A Novel Proximal Element Mediates the Regulation of Mouse Ren-1C Promoter by Retinoblastoma Protein in Cultured Cells

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Selective gene expression is mostly controlled at the level of transcription (1). Regulation of transcriptional activity is achieved through the binding of a series of transcriptional factors to sequence-specific DNA elements (2–4). The identification of both cis-acting elements and nuclear factors in specialized cells facilitates understanding of the molecular mechanisms that underlie tissue- and development-specific gene expression. Renin, an aspartyl protease, plays an important role in the regulation of blood pressure and water-electrolyte balance by catalyzing the rate-limiting step of the renin-angiotensin system, and may be involved in the pathogenesis of hypertension (5–7). The expression of renin gene is regulated in a tissue-specific and development-linked manner (8, 9), and the main site of production of circulating renin is the kidney. Transgenic studies previously demonstrated that the 5′-flanking region of the human renin gene directs tissue-specific expression in the kidney (10, 11), and that 5′-flanking sequences of the mouse renin gene (Ren-2) directed tissue- and development-specific expression of the reporter SV40 T antigen gene (12, 13). In vitro studies, we identified two transcriptionally important promoter elements (RU-1; −224 to −138, and RP-2; −75 to −47) in the mouse renin gene (Ren-1C), and demonstrated that the combination of these elements was responsible for cell type-specific transcriptional activity of Ren-1C gene in transfected human embryonic kidney (HEK) cells (14, 15). Furthermore, recent studies using pituitary and kidney cell lines indicated that novel factors bound to the proximal promoter and regulated transcription of human and mouse renin genes (16, 17). 5′-Flanking sequences of the mouse, rat, and human renin genes show significant homology, suggesting that the promoter regions may be involved in the regulation of renin gene expression in the kidney (18, 19).

The protein product of the retinoblastoma susceptibility gene, RB, is a nuclear phosphoprotein that modulates transcription of genes involved in growth control via interactions with transcription factors. Renin is a rate-limiting enzyme of the renin-angiotensin system that regulates blood pressure and water-electrolyte balance. Renin gene expression is regulated in a tissue-specific and developmentally linked manner. Similarly, the expression of RB is controlled in a differentiation-linked manner. Thus, to investigate whether RB is involved in the regulation of renin gene expression, we examined the effects of RB on transcriptional activity of the mouse renin (Ren-1C) promoter. The Ren-1C promoter contains two transcriptionally important elements; the RU-1 (−224 to −138) and RP-2 (−75 to −47) elements. RB activated the Ren-1C promoter in human embryonic kidney cells. The promoter element responsible for RB-mediated transcriptional regulation was the RP-2 element. The results of DNA-protein binding experiments showed that RB increased nuclear binding activity to the RP-2 element, and site-directed mutation which disrupted binding of nuclear factors to the RP-2 element markedly reduced RB-mediated activation of Ren-1C promoter in human embryonic kidney cells. These results indicate that the RP-2 element plays an important role in RB-mediated transcriptional regulation of Ren-1C promoter activity in human embryonic kidney cells, thereby suggesting an interesting mechanism by which RB may modulate the renin-angiotensin system.
mRNA is also expressed abundantly in the kidney (40), it is possible that RB modulates expression of the renin gene in the kidney.

In the present study, we showed that RB activated transcription of the Ren-IC gene in HEK cells. The proximal promoter region from -75 to +16 of the Ren-IC gene mediated the transcriptional activation by RB. We demonstrated that the RP-2 element (-75 to -47) which overlapped the TATA-like region was the major contributor to the regulation of Ren-IC promoter activity by RB in HEK cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pUCSV3CAT contained the coding sequence for chloramphenicol acetyltransferase (CAT) fused with the simian virus 40 (SV40) enhancer/promoter sequence and polyadenylation signal (41). pUCSV0CAT contained the SV40 polyadenylation signal 5' to the CAT coding gene to efficiently terminate read-through transcription arising from prokaryotic sequences in the constructs (41). pUCSV3CAT was used as a positive control, and pUCSV0CAT was used as a background reference. Plasmid phuRb contained a KpnI (position -240 to EcoRI (position +4602) human cDNA fragment cloned into the Smal and EcoRI sites of the control vector pJ311, which included the SV40 enhancer/promoter sequence and poly(A) signal (kindly provided by Dr. Paul Robbins, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA) (24).

Ren-IC promoter-CAT hybrid genes, R365CAT (-365 to +16), R224CAT (-224 to +16), R183CAT (-183 to +16), R164CAT (-164 to +16), R114CAT (-114 to +16), R75CAT (-75 to +16), and R47CAT (-47 to +16) were constructed as described previously (14). Construction of TK-CAT was described previously (42). The RU-1 or RP-2 elements with or without the other element were linked upstream of R47CAT or TK-CAT in 5' to 3' orientation. R365CAT was used as a template to construct mutations in the RP-2 element (m(RP-2) element) by oligonucleotide-directed mutagenesis (43). The sequences of the oligonucleotide used to create m(RP-2) element were 5'-CCCTGGGTAAAGCAGAGCCT-3'. Once the mutations were obtained and confirmed by sequencing, the altered 381-base pair (-365 to +16) fragment was subcloned into the BglII/HindIII sites of pUCSV0CAT (R365 m(RP-2)-CAT). To construct plasmid pCRP-2, six tandem copies of a synthetic double-stranded RP-2 element (5'-CCCTGGGTAAAGCAGAGCCT-3') were inserted into the BglII site of pUC19 as described previously (44). To construct pCRM/RP-2, six tandem copies of the m(RP-2) element were inserted into the BglII site of pUC19.

Cell Culture—HEK cells were maintained in minimum essential medium containing 10% heat-denatured horse serum (14). HeLa and T98G cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. These cell lines were kept in 5% CO2 and plated approximately 24 h before transfection at a density of 5 x 10^4 cells in 60-mm diameter plastic dishes.

DNA Transfection and CAT Assay—RB expression vector phuRb or pUC19 plasmid with Ren-IC promoter-CAT hybrid genes were transiently cotransfected into cultured cells as described previously (14, 15). Media were replaced with fresh media 24 h after transfection, cells were harvested 48 h after transfection, and aliquots of cell extracts containing equal amounts of total protein (40 μg) were used for CAT assay. CAT assay was performed as described previously (14, 15), and results were normalized on the basis of protein concentration or β-galactosidase activity to correct for differences in transfection efficiency. The conversion ratios of [14C]chloramphenicol were measured with an image analysis system (BAS2000, Fujix, Tokyo, Japan). All experiments were performed at least four times for each construct.

Preparation of Nuclear Extracts—Nuclear extracts from HEK cells were prepared using a modification of the protocol of Dignam et al. (14, 45). The final protein concentration was 5-7 mg/ml. In some instances, cultured cells were transfected with the RB expression vector phuRb 36 h before the preparation of nuclear extracts.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay (EMSA) was performed essentially as described previously (14, 44-46). Briefly, nuclear extracts were preincubated for 15 min on ice in 20-μl reaction mixtures containing 12 μM HEPS, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 12% glycerol, and 500 ng of double-stranded poly(dI-dC). Nonlabeled competitor was included in some of the binding reactions as indicated. The synthetic double-stranded RP-2 element (5'-CCCTGGGTAAAGCAGAGCCT -3'-75 to -47) and the double-stranded DNA fragment of RU-1 element (-224 to -138) were phosphorylated on its 5'-ends by T4 kinase and [γ-32P]ATP, and used as the probes. Aliquots of 0.1 to 0.4 ng (approximately 15,000 cpm) of the probe were added and incubation was continued for 30 min at room temperature. The incubation mixture was loaded on to a 4% polyacrylamide gel in a buffer containing 50 mM Tris-HCl, pH 8.3, 192 mM glycine, and 1 mM EDTA, and electrophoresed at 140 V for 3 h followed by autoradiography. Radioactivity of shifted bands on the gel was quantified using a BAS2000 FUJIX Bio-Imaging Analyzer (Fujix Photo Film). Double-stranded synthetic DNAs containing the consensus binding sequences for Sp-1 were obtained from Stratagene (GELSHIF KIT, La Jolla, CA). Oligonucleotides for the RU-1 element, m(RP-2) element, Pit-1/ GH-1 (47), and CREB/ATF (48) were synthesized on a MilliGen/Bioresearch Cyclone Plus oligonucleotide synthesizer, and purified on OPC columns (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

DNase I Footprinting Analysis—DNase I footprinting was performed essentially as described previously (44, 45). Briefly, the Ren-IC promoter fragment from -114 to +16 relative to the transcriptional start site was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP, followed by digestion with Ddel (-18) to generate a probe. After gel purification, the probe (approximately 15,000 cpm) was incubated with nuclear extracts in 50-μl reaction mixtures containing 12 mM HEPS, pH 7.9, 60 mM KCl, 4 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1 μg of double-stranded poly(dI-dC). The mixtures were incubated for 30 min on ice, followed by 1 min at room temperature with addition of 50 μl of a solution containing 12 mM HEPS, pH 7.9, 5 mM CaCl2, 5 mM MgCl2, and 5-250 ng of DNase I. The reaction was stopped by addition of 100 μl of 12 mM HEPES, pH 7.9, 0.6 mM sodium acetate, pH 7, 0.5% SDS, 0.1 mM EDTA, and 20 μg of tRNA. The DNA was extracted with phenol/chloroform (1:1, v/v) and precipitated with 2.5 volumes of ethanol prior to electrophoresis on a 6% polyacrylamide, 8 M urea sequencing gel. To define the position of the protected region, G + A sequence ladders were prepared (49).

RESULTS

Cell Type-specific and Ren-IC Promoter-dependent Activation by RB—Cotransfection of phuRb activated CAT expression directed by the Ren-IC promoter from -365 to +16 (R365CAT) in HEK cells (Fig. 1). In contrast, this promoter region was not able to mediate the activation by RB in HeLa, T98G, and NIH
The importance of the RU-1 and RP-2 elements in the Ren-1C promoter activity, for RB-induced Promoter Activation—

To assess the functional role of the RU-1 and RP-2 elements in directing RB-induced CAT expression, the Ren-1C promoter was cotransfected with phuRb DNA in HEK cells. The amount of transfected DNA was normalized by pUC19. CAT assay was performed as described under “Experimental Procedures.”

3T3 cells. This inactivity in nonrenal cells was not caused by a lower transfection efficiency, since expression directed by pUCSV3CAT was similar in HEK and these nonrenal cells.

Then, HEK cells were cotransfected with R365CAT and phuRb DNA or pJ3plasmid (Fig. 2). The level of CAT activity was proportional to the amount of cotransfected phuRb DNA in the range of 3 to 6 μg of DNA. In contrast, cotransfection of phuRb did not have any significant effect on CAT expression directed by the TK promoter (TK-CAT).

Effects of RB on the Expression of Renin Promoter-CAT Fusion Genes in HEK Cells—Next, to define DNA sequences responsible for transcriptional activation of the Ren-1C promoter by RB in HEK cells, a series of 5′-deletion mutants extending from −365 to −47 were constructed and tested for their ability to promote transcription. HEK cells were cotransfected with a 5′-deletion construct and phuRb DNA or pJ3plasmid (Fig. 3).

R365CAT activated CAT expression to a significant level (3.2-fold increase) on cotransfection with phuRb, and only the 91-base pair Ren-1C promoter sequences (−75 to +16) were required to elicit the RB-induced expression of the CAT reporter gene in HEK cells (Fig. 3, R75CAT). Although the induction ratios of all mutants with deletion end points up to −75 (R75CAT) were relatively constant, deletion of the sequences from −75 to −48 (RP-2 element) abolished the induction of CAT activity by RB (R47CAT). These results indicated that the induction of CAT expression by RB was dependent on Ren-1C proximal promoter sequences, and suggested that the RP-2 element from −75 to −47 was important for RB-induced Ren-1C gene promoter activity.

Identification of the RP-2 Element (−75 to −47) Responsible for RB-Induced Promoter Activation—To assess the functional importance of the RU-1 and RP-2 elements in Ren-1C promoter activation by RB, we first fused these elements with or without the other element in 5′ to 3′ orientation to the Ren-1C promoter (Fig. 4A). A construct with a combination of the RU-1 and RP-2 elements ([RU-1/RP-2]R47CAT) efficiently activated the Ren-1C promoter on cotransfection with phuRb (3.9-fold).

The RP-2 element alone ([RP-2]R47CAT) also supported RB-inducible activation of Ren-1C promoter (4.3-fold), although the relative CAT activity of [RP-2]R47CAT in the presence or absence of RB was significantly lower than that of [RU-1/RP-2]R47CAT. In contrast, [RU-1]R47CAT, which contained the RU-1 element alone, as well as the control R47CAT, was not able to confer RB inducibility.

To further establish the functional roles of the RU-1 and RP-2 elements in directing RB-induced CAT expression, the RU-1 and/or RP-2 elements were linked upstream of a TK promoter-CAT hybrid gene. As shown in Fig. 4B, [RU-1/RP-2]TK-CAT elicited RB-induced expression of the CAT-reporter gene (3.2-fold activation). In addition, the RP-2 element alone mediated transcriptional activation by RB ([RP-2]TK-CAT, 2.5-fold activation). In contrast, the RU-1 element alone ([RU-1]TK-CAT) did not confer the RB inducibility although this element activated the TK promoter in an RB-independent manner. These results suggested that the RU-1 element functioned as a constitutive activator of TK promoter, and that the RP-2 element was necessary not only for basal Ren-1C promoter activity but also for RB-mediated activation of Ren-1C and TK promoters.

Effects of RB on Nuclear Factors Binding to RP-2 Element—The above results suggested that the RP-2 element was involved in RB-mediated activation of CAT expression directed by the Ren-1C promoter. We previously demonstrated that HEK cell-dominant nuclear factors bound to the RP-2 element
by EMSA (14). To examine the effects of RB on binding of nuclear factors to this element, we first performed DNase I footprint analysis (Fig. 5). A labeled DNA fragment from −114 to −19 was incubated with HEK cell nuclear extracts following DNase I digestion. The results shown in Fig. 5A indicated that a sequence from −68 to −55 in the RP-2 element overlapping the TATA-like region (TAAATAA; −67 to −61, Fig. 5B) was protected from digestion by DNase I (lanes 2−5, denoted by the hatched box, Fig. 5A). The pattern of DNase I footprinting disclosed increased protection on cotransfection with phuRb (lanes 8−11, Fig. 5A).

We next carried out EMSA using the RP-2 element as a probe (Fig. 6A). Incubation of HEK cell nuclear extracts with this element produced a single shifted band, and cotransfection with phuRb tended to increase the intensity of the shifted band derived from nuclear factors binding to the RP-2 element. The shifted band was specifically competed out by the unlabeled RP-2 element and by a DNA fragment including the promoter region from −75 to +16, but not by a promoter fragment from −47 to +16 (lanes 1−3, and lanes 9 and 10, Fig. 6A). Previous studies showed that the human renin gene promoter basal and cAMP-stimulated activities in gonadotropes and chorionic cells were dependent upon the presence of the putative-specific trans-acting factor Pit-1/GH-1-binding site (−79 to −64) bearing partial sequence similarity to the RP-2 element (50−52). In addition, transcription factors Sp-1 and CREB/ATF have been reported to mediate transcriptional activation by RB (27, 28).

Thus, it is possible that a Sp-1, CREB/ATF, or Pit-1 family transcription factors bind to the RP-2 element. However, double-stranded synthetic DNAs containing the consensus binding sites for Sp-1, CREB/ATF, or Pit-1/GH-1 did not compete efficiently with the RP-2 element binding activity (lanes 6−8, Fig. 6A).

We performed EMSA using the RU-1 element as a probe to show binding of phuRb (−) and phuRb (+) extracts to the RU-1 element as a control for extract quality (Fig. 6B). The intensity of the shifted bands derived from nuclear factors binding to the RU-1 element did not show any significant change by cotransfection with phuRb (lanes 2 and 3, Fig. 6B). Therefore, with the result of EMSA using the RP-2 element as the probe, the overexpression of RB is considered to enhance specifically the binding of nuclear factors to the RP-2 element.

Functional Importance of RP-2 Element in RB-mediated Activation—From the above results, the RP-2 element seems to exert a critical influence on RB-mediated promoter activity of the Ren-1C gene in HEK cells. Thus, to evaluate the functional significance of the RP-2 element in RB-mediated Ren-1C promoter activity, we first assayed the effects of a mutation that disrupted binding of nuclear factors to this element. Although the DNA-protein complex formed by the RP-2 element binding activity could be competed out by non-labeled RP-2 element, the m(RP-2) element, that contained substitution mutations interrupting the TATA-like region protected on DNase I footprinting analysis at positions −68 to −55, did not compete out this binding (lanes 2−4, Fig. 6A). In transiently transfected HEK cells, Ren-1C promoter-CAT hybrid gene with this mutated RP-2 element (R365 m(RP-2)-CAT) showed a significant decrease in RB-mediated promoter activity (Fig. 7). We next performed in vivo competition experiments to confirm the functional role of the RP-2 element as an RB-mediated positive regulator. Six tandem copies of RP-2 or m(RP-2) elements were inserted into pUC19. These plasmids, named pC/RP-2 or pC/m(RP-2), were cotransfected with R365CAT into HEK cells, and the CAT activities were analyzed (Fig. 8). The amount of transfected DNA was normalized by pUC19 to 18 µg. The results obtained in this experiment showed that CAT activity decreased with increasing amounts of pC/RP-2, but not with pC/m(RP-2). Therefore, these functional assays further suggest that the RP-2 element overlapping the TATA-like region from −67 to −61 is important for RB-mediated activation of Ren-1C promoter in HEK cells.

**DISCUSSION**

The regulation of renin gene transcription is achieved via the interplay of various signaling stimuli and trans-acting nuclear factors (5−7). All of the mouse renin genes (Ren-1C, Ren-1D, and Ren-2 genes) are expressed abundantly and equivalently in the kidney, and 5’-flanking regions of these genes are highly homologous to around 79 nucleotides upstream from the transcriptional start site (18, 19). We supposed that the highly homologous 5’-flanking regions were involved in the basal and stimulated transcriptional activity of these renin genes. Thus, in this and previous studies we have focused on the promoter region from −365 to +16 of the Ren-1C gene (14, 15, 45). We previously demonstrated that the Ren-1C promoter region from −365 to +16 mediated HEK cell-dominant transcriptional activity and that combination of the RU-1 (−224 to −138) and RP-2 (−75 to −47) elements efficiently induced transcription from the Ren-1C promoter (14). In this study, we showed that the promoter region from −365 to +16 (R365CAT) directed the activation of CAT expression by RB, and indicated that the proximal promoter region from −75 to +16 (R75CAT) was sufficient to confer this inducibility by RB.

The functional role of the RU-1 element in RB-induced
Ren-1C promoter activity was similar to that in basal transcriptional activity. We previously showed that the RU-1 element efficiently directed transcription of Ren-1C promoter only in combination with the RP-2 element (14, 45). Our results in this study showed that the RU-1 element alone did not confer RB responsiveness on Ren-1C and TK promoters, and suggested that the RU-1 element is a constitutive activator-like region. On the other hand, the RP-2 element seemed to be essential for RB-mediated induction. We performed DNase I footprint analysis to identify the nature of the DNA binding activities which occurred at the RP-2 element. We demonstrated that nuclear factors in HEK cell nuclear extracts bound to the region from −268 to −255 in the RP-2 element, overlapping the TATA-like region. The activation of gene transcription in response to stimuli usually involves the induction of nuclear binding factors. DNase I footprinting in this study disclosed increased protection in the RP-2 element by RB, and EMSA showed that the binding activities of nuclear factors to the RP-2 element were increased by RB. The results of deletion analyses and heterologous promoter assay indicated that the RP-2 element was able to confer RB inducibility, and site-directed mutation that disrupted nuclear binding activity to the RP-2 element greatly decreased this RB-mediated activation of CAT expression. Furthermore, in vivo competition of RB binding in transfected HEK cells markedly decreased RB-mediated CAT expression directed by the Ren-1C promoter. These results suggest that the RP-2 element is responsible for RB-induced promoter activity of Ren-1C gene in HEK cells.

Previous studies showed that RB stimulated promoter activity through CREB/ATF and Sp-1 binding sites (27, 28), and increased transcription of the phosphoenolpyruvate carboxykinase gene through an as yet undefined site (53). The RP-2 element has no apparent homology with CREB/ATF or Sp-1-binding sites. The CREB/ATF-binding site is known to confer
cAMP-responsive transcriptional activity. We and others previously demonstrated that the RP-2 element and upstream CREB/ATF sites were involved in cAMP-mediated transcription of the mouse and human renin genes (45, 54–56). However, the synthetic DNA containing the consensus binding site for CREB/ATF did not compete for RP-2 element binding, indicating that CREB/ATF family transcription factors did not participate in RB-mediated activation by the RP-2 element.

Studies have demonstrated that RB can either positively or negatively regulate expression of several genes through cis-acting elements in a cell type-dependent manner (57). At present the exact mechanism by which RB activates transcription of the renin gene through the RP-2 element is unclear. RB may be directly involved in the binding activity of RP-2 element-binding factor through repression of transcription of a repressor protein or a protein that inhibits DNA binding, or through a direct and positive interaction with transcription factors (58). For example, RB stimulates Sp-1-mediated transcription of the c-jun gene by liberating Sp-1 from a Sp-1-negative regulator that specifically inhibits Sp-1 binding to a c-jun Sp-1 site (59). Although the negative regulatory element has not been identified in the RP-2 element, CREB/ATF and negative regulatory element-binding protein bind to the far upstream 5′-flanking region of the mouse renin genes (Ren-1D, −619 to −597; Ren-2, −670 to −648) (54) and an interaction between these nuclear proteins is suggested to play a role in the tissue-specific regulation of renin gene transcription (55). Furthermore, RB may also be indirectly involved in the binding activity of RP-2 element-binding factor. The arrest of cell cycle progression in G1 in response to the overexpression of RB may result in a post-transcriptional modification increasing transcription factor binding to the RP-2 element.

Previous studies indicated binding of nuclear proteins from the pituitary cell line gonadocorticotrope to a region on the human renin promoter that is analogous to the RP-2 element, and suggested that transcription factors related to Pit-1/GH-1 could be responsible for binding to the RP-2-analogous element in the human renin gene (50–52, 60). Although originally identified as a Pit-1/GH-1-binding site, this sequence in the RP-2 element has only partial similarity to the Pit-1/GH-1 recognition sequence. In addition, Germain et al. (16) examined the cis-acting elements of the proximal promoter involved in cAMP-induced human renin gene transcription using renin-producing chorionic cell and kidney cortex cell nuclear extracts. They showed that tissue-specific factors distinct from Pit-1/GH-1 specifically bound the RP-2-analogous element in the human renin gene. Furthermore, Petrovic et al. (17) showed that the human Pit-1/GH-1 sequence did not form complexes with nuclear proteins from renal renin-expressing As4.1 cells nor was it able to compete with the nuclear binding activity in As4.1 cells to the RP-2 element. In this study, double-stranded synthetic DNA containing the consensus binding sites for Pit-1/GH-1 did not compete with the RP-2 element binding activity in HEK cells, supporting the suggestion that the RP-2 element-binding factor is distinct from the Pit-1/GH-1 family transcription factors. Although this factor may still belong to the POU homeodomain class family, its precise identity of the factor is unknown at present.
The data presented here demonstrated that RB activates the Ren-1C gene proximal promoter in HEK cells, and that this effect is mediated mainly via a proximal promoter element from −75 to −47 (RP-2 element) overlapping the TATA-like region. Transcription factors distinct from the CREB/ATF or Pit-1/GH-1 family appear to mediate this RB response, and nuclear factors binding to the RP-2 element seem to be regulated by RB in the activation process. RB-mediated modulation of promoter activity may have a role in the tissue-specific and developmentally linked regulation of renin gene expression. Further studies are obviously warranted to investigate the molecular relationship between RB and regulation of renin gene transcription in response to various stimuli.

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