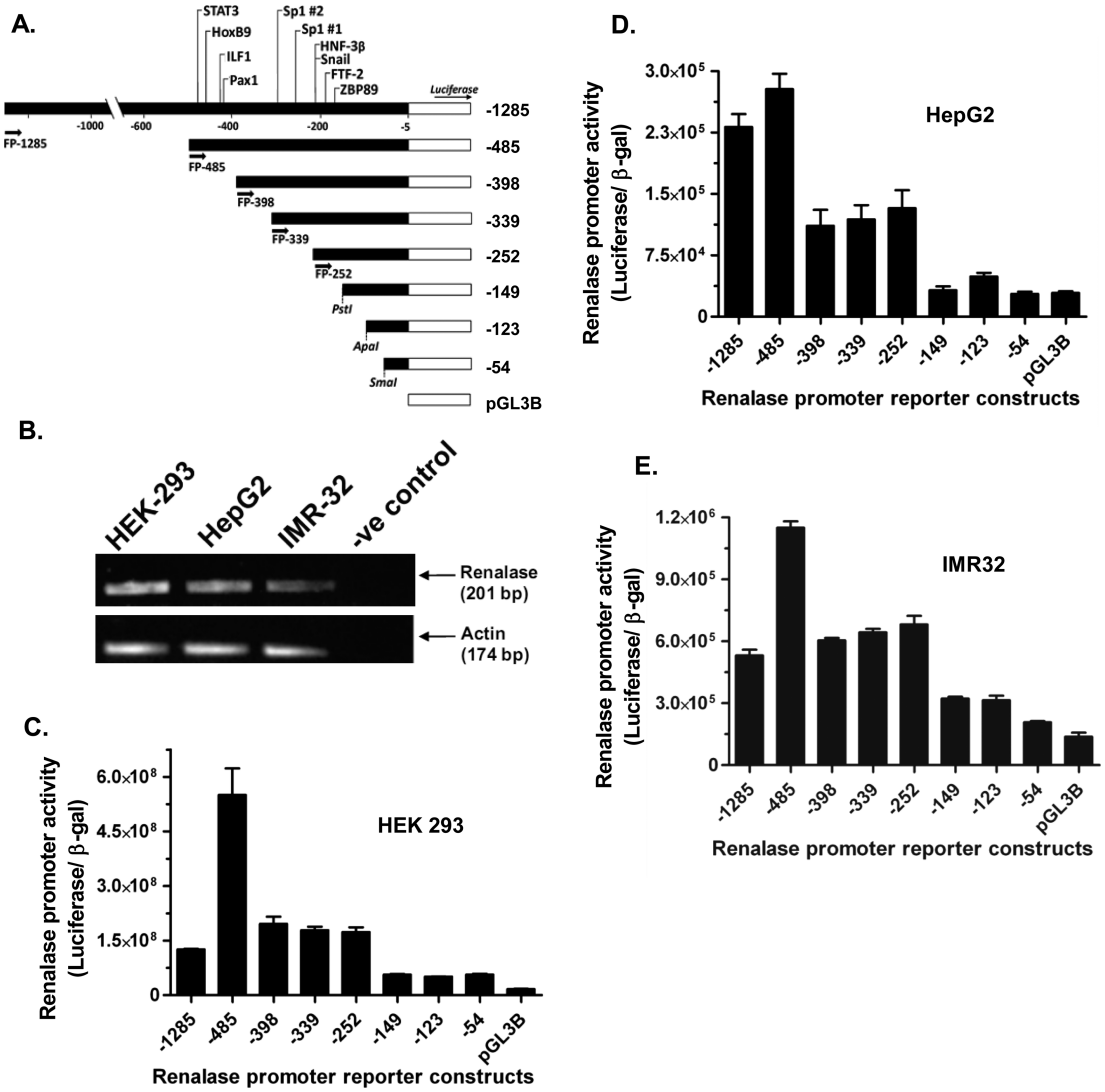


**Figure 1.** Crucial promoter domains of human renalase. (A) Schematic strategy for generation of various renalase promoter reporter constructs. Locations of the crucial cis-regulatory elements are shown on the top, and the numbers at the right indicate the sizes of renalase promoters. (B) Endogenous expression of renalase and actin transcripts in various human cell lines using semiquantitative RT-PCR. Total RNA was isolated from the HEK-293, HepG2, and IMR32 cells, and cDNA was synthesized as described in Experimental Procedures. PCR was performed using human renalase and actin-specific primers, and the PCR products were visualized on an ethidium bromide-stained agarose gel. (C–E) Activity of human renalase promoter reporter constructs in various cell lines. Reporter constructs harboring various lengths of the human renalase promoter were transiently transfected into HEK-293, HepG2, and IMR-32 cells; cells were cotransfected with a $\beta$-galactosidase expression plasmid (pCMV-$\beta$-Gal) for normalization of the promoter activity across various conditions. The cells were lysed 24–30 h after transfection, and cell lysates were assayed for luciferase and $\beta$-galactosidase activities. Results are expressed as means ± the standard error of normalized promoter activity (luciferase/$\beta$-galactosidase).

detection of the promoter region encompassing binding sites for STAT3, Sp1, and ZBP89 transcription factors: forward, 5′-CGGGGTACC[-485 bp]CAAGCATTAGAGGAAAGGTTG-CC[-463 bp]-3′; and reverse, 5′-CCGCTCGAG[-5 bp]AGA-GGGAGCAGCGATCCG[-22 bp]-3′. PCR was performed by a modified version of the touchdown PCR program described previously[16] that consisted of 18 cycles instead of 23 cycles, and all PCR products were analyzed on a 1.8% agarose gel, stained with ethidium bromide, and visualized under a UV trans-illuminator (Bio-Rad).

**siRNA-Mediated Downregulation of Transcription Factors in HEK-293 Cells.** For the downregulation of Sp1, cells in six-well plates were transfected with 1 $\mu$g of pSuper.retro.neo-Sp1 shRNA plasmid[23] expressing Sp1-shRNA or a vector lacking an Sp1-shRNA cassette as a control using Targetfect F2 tranfection reagents (Targeting Systems). For STAT3 or ZBP89 downregulation, cells in six-well plates were transfected with 10 nM STAT3 (sense, 5′-GAAUCAA-GCAGUUUCUUCAGAGCAG-3′; and antisense, 5′-CUGCU-CUGAAGAAACUGCUUGAUUCUU-3′) or ZBP89 (sense, 5′-GCAGCUGUGGCAAGUGUCAUUGATG-3′; and anti-sense, 5′-CAUCAAUGACACUUGCCACAGCUGCUU-3′) dicer substrate siRNA duplexes (Integrated DNA Technologies, Leuven, Belgium) using Targetfect F2. As a control for the siRNA oligos, 10 nM negative control siRNA duplexes (those not having any site in the human genome) were transfected to cells. Forty hours after transfection, the total RNA was isolated from treated cells and cDNA was synthesized from ∼2 $\mu$g of RNA for each condition by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Real-time PCR


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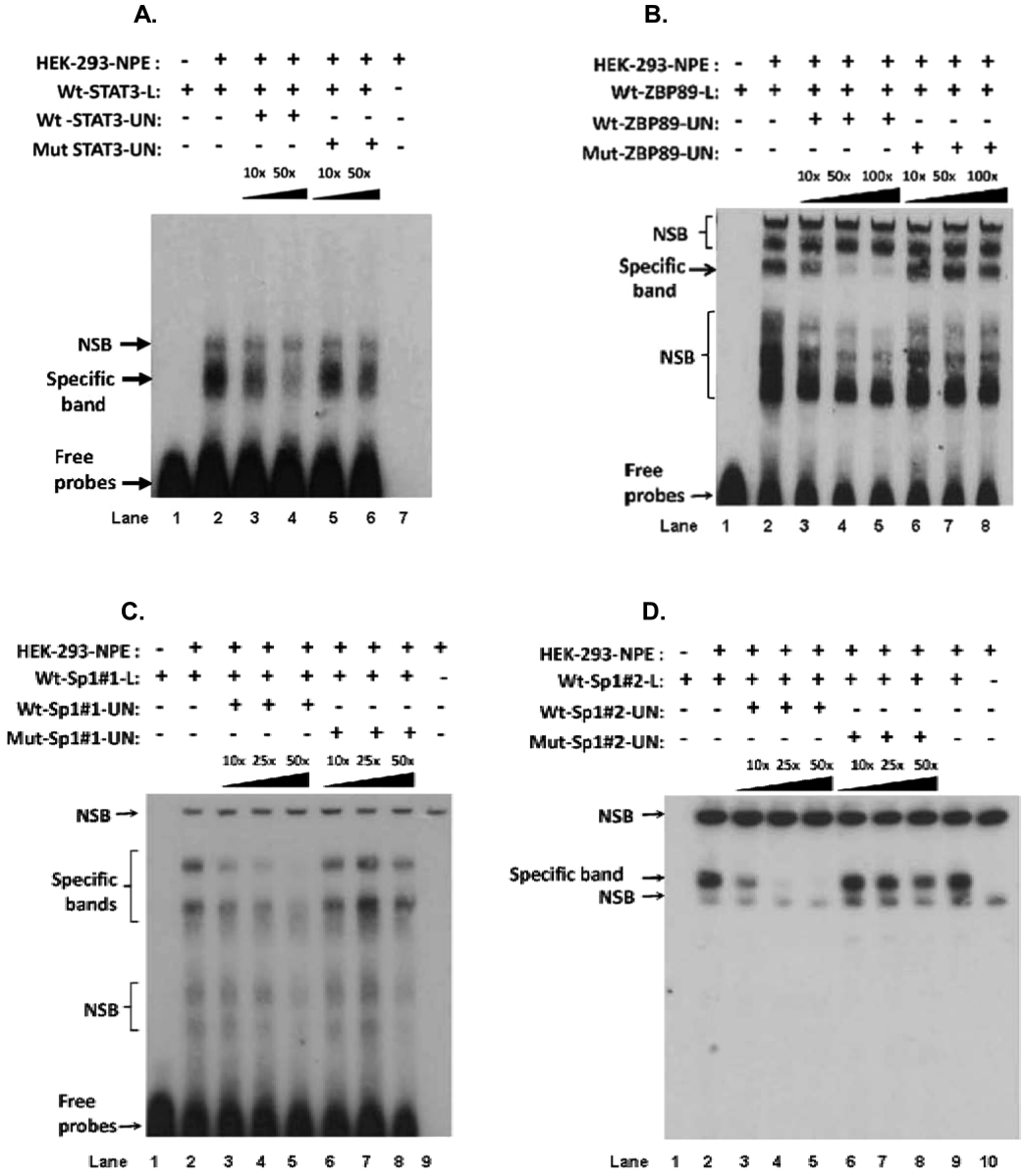


**Figure 2.** Electrophoretic mobility shift assays (EMSAs) display binding of HEK-293 nuclear proteins to renalase promoter domains. (A) EMSA for the STAT3 binding region. Wild-type oligonucleotides harboring STAT3 domains were biotinylated and incubated with nuclear protein extracts as described in Experimental Procedures. Labeled wild-type STAT3 oligos incubated without or with nuclear proteins were loaded (lanes 1 and 2). Competition EMSA experiments were performed using increasing concentrations (10- and 50-fold molar excess) of both wild-type (lanes 3 and 4) and mutant unlabeled oligos (lanes 5 and 6) along with wild-type labeled oligo. (B) EMSA for the ZBP89 binding region. Labeled wild-type ZBP89 probes incubated with or without nuclear proteins extracts (lanes 1 and 2). For competition experiments, labeled probes were incubated with 10-, 50-, and 100-fold molar excesses of unlabeled wild-type oligos (lanes 3–5) and unlabeled mutant oligos (lanes 6–8). (C and D) EMSA for two Sp1 binding regions (indicated as Sp1#1 in panel C and Sp1#2 in panel D). Labeled oligos of wild-type Sp1#1 and Sp1#2 were incubated with or without nuclear proteins (panels C and D, lanes 1 and 2). Competition experiments were performed with 10-, 25-, and 50-fold molar excesses of unlabeled wild-type Sp1 (lanes 3–5) and unlabeled mutant Sp1 oligos (lane 6–8). An equal amount of nuclear proteins without labeled probes was used as another control (lane 9 in panel C, lane 10 in panel D). Specific DNA–protein complexes are indicated as specific bands, whereas nonspecific bands are indicated as NSB. L, labeled oligo; UN, unlabeled oligo; wt, wild-type; Mut, mutant.

was performed with human renalase-specific primers and $\beta$-actin-specific primers, as mentioned above. The relative human renalase transcript level was determined by calculating $2^{-\Delta\Delta Ct}$ for each reaction.[24]

In a parallel set of experiments, cells in 12-well plates were transiently transfected with 0.5 $\mu$g of hRenPro-485 reporter plasmid with or without the Sp1-shRNA or STAT3/ZBP89 siRNA. As controls, a vector lacking Sp1-shRNA or negative control siRNA duplexes were cotransfected, as mentioned above. Forty hours after transfection, cells were harvested for luciferase and Bradford assays.

**Mouse Strains.** Kidney tissue samples (in RNAlater solution) from male BPH (strain BPH/2J, at inbred generation F66) and BPL (strain BPL/1J, at inbred generation F65) mice at the age of 5–7 weeks were procured from the Jackson Laboratory (Bar Harbor, ME) following institutional norms. These strains were developed by a selective inbreeding program solely on the basis of blood pressure; the BPH mice display parallel symptoms of human essential hypertension, with


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elevated blood pressure, increased heart rate, and early mortality compared to those of BPL mice.[25] At the age of ∼4−15 weeks, BPH mice display an ∼120 mm systolic blood pressure whereas BPL mice display an ∼70 mm blood pressure. We intentionally chose young BPH and BPL mice to minimize the effects of age-related confounding factors on gene expression.

**Mouse Kidney RNA Extraction and Real-Time PCR Analysis.** Extraction of total RNA from kidney tissue samples of BPH/BPL mice and cDNA synthesis was conducted as described previously.[15] Real-time PCR was performed using the DyNAmo HS-SYBR Green qPCR Kit (Finnzymes) and mouse renalase-specific primers (forward, 5′-TTTGCCTCAGCCAG-TTGCTACC-3′; and reverse, 5′-TAAATCCATCCCCTCCG-CACAC-3′). Renalase transcript levels among the samples were normalized with respect to 18S rRNA as a housekeeping control using the following primers: forward, 5′-GTAACCC-GTTGAACCCCATT-3′; and reverse, 5′-CCATCCAATCGG-TAGTAGCG-3′. Relative levels of the transcripts were determined as described previously.[24]

**Mouse Kidney Protein Extraction and Western Blot Analysis.** Approximately 40 mg of kidney tissue from BPH/BPL mice was washed twice with PBS (supplemented with protease inhibitors) and homogenized in 1.0 mL of RIPA buffer (supplemented with protease inhibitors) using a Dounce homogenizer. Samples were then sonicated using a Labsonic-M ultrasonic homogenizer (Sartorius) with 30 s on and off for 10 cycles. Homogenized samples were centrifuged at 14000 rpm, and the supernatants were stored in aliquots at −80 °C until further use. Total protein estimation of these samples was done by a Bradford assay. Approximately 20 $\mu$g of protein samples were electrophoresed via denaturing SDS−PAGE. Resolved samples were transferred onto an activated PVDF membrane and probed with specific antibodies for $\beta$-actin, STAT3, ZBP89, and Sp1 as mentioned above. Relative intensities of protein expression were quantified using NIH ImageJ.

**Data Presentation and Statistics.** All promoter reporter transient transfection assays were conducted at least three times, and results are expressed as means ± the standard error (SE) of representative replicates. Statistical significance was determined by a Student's *t* test and ANOVA with Bonferroni's multiple-comparison post-test, as appropriate in different experiments using SigmaPlot (Cranes Software International Ltd., Bangalore, India).

## RESULTS

**Identification of Crucial Promoter Domains That Govern Renalase Transcription.** To identify the proximal promoter domains (*cis*-elements) that may regulate renalase gene expression, progressive human renalase 5′-promoter-deletion/firefly luciferase reporter constructs (from base pair −1285 to −54) were generated (Figure 1A) and transfected into various human cell types [viz., HEK-293 (kidney), IMR32 (brain), and HepG2 (liver)] that displayed abundant expression of renalase transcripts (Figure 1B). While deletion of the promoter region up to base pair −485 preserved (or even enhanced) promoter expression, deletion up to base pair −398 (i.e., deletion of the region from base pair −485 to −399) caused a profound reduction of the luciferase activity across all tested cell types [HEK-293, ∼2.8-fold, $p < 0.01$ (Figure 1C); HepG2, ∼2.5-fold, $p < 0.01$ (Figure 1D); IMR32, ∼1.9-fold, $p < 0.01$ (Figure 1E)]. Computational analyses using CONSITE (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite; with an 85% cutoff)[26] and MatINSPECTOR (www.genomatix.de/matinspector.html; with a 0.9 cutoff)[27] predicted potential binding sites for Hunchback (Pax-1), STAT3, HoxB9, and ILF1 in this region (Table 1 and Figure 1A). Further deletions up to base pair −252 did not alter promoter activity (Figure 1C−E). However, deletion of the region from base pair −252 to −150 led to dramatic reductions in the promoter activities (HEK-293, ∼3-fold, $p < 0.01$; IMR-32, ∼2.1-fold, $p < 0.01$; HepG2, ∼4.1-fold, $p < 0.01$) (Figure 1C-E). Of note, the domain from base pair −252 to −150 harbors potential binding sites for SNAIL, HNF-3$\beta$, FTF-2, and ZBP89 (Table 1 and Figure 1A). Additional deletions in the proximal promoter up to base pair −54 did not result in significant alterations in luciferase activity (Figure 1C−E). Thus, two promoter domains (base pairs −485 to −399 and −252 to −150) are crucial for directing the basal renalase gene expression in several cell types.

**Specific Interactions of Sp1, STAT3, and ZBP89 with Renalase Promoter.** Because the crucial promoter domains (at base pairs −485 to −399 and −252 to −150) governing human renalase gene transcription contained potential binding sites for several transcription factors (Figure 1 and Table 1), we sought to test the interactions of HEK-293 nuclear proteins with synthetic double-stranded oligonucleotides (Table 2) representing the renalase promoter segments by EMSAs. The biotin-labeled STAT3 oligonucleotide yielded one specific broad band [protein/DNA complex (Figure 2A, lane 2)] that might consist of more than one closely spaced band. The specificity of this broad band was determined by a competition EMSA using increasing concentrations (10- and 50-fold molar excess) of unlabeled wild-type STAT3 and mutated oligonucleotides. While the unlabeled wild-type STAT3 oligonucleotide inhibited the formation of the specific complex (Figure 2A, lanes 3 and 4), the unlabeled mutant oligonucleotide showed a profoundly diminished effect on the inhibition of complex formation (Figure 2A, lanes 5 and 6). Similarly, an EMSA with wild-type ZBP89 oligonucleotide displayed formation of one specific complex (Figure 2B, lane 2) that was inhibited by unlabeled wild-type ZBP89 oligonucleotide in a concentration-dependent manner (Figure 2B, lanes 3−5); no concentration-dependent change in the intensity of this specific band was observed in the presence of unlabeled mutant ZBP89 oligonucleotide (Figure 2B, lanes 6−8). Likewise, wild-type Sp1 oligonucleotides (viz. Sp1#1 and Sp1#2, designed on the basis of the putative Sp1 binding sites at base pairs −271 to −262 and −315 to −324 of the renalase promoter) resulted in specific complexes (Figure 2C, lane 2; Figure 2D, lanes 2 and 9) that were inhibited by unlabeled wild-type oligonucleotides in a concentration-dependent manner (Figure 2C, lanes 3−5; Figure 2D, lanes 3−5); unlabeled mutant oligonucleotides did not exhibit consistent concentration-dependent inhibition of complex formation (Figure 2C, lanes 6−8; Figure 2D, lanes 6−8). On the other hand, EMSA experiments using oligonucleotides harboring putative binding sites for Pax-1/ILF-1, HoxB9, SNAIL/HNF-3$\beta$, FTF-2, with HEK-293 nuclear proteins under similar (as STAT3, ZBP89, and Sp1) conditions did not yield any specific DNA/nuclear protein complex (Figure S1 of the Supporting Information). Thus, transcription factors Sp1, STAT3, and ZBP89 appear to exert specific interactions with the human renalase promoter under *in vitro* conditions.

***trans*-Activation of Renalase Promoter Activity by Sp1, STAT3, and ZBP89.** In view of specific interactions of transcription factors Sp1, STAT3, and ZBP89 with renalase

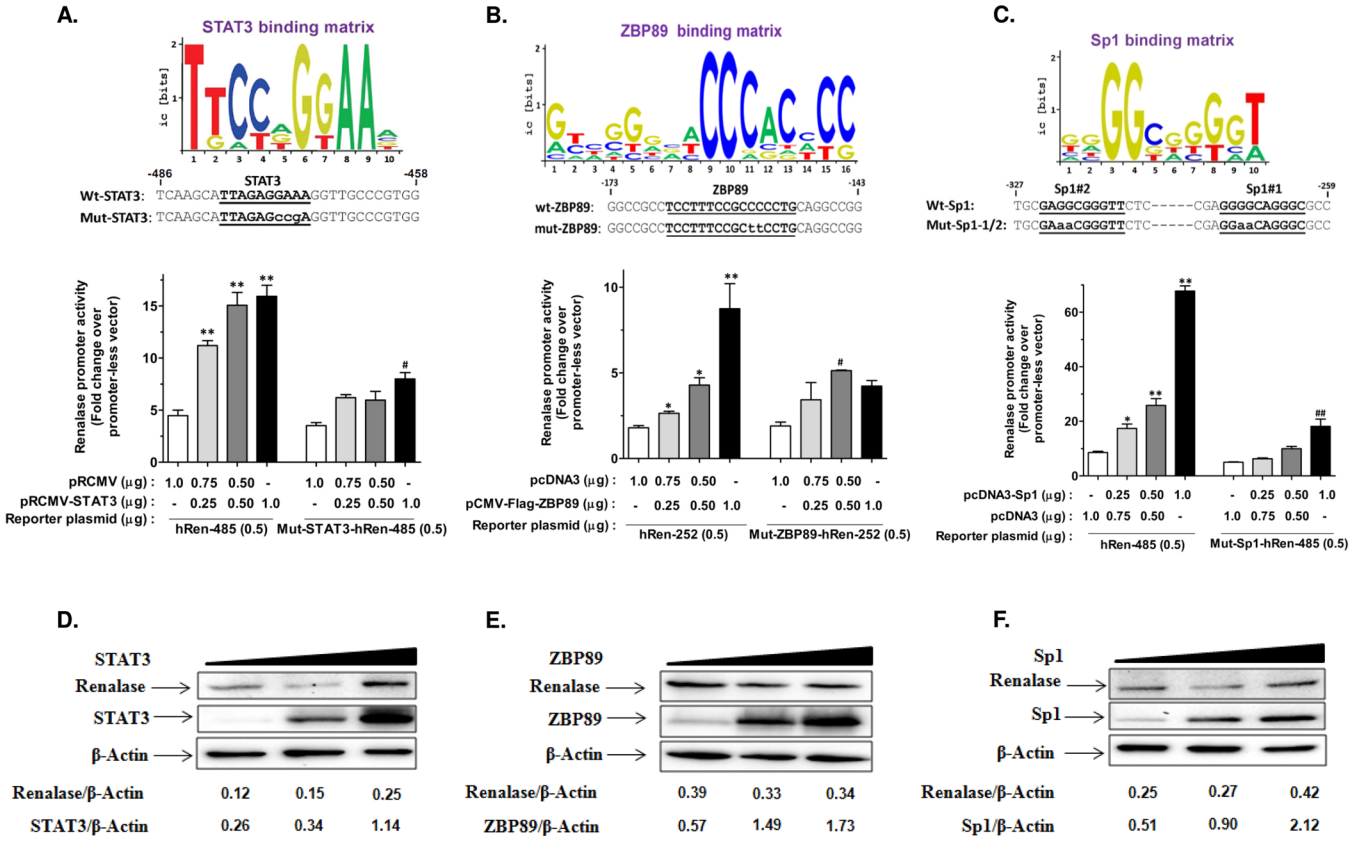


**Figure 3.** Effect of overexpression of STAT3, ZBP89, and Sp1 transcription factors on renalase expression. (A–C) Activation of renalase promoter activity by overexpression of STAT3, ZBP89, and Sp1. HEK-293 cells were transiently transfected with various doses (0–1.0 $\mu$g/well) of an expression plasmid for transcription factor STAT3 (A), ZBP89 (B), or Sp1 (C) along with 0.5 $\mu$g of renalase promoter-luciferase reporter plasmids per well (either wild-type or corresponding mutant plasmid) or pGL3-basic promoter-less reporter plasmid. The total amount of plasmid DNA transfected to each well was made equal by using balancing amounts of the backbone plasmids as indicated in the bottom part of each panel (viz. pRCMV in the cases of STAT3 and pcDNA3 in the cases of Sp1/ZBP89 cotransfection). Luciferase assays were performed 24–30 h after transfection, and luciferase activities were first normalized to per microgram total protein in each well. Renalase promoter activities were then expressed as fold over the normalized luciferase value for the promoter-less pGL3-basic plasmid. Data shown in the bottom region of panels A–C are means ± the standard error of representative replicates. The top region of each panel (A–C) shows the nucleotide binding matrix for the particular transcription factor and the strategy used to generate mutants for the specific domains of the renalase promoter. *$p$ < 0.05 and **$p$ < 0.01 compared with the wild-type promoter reporter plasmid; #$p$ < 0.01 and ##$p$ < 0.05 compared with the mutant promoter reporter plasmid. (D–F) Estimation of renalase protein levels upon overexpression of STAT3, ZBP89, and Sp1. Total proteins from HEK-293 cells transfected with different doses (0, 1.0, and 2.0 $\mu$g/well) of STAT3, ZBP89, or Sp1 were probed for renalase, STAT3, ZBP89, and Sp1 levels. Relative levels of renalase, STAT3, ZBP89, and Sp1 are shown below the representative Western blot images. The values are normalized to $\beta$-actin/GAPDH.

promoter domains as revealed by EMSAs (Figures 2 and 3), we tested the effect of cotransfection of the Sp1/STAT3/ZBP89 expression plasmid with renalase promoter/firefly luciferase reporter plasmids. Concentration-dependent increases (one-way ANOVA $F$ = 36.25, $p$ = 0.0001) in wild-type renalase promoter (hRen-485) activity [up to ~3.8-fold, $p$ < 0.01 (Figure 3A)] were observed in the presence of the STAT3 expression plasmid (pRCMV-STAT3). On the other hand, a renalase promoter plasmid with specific mutations at the STAT3 binding domain (mut-STAT3-hRen-485) displayed significantly less pronounced enhancements in luciferase activity with similar doses of STAT3 plasmid [e.g., ~2.2-fold, $p$ < 0.01 at the highest dose (Figure 3A)]. These observations indicate specific binding of STAT3 with renalase promoter. Similarly, cotransfection of the wild-type renalase promoter (hRen-252) with a ZBP89 expression plasmid (pCMV-Flag-ZBP89) showed a concentration-dependent increase [one-way ANOVA $F$ = 16.24, $p$ = 0.009; up to ~4-fold, $p$ < 0.01 (Figure 3B)] in promoter activity, while the promoter construct mutated at the ZBP89 binding site (mut-ZBP89-hRen-252) caused a much smaller increase in luciferase activity [e.g., ~2.7-fold, $p$ < 0.05 at the preceding dose to the highest dose (Figure 4B)]. Likewise, cotransfection of a Sp1 expression plasmid (pcDNA3-Sp1) with wild-type renalase promoter (hRen-485) showed concentration-dependent increases (one-way ANOVA $F$ = 219.3, $p$ < 0.0001) in luciferase activity with a dramatic augmentation [~7-fold, $p$ < 0.01 (Figure 3C)] at the highest tested dose of Sp1. On the other hand, the promoter plasmid containing mutations at the Sp1 binding sites (mut-Sp1-hRen-485) displayed significantly diminished (e.g., ~3.6-fold, $p$ < 0.01 at the highest dose) promoter activation; the basal (i.e., in the absence of the exogenous Sp1 expression) promoter activity was also diminished (~40%, $p$ = 0.0214) for this mutated plasmid compared to that of the wild-type renalase promoter plasmid (Figure 3C).

**Effect of Overexpression of Sp1, STAT3, and ZBP89 on the Renalase Protein Level.** In light of the profound activation of the renalase promoter by Sp1, STAT3, and ZBP89 in HEK-293 cells (Figure 3A–C), we asked whether the effect is translated to the renalase protein level. As expected,


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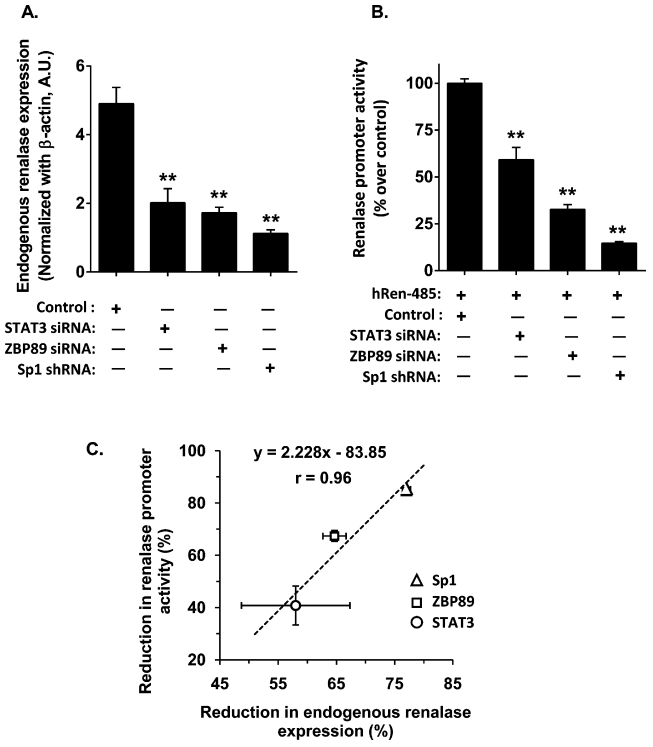


**Figure 4.** Effect of siRNA-mediated downregulation of STAT3, ZBP89, and Sp1 on renalase expression. (A) Downregulation of endogenous renalase expression by STAT3-, ZBP89-, and Sp1-specific siRNA/shRNA. HEK-293 cells were transfected with 1 $\mu$g of Sp1-shRNA expression plasmid per well or 10 nM siRNA duplexes specific to human STAT3 or ZBP89. Equal amounts of an empty vector or negative control siRNA oligos were used under control conditions. Approximately 40 h after transfection, RNA was isolated and cDNA synthesis was conducted as described in Experimental Procedures. Real-time PCR was performed using renalase and $\beta$-actin-specific primers. Results are expressed as arbitrary units (A.U.) of normalized renalase expression to $\beta$-actin. $^{**}p < 0.01$ compared to the respective control. (B). Downregulation of transfected human renalase promoter activity by STAT3-, ZBP89-, and Sp1-specific siRNA/shRNA. HEK-293 cells were cotransfected with human renalase promoter reporter plasmid hRenPro-485 (0.5 $\mu$g/well) and Sp1-shRNA expression plasmid (1 $\mu$g/well) or 10 nM STAT3/ZBP89 siRNA duplex. Equal amounts of an empty vector or negative control siRNA oligos were used as a control. Approximately 40 h after transfection, cells were lysed for luciferase and total protein assays. Results are expressed as the percentage change in the normalized luciferase activity with respect to the corresponding control. $^{**}p < 0.01$ compared to the control. (C) Positive correlation between endogenous renalase expression and transfected renalase promoter activity. The extents of reduction in endogenous renalase transcript and transfected renalase promoter activity upon downregulation of transcription factors in HEK-293 cells were compared.

transfection of each of these transcription factors caused dose-dependent elevation of their intracellular protein levels (Figure 3D−F). Intriguingly, overexpression of Sp1 and STAT3 led to increases (~1.7- and ~2.1-fold over basal, respectively) in the renalase protein level; overexpression of ZBP89, however, did not show any major change in the renalase protein level (Figure 3D−F). Thus, although Sp1, STAT3, and ZBP89 each emerged as an important regulator of renalase transcription, the *trans*-activation effect of ZBP89 appeared to be limited to the transcriptional level.

**Effect of Downregulation of Sp1, STAT3, and ZBP89 on Renalase Expression.** Because overexpression of Sp1, STAT3, and ZBP89 increased the exogenous renalase promoter activity and the extents of activation were significantly reduced upon mutation of their binding sites (Figure 3), we asked whether the endogenous renalase transcript level was also altered by these transcription factors. Indeed, ectopic expression of Sp1-shRNA or dicer substrate siRNA oligos against STAT3 and ZBP89 in HEK-293 cells reduced the level of endogenous human renalase transcript by ~4.3-, ~2.4-, and ~2.8-fold, respectively (Figure 4A), with respect to the control conditions. In corroboration, the siRNA-mediated down-regulation of these transcription factors also diminished the exogenous/transfected human renalase promoter activity in these cells by ~6.8-, ~3.0-, and ~1.7-fold, respectively, as compared to the control (empty vector/control siRNA-transfected) conditions (Figure 4B). Interestingly, the extent of reductions in the endogenous renalase transcript levels by these three transcription factors positively correlated with the extents of reductions of transiently transfected renalase promoter activity (Figure 4C; correlation coefficient of 0.96).

**Binding of Sp1, STAT3, and ZBP89 with Endogenous Renalase Promoter.** Because the human renalase promoter exhibited specific interactions with Sp1, STAT3, and ZBP89 for regulation of gene expression under *in vitro* conditions (Figures 2−4), we next investigated whether these transcription factors bind to the endogenous promoter in the context of chromatin *in vivo* by ChIP assays. Formaldehyde-cross-linked, enzymatic-fragmented chromatins from HEK-293 cells were immunoprecipitated using transcription factor antibodies; the presence of renalase promoter domains in the DNA obtained from these immunoprecipitates was subsequently detected by PCR and confirmed by DNA sequencing. While ChIP-grade rabbit STAT3, Sp1, and ZBP89 antibodies resulted in significant precipitation of endogenous renalase promoter [as revealed by specific PCR amplification (Figure 5, lanes 5−7)], the preimmune rabbit IgG failed to precipitate the renalase promoter (Figure 5, lane 4). As a positive control, fragmented chromatins were also precipitated by the histone antibody that led to robust amplification of the renalase promoter domain (Figure 5, lane 3). Expectedly, DNA extracted from chromatin fragments prior to immunoprecipitation (input DNA) yielded the expected 499 bp band (Figure 5, lane 2) as a positive control for PCR; on the other hand, when water was added to the PCR mixture instead of DNA (as a negative control for PCR), no product was obtained (Figure 5, lane 8).

**Synergistic Regulation of Renalase Expression by Sp1 and STAT3 under Basal and Nicotine-Stimulated Conditions.** Because Sp1 and STAT3 have previously been reported to regulate expression of several genes in a synergistic manner,[28,29] we asked whether these transcription factors also co-activate the renalase gene. Cotransfection of the renalase promoter reporter construct (hRenPro-485) with STAT3 and Sp1 expression plasmids (individually as well as in combination) to HEK-293 cells showed a promoter activity in the presence of both STAT3 and Sp1 significantly higher (~3.5-fold) than the promoter activities [one-way ANOVA $F = 125.5$, $p < 0.01$ (Figure 6A)] under individual conditions (~1.4-fold for STAT3 and ~1.7-fold for Sp1). Thus, Sp1 and STAT3 seem to synergistically augment renalase transcription.

To test whether renalase transcription can be induced by stimuli of cardiovascular relevance such as nicotine (that acts via activation of the nicotinic acetylcholine receptor, the


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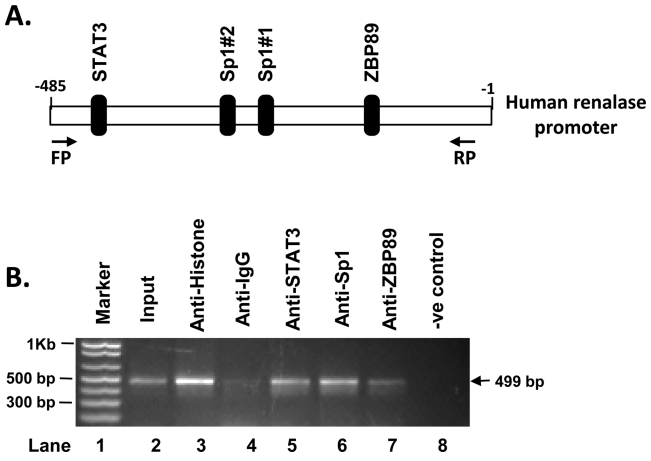


**Figure 5.** ChIP assays demonstrate *in vivo* interactions of STAT3, Sp1, or ZBP89 with the renalase promoter domain. (A) Schematic representation of transcription factor binding sites and strategy used for designing PCR primers. (B) Chromatin preparations from HEK-293 cells were immunoprecipitated with ChIP-grade anti-STAT3, anti-Sp1, anti-ZBP89, preimmune rabbit IgG (as a negative control), and anti-histone antibodies (as a positive control). Immunoprecipitates were purified and de-cross-linked to yield chromatin-bound DNA fragments. PCR was performed using a specific pair of primers, as mentioned in Experimental Procedures. DNA obtained prior to immunoprecipitation was used as Input (as another positive control). Lane 1, DNA marker; lane 8, PCR negative control.

physiological trigger to efferent autonomic outflow) and whether the transcription under stimulated conditions is also synergistically modulated by STAT3 and Sp1 like basal or uninduced (Figure 6A) transcription, two sets of experiments were conducted. In the first set, HEK-293 cells transfected with the renalase promoter plasmid were treated with various concentrations (0.25−1.0 mM) of nicotine. Nicotine displayed a concentration-dependent enhancement of promoter activity [one-way ANOVA $F = 7.05$, $p < 0.05$ (Figure 6B)]. In another set of experiments, STAT3 and Sp1 expression plasmids (individually as well as in combination) were cotransfected into HEK-293 cells with the renalase promoter construct in the presence or absence of 0.5 mM nicotine. Nicotine alone caused an ∼1.5-fold increase in promoter activity; cells cotransfected with STAT3 and Sp1 showed ∼1.8- and ∼2.5-fold activation, respectively, whereas STAT3 with Sp1 displayed ∼3.7-fold activation of promoter activity [one-way ANOVA $F = 148.5$, $p < 0.001$ (Figure 6C)]. These results suggest that nicotine-stimulated activation of renalase expression is governed by synergistic interactions of STAT3 and Sp1 with the renalase promoter.

**Regulation of Renalase Transcription by Catecholamines.** To test whether renalase transcription is modulated by catecholamines, HEK-293 cells were transfected with the renalase promoter reporter plasmid (hRenPro-1285) and treated with various concentrations of catecholamines. Epinephrine showed a dose-dependent increase in the renalase promoter activity [one-way ANOVA $F = 8.23$, $p = 0.0014$, up to ∼2.0-fold (Figure 7A)]. Dopamine also displayed a dose-dependent increase in renalase promoter activity [one-way ANNOVA $F = 10.84$, $p = 0.0004$, up to ∼2.5-fold (Figure 7B)]. As a control for these experiments, HEK-293 cells transfected with the promoter-less pGL3-basic vector did not show any significant change in luciferase activity under identical conditions. In a similar set of experiments, we transfected

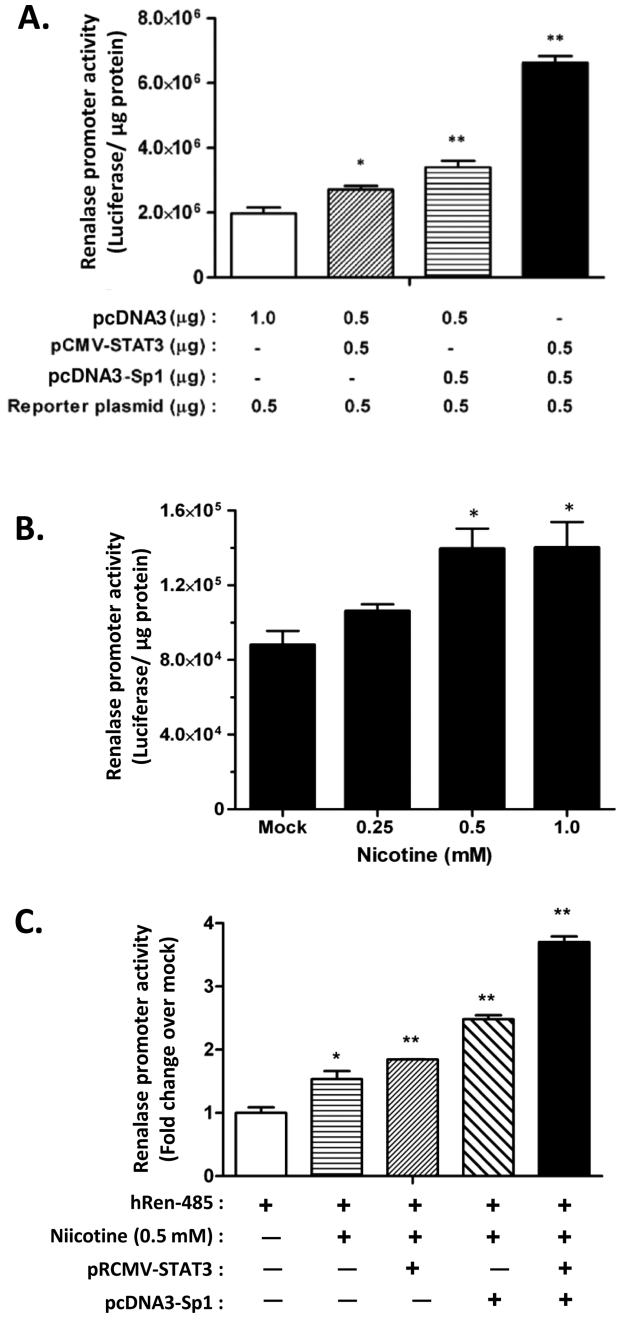


**Figure 6.** Regulation of basal and nicotine-mediated renalase expression by STAT3 and Sp1. (A) STAT3 and Sp1 synergistically activate renalase expression. HEK-293 cells were cotransfected with 0.5 µg of STAT3 and Sp1 expression plasmids individually as well as in combination with renalase promoter reporter plasmid. Results are expressed as luciferase activity per microgram of total protein. *$p < 0.05$ and **$p < 0.01$ with respect to cells transfected without any transcription factor expression plasmid. (B) Nicotine-mediated augmentation of renalase expression. HEK-293 cells were transfected with renalase promoter construct, and 12 h after transfection, the cells were treated with various doses of nicotine (0−1.0 mM). The luciferase assay was performed 30 h after transfection, and results are expressed as luciferase activity per microgram of total protein. *$p < 0.05$ with respect to untreated cells. (C) Nicotine-activated renalase expression is further enhanced by STAT3 and Sp1. STAT3 and Sp1 were cotransfected with renalase promoter plasmid to HEK-293 cells, and 12−16 h after transfection, the cells were treated with 0.5 mmol/L nicotine. The luciferase assay was performed 30 h post-treatment. *$p < 0.05$ and **$p < 0.01$ with respect to untreated cells.


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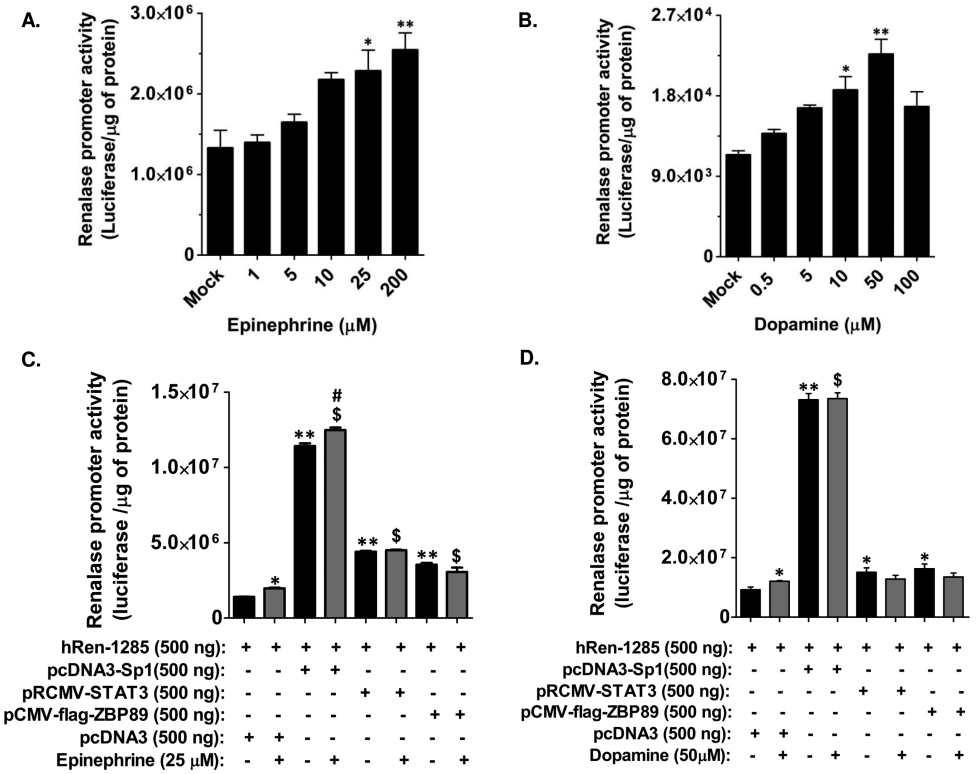


**Figure 7.** Enhancement of renalase promoter activity by catecholamines. (A and B) Epinephrine/dopamine-induced renalase promoter activity. Renalase promoter reporter plasmid hRenPro-1285 was transfected to HEK-293 cells and treated with epinephrine (1−200 $\mu$M) or dopamine (1−100 $\mu$M) as described in Experimental Procedures. Luciferase assays were performed 30 h after transfection, and luciferase activity was normalized to the total number of cells by estimating total lysate proteins. Results are expressed as means ± the standard error of normalized luciferase activity. *$p$ < 0.05 and **$p$ < 0.01 compared to the basal or untreated condition. (C and D) Effect of overexpression of Sp1, STAT3, and ZBP89 on epinephrine/dopamine-induced renalase promoter activity. Expression plasmids for Sp1, STAT3, and ZBP89 were cotransfected with renalase promoter plasmid (hRenPro-1285) into HEK-293 cells. Cells were treated with 25 $\mu$M epinephrine or 50 $\mu$M dopamine. The luciferase assay was performed 30 h post-transfection. *$p$ < 0.05 and **$p$ < 0.0001 with respect to the basal or untreated condition. \$$p$ < 0.0001 compared to the epinephrine or dopamine without overexpression of transcription factor condition. #$p$ < 0.05 compared to the corresponding untreated (without epinephrine) condition.

HEK-293 cells with renalase promoter and treated them with norepinephrine (0−200 $\mu$M). In contrast to dopamine and epinephrine, we did not detect any significant change in the renalase promoter activity at any dose of norepinephrine (data not shown). These observations are consistent with previous reports of augmentation of renalase expression by dopamine and epinephrine in H9c2 and HK2 cells, respectively.[6,14]

Next, to test whether the epinephrine/dopamine activation of renalase transcription in HEK-293 cells is influenced by Sp1, STAT3, and ZBP89, the renalase promoter construct was cotransfected with these transcription factors and treated with epinephrine (25 $\mu$M) or dopamine (50 $\mu$M). Interestingly, while each of these transcription factors by itself caused a profound increase in renalase promoter activity, Sp1 showed a modest additional enhancement in the epinephrine-induced activation (~10%, $p$ < 0.05); STAT3 or ZBP89 did not show any further significant enhancement in renalase promoter activity (Figure 7C). On the other hand, dopamine-induced activation of renalase transcription was not influenced by any of these transcription factors, although each of those displayed a significant increase in renalase promoter activity (Figure 7D).

**Association of Renalase Expression with STAT3 and ZBP89 Levels in Mouse Models of Human Essential Hypertension.** In view of reported associations of renalase with hypertension in human case control studies[1−3,30] as well as in systemic knockout of the renalase gene in mouse,[10] we investigated renalase gene expression levels in mouse models of human essential hypertension [viz. blood pressure high (BPH) versus blood pressure low (BPL) mice]. Real-time PCR analysis showed an ~1.7-fold decreased level of renalase transcripts [$p$ = 0.0085 (Figure 8A)] in the kidney tissues of 5−7-week-old BPH mice compared to those in age- and sex-matched BPL mice.

Because Sp1, STAT3, and ZBP89 play crucial roles in governing human renalase expression (Figures 1−6) and the mouse renalase promoter also harbors the putative binding sites for these transcription factors (Figure 8B), we analyzed the protein levels of Sp1, STAT3, and ZBP89 in the kidney tissue samples of BPH and BPL mice. Interestingly, BPH mice displayed ~28% ($p$ = 0.0196) and ~94% ($p$ = 0.0025) diminished levels of STAT3 and ZBP89, respectively, compared to those of the BPL mice (Figure 8C−F); the Sp1 level remained unchanged between BPH and BPL mice (Figure S2 of the Supporting Information). Such concomitant under-expression of the renalase transcript and STAT3 and ZBP89 proteins in genetically hypertesive BPH mice (vs the genetically hypotensive BPL mice) provided additional evidence of the crucial regulatory roles of these transcription factors in renalase gene expression.


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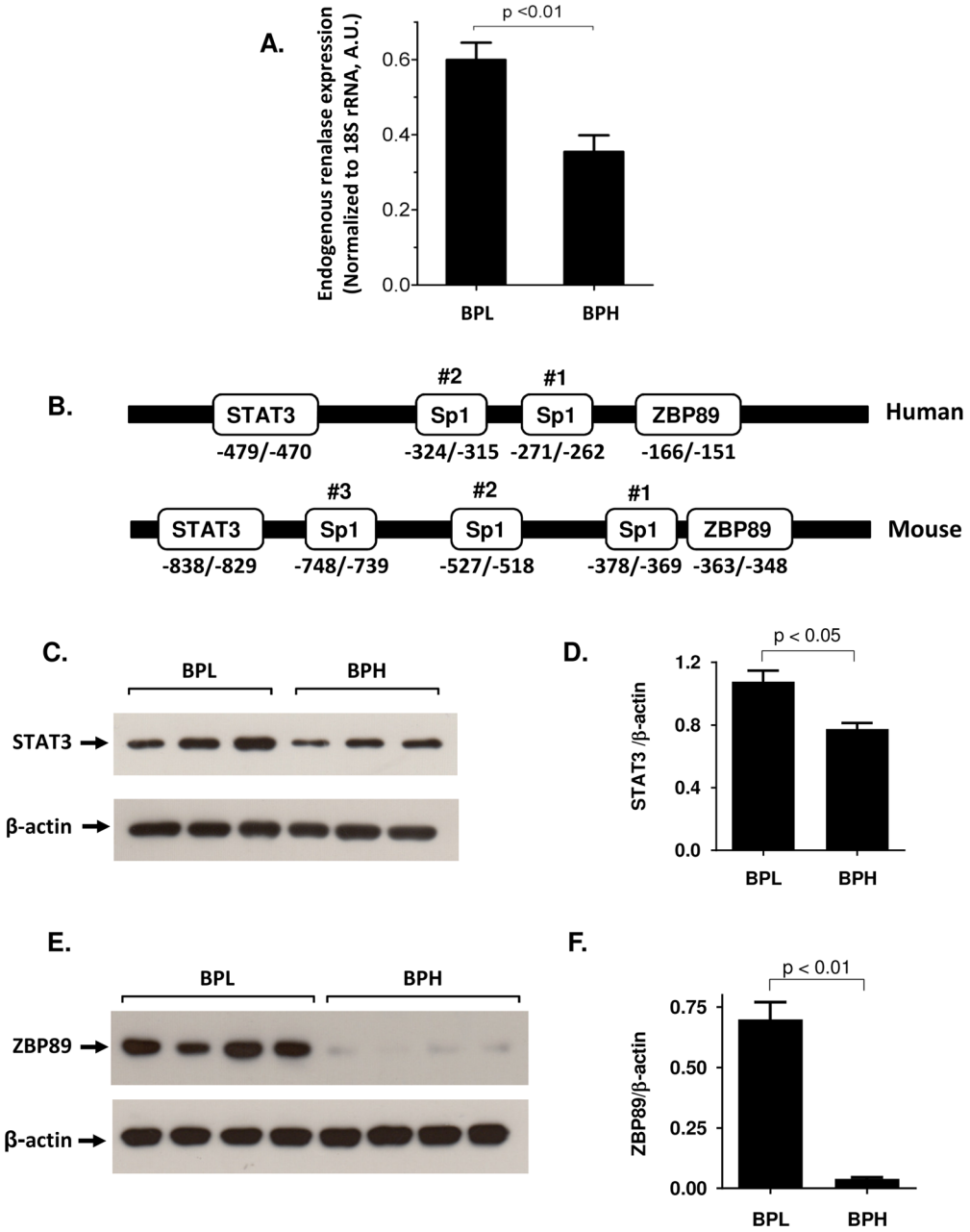


**Figure 8.** Differential expressions of renalase and STAT3 and ZBP89 in genetically hypertensive vs genetically hypotensive mice. (A) Endogenous expression of renalase in BPH and BPL kidney tissues. RNA preparations from BPH ($n$ = 4) and BPL kidney tissue ($n$ = 5) were reverse-transcribed, and quantitative PCR was conducted using renalase- and 18S rRNA-specific primers as described in Experimental Procedures. (B) Schematic representation of human and mouse proximal promoter regions. The conservation of the transcription factor binding domains between these species is shown. The figure is not drawn to scale. (C−F) Abundance of STAT3 and ZBP89 proteins in the kidney tissue of BPH and BPL mice. Approximately 20 $\mu$g of the proteins obtained from BPH and BPL kidney tissues were electrophoresed, blotted on a PVDF membrane, and immunodetected using the antibodies specific for STAT3 (C), ZBP89 (E), or $\beta$-actin (C and E) proteins, as described in Experimental Procedures. Quantitative analysis of relative levels of STAT3 and ZBP89 proteins is shown in panels D and F, respectively. The values are normalized to $\beta$-actin.

## DISCUSSION

Although several recent reports strongly associated an altered expression of the renalase gene[1−5,10,30] with cardiovascular/renal disease states, the molecular mechanism of regulation of this newly discovered gene remains unknown. In this study, we set out to systematically elucidate the crucial *cis*-regulatory elements in the human renalase proximal promoter and the transcription factors that may govern renalase expression. Two promoter domains located between base pairs −485 to −398 and base pairs −252 to −149 were observed to be crucial for basal expression of renalase in several cell types (Figure 1C−E). Interestingly, the renalase promoter activity was much higher in the HEK-293 kidney cell line than in HepG2 liver and IMR32 brain cell lines. The differential renalase promoter activities among these cell types may be due to the altered level of expression and/or interaction of important transcription factors with the crucial promoter domains mentioned above. However, the higher renalase promoter activity in kidney cells as compared to those in other cell types is consistent with the tissue-specific expression pattern of the renalse transcript in


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these tissue types.[1] Importantly, these promoter domains harbored putative binding sites for several putative transcription factors (Table 1). Of note, apart from these two promoter domains, two putative Sp1 binding sites [between base pairs −324 and −262 (Table 1)] were predicted by two independent transcription factor prediction programs (viz. MatInspector and Consite). Among these several transcription factors, which are crucial for basal or inducible renalase expression? Our EMSA analyses showed specific binding of nuclear proteins to renalase promoter domains harboring binding sites for transcription factors Sp1 (specificity protein 1), STAT3 (signal transducer and activator of transcription 3), and ZBP89 (zinc binding protein 89) *in vitro* (Figure 2); ChIP assays also provided evidence of interaction of these transcription factors with endogenous renalase promoter domains *in vivo* in the context of chromatin (Figure 5). In corroboration, overexpression of Sp1, STAT3, and ZBP89 upregulated renalse promoter activity as well as the endogenous renalase protein level (Figure 3), whereas siRNA-mediated downregulation of Sp1, STAT3, and ZBP89 diminished endogenous human renalase transcripts as well as transfected promoter activity (Figure 4). Taken together, Sp1, STAT3, and ZBP89 emerged as the crucial transcription factors for renalase gene expression.

Sp1 is known to interact with several nuclear proteins and govern the transcription of various genes, including chromogranins and monoamine oxidase A (MAO-A) and B (MAO-B) that are important for catecholamine storage and/or metabolism.[22,31−33] Renalase appears to be similar to MAO-A and MAO-B in this respect because its expression is activated by Sp1 under basal as well as nicotine-stimulated conditions (Figures 4 and 6). On the other hand, it is not known whether STAT3, a crucial transcription factor in the JAK-STAT signaling pathway that is activated in response to various extracellular stimuli,[34,35] augmenting both basal and nicotine-evoked expression of renalase (Figures 4 and 6), may regulate the expression of other members of the monoamine oxidase family (viz. MAO-A and MAO-B). Likewise, renalase is the only monoamine oxidase that is regulated by ZBP89, a kruppel-like zinc figure protein known to be involved in the regulation of many genes.[36,37] Interestingly, alignment of the orthologous mammalian sequences around the STAT3, Sp1, and ZBP89 binding domains in the renalase gene revealed a high degree of homology among mammals. Each of the crucial transcription factors for renalase gene expression has at least one highly conserved binding site, attesting to their importance in regulating the renalase gene (Figure S3 of the Supporting Information).

Can renalase gene expression be induced by physiological and/or pharmacological agents? Treatment of HEK-293 cells expressing the renalase promoter or luciferase reporter by nicotine caused augmentation of renalase promoter activity in a dose-dependent manner (Figure 6B). Although we have not studied the detailed signal transduction for nicotine-evoked renalase gene regulation, the Sp1 and STAT3 binding domains in the renalase proximal promoter in *cis* and Sp1 and STAT3 in *trans* are likely to be involved because overexpression of each of these transcription factors activated gene expression (Figure 6A,C). This observation is consistent with the report that nicotine activates STAT3 and Sp1 in several cell types.[38−40] In addition to nicotine, the catecholamines (viz. epinephrine and dopamine) also significantly enhanced renalase promoter activity in HEK-293 cells (Figure 7). Although the detailed molecular mechanism of catecholamine-evoked renalase gene activation (viz. identification of *cis*-elements, transcription factors, and signaling molecules) remains to be investigated, epinephrine-induced renalase transcription appeared to be *trans*-activated by Sp1 (Figure 7). Of note, consistent with these observations in HEK-293 human embryonic kidney cells, upregulation of endogenous renalase transcripts by dopamine in rat cardiomyoblast H9c2 cells and upregulation of renalase mRNA as well as protein by epinephrine in human kidney proximal tubular epithelial HK2 cells have been reported.[6,14]

A recent study demonstrated that recombinant renalase or a 20-amino acid peptide fragment (that is conserved in all known human renalase isoforms but lacks detectable amine oxidase activity) activated AKT, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinases, and downregulated c-Jun N-terminal kinase in HK2 cells to protect against cisplatin- and hydrogen peroxide-induced necrosis.[11] In view of this observation suggesting a new role for renalase to act as a signaling molecule, independent of its catecholamine metabolizing ability, we asked whether our observations on renalase transcriptional regulation might be influenced by fetal bovine serum (FBS) in the cell culture medium because FBS is likely to contain a significant amount of renalase. Accordingly, we assessed the transcriptional regulation of renalase in HEK-293 cells in the presence or absence of FBS in the cell culture medium. No significant change in the basal renalase promoter activity was observed upon serum deprivation; the Sp1-, STAT3-, and ZBP89-mediated *trans*-activations of the renalase promoter also remain unaffected under FBS-deprived conditions (Figure S4 of the Supporting Information). Thus, the transcriptional regulation of renalase in HEK-293 cells was independent of FBS in the culture medium.

Because several recent studies associated the renalase gene with hypertension,[1−3,30] we asked whether renalase gene expression is altered between the mouse models of human essential hypertension (viz. genetically hypertensive BPH *vs* genetically hypotensive BPL mice) and whether any of the transcription factors demonstrated to regulate the human renalase gene (Figures 1−6) are differentially expressed in these mouse models. Interestingly, the renalase transcript was underexpressed in the kidney tissue samples of BPH mice as compared to BPL mice (Figure 8). This observation is consistent with a recent study reporting 1.73-fold diminished expression of renalase protein in kidneys of the well-studied rat model of human hypertension [viz. spontaneously hypertensive rat (SHR) *vs* its normotensive control Wister Kyoto rat (WKY)]; the plasma renalase content in SHR was also significantly lower than in WKY.[41] Of note, these hypertensive rodent models (SHR and BPH) also displayed underexpression and reduced enzyme activity of other monoamine oxidases MAO-A and MAO-B[42−44] in the kidneys as compared to the normo- and hypotensive WKY and BPL. Such parallel reductions in the level of expression and activity of all three members of the monoamine oxidase family (that play crucial roles in catecholamine degradation *in vivo*) are consistent with the elevated catecholamine levels in SHR.[45,46] What might be the molecular basis for the underexpression of renalase in BPH mice? Previous studies revealed that polymorphisms in the regulatory domains (e.g., promoter or 3′-UTR) could govern differential expression of several genes in these rodent models of human hypertension.[15,47,48] Sequencing of the ∼1 kb upstream renalase promoter and 3′-UTR regions using gene-specific primers (Table S1 of the Supporting Information), however, did not show any variation between the BPH and

BPL sequences. Next, we analyzed the protein levels of transcription factors STAT3, ZBP89, and Sp1 [that were observed to regulate renalase gene expression in cultured cells (Figures 1−6)] in the kidneys of BPH and BPL mice. Interestingly, levels of expression of STAT3 and ZBP89 were observed to be diminished in BPH compared to BPL (Figure 8). The concomitant underexpression of renalase, and STAT3 and ZBP89 suggests a crucial role for these transcription factors in renalase gene regulation. It must, however, be noted that because transcription factors regulate several genes at a time, the diminished renalase expression level in BPH mice could also be mediated by additional signaling pathways.

In conclusion, this study identified the key molecular players (viz. Sp1, STAT3, and ZBP89) necessary for the regulation of basal or inducible expression of the human renalase gene, for the first time to the best of our knowledge. Additionally, diminished levels of STAT3 and ZBP89 along with reduced renalase transcript in the kidneys of the genetically hypertensive mouse BPH that closely paralleled and exhibited many comorbidities of human essential hypertension[25,43] provided evidence of crucial roles of these transcription factors in renalase gene regulation in a pathophysiological context. Further studies of the molecular basis of renalase gene expression *in vivo* may provide additional insights into cardiovascular and renal disease pathogenesis.

## ■ ASSOCIATED CONTENT

### Supporting Information

Four supplementary figures and one supplementary table. This material is available free of charge via the Internet at http://pubs.acs.org.

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P.J.S. and V.G. contributed equally to this work.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

BPH, blood pressure high; BPL, blood pressure low; MAO, monoamine oxidase; SHR, spontaneously hypertensive rat; Sp1, specificity protein 1; STAT3, signal transducer and activator of transcription 3; WKY, Wistar Kyoto rat; ZBP89, zinc binding protein 89.

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