Transcriptional Regulation of the Rat Renin Gene by Regulatory Elements in Intron I

Till Voigtländer, Detlev Ganten, Michael Bader

Abstract—Renin catalyzes the rate-limiting step in the enzymatic cascade leading to the vasoactive peptide angiotensin II. Therefore, the activity of the renin-angiotensin system in a tissue is regulated significantly at the level of transcription of the renin gene. Besides transcription factor binding sites in the promoter region, the renin genes of human and rat contain regulatory elements also in intron I. Inclusion of intron I in reporter gene constructs with the renin promoter leads to a marked down-regulation of gene expression in nonrenin expressing 293 human embryonic kidney cells but has hardly any effect in renin-expressing L8 rat skeletal myoblasts. In combination with the cytomegalovirus immediate early gene promoter, the silencing occurs in both cell lines but is less pronounced in L8 cells. By partially deleting intron I in these constructs, we describe 5 negative (I-NRE) and 2 positive (I-PRE) regulatory elements responsible for these effects. Using gel-retardation and methylation-interference assays with 293-nuclear extracts, we detected a pseudo-palindromic protein-binding sequence between position +159 and +171 relative to the transcriptional start site. Binding of transcription factors to this sequence may be important for the tissue-specific silencing of the renin gene outside the juxtaglomerular cells of the kidney. (Hypertension. 1999;33[part II]:303-311.)

Key Words: angiotensin ■ renin ■ transcription ■ silencer ■ intron ■ gene

The renin-angiotensin system plays a key role in the control of blood pressure and electrolyte homeostasis in mammals. In rats and humans, renin represents the rate-limiting factor in the catalytic generation of the effector peptide angiotensin (ANG) II. Therefore, the understanding of its transcriptional regulation under basal as well as stimulatory conditions is of particular scientific interest and has been a major goal for the past 10 years. Renin-gene expression studies are confronted with several difficulties. The major problem is the lack of suitable renin-producing cell lines. Using a transgenic approach, Sigmund et al1 have generated As4.1 cells that resemble juxtaglomerular cells and secrete renin in the culture medium. However, these cells have not yet been utilized broadly for gene-regulation studies. Instead, renin-secreting cell lines from extrarenal renin production sites as well as nonrenin secreting cell lines have often been used in the past to study transcriptional regulation of the renin gene. So far the interest has focused primarily on the 5′ region and several different cis-acting regulatory elements like RP-2, RU-1,2 PRE, NRE,3 cAMP response element (CRE)4 and Pit-1 sites5 have been described in mouse and human renin genes. However, because of the use of different cell lines and renin genes of different species, the results concerning the location of a functional CRE are contradictory, whereas some functional elements have been demonstrated only in 1 defined cell type (for review see reference 5).

When the human and the rat renin gene were compared, a high degree of interspecies homology was discovered not only in the promoter and the coding region, but also in at least the first 120 bp of intron I.6 The degree of homology within the intronic sequences even exceeded that of the promoter and of exon I sequences. In a previous study,7 we have extended this analysis to the mouse Ren-1′ sequence, confirming the former results. A high degree of homology can be an indicator for a specific function of a sequence even if it is located in an intron. In recent years, an increasing number of intronic sequences has been identified exerting a stimulator-y9 or inhibitory10,11 function in terms of gene regulation. Further evidence for regulatory elements in the transcribed region of renin genes comes from transgenic animals. Several studies12 in transgenic mice and rats have demonstrated that correct tissue specificity of the Ren-2 gene expression is directed by DNA sequences residing between 2.5 kb 5′-flanking and 3 kb 3′-flanking regions. Likewise, 4.6 kb of the 5′-flanking sequence of the Ren-2 gene conferred tissue specificity to a SV40 large T-antigen reporter gene in transgenic mice.13 In contrast, 2.5 kb 5′-flanking region of Ren-2 failed to direct tissue specificity to the same reporter gene,14 leading to the hypothesis that functional important
regulatory elements may reside downstream of the transcription start site.

Taken together, these results suggest that intron I of the renin gene may be an interesting sequence to search for regulatory elements. In a previous report,15 we have presented some preliminary results concerning the effect of rat renin intron I on gene regulation, demonstrating for the first time the existence of a potent silencer within the intron I. In the meantime, the presence of a silencer has been demonstrated by other groups in the human renin gene.15,16 We now report a more detailed study dissecting several regulatory regions in intron I and identifying by gel-retardation experiments and methylation-interference assays a DNA sequence that is bound by nuclear proteins.

Methods

Library Screening

A Sprague-Dawley (SD) genomic library (Stratagene) was screened by the use of standard methods17 and an EcoRI/XbaI fragment of the rat renin gene comprising 1.2 kb 5'-flanking region, exon I, and 316 bp intron I as a radiolabeled probe. Four clones (r1, r4.1, r4.2, and r8) could be isolated and mapped by restriction analysis (Figure 1). A 9.7 kb SacI fragment from clone r8 containing 3.9 kb 5'-flanking region, exon I, and the entire intron I was then subcloned to generate the basic renin gene vector.

Site-Directed Mutagenesis of the 2 Translation Start Codons

PCR-directed site-specific mutagenesis of the 2 translation start codons ATG I and ATG II (at position +33 bp and +48 bp with respect to the transcription start site18 was performed by the use of a 4 primer system with a pair of overlapping internal primers. The total length of the sequence remained unchanged. The primers were designed as follows: 5' primer rR12 [5' TCT AGA GAC ACG CAT AAT CAG TGC 3'] and 3' primer rR13 [5' CAG AGA GGC GGC CGC CTC CGG ATC TGG TCC 3'] for mutagenesis of start codons ATG I/II and 5' primer rR14 [5' CGG GAG GGC GCC TCT CTG GG 3'] and 3' primer rR15 [5' CCT GCA AAA CTC TAG AGG GCC CG 3'] for mutagenesis of start codon ATG II (bold letters indicate mutated residues). In addition to the mutagenesis of ATG II, a new NolI restriction site was introduced in the sequence. PCR products were subcloned in a T-vector system (Promega), sequenced, combined at the NolI restriction site, and integrated as a 1.8 kb XbaI fragment in the basic renin gene vector, replacing the former genomic part with functional ATG start codons.

Reporter Gene Constructs

Two reporter genes, β-galactosidase from E. coli (lacZ) and firefly luciferase, were used in parallel. The basic lacZ vectors contained a 121 bp nuclear translocation signal (NTS) fused in frame to the lacZ gene and either a 850 bp intron and polyadenylation signal (pA) cassette from SV40 or a splice acceptor site (SAS) from the c-myc gene upstream of the luciferase gene. Reporter gene constructs with parts of the renin gene or the cytomegalovirus immediate early gene (CMV) promoter were cloned by standard methods. Restriction enzymes or Bal31 nuclease11 was applied for the shortening of the intron-I fragments.

Cell Culture and Transfection Experiments

Two cell lines, 293 (human embryonic kidney cells19) and L8 (rat skeletal muscle myoblasts20) were used in the present study. Both were cultured under optimal conditions and transfected with 5 μg of each expression plasmid (all described in the methods section) by the CaPO4 coprecipitation method.20 As an internal control, 2 μg of pUC13-122 (for lacZ plasmids) or pCH110 (Pharmacia, for luciferase plasmids) containing the lacZ gene under control of the SV40 early gene promoter were added to the precipitation mixture. Both reporter gene activities were measured in each cell extract 40 hours after transfection as described.19 The lacZ activity measured was normalized to the luciferase activity of the same extract if the promoter to test was linked to lacZ and vice versa. The values were statistically analyzed by use of ANOVA.
Preparation of Nuclear Extracts and Gel-Retardation Experiments

293 and L8 cells were grown to 75% confluence, harvested after a short trypsin treatment, and pelleted at low speed. Nuclear extracts were prepared as described by Dignam et al.,23 except that HEPES was replaced by Tris-HCl, pH 7.9. Protein content was quantified according to Bradford24 by the use of the BioRad protein assay system. Salt content was determined by conductivity measurement.

Gel-retardation experiments were performed as follows: In 20 μL total reaction volume shift buffer (20 mmol/L HEPES, pH 8.4, 60 mmol/L KCl, 4% Ficoll) was mixed with 2 mmol/L DTT, 2 mg bovine serum albumin, 2 mg poly(dI-dC) heteropolymer, 2.5 to 7 mg nuclear extract, and 15 000 to 25 000 cpm end-labeled probe. After 30 minutes incubation at 30°C, samples were loaded to a 4% to 6% (39:1) polyacrylamide gel in 0.25 mol/L TBE (25 mmol/L Tris-HCl, 25 mmol/L boric acid, 0.05 mmol/L EDTA) and electrophoresed at 15 V/cm. Gels were dried and exposed to Kodak XAR-5 X-ray film.

DE 52 Purification and Heparin-Sepharose Chromatography of Nuclear Extracts

Purification and chromatographic separation was carried out at 4°C essentially as described by Scheidereit et al.25 A DEAE cellulose column (DE 52, Whatman, 1 mL packed column volume [cv] per 4 to 10 mg protein load) was equilibrated in buffer C (20 mmol/L Tris-HCl, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and 0.5 mmol/L PMSF). Crude nuclear extracts were adjusted to 0.42 M NaCl, passed over the column, eluted with 1 additional cv buffer BC 500 (20 mmol/L Tris-HCl, pH 7.9, 20% glycerol, 0.5 M KCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and 0.5 mmol/L PMSF) and dialysed against 60X volume buffer BC 100 (buffer BC with 0.1 M KCl). The purified material was loaded onto a heparin-sepharose column (1 mL cv per 3.5 mg protein load, equilibrated with 5 cv BC 100) at 2 cv per hour, washed with 2 cv BC 100 at 2 cv per hour and eluted with a linear gradient (5 cv) from 0.1 to 0.5 M KCl, again at 2 cv per hour. In a final step, the column was washed with 0.3 cv of BC 1000 (buffer BC with 1 M KCl) to elute remaining proteins. The gradient was collected in 32 fractions and protein and salt content was analyzed as described above.

Methylation-Interference Assay

For the methylation-interference assay, probes were labeled by a fill-in reaction of restriction sites by the use of exo-Klenow polymerase (Stratagene) and [32P]dGTP or [32P]dATP. Partial methyl-ation of the probes was subsequently performed essentially as described,17 by the use of 2X107 cpm probe and 1 μg poly(dI-dC) as carrier for the methylation reaction at 25°C and with 1.5 M sodium acetate/1 M 2-mercaptoethanol for reaction termination. After 2 ethanol precipitations and rinsing, the partially methylated DNA was used for gel-retardation assays as described above except that the gels were not dried before X-ray film exposure. All visible retarded complexes as well as the unbound DNA fraction were cut out from the gel, purified, and cleaved in 100 μL 10% piperidine at 90°C for 30 minutes. After precipitation and rinsing, the DNA was loaded...
onto an 8% polyacrylamide-sequencing gel and visualized by autoradiography.

Results

Cloning of the Rat Renin Gene

Previously, 1.2 kb of the rat renin promoter region had been cloned. For the present study, a longer 5'-flanking region was considered to be necessary to include as many regulatory elements of the promoter region as possible. By screening of a rat genomic library (Stratagene), 4 clones (r1, r4.1, r4.2, and r8) could be isolated, comprising 16.5 kb 5'-flanking region, all exons and introns and 5.8 kb 3'-flanking region (Figure 1). A comparison between these clones revealed a BgIII restriction fragment length polymorphism probably due to different amounts of the tandem repeats present at this site in intron I of the rat renin gene.

Transcriptional Effects of Intron I

Conversion of the coding exon I of the rat renin gene into a noncoding one by PCR-directed site-specific mutagenesis of the 2 translation start sites at position +33 and +48, respectively, served as a starting point for the subsequent construction of 30 different reporter gene constructs, 16 containing the 3.9 kb renin 5'-flanking region and 14 containing the 760 bp CMV promoter element. For each promoter fragment, experiments with 2 reporter genes (lacZ and luciferase) were performed yielding essentially identical results.

All constructs were transfected into 2 different cell types, the human embryonic kidney cell line 293 not expressing renin and the rat skeletal myoblast cell line L8 containing renin transcripts detectable by RT-PCR (not shown). Schematic representations of all constructs used in the transfection experiments and their transcriptional activity are shown in Figure 2 (renin promoter) and Figure 3 (CMV promoter).

In 293 cells, addition of parts of the rat renin intron I to reporter gene constructs driven by the homologous renin 5'-flanking region resulted in a down-regulation of reporter gene activity (Figure 2A). Integration of the first 65 bp of intron I (rRI D1) led to a significant 2-fold decrease of lacZ or luciferase activity from 100 ± 6.7% to 51.5 ± 2.5%. Elongation of intron I to 117 bp (rRI D3) and further to 275 bp (rRI D5) resulted in additional reductions of reporter gene activities to 17.2 ± 1.9% and 9.0 ± 2.8%, respectively. Although longer sequences of intron I (rRI D7,9,10,11) showed only insignificant additional changes in reporter gene activity,

Figure 3. Expression of CMV promoter/renin intron constructs. Constructs containing 760 bp of the CMV promoter (C1) and renin exon 1 and the indicated segments of intron I (CI D1 to 11, CI 1) were transfected into 293 (A) or L8 (B) cells and reporter gene activities were measured after 40 hours. The data were normalized to a cotransfected construct carrying another reporter gene under the control of an ubiquitously active promoter and are given as percentage of the activity of C1 (set as 100%). Mean ± SE of at least 3 independent experiments performed in triplicate are shown. The reporter gene activities for all intron-bearing constructs was significantly different than the 1 of C1. * indicates P < 0.05 versus the next shorter construct.

Transcriptional Silencer in Rat Renin Intron I

by guest on August 24, 2017 http://hyper.ahajournals.org/ Downloaded from
inclusion of almost the entire intron I, except for the last 40 bp at the 3′ end, (rRI1) led to a further significant reduction of the transcription level to 2.7±0.9%. This is exactly in the range of negative control constructs bearing the renin sequences in antisense orientation (as1 and asI1).

Transfection of the same constructs into L8 cells revealed a completely opposite pattern of reporter gene activity (Figure 2B). Integration of the first 65 bp of intron I (rRI1) increased lacZ or luciferase activity 3-fold from 100±2.0% to 296±12%. Elongation of intron I to 117 bp (rRI3) decreased transcription activity by a factor of 2.3 to 131.5±6.5%. Variations between 117 and 410 bp length of intron I did not display any significant changes. Further elongation of intron I to 538 bp (rRI9) and 750 bp (rRI11), however, resulted first in a significant decrease in reporter gene activity to 79.6±2.0%, followed by an increase to 178.5±3.5%. Inclusion of the full-length intron (rRI1) significantly decreased reporter gene activity to 77.9±4.2%, compared with the intron-less construct (rR1).

Transfection experiments with renin intron constructs controlled by the heterologous CMV promoter in 293 cells displayed a similar but even stronger inhibitory influence of intron I on transcriptional activity as seen in the corresponding renin constructs (Figure 3A). Integration of the first 65 bp of intron I (CIA1) decreased the lacZ or luciferase activity 5-fold to 16.9±1.5% (instead of 2-fold in the case of construct rRI1). Elongating intron I to 103 bp caused a reduction of reporter gene activity to 6.3±0.4%. However, further elongation elicited no additional significant effect except for the addition of the full-length intron I lowering the transcription level to 1.1±0.3%.

The transcriptional activities of the CMV promoter/renin intron constructs transfected into L8 cells were basically comparable to the ones in 293 cells, but con-
Transcriptional Silencer in Rat Renin Intron I

Figure 5. Sequence-specific DNA-binding in rat renin intron I. Protein-DNA complexes were formed with the first 65 nucleotides of intron I partially methylated and endlabelled with $^{32}P$ and 293 nuclear extracts. After polyacrylamide gel electrophoresis, complexes B4 and B12 as well as free probes from 2 lanes (see Figure 4) were eluted from the gel, cleaved at the methylated residues and analyzed on a sequencing gel in comparison to the untreated probe. Only complex B4 showed selective binding to DNA that is unmethylated at the positions indicated by the white letters. The pseudo-palindromic sequence surrounding these positions is indicated by arrows. Positions are numbered relative to the transcriptional start site.

Contrasted to the results obtained with the corresponding renin promoter/renin intron constructs in L8 cells (Figure 3B). Integration of the first 65 bp of intron I (ClΔ1) decreased reporter gene activity by a factor of 3.2 to 30.8±5.2%, an activity level that was also found in intron deletion constructs with a length of intron I between 103 and 432 bp. Further elongation of intron I to 538 bp (ClΔ9), 750 bp (ClΔ11), and 5.3 kb (ClI) resulted again in the wavy pattern of reporter gene activity seen with renin promoter/renin intron constructs with activity levels of 9.4±1.6%, 28.5±2.8%, and 9.5±2.1%, respectively. Thus, the addition of the full-length renin intron reduced transcriptional activity 10-fold for the CMV promoter but only by 22% for the renin promoter in L8 cells.

DNA-Protein Interactions in Intron I

The sequence causing the most obvious changes in reporter gene activity, ie, the coding part of exon I (79 bp) and the first part of intron I (65 bp), was further analyzed for possible DNA-protein interactions. Nuclear extracts of both cell lines were fractionated on heparin-sepharose columns and the fractions were analyzed by gel-retardation assays. As probes, different end-labeled fragments were used containing either the exon I-intron I sequence mentioned above (+45 to +189 bp) or a part of it.

The elution profile of the heparin-sepharose column using 293 nuclear extracts is depicted in Figure 4A, a typical gel-retardation experiment with gradient-separated extracts and a fragment covering the first 65 bp of intron I (+124 to +189 bp) as probe is shown in Figure 4B. The protein-DNA complexes separated by the shift experiment were named B1 to B18 with regard to their degree of retardation and their affiliation to low salt (B1-B8) or high salt (B9-B18) portions of the gradient. Although most complexes were common to both 293 and L8 nuclear extracts, B4, represented by faint (Figure 4B) to moderate (data not shown) signals, was exclusively restricted to 293 extracts. On the other hand, a complex named B15′, presenting a weak and variable signal, could only be observed in L8 extracts (data not shown).

To determine the sequence specificity of the different binding activities methylation-interference assays were performed. The first 65 bp of intron I were labeled at the 5′-end and randomly methylated at an average of 1 guanine (G) or (to a lesser extent) adenine (A) residue per probe molecule. After incubation with the heparin-sepharose fractions 14, 20, 24, and 30 derived from 293 nuclear extract, protein-DNA complexes (B1-B18) were separated by polyacrylamide-gel electrophoresis, purified from the gel, cleaved at methylated residues and subsequently analyzed on a denaturing sequencing gel as outlined in Methods (Figure 5). The methylated probe cleaved only by piperidine without prior incubation with nuclear extracts and separation by electrophoresis as well as the free probe served as controls. Both displayed an identical G/A sequence pattern indicating that the reaction procedures have no effect per se on the methylated probe. Analyzing the sequence pattern of all complexes only B4 showed significant differences in the signal intensity of G and A residues (Figure 5). The signal intensities of the residues at positions +162 (G), +163 (A), +166 (G), and +167 (G) decreased markedly whereas that of +168 (A) increased significantly indicating that methylation at these nucleotide positions interfered with sequence-specific protein binding. Analysis of the surrounding sequence profile of the putative binding region revealed a dodecamer sequence (positions +159 to +171) with 8 out of 12 nucleotide positions arranged in a palindrome-like configuration. Neither B12 (Figure 5) nor any other protein-DNA complex in the gel-retardation experiments exhibited sequence-specific binding in the methylation-interference assay (data not shown).

Discussion

In the present study, nonrenin-expressing 293 cells and renin-expressing L8 cells were used in parallel to investigate transcriptional regulation of reporter-gene constructs. The 293 cells are well established regarding transcription analysis of renin gene constructs.2,27 The paradox that their endogenous renin gene is silent while most transfected renin-gene constructs can be transcribed with high efficiency may be explained by the expression of the adenoviral E1A protein,20 which is capable of activating many eukaryotic promoters when transfected as episomal DNA constructs, whereas the activation of endogenous genes is variant between different genes.28 L8 cells were used for the first time in expression studies of the renin gene. However, a related rat skeletal muscle myoblast cell line, L6, has already been shown to express renin.29

Transcription analysis of the different constructs revealed a complex pattern of 7 potential cis-regulating elements within the first 850 bp of intron I, 5 negative regulatory elements...
I-NRE 1 to 5 and 2 positive regulatory elements I-PRE 1 and 2. Their approximate positions within intron I and their functional relevance considering cell type and promoter region are outlined in Figure 6. In summarizing our data, we propose the following hypothetical model for the transcriptional control of the rat renin gene by intron I:

Two regulatory elements (I-PRE 1 and I-NRE 1) are located within the first 65 bp of intron I demonstrating antagonistic regulatory effects. Whereas I-NRE 1 acts promoter-independently, I-PRE 1 represents as a renin-promoter dependent enhancer in both cell types with barely any effect in constructs controlled by the CMV promoter. I-NRE 2, located in the segment between 66 and 103 bp of intron I, represents a promoter-independent negative regulatory element in both cell types. I-NRE 3, located between nucleotides 138 and 275 bp of intron I, acts as a renin-promoter and 293-cell specific negative regulatory element. Almost promoter independent but L8-cell specific are the regulatory effects of I-NRE 4 and I-PRE 2. I-NRE 4, located in the DNA segment between 432 and 538 bp of intron I, reduces the transcription activity of the renin and the CMV-promoter, whereas no effect is seen in 293 cells. I-PRE 2 however, located more downstream between nucleotides 618 and 750 of intron I, antagonizes the silencing effect of I-NRE 4 in L8 cells irrespective of the origin of the controlling promoter region. Again, there is no obvious regulatory effect in 293 cells. I-NRE 5 was localized to the segment between 750 and 848 bp of intron I as reporter gene activities of Δ12 deletion mutants (Figure 1) exhibited the same transcriptional activities as full-length intron constructs (data not shown). I-NRE 5 influences transcription regulation of either promoter in either cell line. Taken together, I-NRE 2, 4, and 5 may be responsible for the low expression of the endogenous renin gene in L8 cells and, together with I-NRE 1 and 3, also for the inactivity of this gene in 293 cells.

Splicing artifacts as explanation for the differential reporter gene expression in both cell lines are unlikely as all intron-bearing constructs were cloned in a modular manner guaranteeing an identical nucleotide sequence around the SAS. Moreover, RT-PCR analysis of RNA isolated from cells transfected with the different constructs revealed amplification products of the expected size for a correct splicing process (data not shown). Furthermore, the differential interactions of some of the intron-I elements with both promoters can hardly be explained on a post-transcriptional level.

Regulatory elements in intronic sequences of mammalian genes are a common phenomenon.8–11 After our initial report of a transcriptional silencer in the rat renin gene,7 Lang et al15 described a strong orientation- and position-independent repressor in intron A of the human renin gene active in Calu-6 cells, a renin-producing human lung carcinoma cell line. In contrast to our study, the authors tested the transcriptional
influence of intron A only in combination with the homologous human renin promoter and could only demonstrate a regulatory effect on a promotor fragment of at least 0.9 kb in length. More recently, Germain et al. published a similar study confirming the existence of this silencer active in Calu-6 cells. In addition, they demonstrated a comparable silencing effect of human renin intron A on the heterologous SV40 promoter in these cells. In contrast to our results, the authors found a stimulatory effect of the first intron transfecting these constructs into the nonrenin expressing cell lines CHO and HeLa. The discrepancy regarding nonrenin producing cell lines may be explained by the use of different cell lines and renin genes of different origin. Nevertheless, it should be emphasized that it is difficult to understand why nonrenin expressing cell lines should express the transfected constructs.

Analysis of protein binding to the DNA region containing I-PRE 1 and I-NRE 1 by gel-retardation experiments and methylation-interference analyses revealed a number of complexes, 1 of them demonstrating sequence-specificity. The responsible cis-acting DNA sequence consisted of 12 nucleotides (AACCTGGCCCCCT, +160 to +171 bp) of which 8 (underlined) form a palindromic structure, which is typical for binding sites of dimeric transcription factors. The protein-binding DNA sequence in the rat renin intron I contains (on the opposite strand) the pentamer GAGGC (underlined) for binding sites of dimeric transcription factors. A combination of upstream and proximal elements is required for efficient expression of the mouse renin promoter in cultured cells. Nucl Acids Res. 1993;20: 3617–3623.

References


