Transcription Factor Decoy to Study the Molecular Mechanism of Negative Regulation of Renin Gene Expression in the Liver In Vivo

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Abstract—Renin is synthesized in high quantities in the juxtaglomerular cells of the kidney, but little or none is synthesized in the liver. Our previous in vitro and biochemical studies have demonstrated that tissue-specific expression of the mouse renin gene is regulated by the specific interaction between negative regulatory element (NRE) in the 5'-flanking region of the renin gene and NRE binding protein (NREB). In this study, we examined the hypothesis that this interaction between the NRE in the promoter region of the rat renin gene and the NREB in the liver contributes to the suppressed renin gene expression in this tissue in vivo. We used in vivo transfection of NRE transcription factor decoy (TFD) double-stranded oligonucleotide into the rat liver via portal vein infusion. A gel mobility shift assay showed that transfected NRE TFD blocked endogenous NREB binding with the rat renin gene. This resulted in enhanced hepatic renin mRNA expression, immunohistochemical detection of renin in the liver, and consequently, increased plasma renin concentration. Taken together, these results document the importance of NREB in the inhibition of renin gene expression in rat liver in vivo and suggest the possibility of in vivo renin gene modulation by the TFD approach. (Circ Res. 1999;84:1059-1066.)

Key Words: renin ■ negative regulatory element ■ transcription factor decoy ■ hemagglutinating virus of Japan liposome ■ gene expression

R enin, an aspartyl proteinase involved in the regulation of cardiovascular homeostasis, exhibits tissue-specific expression. Renin is mainly produced by the juxtaglomerular cells of the kidney, where it is stored in granules and released into circulation. Circulatory renin has a well-established role in blood pressure and volume homeostasis. On the other hand, renin is minimally expressed, or not expressed, in the liver. Tissue-specific gene expression is mainly dependent on the specific interaction of cis DNA sequences with nuclear trans-acting proteins. Certain trans-acting factors are ubiquitous, whereas others exhibit temporal tissue-specific or cell-specific expression. Recently, it has been shown that the interaction of a silencer sequence with corresponding specific nuclear trans-acting proteins plays an important role in tissue-specific regulation of expression of genes such as interleukin-2 or T cell receptor.

Previously, we have identified the presence of a putative consensus negative regulatory element (NRE) in mouse, rat, and human renin genes. We have reported that the tissue-specific expression of the mouse renin gene is regulated by the interaction of NRE and NRE binding protein (NREB). To test the hypothesis that this interaction plays an important role in the negative regulation of renin gene expression in the rat liver, we took the novel approach of using double-stranded oligonucleotide (ODN) as a transcription factor decoy (TFD) to block hepatic NREB interaction with renin gene. The decoy method is based on the competition for a nuclear transcription binding protein between a specific cis element present in the target gene and the exogenously introduced double-stranded decoy ODN corresponding to that cis sequence. To transfer double-stranded ODN efficiently in vivo, we used the Hemagglutinating Virus of Japan (HVJ)–liposome method as the mode of delivery. Our results demonstrated that the infusion of HVJ-liposome complex with NRE TFD into rat portal vein resulted in the inhibition of NRE and NREB interaction that consequently activated hepatic renin gene expression.

Materials and Methods

Synthesis of Oligonucleotides and Selection of Sequence Targets

Phosphorothioate ODNs spanning rat renin NRE and scrambled NRE ODN, which contains the same composition of nucleotides as rat renin NRE, were synthesized by an automated solid-phase
Mouse Renin (Ren1)  

5’ TCTATACCTACCTAATTTGTCACAGGGCTAGAATTATCA 3’  

5’ CCGCAGCTTGGCCTACAGGGAATGTTATGAGA 3’  

NRE  

Rat Renin  

5’ CCCTACCCACCTAGCTTTGCCCCTACCGGCTAGAATTATGAGA 3’  

Human Renin  

5’ CCCTACCCACCTAGCTTTGCCCCTACCGGCTAGAATTATGAGA 3’  

Preparation of HVJ-Liposome Solution  

Lipids (phosphatidylcholine, phosphatidylserine, and cholesterol) were mixed at a ratio of 4.8:1:2 (wt/wt/wt) as described previously. The lipid mixture (10 mg) in tetrahydrofuran was deposited in a rotary evaporator. NRE and scrambled NRE ODNs were incorporated into liposomes by vortex, shaking, and sonication as previously reported. The liposomes and HVJ, inactivated by UV irradiation, were mixed at a ratio of 4.8:1:2 (wt/wt/wt) as described previously. The lipid mixture was deposited in a rotary evaporator. NRE and scrambled NRE ODNs were incorporated into liposomes by vortex, shaking, and sonication as previously reported. The liposomes and HVJ, inactivated by UV irradiation, were mixed at a ratio of 4.8:1:2 (wt/wt/wt) as described previously.

Preparation of Nuclear Extracts  

Rats were killed by decapitation 2 days after NRE TFD or scrambled ODN infusion. After in situ perfusion with PBS (containing, in mmol/L, NaCl 137, KCl 3, NaHPO4 8, and KH2PO4 1), the liver was removed and snap-frozen in liquid nitrogen. Nuclear extracts were prepared as previously described. In brief, the liver was homogenized with a Potter-Elvehjem homogenizer in 4 volumes of ice-cold homogenization buffer containing, in mmol/L, HEPES (pH 7.5) 10, spermidine 0.5, spermine 0.15, EDTA 5, EGTA 0.25, β-mercaptoethanol 7, and PMSF 1, and 0.5 mol/L sucrose. After centrifugation at 12 000g for 30 minutes at 4°C, the supernatant fraction was brought to 45% (NH4)2SO4 and stirred for 30 minutes at 4°C. The precipitated proteins were collected at 17 000g for 30 minutes and resuspended in homogenization buffer containing 0.35 mol/L sucrose. The supernatant fraction was brought to 45% (NH4)2SO4 and stirred for 30 minutes at 4°C. The precipitated proteins were collected at 17 000g for 30 minutes and resuspended in homogenization buffer containing 0.35 mol/L sucrose.

Preparation of nuclear extracts of liver treated with NRE ODN or scrambled ODN was performed as previously described. A gel mobility shift assay was performed as described previously. A double-stranded renin NRE ODN was labeled with T4 kinase (GIBCO-BRL) and [γ-32P]dATP (specific activity, 3000 Ci/mm, Amersham International plc) and purified by PAGE. Binding reactions (10 μL) including 32P-labeled NRE ODN (0.5 to 1 ng, 20 000 cpm), 1 μg of polydeoxyinosinic-polydeoxyctydilic acid (Sigma), and 10 μg of liver nuclear extracts were incubated for 30 minutes at room temperature before loading onto a 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and autoradiographed. Gel Mobility Shift Assay  

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis  

Two days after infusion of NRE TFD or scrambled ODN, RNA was extracted from rat livers using RNezol (Tel-Test), reverse transcribed by reverse transcriptase and random hexamers (Perkin Elmer Cetus), and applied to PCR. The primers for RT-PCR for renin were 5’ GCT AGC TAG TGT TGA CAG GCC AGT TAG TCG -3’ (5’ primer) and 5’ CAG TCG ATC ACA ACT GTC CGG TCA ATC CAG AGC -5’. The PCR reaction was carried out with 32 P-labeled probe. A gel mobility shift assay was performed as described previously. A double-stranded renin NRE ODN was labeled with T4 kinase (GIBCO-BRL) and [γ-32P]dATP (specific activity, 3000 Ci/mm, Amersham International plc) and purified by PAGE. Binding reactions (10 μL) including 32P-labeled NRE ODN (0.5 to 1 ng, 20 000 cpm), 1 μg of polydeoxyinosinic-polydeoxyctydilic acid (Sigma), and 10 μg of liver nuclear extracts were incubated for 30 minutes at room temperature before loading onto a 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and autoradiographed. The gel was baked, prehybridized, and hybridized to rat renin probe (kindly donated from Dr Kazuo Murakami, Tsukuba University, Tsukuba, Japan) and GAPDH ODN probe (Clontech Laboratories, Inc). The filter was then washed and exposed to x-ray film. The filter was then washed and exposed to x-ray film.

Immunohistochemistry for Renin in the Liver  

A portion of the liver was fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm-thick sections. After deparaffinization, tissue sections were incubated with rabbit polyclonal anti-renin antiserum (kindly provided by Dr Tadashi Inagami, Vanderbilt University, Nashville, Tenn) diluted 1:500 with distilled water at 4°C overnight. After washing with PBS, they were incubated with biotinylated anti-rabbit IgG antiserum followed by
incubation with avidin-biotinylated horseradish peroxidase complex and counterstained with hematoxylin.

**Measurement of Hepatic and Plasma Renin Concentration**

Animals were anesthetized 2 days after transfection of NRE TFD or scrambled ODN. Livers were promptly removed and frozen in liquid nitrogen and stored at −70°C until use. On the day of extraction, the liver was thawed at 4°C, weighed, and homogenized by polytron in 2.6 mmol/L EDTA and 1 mmol/L PMSF. Samples were centrifuged at 10 000 rpm for 30 minutes at 4°C. Supernatants were used for the assay. Blood was drawn from the aorta directly into a syringe using a 21-gauge needle. The blood sample was centrifuged at 5000 rpm for 10 minutes, and plasma was collected and frozen at −80°C until use. Hepatic renin and plasma renin concentrations were measured as described previously. To activate inactive renin in rat plasma, we used the method as previously reported. Briefly, trypsin (type 1-S, 10 000 BAEE U/mg at 50 mg/mL) (Sigma) dissolved in 0.2 mol/L sodium acetate buffer (pH 4.5) containing 0.2 mol/L CaCl2 was added at a concentration of one tenth of the plasma volume. The mixture was incubated at 27°C for 1 hour. Soybean trypsin inhibitor (type 1-S, 100 mg/mL) (Sigma) at a concentration of one tenth of the plasma volume was then added. Soybean trypsin inhibitor was dissolved in 0.1 mol/L sodium phosphate buffer, pH 7.4.

**Statistical Analysis**

All values are expressed as mean±SEM. ANOVA was used to determine the significance of differences in multiple comparisons. P<0.05 was considered to be statistically significant.

**Results**

**Existence of Specific Nuclear Protein Binding With Rat Renin NRE in the Liver**

As shown in Figure 1, we have identified a putative NRE sequence in the rat and human renin genes by comparison with the mouse renin genomic sequence. To examine whether this putative NRE in the rat renin gene recognizes specific NREB in the rat liver, we performed a gel mobility shift assay by incubating rat liver nuclear extract with 32P-labeled rat renin NRE probe. Using this assay, we observed the complex formation of 32P-labeled rat renin NRE probe with specific nuclear protein (Figure 2A). This binding was not inhibited by the preincubation of nuclear extracts with a 100-fold excess of MHC-I NRE ODN, but was competitively inhibited by preincubation with rat renin NRE and human c-myc NRE ODN, which has a high degree of sequence homology with renin NRE. This binding was not inhibited by the preincubation of nuclear extracts with a 100-fold excess of unlabeled double-stranded scrambled NRE ODN, but was competitively inhibited by preincubation with rat renin NRE and human c-myc NRE ODN, which has a high degree of sequence homology with renin NRE. Also, to ensure the specificity of binding, we performed the competition assay with 100-fold and 200-fold excess of unlabeled renin and c-myc NRE ODN, as shown in Figure 2B. However, major histocompatibility complex class I (MHC-I) NRE ODN, the sequence of which is totally different from renin NRE, did not affect the binding, which suggests that rat liver nuclear extract contains a specific nuclear binding protein that recognizes the rat renin NRE sequence.

Figure 2. Existence of specific nuclear protein binding with rat renin NRE in the liver. A, Gel mobility shift assay was performed using 32P-labeled rat renin NRE probe and nuclear extract (10 μg) prepared from untreated rat liver, with or without 100-fold excess of competitors, rat renin NRE ODN, c-myc NRE ODN, MHC-I NRE ODN, and scrambled ODN. NREB binding with 32P-labeled rat renin NRE probe was competed by the preincubation with rat renin NRE, c-myc NRE but not with MHC-I NRE. B, To confirm the specificity of NREB binding, competition assay was performed with 100-fold and 200-fold excess of rat renin NRE ODN and c-myc NRE ODN. Lane 1, No nuclear protein present (control); lane 2, nuclear protein from untreated rat liver; lane 3, nuclear protein from untreated liver with unlabeled 100-fold excess of c-myc NRE ODN; lane 4, nuclear protein from untreated liver with unlabeled 200-fold excess of c-myc NRE ODN; lane 5, nuclear protein from untreated liver with unlabeled 100-fold excess of rat renin NRE ODN; lane 6, nuclear protein from untreated liver with unlabeled 200-fold excess of rat renin NRE ODN; lane 7, nuclear protein from untreated liver with unlabeled 200-fold excess of MHC-I NRE ODN; lane 8, nuclear protein from untreated liver with unlabeled 200-fold excess of scrambled NRE ODN.
Examination of FITC-Labeled ODN Uptake in the Liver by the HVJ-Liposome Method

To verify that the interaction of NREB with NRE attenuates renin gene expression in the rat liver in vivo, we infused NRE TFD into the portal vein using HVJ-liposome as the in vivo delivery method. To examine the transfection efficiency of HVJ-liposome, we labeled double-stranded ODN with FITC and infused FITC-labeled ODN (5 µmol/L) in HVJ-liposome solution into the portal vein. Rats were euthanized 24 hours after injection, and liver sections were embedded in paraffin and examined with fluorescent microscopy. As shown in Figure 3, fluorescence was observed in the cytoplasm and nuclei of hepatocytes, as well as in endothelial and Kupffer cells. Quantitative analysis showed that when FITC-labeled double-stranded ODNs were infused without HVJ-liposome, fluorescence was detected in a few hepatocytes (8.9 ± 1.2% of hepatocytes), and that >50% of hepatocytes exhibited fluorescence after transfection of FITC-labeled ODN with the HVJ-liposome method (54.2 ± 8.4% of hepatocytes).

Interference of the Binding of NREB With NRE In Vivo After Decoy Transfection

To examine whether NRE TFD sequestered NREB in the liver in vivo, we performed a gel mobility shift assay using nuclear extract prepared from liver treated with HVJ-liposome solution alone as a control. As shown in Figure 4, gel mobility shift assay was performed using 32P-labeled rat renin NRE probe and nuclear extract prepared from rat liver treated with HVJ-liposome containing no ODN; lane 4, nuclear protein from rat liver transfected with scrambled ODN; lane 5, nuclear protein from rat liver transfected with NRE TFD; lane 6, nuclear protein from rat liver transfected with NRE TFD with 100-fold excess of unlabeled rat renin NRE ODN; lane 7, nuclear protein from rat liver transfected with NRE TFD with 200-fold excess of unlabeled rat renin NRE ODN. Data are representative of 5 separate experiments.
after NRE TFD transfection. The binding of NREB with 32P-labeled NRE was decreased significantly after the transfection of NRE TFD. In contrast, no change in NREB binding was observed in the liver nuclear extract of rats receiving HVJ-liposome solution without NRE TFD, nor from rats receiving scrambled ODN transfection (Figure 4). These data demonstrated that in vivo TFD transfection could result in the inhibition of the binding of nuclear protein (NREB) with the corresponding cis element (NRE).

Expression of Renin mRNA in Rat Liver

To investigate whether NRE TFD modulates renin gene expression in vivo, we first examined the changes in hepatic renin mRNA level. Two days after transfection with NRE TFD, total RNA was extracted from the liver, and RT-PCR was performed with primers for rat renin gene and rat β-actin gene. As shown in Figure 5A, we observed a significant increase in rat renin mRNA level in the liver in response to NRE TFD transfection. In contrast, no significant change in renin mRNA was detected after scrambled ODN treatment. In our experiments, renin mRNA was not detectable by RT-PCR in the liver of untreated Wistar rats. As a control, we examined the mRNA levels of β-actin and observed that there were no differences between samples. The quantitative analysis cannot be performed on RT-PCR; therefore, we also tried Northern blot analysis. As shown in Figure 5B, rat renin mRNA was detected from the renin NRE TFD transfected liver. On the other hand, no renin mRNA was observed from the scrambled ODN–treated liver. These data from both RT-PCR and Northern blot analysis showed the expression of rat renin mRNA after renin NRE TFD transfection into the liver.

Immunohistochemical Detection of Renin in Rat Liver

To evaluate the effect of NRE TFD on renin gene product in the liver, we performed immunohistochemical analysis using rat renin antibody. As shown in Figure 6, positive staining was observed in rat livers at 2 and 5 days after NRE TFD transfection, whereas immunoreactive renin was not detected...
in the livers of rats receiving scrambled ODN. No staining was observed in the livers of untreated rats nor in control rats treated with HVJ-liposome solution alone (data not shown).

Changes in Hepatic and Plasma Renin Concentration
To examine whether the increased renin expression in the liver resulted in an increase in hepatic and plasma renin concentration, we measured total renin and active renin concentration in liver homogenates and plasma samples 2 days after NRE TFD transfection. As shown in Figure 7, both prorenin and active renin concentrations were increased in NRE TFD–treated rats as compared with untreated or scrambled ODN–treated ones. We also examined changes in hepatic renin concentration 2 days after decoy treatment. The active hepatic renin concentration is very low in untreated rats (4.02 ± 0.7 ng angiotensin I [Ang I] per mL/[hour · g protein]; n = 3) and did not change significantly with scrambled ODN treatment (2.50 ± 0.67 ng Ang I per mL/[hour · g protein]; n = 3). Even after the treatment with NRE TFD, this value did not change significantly (3.76 ± 0.74 ng Ang I per mL/[hour · g protein]; n = 3). However, the hepatic prorenin concentration increased significantly after NRE TFD treatment (13.82 ± 5.15 ng Ang I per mL/[hour · g protein]; n = 3; P < 0.01) from the baseline of untreated rats (6.22 ± 2.11 ng Ang I per mL/[hour · g protein]), whereas scrambled ODN treatment did not have any effect on hepatic prorenin concentration (6.28 ± 2.15 ng Ang I per mL/[hour · g protein]; n = 3; P = NS).

Discussion
The renin-angiotensin system is traditionally thought to be a blood-borne biochemical cascade the product of which, angiotensin II, is a potent vasoconstrictor and primary stimulus for aldosterone secretion. Numerous studies have shown an important role for the circulating renin-angiotensin system in blood pressure and electrolyte, as well as fluid, homeostasis.1,6 Renin is expressed at high levels in the kidney.6 In contrast, in the liver, the renin gene is expressed at a very low level or not at all.2 We have reported from in vitro studies that this tissue-specific renin gene expression is regulated by the interaction between NRE and NREB.7,8 Recently, using in vivo gene transfer approach, we studied this interaction in vivo in the mouse submandibular gland (SMG).9 We observed that chloramphenicol acetyltransferase expression driven by the renin gene promoter is negatively regulated in the mouse SMG by NRE and NREB interaction. We documented further that this interaction is responsible for the negative regulation of Ren-1d gene in mouse SMG.9
We hypothesized that the interaction between NRE and NREB also regulates rat renin gene expression in a tissue-specific manner. Rat renin gene contains the putative NRE with a high degree of homology to the mouse sequence. To prove our hypothesis in vivo, we used rat liver as a target organ that exhibits a very low level of renin gene expression.2 We showed the therapeutic effect of E2F decoy on neointimal hyperplasia in vivo by blocking the activation of genes mediating cell cycle progression.14 Recently, we have also shown the usefulness of the decoy approach in inhibiting chloramphenicol acetyltransferase expression driven by the promoter of mouse renin gene in vivo.5 However, these studies did not evaluate direct target gene expression. In the present study, with the use of in vivo TFD approach, we examined the direct effect on the renin gene expression in vivo.

At baseline, we could not detect renin mRNA by RT-PCR in Wistar rat liver, although Samani et al32 reported the existence of renin mRNA using RNase protection assay. In response to NRE TFD treatment, hepatic renin mRNA was detected. In contrast, hepatic renin mRNA was not detected in response to scrambled ODN transfection. The NRE TFD–induced hepatic renin expression was confirmed at the protein level by immunohistochemical documentation at 2 and 5 days after transfection of NRE TFD. We measured hepatic and plasma renin concentration 2 days after transfection of NRE TFD. Heparic and plasma prorenin concentration after NRE TFD treatment increased 2- to 3-fold as compared with scrambled ODN–treated rats or untreated rats. Interestingly, only a small but significant increase was seen in plasma active renin concentration, although hepatic active renin was not increased significantly. This suggests that most of the renin synthesized and secreted from the hepatocytes was in the precursor form, prorenin. This may reflect the limited ability of hepatocytes to process prorenin to renin. The increase in plasma active renin suggests that there is some activation of circulating prorenin in peripheral tissues. The physiological effects of increased renin gene expression in the liver after NRE TFD treatment is interesting and will be addressed in future studies in terms of blood pressure, electrolyte homeostasis, and local effects on the liver.

In summary, we have identified a specific protein (NREB) in the liver that binds to NRE sequence in the promotor region of the rat renin gene and have demonstrated that the interaction between NRE and NREB suppresses renin gene expression in the rat liver. Furthermore, we have provided direct evidence that an in vivo TFD approach can be used to study the regulation of endogenous gene expression and to modulate the expression of a specific gene in vivo.

Acknowledgments
This work was supported by NIH Grants HL07708, HL35252, HL35610, HL46631, HL48638, and HL58616 and a grant from Longwood Foundation for Translational Research. V.J.D. is a recipient of NIH Merit Award HL35610. We acknowledge I. Morales for secretarial assistance.

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Role of NRE in Renin Gene Expression in Rat Liver


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Circ Res. 1999;84:1059-1066
doi: 10.1161/01.RES.84.9.1059

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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