Journal of Cellular Biochemistry 93:327–336 (2004)

Candidate *cis*-Elements for Human *Renin* Gene Expression in the Promoter Region

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Abstract The regulation of *renin* gene expression, the rate-limiting enzyme of the system, is thought to be fundamental to the total system. Previously, we mapped six putative *cis*-elements in the promoter region of the human *renin* gene with nuclear proteins from human chorionic cells and human renal cortex by DNase I protection assay (footprint A–F). Each footprint contains Ets motif like site (A), HOXñPBX recognition sequence (B), unknown sequence as DNA binding consensus (C), CRE (D), COUP-TFII (ARP-1) motif like site (E), and AGE3 like site (F). Footprint D has been characterized by means of functional studies as the genuine human *renin* gene CRE interacting with CREB in cooperation with the site of footprint B. To obtain further clues to the specific expression in the promoter region, these putative *cis*-elements were conducted to a consensus-specific binding assay to compare renin-producing and non-renin-producing cells by EMSA and electromobility super-shift assay. Different sequence-specific DNA/protein binding was obtained among the different cell lines with footprint B site, with COUP-TFII (ARP-1) motif like site and possibly with footprint F site. The results implicate these putative *cis*-elements and each corresponding *trans*-factor in the specific expression of the human *renin* gene in the promoter region. Further functional characterization of these elements would provide important data for a better understanding of human *renin* gene expression. J. Cell. Biochem. 93: 327–336, 2004.

Key words: renin; renin-angiotensin system; Ets; HOX; CRE; CREB; COUP-TFII

The renin-angiotensin system plays major roles in blood pressure regulation and electrolyte metabolism [Corvol et al., 1997]. The rate-limiting step of the system is the conversion of angiotensinogen to angiotensin I catalyzed by renin. Thereby, the regulation of *renin* gene expression is thought to be fundamental to the total system. Several human cell lines have been used as models of renin-producing cells, one being human chorionic cell [Pinet et al., 1988], as there is no established human JG cell line despite the existence of the established mouse cell line As4.1 [Sigmund et al., 1990]. Recently, Germain et al. [1998] showed that 250-bp region located about 6 kb upstream from the transcription starting point of the human *renin* gene gave up to 57-fold higher transcription rates as a distal enhancer with human chorionic cells. Fuchs et al. [2002] showed that 12 kb of 5′ region of human *renin* gene is needed for tissue-specific and regulated human *renin* gene expression with human renin/LacZ construct for *trans*-gene experiment. These data indicate the importance of the distal enhancers for tissue-specific and regulated human *renin* gene expression. On the other hand, it is well known that proximal promoter regions of genes are indispensable for the regulation of the basic portion of gene expression. Recently, proximal promoters of mouse *renin* gene have been well investigated [Petrovic et al., 1996; Pan et al.,

Abbreviations used: Pit-1, pituitary-specific *trans*-acting factor; CRE, cAMP response element; CREB, CRE binding protein; CREM, CRE modulato1; ATF-1, activating transcription factor-1; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; ARP-1, apoAI regulatory protein-1; AGE3, angiotensinogen gene-activating element 3; EMSA, electromobility shift assay.

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Received 16 January 2004; Accepted 31 March 2004

DOI 10.1002/jcb.20151

2001a,b], whereas human proximal promoters remain to be elucidated. Previously, we mapped six protein-binding sites in the proximal promoter region of the human *renin* gene (−336 to +16) by DNase I footprint assay with nuclear extract of human chorionic cell and ischemic human renal cortex in common [Borensztein et al., 1994; Germain et al., 1996; Konoshita et al., 1996]. Human renal cortex does not consist of homogeneous cells, but, at least, the presence of nuclear factors reacting to human renin promoter has been proven with this method. Referring to consensus sequences to known transcription factors, some of these footprints designated from A to F were revealed to contain putative *cis*-elements; Ets motif like site (A: −29/−6) [Wasylyk et al., 1993], CRE (D: −234/−214) [Montminy et al., 1986], COUP-TFII (ARP-1) motif like site (E: −259/−245) [Ladias and Karathanasis, 1991]. The candidate human renin CRE was proven to be genuine CRE [Borensztein et al., 1994; Smith et al., 1994]. At first, footprint B (−79/−62) was regarded to contain Pit-1 [Nelson et al., 1988] motif like sequence [Borensztein et al., 1994; Catanzaro et al., 1994]. We demonstrated that the CRE and footprint B are implicated in the regulation of human *renin* gene transcription by cAMP, but, at the same time demonstrated that the protein from chorionic cell binding to this region is distinct from Pit-1 [Germain et al., 1996]. A recent report indicates that this corresponding region of mouse *Ren-1^c* is HOXñPBX recognition sequence although whether HOX and PBX proteins bind to the human proximal promoter element has not been examined [Pan et al., 2001b]. Footprint C (−116 to −80) contains no known consensus sequence. Footprint F (F: −293/−272) contains AGE3 like site but the corresponding factor has not been identified [Tamura et al., 1994].

In the present study, to obtain further clues to the mechanism of human *renin* gene expression in the promoter region, these putative *cis*-elements were conducted to consensus-specific binding assay with the nuclear protein from renal cortex and chorionic cells as renin-producing cells and from the human choriocarcinoma cell line JEG3 and human hepatoblastoma cell line HepG2 as non-renin-producing cells for comparison. Almost the same DNA/protein complexes were obtained with Ets motif like site, footprint C site, and CRE, whereas different DNA/protein complexes were obtained with footprint B, with COUP-TFII (ARP-1) motif like site and possibly with AGE3 like site among the different cell lines. These results implicate several putative *cis*-elements and each corresponding *trans*-factor in the human *renin* gene expression in the proximal promoter region.

METHODS

Preparation of Nuclear Extract From Human Renal Cortex, Chorionic Cells, JEG3, HepG2, and of Ets-1 or COUP-TFII (ARP-1) Containing Cellular Extract From COS-1 Cells and Purified CREB Protein

Nuclear extracts were prepared from human renal cortex and human chorionic cells as renin producing cell and from human choriocarcinoma cell line (JEG3) and human hepatoblastoma cell line (HepG3) as non-renin producing cells.

Nuclear extracts from renal cortex were prepared essentially according to Gorski's method [Gorski et al., 1986] with minor modifications from ischemic kidneys removed because of renovascular hypertension. All manipulations were performed in the cold. Minced kidney (10−20 g) was brought up to 30 ml with homogenization buffer [10 mM Hepes (pH 7.6), 15 mM KCl, 2 mM EDTA, 2.4 M sucrose, 0.5 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM PMSF, 2 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin] and was homogenized using a motor-driven 30 ml Teflon-glass homogenizer. The homogenate was filtered through four layers of gauze, diluted 85 ml with homogenization buffer, layered in three 27 ml aliquots over three 10 ml cushion solutions (2 M sucrose, 10% bidistilled glycerol, 0.5 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM PMSF, 2 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin), and centrifuged at 24,000 rpm for 30 min at 0°C in an SW27 rotor. The combined nuclear pellets were resuspended in 50 ml of a 9: 1 (v/v) mixture of homogenization buffer and H_2O, again using a Teflon-glass homogenizer. This homogenate was layered over two 10 ml cushion solutions and recentrifuged under the same conditions. The pelleted nuclei were resuspended in 20 ml of nuclear lysis buffer [20 mM Hepes (pH 7.9), 0.2 mM EDTA, 2 mM EGTA, 0.75 mM Spermidine, 0.15 mM Spermine, and 2 mM DTT]. One-tenth volume of 3.9 M $(NH_4)_2SO_4$ was added drop wise, and the extract was gently shaken for 40 min at 4°C. The lysate was then centrifuged

at 40,000 rpm for 30 min in a Ty 65 rotor (4°C) to pellet chromatin. Solid $(NH_4)_2SO_4$ was added to the supernatant and was slowly dissolved and gently shaken for 30 min at 4°C. The precipitated proteins were sedimented by 30 min centrifugation at 40,000 rpm in a Ty 65 rotor. The protein pellet was resuspended in dialysis buffer [25 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, and 5% glycerol] and dialyzed twice for 2 h each time against 500 ml of the same buffer. The protein concentration was determined according to Lowry's method and then, the protein was stored in small aliquots in liquid nitrogen.

Nuclear extracts of human chorionic cells, JEG3 and HepG2 were prepared essentially according to Shapiro's method [Shapiro et al., 1988] with minor modifications as previously described [Borensztein et al., 1994].

Ets-1 or COUP-TFII (ARP-1) containing cellular extracts were prepared fundamentally with Kumar's method [Kumar and Chambon, 1988] with minor modifications. COS-1 cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fifty to 60% confluent dishes were transfected with an appropriate quantity of Ets [Wasylyk et al., 1991] or COUP-TFII (ARP-1) [Ladias et al., 1992] cDNA containing expression vector. Forty hours after transfection, the cells were collected in 40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl, and pelleted by low speed centrifugation. Cells were resuspended in 400 μl of a buffer containing 20 mM Tris-HCl, pH 7.4, 0.4 M KCl, 2 mM DTT, and 10% glycerol, and broken by freezing and thawing. Debris was removed and the supernatant was aliquoted and stored at −70°C.

Purified CREB protein was provided by Dr. M. Montminy.

Electromobility Shift Assays (EMSA)

Table I summarizes the sequences of oligonucleotides for each footprint used for EMSA. Double-stranded oligonucleotides were synthesized (Applied Biosystems, Foster City, CA) and end-labeled with (γ-^{32}P) ATP and T4 polynucleotide kinase.

Binding reactions for Ets-REN, footprint B, footprint C, and CRE-REN were performed by incubating 1 μl Cos-1 cell extract (for Ets-REN) or 3 μg of each nuclear extract with 20 fmole (about 20,000 cpm) of end-labeled double-stranded oligonucleotides for 15 min at 4°C in 18 μl buffer containing 10 mM Hepes (pH 7.8), 1 mM Na_2HPO_4 (pH 7.2), 0.1 mM EDTA, 50 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 2.5% glycerol, 0.75–2 μg double-stranded poly (dI-dC) and 0–1 μg sonicated salmon sperm DNA. The specificity of the DNA/protein binding was determined by running the same binding reaction using different competitor oligonucleotides (homologous and heterologous, Table I). The bound and free elements were separated by

TABLE I. Alignment of Oligonucleotide Sequences Used in This Study

Footprint A	
Ets consensus	**GGAW GGAW**
Ets-REN	−34GAGTGTATAAAAGG**GGAA**GGGCTAAG**GGA**GCCACAG+2
Ets-M1	GAGTGTATAAAAGG***TTG***AGGGCTAAG**GGA**GCCACAG
Ets-M2	GAGTGTATAAAAGG**GGAA**GGGCTAAG***TTG***GCCACAG
Footprint B	
HOX·PBX consensus	**NNATAAATCAN**
Pit1 consensus	**ATGNATAAWT**
FP-B-REN	−88CAGGG**TAATAAATCAG**GGCAGAGCAGAATTGCAAT−54
FP-B-MUT	−88CAGGG***CGGC*AAATCAG**GGCAGAGCAGAATTGCAAT−54
Footprint C	
FP-C-REN	−116GAGATTTATTGCTGACTGCCCTGCCATCTACCCCAG−80
Footprint D	
CRE consensus	**TGACGTCA**
CRE-REN	−235GAGGGCTGC**T**AG**CGTCA**CTGGACACAAGATTGCTTT−199
CRE-MUT	−235GAGGGCTGC**T**AG***TCGGA***CTGGACACAAGATTGCTTT−199
CRE-SOM	−68CTGGGGGCGCCTCCTTGGC**TGACGTCA**GAGAGAGAG−32
Footprint E	
COUP-TFII consensus	**AGGGGTCANAGGGNTCA**
COUP-TFII-REN	−264GCTCC**AGGGGTCACAGGGC**CAAGCCAGATAGAGGGC−228
COUP-TFII-MUT	GCTCCAGGG***CAGGGGTCCC*C*GGGTT***CAGATAGAGGGC
Footprint F	
AGE3 consensus	**AGCTGTGCTTGT**
FP-F-REN	−299CCCTG**AGC**A**GTGCT**GTTTCTCATCAGCCTCTGC−266
FP-F-MUT	CCCTG***GTTGA*TGC*G***GTTTCTCATCAGCCTCTGC

Each consensus sequence is indicated in bold letters above the oligonucleotides. Bold letters in oligonucleotides indicate homologies with each consensus.

electrophoresis in 6% non-denaturing polyacrylamide gels run in 22 mM Tris/borate/0.5 mM EDTA at 250 V for 2–3 h.

Binding reactions for COUP-TFII-REN were performed according to Ladias et al. [1992] incubating 60 nl COUP-TFII-REN (ARP-1) transfected Cos-1 extract or 3 μg of each nuclear extract. These were incubated with 20 fmole (about 20,000 cpm) labeled oligonucleotides for 30 min at 4°C in 20 μl of buffer containing 25 mM Hepes (pH 7.6), 8% Ficoll 400, 40 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 1 μg of double-stranded poly (dI-dC) with or without competitor oligonucleotides (Table I). The components were separated by electrophoresis in 4% non-denaturing polyacrylamide gels run in 44 mM Tris/borate 1 mM EDTA at 10 V/cm for 2–3 h.

Binding reactions for footprint F were performed according to Tamura et al. [1993] incubating 3 μg of each nuclear extract with 20 fmole (about 20,000 cpm) labeled oligonucleotide for 30 min at room temperature in 20 μl buffer containing 12 mM Hepes (pH 7.9), 60 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 12% glycerol, and 0.5 μg of double-stranded poly (dI-dC) with or without competitor oligonucleotides (Table I). The components were separated by electrophoresis in 5% non-denaturing polyacrylamide gels run in 88 mM Tris/borate 2 mM EDTA at 140 V for 2–3 h.

Electromobility Super-Shift Assay

The electromobility super-shift assay for CRE-REN consensus was performed as previously described [Germain et al., 1996]. One microliter of anti-sera against CREB, CREM, and ATF-1 kindly provided by Dr. M. Montminy [Hagiwara et al., 1992] was added to the EMSA reaction mixture and then incubated for 1 h at 4°C before electrophoresis under the same conditions as described above for EMSA.

RESULTS

Ets-1 Transfected Cos-1 Cell Extracts and Nuclear Extracts From Different Cellular Origins and Ets Motif Like Site

The binding of Ets-1 transfected Cos-1 cell extracts to the Ets motif like site of the human *renin* gene promoter was examined by EMSA using double-stranded oligonucleotide Ets-REN (−34 to +2) (Fig. 1). Three specific high-affinity DNA/protein complexes were formed which could be blocked by 100-fold excess of homologous competitor but not by 100-fold excess of consensus mutated competitor (Ets-M1, Ets-M2). With each of the nuclear extracts from four different cellular origins, the same consensus-specific DNA/protein complexes were observed. There was no difference in the existence or consensus-specificity of the complexes between renin producing cells and non-renin producing cells.

Nuclear Extracts From Different Cellular Origins and Footprint B Site

The binding of each nuclear extract from four different cellular origins to footprint B of the human *renin* gene promoter was examined by EMSA using double-stranded oligonucleotide

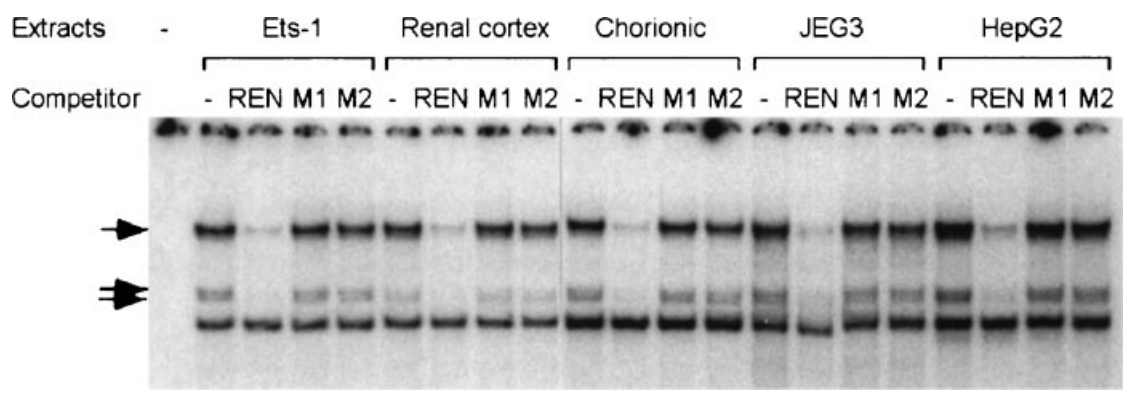

Fig. 1. Electromobility shift assay (EMSA) of Ets-1-transfected Cos-1 extracts and nuclear extracts from different cellular origins with the Ets motif like site of the human *renin* gene promoter (−34 to +2). A double-stranded labeled oligonucleotide containing the renin Ets protein recognition site like region (Ets-REN: −34GAGTGTATAAAAGGGGAAGGGCTAAGGGAGCCACAG +2) was used as the probe. Competition experiments were performed with a 100-fold molar excess with homologous DNA (Ets-REN) or with mutated oligonucleotides (Ets-M1: −34GAGTGTATAAAAGGTTGAGGGCTAAGGGAGCCACAG+2, Ets-M2: −34GAGTGTATAAAAGGGGAAGGGCTAAGTTGGCCACAG +2). Specific DNA/protein complexes are indicated by arrows.

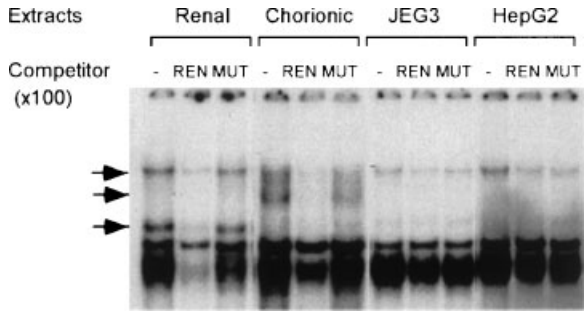

Fig. 2. EMSA of nuclear extracts from different cellular origins with the footprint B site of the human *renin* gene promoter (−88 to −54). A double-stranded labeled oligonucleotide containing the footprint B (FP-B-REN: −88CAGGGTAATAAATCAGGGCAGAGCAGAATTGCAAT−54) was used as the probe. Competition experiments were performed with a 100-fold molar excess with homologous DNA (FP-B-REN) or with mutated oligonucleotides (FP-B-MUT: −88CAGGGCGGCAAATCAGGGCAGAGCAGAATTGCAAT−54). Specific DNA/protein complexes are indicated by arrows.

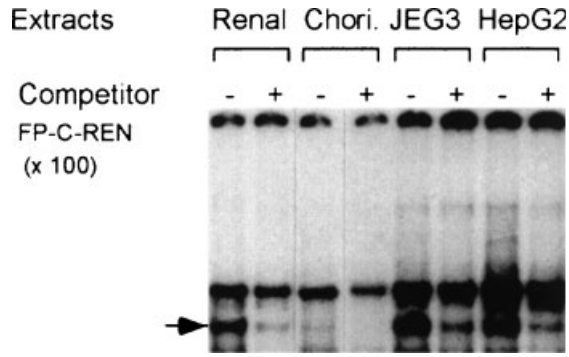

Fig. 3. EMSA of nuclear extracts from different cellular origins with the footprint C site of the human *renin* gene promoter (−116 to −80). A double-stranded labeled oligonucleotide containing the footprint C (FP-C: −116GAGATTTATTGCTGACTGCCCTGCCATCTACCCCAG−80) was used as the probe. Competition experiments were performed with a 100-fold molar excess with homologous DNA (FP-C). Specific DNA/protein complexes are indicated by an arrow.

FP-B (−88 to −54) (Fig. 2). With renal cortex nuclear extracts, two specific high-affinity DNA/protein complexes were formed which could be blocked by 100-fold excess of homologous competitor (FP-B) but not by 100-fold excess of consensus mutated competitor (FP-B-MUT). With chorionic cell nuclear extract, two specific complexes were observed, one of which at a position distinct from that of renal cortex. With nuclear extracts from JEG3 and HepG2, only one specific complex was observed at the same position as the complexes with renal cortex and chorionic cells.

Nuclear Extracts From Different Cellular Origins and Footprint C Site

The binding of each nuclear extract from four different cellular origins to the Element C of the human *renin* gene promoter was examined by EMSA using double-stranded oligonucleotide FP-C (−116 to −80) (Fig. 3). One specific high-affinity DNA/protein complex was formed which could be blocked by 100-fold excess of homologous competitor (FP-C). There was no difference in the existence or specificity of the complexes between renin producing cells and non-renin producing cells.

Purified CREB Protein and Nuclear Extracts From Different Cellular Origins and CRE

The binding of purified CREB protein to the CRE of the human *renin* gene promoter was examined by EMSA using double-stranded oligonucleotide CRE-REN (−235 to −199) (Fig. 4). With purified CREB and human renin CRE oligonucleotide, two specific high-affinity DNA/protein complexes were formed which could be blocked by 100-fold excess of homologous competitor (CRE-REN) and rat somatostatin promoter containing CRE (CRE-SOM) but not by 100-fold excess of consensus mutated competitor. The purified CREB protein seemed to migrate a little faster than the complexes formed with nuclear proteins. Possibly, this phenomenon is attributable to modifications of proteins like glycosilation and phosphorylation. With each nuclear extract from four different cellular origins, almost the same consensus-specific DNA/protein complexes were observed (Fig. 4). There was no difference in the existence or consensus-specificity of the complexes between renin producing cells and non-renin producing cells. The two bands are thought to be a monomer and a dimer of CREB protein. There seemed to exist other bands with JEG3 and HepG2. It is not sure but possibly these are several homo-complexes or fragments of CREB protein.

Super-shift assays were performed to further characterize the protein binding to CRE of the human *renin* gene (Fig. 5). With purified CREB and human renin CRE oligonucleotide, two specific high-affinity DNA/protein could be supershifted with anti-CREB serum. With each nuclear extract from four different cellular origins, almost the same super-shift was observed without special differences. Experiments conducted with anti-sera directed against

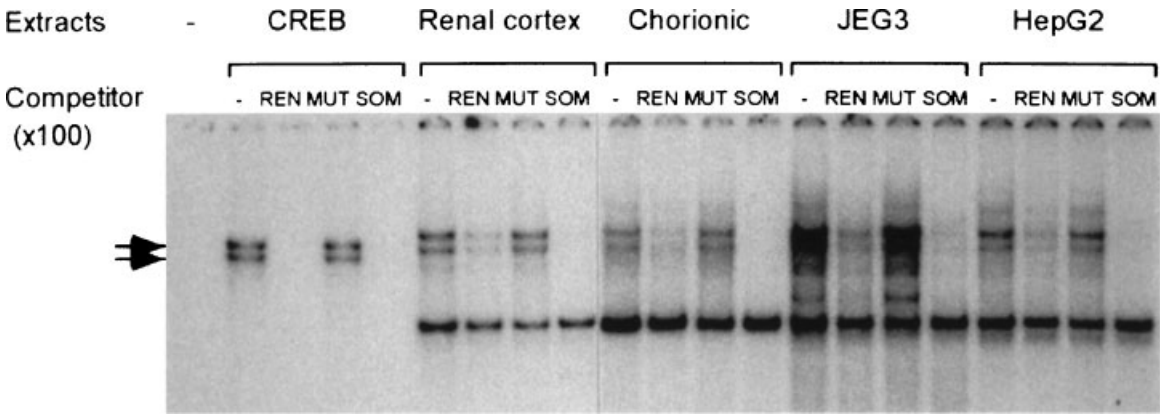

Fig. 4. EMSA of purified CREB protein and nuclear extracts from different cellular origins with the CRE of the human *renin* gene promoter (−235 to −199). A double-stranded labeled oligonucleotide containing the CRE (CRE-REN: −235GAGGGCTGCTAGCGTCACTGGACACAAGATTGCTTT−199) was used as the probe. Competition experiments of purified CREB protein provided by Dr. M. Montminy and nuclear extracts from various origins were performed with a 100-fold molar excess with homologous DNA (CRE-REN) or with somatostatin CRE site (CRE-SOM: −68CTGGGGGCGCCTCCTTGGCTGACGTCAGAG−32) or with mutated oligonucleotides (CRE-MUT: −235GAGGGCTGCTAGTCGGACTGGACACAAGATTGCTTT-199). Specific DNA/protein complexes are indicated by arrows.

CREM and ATF-1, which belong to the same family of bZIP transcription factors, showed no super-shift with purified CREB protein or with nuclear extracts from four different cellular origins.

COUP-TFII (ARP-1) Containing Cos-1 Cell Extracts and Nuclear Extracts From Different Cellular Origins and COUP-TFII (ARP-1) Motif Like Site

The binding of COUP-TFII (ARP-1) transfected Cos-1 cell extracts to the COUP-TFII (ARP-1)-1 motif like site of the human *renin* gene promoter was examined by EMSA using double-stranded oligonucleotide COUP-TFII-REN (−264 to −228) (Fig. 6). One specific high-affinity DNA/protein complex was formed which could be blocked by 100-fold excess of homologous competitor but not by 100-fold excess of consensus mutated and non-specific competitors (COUP-TFII-MUT, CRE-REN). With renal cortex nuclear extracts and chorionic cell nuclear extract, the same consensus-specific DNA/protein complexes were observed. With nuclear extracts from JEG3, DNA/protein complex was not formed at the same position as that with COUP-TFII (ARP-1) transfected Cos-1 cell extracts. With nuclear extracts from

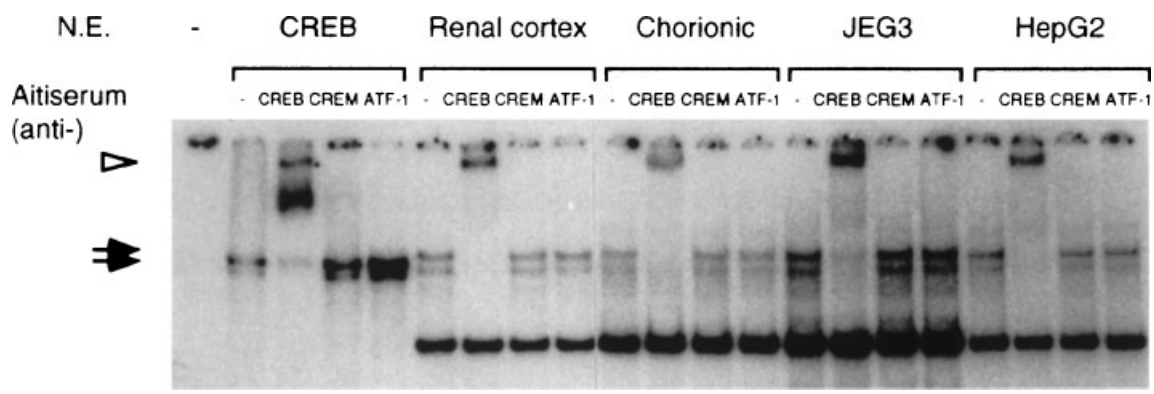

Fig. 5. Electromobility super-shift assay of purified CREB protein and nuclear extracts from different cellular origins with the CRE of the human *renin* gene promoter (−235 to −199). A double-stranded labeled oligonucleotide containing the CRE (CRE-REN:−235GAGGGCTGCTAGCGTCACTGGACACAAGATTGCTTT−199) was used as the probe. Super-shift assay of purified CREB protein provided by Dr. M. Montminy and nuclear extracts from various origins were performed with anti-CREB, anti-CREM, and anti-ATF-1 serum. The arrows indicate the specific DNA–protein complexes. The open arrowhead indicates the supershifted complex.

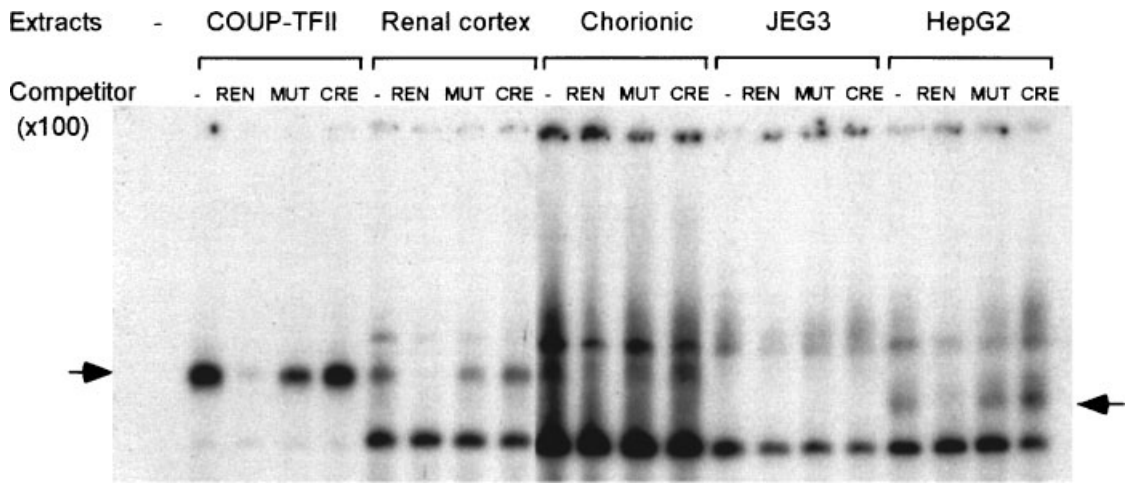

Fig. 6. EMSA of COUP-TFII (ARP-1)-transfected Cos-1 extracts and nuclear extracts from different cellular origins with the COUP-TFII (ARP-1) motif like site of the human *renin* gene promoter (−264 to −228). A double-stranded labeled oligonucleotide containing the renin COUP-TFII (ARP-1) recognition site like region (COUP-TFII-REN: −264GCTCCAGGGGTCACAGGGCCAAGCCAGATAGAGGGC−228) was used as the probe. Competition experiments were performed with a 100-fold molar excess with homologous DNA (COUP-TFII-REN) or with mutated and non-specific oligonucleotides (COUP-TFII-MUT:−264GCTCCAGGGCAGGGGTCCCGGGTTCAGATAGAGGGC−228, CRE-MUT:−235GAGGGCTGCTAGCGTCACTGGACACAAGATTGCTTT−199) Specific DNA/protein complexes are indicated by arrows.

HepG2, DNA/protein complex was not formed at the same position as that with COUP-TFII (ARP-1) transfected Cos-1 cell extracts. However, consensus-specific DNA/protein complex, which migrated more rapidly, was observed.

Nuclear Extracts From Different Cellular Origins and Footprint F Site

The binding of each nuclear extract from four different cellular origins to the footprint F of the human *renin* gene promoter was examined by EMSA using double-stranded oligonucleotide FP-F-REN (−299 to −266) (Fig. 7). With renal cortex nuclear extracts, one specific DNA/protein complex was formed which could be blocked by 100-fold excess of homologous competitor (FP-F-REN) and slightly blocked by 100-fold excess of consensus mutated competitors (FP-F-MUT), the complex of chorionic cell nuclear extract being invisible. With nuclear extracts from JEG3 and HepG2, one specific complex was observed which seemed to migrate more slowly, although this was not sure.

DISCUSSION

Recently, new knowledge about the distal enhancers of human *renin* gene has been

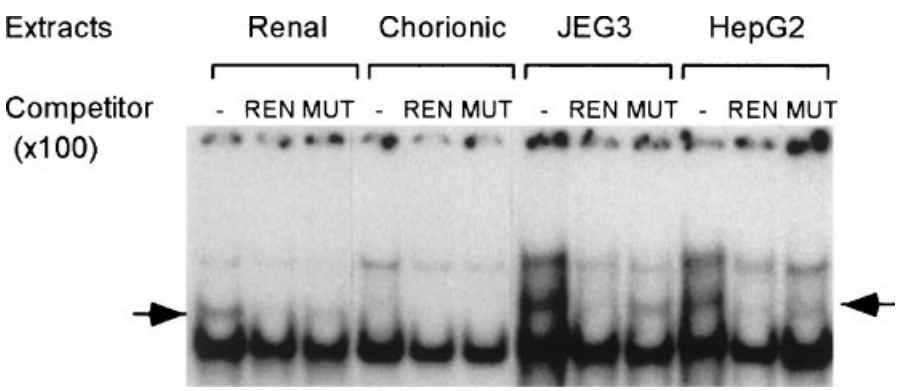

Fig. 7. EMSA of nuclear extracts from different cellular origins with the footprint F site of the human *renin* gene promoter (−299 to −266). A double-stranded labeled oligonucleotide containing the AGE3 like region of the human *renin* gene (FP-F-REN: −299CCCTGAGCAGTGCTGTTTCTCATCAGCCTCTGC−266) was used as the probe. Competition experiments were performed with a 100-fold molar excess with homologous DNA (AGE3-REN) or with mutated oligonucleotides (FP-F-MUT:−299-CCCTGGTTGATGCGGTTTCTCATCAGCCTCTGC−266). Specific DNA/protein complexes are indicated by an arrow.

obtained [Germain et al., 1998; Fuchs et al., 2002]. On the other hand, it is well known that proximal promoter regions of genes are indispensable for the regulation of the basic portion of gene expression and the proximal region of human *renin* gene should be well characterized. Previously, we mapped six protein-binding sites in the proximal promoter region of the human *renin* gene (−336 to +16) by DNase I footprint assay with nuclear extract of human chorionic cell and human renal cortex [Borensztein et al., 1994; Konoshita et al., 1996] in common (footprint A–F). Footprint D (−234/−214) site contains CRE like sequence and was characterized as CRE of human *renin* gene in human chorionic cells [Borensztein et al., 1994; Smith et al., 1994]. Footprint B (−79/−62) site was demonstrated to be implicated in the cAMP mediated *renin* gene transcription with CRE synergistically in chorionic cells [Germain et al., 1996]. In the evaluation with renal cortex, we demonstrated that all other sites of the footprints interact with nuclear protein in a sequence-specific manner; footprint A (−29/−6), footprint E (−259/−245) seemed to be Ets binding site and COUP-TFII (ARP-1) binding site, respectively. Footprint C (−107/−83) contains no known consensus as *cis*-element. Footprint F (−293/−272) contains AGE3 like sequence of which the corresponding factor (AGF3) has not been identified [Konoshita et al., 1996]. However, the specific bindings between chorionic cell nuclear protein and non-renin producing cells and these footprint sites of human *renin* gene have not been examined. Accordingly, this study was undertaken to obtain further clues to the regulation of human *renin* gene expression in the promoter region. For this sake, these putative proximal *cis*-elements were subjected to consensus-specific binding analysis, and the pattern of DNA/protein binding was compared between renin-producing and non-renin producing cells. Because there is no established human JG cell line, renal cortex cells from ischemic kidneys were applied to the experiments despite the limited number of JG cells in cortex (less than 0.01% of renal cortex cells) and the heterogeneous constitution, at least, to confirm the presence of reacting proteins to the human renin promoter.

Ets is one of the proto-oncogenes that contain Ets domain. It is thought to be involved in constitutive transcription [Sato, 2001]. Our results suggest that the nuclear protein binding to Ets binding motif like site of the human *renin* gene (footprint A) is Ets-1 itself. It is unlikely that the site is implicated in the specific *renin* gene expression as no apparent distinct DNA/protein binding was observed among cell lines. Ets specific sequences are not found in rodents' *renin* gene, but the corresponding site of mouse *Ren-1^c* reacts with As4.1 [Petrovic et al., 1996]. Further evaluation should provide data on this issue. As a previous reporter assay did not indicate special activity in footprint C site [Borensztein et al., 1994], and no apparent distinct DNA/protein binding was observed among cell lines in the present study, it is unlikely that the site is implicated in the specific *renin* gene expression. CREB is one member of the CRE/ATF family and contains b-ZIP structure [Hai and Hartman, 2001]. We have already indicated that the CRE like site of the human *renin* gene (footprint D) functions as genuine CRE [Borensztein et al., 1994; Germain et al., 1996]. CRE is thought to be involved in inducible gene expression. However, it is unlikely that the site is implicated in the specific *renin* gene expression, as no apparent distinct DNA/protein binding was observed among different cell lines in the present study. Our investigations showed two specific DNA/protein complexes consisting of CREB but not of ATF-1, whereas with Calu-6 cells, which are one type of human renin producing cell, five specific DNA/protein complexes consisting of ATF-1 and CREB-1 have been demonstrated [Ying et al., 1997].

Initially, footprint B (−79/−62) was regarded to contain Pit-1 [Nelson et al., 1988] motif like sequence [Sun et al., 1993; Borensztein et al., 1994; Catanzaro et al., 1994]. Pit-1 is one of the POU-domain transcription factors [Andersen and Rosenfeld, 2001]. POU family proteins are thought to be implicated in differentiation and tissue-specific gene expression. The footprint B site of the human *renin* gene and corresponding site of the mouse *Ren-1^c* have been regarded as important sites for gene expression [Tamura et al., 1992; Germain et al., 1996; Petrovic et al., 1996]. However, we demonstrated that Pit-1 containing GH_3B6 nuclear extracts did not interact with the human *renin* gene and that the binding protein of chorionic cell is distinct from Pit1 itself [Germain et al., 1996]. It has been suggested that members of the POU family of transcription factors, or some other closely related group such as the Hox proteins, parti-

cipate in directing *renin* gene expression to renin-producing cells [Catanzaro et al., 1994]. A recent report indicated that this corresponding region of mouse *Ren-1^c* is HOXñPBX recognition sequence, although whether HOX and PBX proteins bind to the human proximal promoter element has not been examined [Pan et al., 2001b]. *Hox* genes are members of the homeobox family of transcription factors and control many aspects of morphogenesis and cell differentiation in animals [Botas, 1993]. In vertebrates, there are 39 *Hox* genes organized in four clusters (A, B, C, and D) on separate chromosomes, with members of each cluster classified into as many as 13 paralog groups based on sequence similarity [Scott, 1993]. In the present study, apparent distinct DNA/protein binding was observed with the footprint B site of the human *renin* gene among different cell lines. These heterogeneity suggests a possibility that this site of the human *renin* gene is implicated in specific gene expression.

COUP-TFII (ARP-1) is one of the orphan receptors that are members of the nuclear receptor superfamily of ligand-activated transcription factors [Ladias et al., 1992]. In this study, the nuclear protein from kidney and chorionic cells which bind to *renin* gene (footprint E) seems to be COUP-TFII (ARP-1) itself. On the other hand, apparent distinct DNA/protein binding was observed with non-renin producing cell lines. Considering the central role that nuclear receptors play in differentiation, development, metabolic regulation, homeostasis, and disease [Mangelsdorf et al., 1995; Cooney et al., 2001], presumed crucial roles in angiogenesis indicated by knockout mice model [Pereira et al., 1999] and suggested roles in the regulation of steroidogenesis in human adrenal cortex and its disorders [Suzuki et al., 2000; Shibata et al., 2001], this site of the human *renin* gene may play an important role in the specific gene expression of the human renin.

Footprint F contains AGE3 like sequence. AGE3 is a sequence found in mouse *angiotensinogen* gene and is thought to be involved in constitutive gene regulation [Tamura et al., 1994]. In the present study, distinct DNA/protein binding seemed to be formed with the site of the human *renin* gene among cell lines. Accordingly, it is possible that the site of the human *renin* gene is involved in specific expression. However, the complex of chorionic cells was invisible by EMSA, and the *trans*-factor has not been identified so far. Thus, role of the element of the human *renin* gene remain unclear.

In conclusion, the results of the study implicate three putative *cis*-elements, footprint B site, COUP-TFII (ARP-1) motif like site, and footprint F site, and each corresponding *trans*-factor in gene expression of human renin in the proximal region. Further functional characterization of these elements would provide important data for a better understanding of human *renin* gene expression.

ACKNOWLEDGMENTS

We thank Dr. B. Wasylyk (INSERM U184) for Ets-1 cDNA containing expression vector, Dr. J. Ladias (Harvard Medical School, Boston, MA) for ARP-1 (COUP-TFII) cDNA containing expression vector, Dr. M. Montminy (The Salk Institute for Biological Studies, San Diego, CA) for the CREB, CREM, and ATF-1 antiserum and purified CREB protein. We are thankful to Mr. J.S. Gelblum for a critical reading of the article.

REFERENCES

Andersen B, Rosenfeld MG. 2001. POU domain factors in the neuroendocrine system: Lessons from developmental biology provide insights into human disease. Endocr Rev 22:2–35.

Borensztein P, Germain S, Fuchs S, Philippe J, Corvol P, Pinet F. 1994. *cis*-regulatory elements and *trans*-acting factors directing basal and cAMP-stimulated human *renin* gene expression in chorionic cells. Circ Res 74: 764–773.

Botas J. 1993. Control of morphogenesis and differentiation by *HOM/Hox* genes. Curr Opin Cell Biol 5:1015–1022.

Catanzaro DF, Sun J, Gilbert MT, Yan Y, Black T, Sigmund C, Gross KW. 1994. A Pit-1 binding site in the human *renin* gene promoter stimulates activity in pituitary, placental and juxtaglomerular cells. Kidney Int 46:1513–1515.

Cooney AJ, Lee CT, Lin SC, Tsai SY, Tsai MJ. 2001. Physiological function of the orphans GCNF and COUP-TF. Trends Endocrinol Metab 12:247–251.

Corvol P, Soubrier F, Jeunemaitre X. 1997. Molecular genetics of the renin-angiotensin-aldosterone system in human hypertension. Pathol Biol (Paris) 45:229–239.

Fuchs S, Germain S, Philippe J, Corvol P, Pinet F. 2002. Expression of renin in large arteries outside the kidney revealed by human renin promoter/LacZ transgenic mouse. Am J Pathol 161:717–725.

Germain S, Konoshita T, Philippe J, Corvol P, Pinet F. 1996. Transcriptional induction of the human *renin* gene by cyclic AMP requires cyclic AMP response element-binding protein (CREB) and a factor binding a pituitary-specific *trans*-acting factor (Pit-1) motif. Biochem J 316:107–113.

Germain S, Bonnet F, Philippe J, Fuchs S, Corvol P, Pinet F. 1998. A novel distal enhancer confers chorionic

expression on the human *renin* gene. J Biol Chem 273: 25292–25300.
Gorski K, Carneiro M, Schibler U. 1986. Tissue-specific in vitro transcription from the mouse albumin promoter. Cell 47:767–776.
Hagiwara M, Alberts A, Brindle P, Meinkoth J, Feramisco J, Deng T, Karin M, Shenolikar S, Montminy M. 1992. Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. Cell 70:105–113.
Hai T, Hartman MG. 2001. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: Activating transcription factor proteins and homeostasis. Gene 273:1–11.
Konoshita T, Germain S, Philippe J, Corvol P, Pinet F. 1996. Evidence that renal and chorionic tissues contain similar nuclear binding proteins that recognize the human renin promoter. Kidney Int 50:1515–1524.
Kumar V, Chambon P. 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145–156.
Ladias JA, Karathanasis SK. 1991. Regulation of the apolipoprotein *AI* gene by ARP-1, a novel member of the steroid receptor superfamily. Science 251:561–565.
Ladias JA, Hadzopoulou-Cladaras M, Kardassis D, Cardot P, Cheng J, Zannis V, Cladaras C. 1992. Transcriptional regulation of human apolipoprotein genes *ApoB*, *ApoC-III*, and *ApoAII* by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3. J Biol Chem 267:15849–15860.
Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. 1995. The nuclear receptor superfamily: The second decade. Cell 83:835–839.
Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH. 1986. Identification of a cyclic-AMP-responsive element within the rat *somatostatin* gene. Proc Natl Acad Sci USA 83:6682–6686.
Nelson C, Albert VR, Elsholtz HP, Lu LI, Rosenfeld MG. 1988. Activation of cell-specific expression of rat growth hormone and *prolactin* genes by a common transcription factor. Science 239:1400–1405.
Pan L, Black TA, Shi Q, Jones CA, Petrovic N, Loudon J, Kane C, Sigmund CD, Gross KW. 2001a. Critical roles of a cyclic AMP responsive element and an E-box in regulation of mouse *renin* gene expression. J Biol Chem 276:45530–45538.
Pan L, Xie Y, Black TA, Jones CA, Pruitt SC, Gross KW. 2001b. An Abd-B class HOX.PBX recognition sequence is required for expression from the mouse *Ren-1c* gene. J Biol Chem 276:32489–32494.
Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY. 1999. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Dev 13: 1037–1049.
Petrovic N, Black TA, Fabian JR, Kane C, Jones CA, Loudon JA, Abonia JP, Sigmund CD, Gross KW. 1996. Role of proximal promoter elements in regulation of *renin* gene transcription. J Biol Chem 271:22499–22505.
Pinet F, Corvol MT, Bourguignon J, Corvol P. 1988. Isolation and characterization of renin-producing human chorionic cells in culture. J Clin Endocrinol Metab 67: 1211–1220.
Sato Y. 2001. Role of ETS family transcription factors in vascular development and angiogenesis. Cell Struct Funct 26:19–24.
Scott MP. 1993. A rational nomenclature for vertebrate homeobox (*HOX*) genes. Nucleic Acids Res 21:1687–1688.
Shapiro DJ, Sharp PA, Wahli WW, Keller MJ. 1988. A high-efficiency HeLa cell nuclear transcription extract. Dna 7:47–55.
Shibata H, Ikeda Y, Mukai T, Morohashi K, Kurihara I, Ando T, Suzuki T, Kobayashi S, Murai M, Saito I, Saruta T. 2001. Expression profiles of COUP-TF, DAX-1, and SF-1 in the human adrenal gland and adrenocortical tumors: Possible implications in steroidogenesis. Mol Genet Metab 74:206–216.
Sigmund CD, Okuyama K, Ingelfinger J, Jones CA, Mullins JJ, Kane C, Kim U, Wu CZ, Kenny L, Rustum Y, Dzau VJ, Gross KW. 1990. Isolation and characterization of renin-expressing cell lines from transgenic mice containing a renin-promoter viral oncogene fusion construct. J Biol Chem 265:19916–19922.
Smith DL, Morris BJ, Do YS, Law RE, Shaw KJ, Hseuh WA. 1994. Identification of cyclic AMP response element in the human *renin* gene. Biochem Biophys Res Commun 200:320–329.
Sun J, Oddoux C, Lazarus A, Gilbert MT, Catanzaro DF. 1993. Promoter activity of human renin 5′-flanking DNA sequences is activated by the pituitary-specific transcription factor Pit-1. J Biol Chem 268:1505–1508.
Suzuki T, Takahashi K, Darnel AD, Moriya T, Murakami O, Narasaka T, Takeyama J, Sasano H. 2000. Chicken ovalbumin upstream promoter transcription factor II in the human adrenal cortex and its disorders. J Clin Endocrinol Metab 85:2752–2757.
Tamura K, Tanimoto K, Murakami K, Fukamizu A. 1992. A combination of upstream and proximal elements is required for efficient expression of the mouse renin promoter in cultured cells. Nucleic Acids Res 20:3617–3623.
Tamura K, Tanimoto K, Ishii M, Murakami K, Fukamizu A. 1993. Proximal and core DNA elements are required for efficient angiotensinogen promoter activation during adipogenic differentiation. J Biol Chem 268:15024–15032.
Tamura K, Umemura S, Ishii M, Tanimoto K, Murakami K, Fukamizu A. 1994. Molecular mechanism of transcriptional activation of angiotensinogen gene by proximal promoter. J Clin Invest 93:1370–1379.
Wasylyk C, Gutman A, Nicholson R, Wasylyk B. 1991. The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. Embo J 10:1127–1134.
Wasylyk B, Hahn SL, Giovane A. 1993. The Ets family of transcription factors. Eur J Biochem 211:7–18.
Ying L, Morris BJ, Sigmund CD. 1997. Transactivation of the human renin promoter by the cyclic AMP/protein kinase A pathway is mediated by both cAMP-responsive element binding protein-1 (CREB)-dependent and CREB-independent mechanisms in Calu-6 cells. J Biol Chem 272:2412–2420.