Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 113-122

Regulatory Elements in the Promoter Region of the Renal Kallikrein Gene in Normotensive *vs* Hypertensive Rats

Cindy Wang, Yin-Peng Chen, Lee Chao and Julie Chao*

Department of Biochemistry and Molecular Biology
Medical University of South Carolina, Charleston, SC 29425

Received October 6, 1995

The renal kallikrein-kinin system has been implicated in the pathogenesis of hypertension. The expression level of the renal kallikrein gene in the kidney is significantly lower in spontaneously hypertensive rats (SHR) as compared with that of normotensive (SD and WKY) rats. Deletion analysis showed that the fragment -356/-188 of the promoter contains a transcriptional silencer(s) and the GC rich region located between -77 and -187 is the minimal essential element for directing the expression of the CAT reporter gene in mouse L cells. In the kidney of normotensive *vs* hypertensive rats, the nuclear protein factors NF1/CTF and SP1 bind differently to the renal kallikrein promoter, but similarly in the salivary gland. The differential transcriptional regulation of the rat renal kallikrein gene in the kidney may be responsible for the genetic difference between normotensive and hypertensive rats.

Tissue kallikreins (E.C. 3.4.21.35) are composed of a multigene family which encodes for a subgroup of serine proteinases. Kallikrein family members perform diverse physiological roles through the liberation of various peptide hormones and growth factors. The best characterized function of kallikreins is their ability to release vasoactive kinins from kininogens. The rat renal kallikrein gene rKLK7 (RSKG7) is specifically expressed in the kidney and salivary gland (1). In the kidney, renal kallikrein participates in the increase of the renal blood flow, diuresis and natriuresis, and the regulation of prostaglandin synthesis. In the salivary gland, renal kallikrein is possibly involved in the local regulation of blood flow and the ionic composition of saliva (2, 3). Recent studies have demonstrated that the renal kallikrein-kinin system plays an important role in the regulation of blood pressure, control of sodium and water excretion and renin release through generation of kinins. It has been shown that kinins and the kinin receptors play an important role in the development of kidney (4). An association between tissue kallikrein and blood pressure regulation has been established by studies using experimental animal models (5-7). A restriction fragment length polymorphism (RFLP) at tissue kallikrein loci in normotensive *vs.* hypertensive rats was identified (5) and the RFLP cosegregates with a high blood pressure phenotype (6). In addition, a decreased level of urinary kallikrein excretion was reported in spontaneously hypertensive rats (SHR) when compared with normotensive Wistar-Kyoto (WKY) rats (7). These findings suggest that transcriptional regulation of the rat renal kallikrein gene is involved in blood

*Corresponding author. Fax: (803)-792-4322.

0006-291X/95 $12.00

pressure regulation. At the present time, little information is available concerning the basal transcriptional regulation of the rat renal kallikrein gene and its hypertensive phenotype.

We previously demonstrated that the 300 bp from the immediate 5'-flanking region of the human tissue kallikrein gene was capable of directing tissue-specific expression of the human tissue kallikrein gene in transgenic mice (8). In this study, we have analyzed the expression levels, the 5'-flanking region sequence, *cis*-regulatory elements and *trans*-acting factors that are important for differential expression of the renal kallikrein gene in normotensive *vs.* hypertensive rats.

MATERIALS AND METHODS

Northern blot analysis of renal kallikrein gene expression in the kidney of normotensive vs. hypertensive rats. Northern blot analysis was performed using twenty five μg of total kidney RNA extracted from Sprague Dawley (SD), Wistar-Kyoto (WKY), and spontaneously hypertensive rats (SHR) according to a previously described method with some modifications (9). The renal kallikrein specific oligonucleotide (5'-TGAGGAATGGTTTGTAGT CAGGGTGAG-3') was end-labeled by α-^{32}P-ATP with TdT (terminal deoxynucleotide transferase, GIBCO/BRL, Bethesda, MD) and used as a probe at a final concentration of 3 x 10^5 cpm/ml at 56°C for 20 hr. The membrane was washed with 0.1 x SSPE at 40°C and exposed to X-ray film at -70°C. The X-ray film was scanned into Adobe Photoshop 2.5 with a Hewlett Packard Scan Jet $IIc_{X/T}$ and the mean intensity of respective bands was quantitated by Image 1.47.
Sequencing of the 5'-flanking region of the renal kallikrein gene in normotensive vs. hypertensive rats. The promoter region, from -1182 to +5, was amplified by PCR from WKY and SHR genomic DNA. The reaction mixture contained 1 μg of WKY and SHR genomic DNA, 20 pmoles of the 3' primer (5'-TGGAGCTTGAGGAGCCTCCT-3' located at the transcription initiation site), 20 pmoles of the 5' primer (5'-GGAGGTACCAGATTTTGTGG-3' located on -1183 to -1164 of the rat renal kallikrein gene), 20 nmoles of dNTP, 5 μl of 10 x PCR buffer, and 0.5 U of *Taq* DNA polymerase in a total volume of 50 μl, followed by 35 cycles of hot-start polymerase chain reaction (PCR) (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) with Ampliwax (Perkin Elmer Cetus, Norwalk, CT) in a thermal cycler. Five μl of the amplified rat renal kallikrein gene promoter region of SHR, WKY and SD rats was sequenced with a dsDNA PCR sequencing kit (GIBCO/BRL, Bethesda, MD).
Cell line and culture. The mouse L cells were maintained in Dulbecco's Modified Eagle medium (D-MEM) containing 10% (vol./vol.) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.
Plasmid constructions. To define DNA sequences in the renal kallikrein promoter responsible for specific transcription in mouse L cells, a 1188 bp DNA fragment of the rat renal kallikrein gene containing the 1183 bp of 5'-flanking region, the transcriptional start site, and 5 bp of exon 1 was inserted to a Sma I site of the pUC-CAT vector in the sense and antisense orientations with respect to the CAT (chloramphenicol acetyltransferase) gene {rKLK7-CAT (+) and rKLK7-CAT (-)}. A series of 5'-deletion mutants extending from -780 to -76 (Δ780-CAT, Δ657-CAT, Δ356-CAT , and Δ187-CAT, and Δ76-CAT) were constructed and tested for their ability to promote transcription. The plasmid vector p0GH, featuring a promoterless human growth hormone gene (hGH), was also used to characterize the regulatory sequences of the renal kallikrein gene (10). Deletional mutations of hGH reporter gene constructs, Δ342-p0GH, Δ182-p0GH and Δ76-p0GH, were constructed by linking respective renal kallikrein gene 5'-flanking regions with the human growth hormone reporter gene at Bam HI site in the sense orientation.
Transfection and reporter gene assay. Mouse L cells at 80% confluency were transfected with supercoiled plasmid DNA by the standard calcium phosphate precipitation method (11). A calcium phosphate coprecipitate containing 8 μg of DNA was added to the cultured cells. Mouse L cells were co-transfected with 5 μg of a β-galactosidase expression plasmid under the control of Rous sarcoma virus long terminal repeat, which allowed for adjustments of transfection efficiencies. The level of CAT enzyme in cell extracts was quantitated with a CAT ELISA kit (5 prime → 3 prime, Boulder, CO). For the human growth hormone reporter gene, DEAE-dextran-mediated plasmid DNA transfection was performed essentially as described by Selden *et al.* (10). The level of human growth hormone secreted in culture medium was assayed by radioimmunoassay which is specific for human growth hormone (12).

Preparation of nuclear extracts. Salivary gland and kidney nuclear extracts were prepared according to the method of Gorski *et al.* (13). The salivary nuclear extract was prepared from 2 g of SD and SHR salivary gland. The kidney nuclear extract was prepared from 10 g of SD or SHR rat kidney. The final protein concentration was 1-2 mg/ml.

Gel mobility shift assay. The 5'-flanking regions (-356 to -188 and -187 to +5) of the renal kallikrein gene were cloned into Hinc II site of pUC 19 vector. DNA fragments were prepared by Bam HI and Hind III digestion of rKLK7 (-356 to -188)-pUC19 and rKLK7 (-187 to +5)-pUC19 dsDNA and purified by agarose gel electrophoresis. Double-stranded promoter sequences were radiolabeled at both ends with Klenow and [α-^{32}P] dATP. DNA binding assays were performed essentially as described by Hennighausen and Lubon (14) using 1 ng of radiolabeled oligonucleotide and 5 µg of rat salivary gland nuclear extract or 1 µg of rat kidney nuclear extract. The binding mixtures were loaded directly onto a 4% polyacrylamide gel (30:1 acrylamide/bisacrylamide) in 50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.3, and electrophoresed at 10 V/cm for 2 hr at room temperature. The following double-stranded DNA were used as specific competitors in gel mobility shift assays and obtained from Stratagene (La Jolla, CA): Sp1 competitor, GATCGATCGGGGCGGGGGGCGATC, which contains a GC box sequence; Oct-1 competitor, GATCGAATGCAAATCACTAGCT, which contains an Oct-1 sequence; and NF1/CTF, ATTTTGGCTTGAAGCCAATATG, which contains a CCAAT sequence.

RESULTS

Expression levels of renal kallikrein mRNA in normotensive vs. hypertensive rats. The Northern blot analysis using a specific oligonucleotide probe showed that the mRNA levels of renal kallikrein in the kidney of normotensive (SD and WKY) rats were similar, whereas its expression in the hypertensive (SHR) rat kidney is about 20% of the level in SD or WKY, as determined by quantitative densitometry (Figure 1). In addition, a semi-quantitative RT-PCR specific for renal kallikrein also showed that its mRNA level is higher in the kidney of

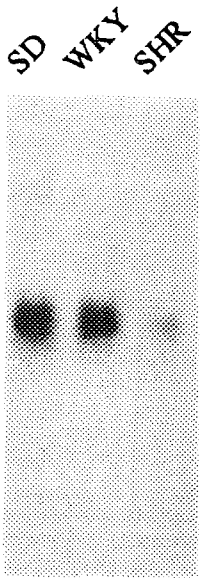


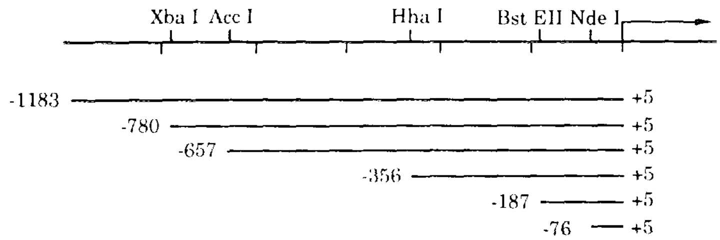
Figure 1. Northern blot analysis of the rat renal kallikrein (rKLK7) mRNA expression in the kidney of SD, WKY, and SHR. SD, WKY, or SHR indicates total RNA extracted from the kidney of SD, WKY, or SHR.

normotensive rats (SD and WKY) than hypertensive (SHR) rat. However, transcripts of the renal kallikrein gene in the salivary gland were similar between normotensive (SD and WKY) and hypertensive (SHR) rats as analyzed by Northern blot analysis using a specific oligonucleotide probe (data not shown).

Sequence homology in the 5'-flanking region of normotentive vs. hypertensive rats. To delineate the sequence homology in the 5'-flanking region of the renal kallikrein gene between WKY and SHR, the promoter region (-1183 to +5) of the renal kallikrein gene was amplified by PCR from genomic DNA and the region from -500 to +5 was sequenced. The sequences located from -500 to +5 of the renal kallikrein gene 5'-flanking region are identical between WKY and SHR. This result indicates that *cis*-acting elements at the proximal promoter region of the renal kallikrein gene are identical between normotensive and hypertensive rats.

Sequence analysis of the 5'-flanking region of the renal kallikrein gene. A partial restriction map of this genomic fragment is shown in Figure 2A. Putative *cis*-acting regulatory elements in the renal kallikrein gene (rKLK7) were identified by comparing its 5'-flanking sequences with that of rat tissue kallikrein (rKLK1) gene. The most proximal region corresponds to and includes the variant TATA box (-28 to -22), two variant GC boxes at -55 to -50, and -94 to -89 and three CCAAT consensus sites at 217 to -209, -83 to -75, and -69 to -61 (Figure 2B), which are conserved in rat kallikrein gene family.

A



B

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         CCAAT
rKLK7:  GACCTACCTT AGAGCTGTGC GCTGGGGGTC ACTTTCTCCT CCATCCACCT ATTACACCCC TTCACTATTA
rKLK1:       T           T  G  T             G  T   GT C  GA     C            G   C

rKLK7:  CCCCTGACCT TGCCATTTGG CTTTAATCAC CAAAACATGA ACAGGTACTG GAGAACCAGG CACTGCCCTA
rKLK1:                   C        GG  C    G              C A    AC G      C  T   C
         CCAAT
rKLK7:  CCCAATCTCA GCCCTGGGAA GGTATCCAGG GTGACCTAGA GCCTACTGGA CAGCAGGTGC AACAGGGCCC
rKLK1:          A                     C      A    G         A              G
                                                                 GC box        CCAAT
rKLK7:  TCCCCTCCCA CAGCCAAGAG GGTGAGAGCA CGGAATCCAC CTCCGTCAGC AAGGGCAGGG CTTAGCCAGC
rKLK1:                   T     T         GAA  T                - C    G
                  CCAAT        GC Box                       TATA box
rKLK7:  ATATGAGTGC CAACAGGACA GGGGTGGGGC TGGGGGAGAA CTAGGAGTTT TTAAAGGCTC CCCAAGGAGG
rKLK1:    C  G            G       C           A      TG    A-
               +1
rKLK7:  CTCCTCAGCT C
rKLK1:
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Figure 2. Restriction map and sequence comparison of the 5'-flanking region of the rat renal kallikrein (rKLK7) gene. A, partial restriction map of the rat renal kallikrein promoter region. The solid arrow indicates that transcription start site and the direction of transcription. The six DNA fragments were cloned into pUC-CAT polylinker and used in transient transfection assay. B, nucleotide sequence comparison of the 5'-flanking region. The 5'-flanking regions of rat renal kallikrein (rKLK7) and rat tissue kallikrein (rKLK1) genes are aligned with gaps (-) to maximize homology. Blanks indicate sequence identity. TATA, CCAAT, and GC box homologues are underlined.

Rat renal kallikrein proximal promoter directs reporter gene transcription in mouse L cells. To define DNA sequences responsible for the transcription of the renal kallikrein gene, a series of chimeric constructs were tested for their ability to promote the transcription of CAT reporter gene and human growth hormone gene in mouse L cells (Table 1). The initial deletion of sequences between -1183 and -781, -780 and -658, -657 and -338, had no effect on the CAT expression level. However, the CAT expression level was increased when the sequence between -337 and -188 was removed. This result suggests that a negatively regulatory element or silencer resides in the region from -337 to -188. A further deletion between -187 and -77 resulted in a decrease of CAT expression. Similar results were observed when human growth hormone was used as a reporter gene in p0GH vector. These results suggest that the region located between -187 and -77 of the 5'-flanking region contains most of the necessary renal kallikrein promoter sequences. This region contains a variant TATA box at -28 to -22 and two consensus CCAAT sequences at -85 to -75 and at -69 to -61 that is commonly found within 60-80 bp upstream of the cap site in many mammalian promoters.

Tissue-specific interaction of salivary nuclear factors to cis-acting elements upstream of the renal kallikrein gene transcription initiation site. In gel mobility shift assay, we identified two protein-DNA complexes formed by SD salivary nuclear extracts interacting with -356/-188 probe as shown in Figure 3. Similar results were observed when protein-DNA complexes formed by salivary nuclear extracts interacting with -187/+5 probe were examined (Figure 3). The lower complex but not the higher complex formed between salivary nuclear extract and -356/-188 or -187/+5 probe was specifically competed by the addition of NF-1/CTF consensus oligonucleotide (Figures 4 and 5), which suggests that NF-1/CTF is necessary for the transcription of the renal kallikrein gene in the salivary gland. In addition, these results suggest that the formation of lower complex was not due to nonspecific binding although it cannot be replaced by a hundred-fold molar excess of the unlabeled probe. The upper complex can not be competed by the addition of NF-1/CTF, SP1 or Oct-1 consensus oligonucleotides. Furthermore, in the salivary gland, transcription factors interact similarly with the renal kallikrein promoter in normotensive (SD and WKY) and hypertensive (SHR) rats.

Table 1. CAT assays of pCAT constructs in mouse L cells and human growth hormone (hGH) levels produced in mouse L cells transfected with p0GH derivatives

pCAT construct	Relative CAT activity	p0GH construct	hGH (ng/ml)
pUC-CAT	1		
RSV-CAT	2.56 ± 0.55		
rKLK7-CAT (-)	1.00 ± 0.11		
rKLK7-CAT (+)	1.04 ± 0.14		
Δ780-CAT	1.08 ± 0.07		
Δ657-CAT	1.13 ± 0.10		
Δ356-CAT	1.03 ± 0.07	Δ342-p0GH	<1
Δ187-CAT	1.60 ± 0.27	Δ182-p0GH	18.8
Δ76-CAT	1.12 ± 0.08	Δ76-p0GH	7.6

CAT assays are corrected for transfection efficiencies by performing assays on extracts with equivalent β-galactosidase activity. Relative CAT activities ± standard error are calculated by standardized percent CAT conversion to the activity of mouse L cells transfected with promoterless vector pUC-CAT, which were averaged for each experiment and set at 1. Levels of human growth hormone produced by mouse L cells are expressed as ng/ml as determined by radioimmunoassay.

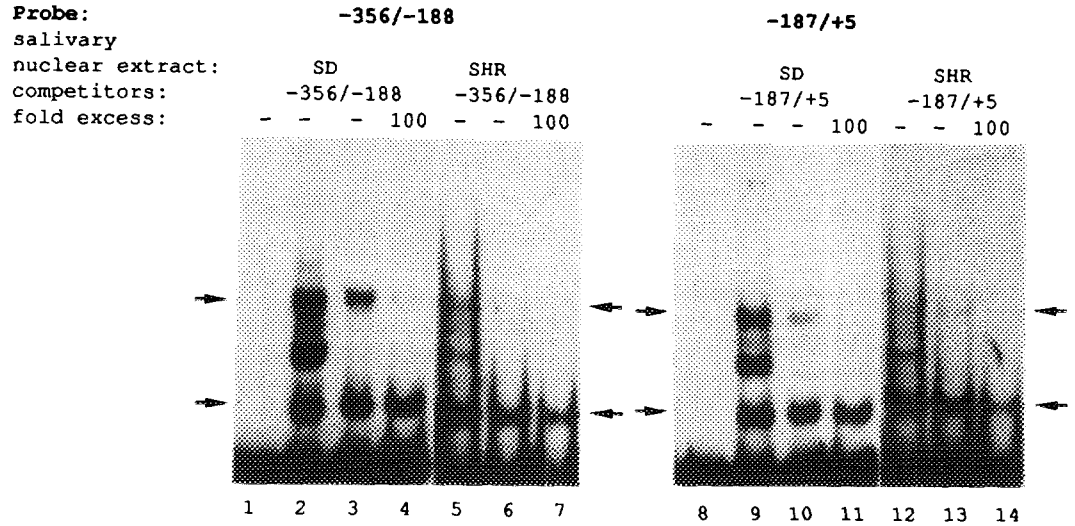

Figure 3. Sequence-specific association of salivary nuclear protein with 5'-flanking region of the rat renal kallikrein (rKLK7) gene. The arrow indicates the position of the protein-DNA complex. Lanes 1 and 8 represent free probe alone. Lanes 3-4 and 6-7 show the specific binding of SD and SHR salivary nuclear protein to fragment -356/-188 in the presence of poly(dI-dC). Lanes 10-11 and 13-14 show the specific binding of SD and SHR salivary gland nuclear protein to fragment -187/+5 in the presence of poly(dI-dC).

Tissue-specific interaction of kidney nuclear factors to cis-acting elements upstream of the renal kallikrein gene transcription initiation site. Using kidney nuclear extracts from normotensive SD rats, we identified three different protein-DNA complexes

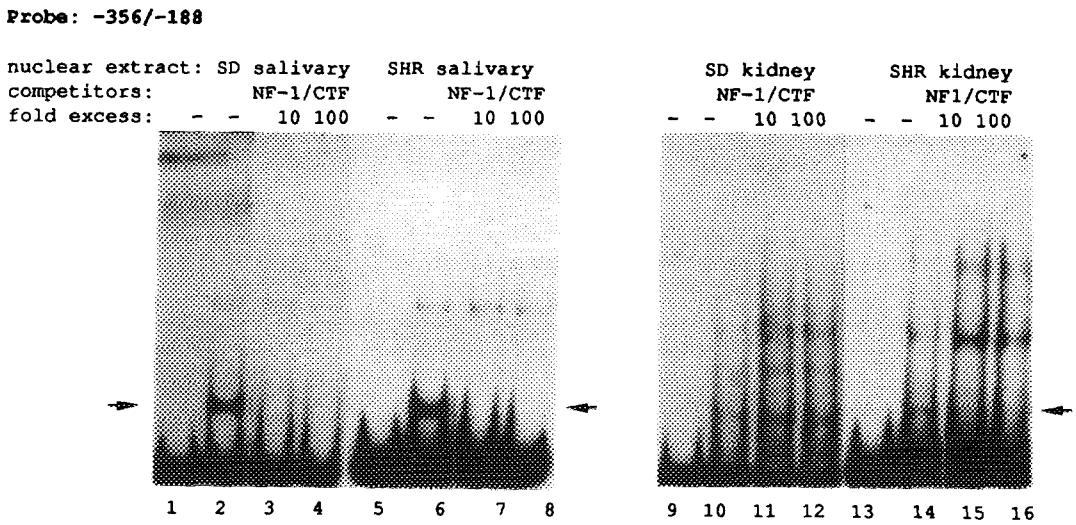

Figure 4. Nuclear factor binding to the SP1 and CCAAT box on -356/-188 of the rat renal kallikrein (rKLK7) gene. Lanes 1, 5, 9, and 13 represent free probe alone. Lanes 2, 6, 10, and 14 show the specific binding of SD, SHR salivary gland, SD, SHR kidney nuclear protein to fragment -356/-188 in the presence of poly(dI-dC), respectively. Lanes 3-4, 7-8, 11-12 and 15-16 also include the unlabeled double-stranded oligonucleotide competitors that contain the nuclear factor binding sites specific for NF-1/CTF or SP1. Indicated are the competitor and approximate molar excess over radiolabeled probe. Arrows indicate complexes eliminated by NF-1/CTF or SP1 competitors.

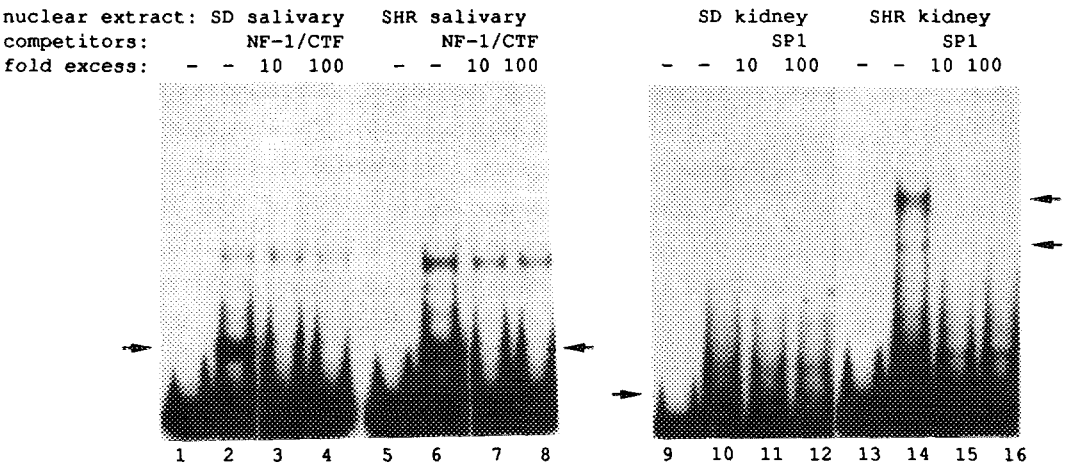

Figure 5. Nuclear factor binding to the SP1 and CCAAT box on -187/+5 of the rat renal kallikrein (rKLK7) gene. Lanes 1, 5, and 9 represent free probe alone. Lanes 2, 6, 10, -187/+5 fragments plus SD salivary gland, SHR salivary gland, and SHR kidney nuclear protein, respectively. Lanes 3-4, 7-8, and 11-12 also include the unlabeled double-stranded oligonucleotide competitors that contain the nuclear factor binding sites specific for NF-1/CTF. Indicated are the competitor and approximate molar excess over radiolabled probe. Arrows indicate complexes eliminated by NF-1/CTF competitors.

with -356/-188 probe as shown in Figure 6. None of the complexes formed between SD kidney nuclear extract and -356/-188 probe can be competed by the addition of NF-1/CTF, SP1 or Oct-1 consensus oligonucleotides (Figure 4). When -187/+5 DNA probe was used in the gel mobility

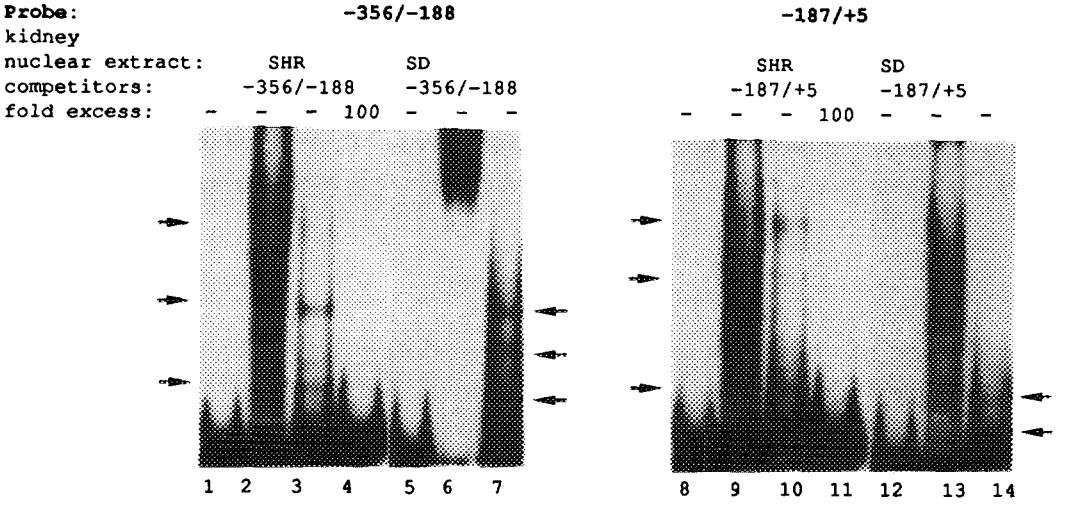

Figure 6. Sequence-specific association of kidney nuclear protein with 5'-flanking region of the rat renal kallikrein gene (rKLK7). The arrows indicate the positions of the protein-DNA complexes. Lanes 1 and 8 represent free probe alone. Lanes 3-4 and 6-7 show the specific binding of SD and SHR kidney nuclear protein to fragment -356/-188 in the presence of poly(dI-dC). Lanes 10-11 and 13-14 show the specific binding of SD and SHR kidney nuclear protein to fragment -187/+5 in the presence of poly(dI-dC).

shift assay using nuclear extracts from the kidney of SD rats, two complexes were identified (Figure 6). The lower complex formed between SD kidney nuclear extract and -187/+5 probe was competed by the addition of SP1 consensus oligonucleotides (Figure 5). However, the upper complex formed between SD kidney nuclear extract and -187/+5 probe cannot be competed by the addition of NF-1/CTF, SP1 or Oct-1 consensus oligonucleotides. The kidney nuclear extract of WKY rats formed the same protein-DNA complexes with -356/-188 or -187/+5 probe as that of SD rats (data not shown). Our results on tissue-specific interactions of -356/-188 and -187/+5 probes indicate that different *trans*-acting factors are involved in the tissue-specific transcriptional regulation of the renal kallikrein gene in the salivary gland and kidney. In SD rats, NF-1/CTF interacts with the renal kallikrein promoter in the salivary gland, whereas SP1 interacts with the minimal promoter of renal kallikrein in the kidney.

Differential regulatory mechanisms of the rat renal kallikrein gene in the kidney of normotensive vs. hypertensive rats. Analysis of the *trans*-acting factors in the kidney of SHR interacting with the renal kallikrein promoter showed that three complexes were resolved with -356/-188 probe and their migration rates were different from those complexes formed with SD kidney nuclear extract (Figure 6). The lowest complex formed between kidney nuclear extracts of SHR and -356/-188 probe was competed by the addition of NF-1/CTF consensus oligonucleotide. These results indicate that NF-1/CTF interacts with the renal kallikrein gene promoter in hypertensive but not normotensive rats. When the interactions of SHR kidney nuclear extract and -187/+5 probe were examined, three complexes were resolved on gel mobility shift assay. These three complexes migrated differently as compared with the two complexes formed by SD rat kidney nuclear extract (Figure 6). The two upper complexes formed by SHR kidney nuclear extract were identified to be complexed with SP1 *trans*-acing factor, as indicated by the replacement of these two complex by SP1 consensus oligonucleotide. Although one of the complexes formed by SD rat kidney nuclear extract and -187/+5 probe was also identified to be complexed with SP1 *trans*-acing factor, it migrated much faster than those formed with SHR kidney nuclear extract (Figure 5). These results indicate that the mechanism of the interaction between *trans*-acting factors and the renal kallikrein gene promoter in normotensive (SD and WKY) rats is different from that in hypertensive (SHR) rats. Therefore, the transcription of the renal kallikrein gene is differentially regulated in normotensive *vs.* hypertensive rats.

DISCUSSION

The low urinary kallikrein excretion in essential hypertensive patients and genetically hypertensive rats has been implicated in the pathogenesis of hypertension (15, 16). The primary source of urinary kallikrein is the kidney (17), and the existence of the renal kallikrein-kinin system was demonstrated by localizing all kallikrein-kinin system components in kidney. In the kidney, renal kallikrein specifically cleaves low molecular weight kininogen to generate bradykinin in the tubular lumen. The released kinin accelerates renal excretion of sodium, chloride, and water and regulates prostaglandin synthesis [2, 3]. However, the basal transcriptional mechanism of the renal kallikrein gene in the kidney is not completely understood. In this study, we show that the

rat renal kallikrein transcription is differentially regulated in the kidney between normotensive and hypertensive rats.

The renal kallikrein gene 5'-flanking region, between -187 and +5, contains two potential GC-rich sequences located at -46 to -41 and at -97 to -89, respectively. The *trans*-acting factor SP1, that binds to the GC box, can cooperate with other *trans*-acting factors resulting in higher transcription efficiency. The abnormal interaction with the *trans*-acting factor SP1 in the kidney of SHR could be responsible for the reduced renal kallikrein expression level in the kidney of SHR. Further studies will be needed to establish a direct link between the differential interaction to nuclear factor(s) and the reduced expression level of the renal kallikrein gene in the kidney of spontaneously hypertensive rats.

The involvement of transcription factors in the pathogenesis of hypertension has been demonstrated previously in both *in vivo* and *in vitro* studies (18-21). Recent evidence suggests that the regulation of protooncogene expressions mediates the vascular smooth muscle cell growth and proliferation, which may be a crucial step in the pathogenesis of hypertension and atherosclerosis. Thus, the abnormal expression of protooncogenes and transcription factors in hypertensive state may affect the expression of genes which are important in the regulation of blood pressure.

In conclusion, the experiments reported here indicate that the proximal promoter region of the rat renal kallikrein gene interacts differently with *trans*-acing factors in the kidney of normotensive and hypertensive rats, which could be responsible for the low urinary kallikrein excretion in SHR. It is likely that the a defect in the transcriptional regulation of the renal kallikrein gene may also be implicated in human hypertensive patients. Further studies will be needed to define the role of *trans*-acting factors in low urinary kallikrein excretion and provide a molecular basis for the relationship between low urinary kallikrein excretion and hypertension.

ACKNOWLEDGMENTS

We thank Dr. Carmelann Zintz and Gary P. Richards for their critical review of this manuscript. This study was supported by National Institutes of Health grants HL 29397 and HL 44083.

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