cis-Regulatory Elements and trans-Acting Factors Directing Basal and cAMP-Stimulated Human Renin Gene Expression in Chorionic Cells

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Abstract  Much knowledge was accumulated in the regulation of plasma renin activity and renin secretion during recent years. However, the mechanisms of renin gene transcription, especially for the human gene, have been poorly studied because of the lack of cell lines expressing renin. Cells derived from chorion tissue were used to study renin gene transcription because these cells express renin and regulate renin secretion in a similar way to JG cells. The present study was performed to determine the cis-regulatory elements and the trans-acting factors involved in human renin gene expression using chorionic cells. Transient DNA transfections were performed with various constructs containing the 5'-flanking region of the human renin gene. 5'-Deletion analysis of the human renin promoter (from -2616 to -67 bp) revealed the presence of two proximal negative cis-regulatory elements between -374 and -273 bp and between -273 and -137 bp. These elements were not present in a non-renin-producing cell line, JEG-3 cells. DNase I footprinting revealed that two sequences located within these regions bind trans-factors present in chorionic cellular nuclear extract: AGE3-like sequence (-293/-273) and apolipoprotein A1 regulatory protein-1-like sequence (-259/-245). The first 110 bp of the renin promoter were sufficient to direct specific expression in chorionic cells and contained two footprints sharing homology with ets (-29/-6) and pituitary-specific factor (Pit-1) (-70/-62) sequences. Furthermore, one footprint (-234/-214) contained the sequence TAGCGTCA, which shares strong homology to the cAMP-responsive element (CRE) binding site. Gel shift analysis showed specific DNA/protein complexes within this region, which were displaced by the somatostatin consensus CRE. Finally, luciferase analysis of 5'-deletion mutant revealed that -273 to +16 bp of the renin promoter was sufficient to confer complete forskolin stimulation, whereas deletion to -130 (deletion of the CRE) decreased cAMP responsiveness by 50% and those to -67 bp (deletion of the CRE and Pit-1-like sequences) suppressed it. Thus, these latter two sequences probably act together to confer complete cAMP responsiveness. (Circ Res. 1994;74:764-773.)

Key Words  • renin gene • chorionic cells • luciferase • cis-acting elements • trans-acting factors • cAMP-responsive element • pituitary-specific factor • ets

Renin (E.C.3.4.23.15), an aspartyl protease that cleaves angiotensin I from angiotensinogen in the rate-limiting step of the renin-angiotensin system, plays a major role in blood pressure regulation and fluid and electrolytic homeostasis.

During recent years, much knowledge has accumulated concerning the numerous factors influencing renin secretion, such as β-adrenergic agonists, peptidic hormones, and drugs. Several intracellular mediators influencing renin release, such as cAMP, cGMP, and intracellular calcium, have also been identified. Most of these studies were performed in isolated perfused kidney, kidney slices, or juxtaglomerular cell cultures because kidney-derived renin represents most of the circulating enzyme. Another important step in the control of renin secretion is the rate of transcription of the renin gene. However, studies examining this step are difficult to perform, in particular because of the lack of established renin-producing cell lines.

The transcriptional regulatory elements of the mouse renin genes have been largely characterized. One of the interesting features of this species is the presence of a duplicated renin gene in some strains, eg, DBA/2J. The Ren1 gene is expressed mainly in the kidney, whereas the Ren2 gene is expressed in both the kidney and the submandibular gland. Nakamura et al have shown the presence of a negative regulatory element in the 5'-flanking region of both mouse renin genes; this element is functional in the Ren1 but not the Ren2 gene, probably because of the interference by an adjacent 150-bp insertion in this latter gene. This difference in functionality may play a role in the differential expression of the renin genes in the submandibular gland. First, this negative regulatory element could bind a specific nuclear protein, present in the submandibular gland, resulting in the inhibition of Ren1 expression in this tissue. Second, the high expression of submandibular Ren2 could be due to the nonfunctionality of the negative regulatory element. Recently, Tamura et al identified, by transient DNA transfection, two positive cis-acting sequences located within a large insertion in the mouse gene and not present in the human gene, suggesting that transcriptional regulation of the renin gene could differ between species. In addition, they showed that renin gene expression depends strongly on the cell model used and on the presence or absence of trans-acting factors in these cells.

Very little is known about the mechanisms controlling human renin gene expression, mainly because of the lack of established renin-producing cell lines. Human
renin is expressed in many cell types, but its expression is particularly high in juxtaglomerular and chorionic cells. Juxtaglomerular cells are difficult to isolate and to culture, and they lose their ability to produce renin after a single passage. Attempts to immortalize these cells by the use of different SV40 mutants resulted in limited expression of the human renin gene. Although these cells allowed the study of some of the mechanisms involved in renin secretion, they cannot be used for renin gene expression studies. Chorion tissue represents the second major site of production, after kidney, in human tissues. Moreover, chorionic cells in primary and secondary cultures produce large quantities of renin, predominantly in the prorenin form, and are easily accessible. Renin regulation in this model was studied and showed a role of cAMP and protein kinase C. This is similar to the regulation demonstrated in primary cultures of JG cells. Therefore, these cells may provide a useful model for studying the regulation of renin gene expression.

Using transient DNA transfections, Duncan et al. reported that human chorionic cells are able to use the first 600 bp of the renin promoter to direct chloramphenicol acetyl transferase (CAT) expression without needing an enhancer. Other studies have been performed using the JEG-3 cell line, which is derived from a human choriocarcinoma. However, this model has several limitations: these cells do not express the renin gene, and analysis of the 5'-flanking region of the gene required the use of the herpes simplex virus thymidine kinase (tk) promoter to direct CAT expression.

There is a single recent study on a trans-acting factor controlling human renin gene expression showing that nuclear extracts isolated from a rat lactotrope precursor cell line (GC) interact with a pituitary-specific factor (Pit-1) binding site consensus sequence. Since Pit-1 is expressed only in pituitary cells, the functionality of this cis-acting sequence remains to be shown in renin-expressing cells.

The present study was performed to determine the cis-regulatory elements and the trans-acting factors involved in human renin gene expression, using renin-producing cells derived from chorionic tissue. The promoter elements involved in the transcription of the human renin gene in chorionic cells were localized by different approaches. Luciferase assays in human chorionic cells transiently transfected with constructs made by sequential deletions of the promoter region allowed the mapping of basal and cAMP-induced renin gene expression. Footprinting analysis of the human renin promoter, reported here for the first time, revealed the presence of several cis-regulatory elements within the proximal renin promoter. These consisted of sequences with varying degrees of homology with, respectively, the Pit-1-like binding site consensus sequence, the cAMP-responsive element (CRE), the apolipoprotein A1 regulatory protein-1 (ARP-1) binding site, an ets binding site, and the AGE3 sequence on the angiotensinogen gene. Finally, gel mobility shift analysis showed that the CRE sequence specifically binds nuclear extracts from chorionic cells.

Materials and Methods

Materials

All DNA-modifying enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized with an Applied Biosystems synthesizer. Reverse transcriptase, Tag DNA polymerase, ATP, and culture media were obtained from Boehringer Mannheim. [α-32P]CTP and [γ-32P]ATP were purchased from Amersham. Purified luciferase from Photinus pyralis, synthetic D-luciferin, and forskolin were obtained from Sigma.

Cell Culture and Transfections

Primary and secondary cultures of human chorionic cells were prepared essentially as previously described. Briefly, the chorion was separated from other membrane layers and digested by successive incubation periods in 0.05% collagenase and 0.05% trypsin. After filtration (45-um nylon mesh) and centrifugation, the cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (10 μg/mL), penicillin (10 U/mL), 4 mmol/L glutamine, and 10% fetal calf serum (FCS) and then plated onto the 25-cm² flasks containing 4 mL DMEM. When the cells were confluent, the first subculture was carried out: chorionic cells were treated with 0.05% collagenase and then with 5 mmol/L EDTA. The cell suspension was centrifuged, and the resultant cell pellets were resuspended in DMEM supplemented as above and then plated at a 1:4 dilution into similar flasks containing DMEM.

The human choriocarcinoma cell line (JEG-3) was obtained from the American Type Culture Collection and maintained in DMEM supplemented as above.

Transient DNA transfections into chorionic and JEG-3 cells were performed by calcium phosphate precipitation using 25 μg DNA per plate consisting of 5 μg RSV-CAT (as an internal control for plate-to-plate transfection efficiency) and 20 μg renin luciferase reporter plasmid. After overnight incubation, the cells were treated for 2 minutes with a 15% glycerol shock. The medium was then replaced with serum-free defined medium containing 50% Dulbecco's medium and 50% Ham F12 supplemented with streptomycin (10 μg/mL), penicillin (10 U/mL), selenium (3 x 10⁻¹⁵ mol/L), palmitic acid (1 μg/mL), oleic acid (5 μg/mL), linoleic acid (5 μg/mL), bovine serum albumin free fatty acid (1 mg/mL), transferrin (5 μg/mL), and glucose (4 mmol/L). The cells were incubated for 24 hours with or without forskolin 10⁻⁷ mol/L, and then the luciferase activity was determined and the CAT immunosassay performed.

Direct renin immunosassay was performed on culture medium by enzyme-linked immunosorbent assay (ELISA) with a sensitivity limit of 20 pg/mL.

Reporter Assays

Transfected cells were washed three times with phosphate-buffered saline (PBS), lysed by addition of 0.1 mL 1% Triton, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 25 mmol/L Tris-phosphate (pH 7.8), 15% glycerol, 1 mmol/L dithiothreitol (DTT), and 0.2 mmol/L phenylmethy1sulfonyl fluoride, and harvested by scraping. The lysates were transferred to Uppendorf tubes, and after centrifugation, the supernatant was saved. Luciferase activity was measured by a liquid scintillation counter (model 1211 Rackbeta, LKB). Luminescence was integrated for 1 minute after addition of 150 μmol/L luciferin and 400 μmol/L ATP.

Quantitative determination of CAT was performed by a sandwich enzyme immunoassay (Boehringer Mannheim). During the first step, CAT contained in cell extracts binds specifically to modulates coated with anti-CAT antibodies. Then, a digoxigenin-labeled anti-CAT antibody is bound to the fixed CAT, and during the last step, the digoxigenin-labeled anti-CAT antibody is detected by a peroxidase-labeled anti-digoxigenin antibody.

Plasmid Construction

Human renin gene fragments were isolated from the two plasmids phrNCAT30 and phrCAT06, kindly provided by Dr. Fukamizu (University of Tsukuba, Japan) and derived from the plasmid subclone AHRn88. The plasmid pRSVL, desig-
nated here as pRSV-luciferase and in which luciferase transcrip-
tion is driven by the Rous sarcoma virus long-terminal promoter,
was provided by Dr Swesh Subramani (version LpJD201).26 The promoterless plasmid (BS luci) was con-
structed by subcloning a HindIII/BamHI-digested fragment of LpJD201 (corresponding to the intronless luciferase construct with the SV40 small t antigen intron and an SV40 polyadenyl-
ation signal) into the polylinker region of the plasmid Blue-
Script SK (Stratagene). The plasmids p582+ and p582− were con-
structed as follows: a 598-bp fragment of the human renin gene
(−582 to +16) was isolated by Bgl II/HindIII digestion of phnCAT6 and subcloned in either orientation into the
HindIII site of BS luci. The resulting p582+ plasmid contained nucleotides −582 to +16 of the renin gene followed by the luciferase coding sequence. The plasmid containing the an-
tisense insert (+16 to −582) was designated as p582−. The
plasmid p892+ was constructed as follows: a 908-bp fragment of the human renin gene (−892 to +16) was isolated by HindIII digestion of phnCAT30 and inserted into the HindIII site of BS luci. The plasmid p2616+ was constructed by iso-
lat ing a 2632-bp fragment of the human renin gene (−2616 to +16) by Bgl II/BamHI/PstI 1 digestion of phnCAT30 and inserted into the HindIII site of BS luci.

5′-Deletions were generated by linearization of the p582+ plasmid by Xho I and Apa I, followed by exonuclease III digestion, S1 nuclease digestion, repair, and self-ligation. The 5′-extent of the exonuclease III digestion is given in nucleotide pairs corresponding to the published sequence.25 The plasmid p10− was constructed as follows: a 127-bp fragment (−110 to +16) of the renin gene was isolated by Kpn I and HindIII digestion of the plasmid p110+ (previously obtained by the exonuclease III digestion above) and inserted into the HindIII site of BS luci. All constructions were verified by the dyelex sequencing method (Sequenase Version 2.0 DNA Sequencing Kit, USB).

**Reverse Transcriptase Reaction and Polymerase Chain Reaction**

Total RNA was extracted from chorionic and JEG-3 cells by the method of Chomezynski and Sacchi.27 Total RNA (500 and 50 ng) was used to prepare cDNA with the M-MLV reverse transcriptase (200 U/μL GibCO-BRL) in the appropriate buffer (Boehringer Mannheim) in the presence of 7.5 mmol/L oligo(dT), 0.5 μmol/L of the RNase inhibitor rRNasin (40 000 U/mL, Promega), 50 mmol/L DTT, 1.0 μg yeast tRNA, and 2.5 mmol/L of each deoxyribonucleotide. After 1 hour of incuba-
tion at 37°C, the reaction was stopped by heating the samples for 2 minutes at 95°C.

The polymerase chain reaction (PCR) was carried out as described by Caroff et al.28 Briefly, 3 μL of the final cDNA solution was mixed with 1 μL (10 pmol) of both primers, 4.5 μL of 25 mmol/L MgCl₂ solution, 1 μL of a 25 mmol/L solution of each deoxyribonucleotide, 5 μCi of [α-32P]dCTP, 14 μL H₂O₂, and 2.5 μL of 10× PCR buffer (supplied with 1.25 U of Taq polymerase). Thirty cycles of PCR were performed, consisting of denaturation at 94°C, annealing at 48°C, and extension at 72°C. After PCR, polyacrylamide gel electropho-
resis was performed with the amplification products originating from the renin mRNA, followed by autoradiography. To avoid amplification of genomic DNA coding for renin, the two primers
5′(GTGTCTGTTGGGCTACACCCATGTTG)₃ and
5′(GATTCTCTGAAATACATAGTCCGTG)₃ were chosen, the first sequence being present in exon 7 of the renin gene and the second spanning the exon 8/exon 9 border.

Quantitative reverse transcriptase PCR was also carried out as described previously28 by use of an internal standard. Serial dilutions of total RNA (from 500 to 30 ng) from chorionic and JEG-3 cells were used with a fixed amount of internal standard (10 pg) to prepare cDNA before PCR. Bands corresponding to PCR products were excised and counted in a β-counter.

**Primer Extension Analysis**

Total RNA was isolated from human chorionic cells, after transcription as described above with the plasmids BS luci, p582+ and p110+, according to the method of Chomezynski and Sacchi.27 Primer extension reaction was carried out ac-
cording to the protocol of the AMV reverse transcriptase primer extension system (Promega). Briefly, RNA was resus-
pended in 5 μL H₂O and incubated with 1 μL (100 fmol) of
32P-labeled luciferase primer (corresponding to the sequence +37 to +63 of the luciferase gene) and 5 μL of AMV primer extension reaction 2X buffer. Primer and RNA were annealed by heating at 85°C for 5 minutes, then at 60°C for 2 hours. AMV reverse transcriptase was added to the same buffer with sodium pyrophosphate (6 mmol/L) and incubated at 42°C for 1 hour. The reaction was stopped by addition of loading dye, and the primer extension products were analyzed on denatur-
ing 6% polyacrylamide/7 mol/L urea gel.

**Preparation of Nuclear Extracts and DNase I Footprinting Assays**

Human chorionic cell nuclear extracts were prepared as previously described.29 The −336 to +16 fragment of the renin gene promoter was isolated by Avr II/HindIII digestion of the p582+ plasmid and inserted into the EcoRV site of Blue-
Script SK. The synthetic DNA fragment corresponding to
−336 to +16 was obtained by PCR amplification using the Blue-Script SK and KS universal primers. The SK or KS primer was labeled with [γ-32P]ATP and T4 polynucleotide kinase before PCR amplification. Footprint analysis was per-
fomed in a 10 μL reaction mixture containing 4 mmol/L MgCl₂, 4 mmol/L spermidine, 10 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 2.5% glycerol, 0.5 μg of double-stranded poly(dL-dC), 30 μg of nuclear extract, and 15 000 cpm of end-labeled fragment. After 20 minutes of incubation at 4°C, 2 μL of DNase I (Boehringer Mannheim, 10 IU/μL) at various dilutions ranging from 1/50 to 1/400 was added, and digestion was allowed to proceed for 1 minute at 20°C. The reaction was stopped by addition of 30 μL of a solution containing 50 mmol/L EDTA, 0.1% sodium dodecyl sulfate, 0.2 mg/mL yeast tRNA, and 10 mg/mL of proteinase K. The reaction mixture was incubated for 45 minutes at 42°C. The DNA was extracted once with phenol/chloroform, precipitated with 2 volumes of ethanol, resuspended in 98% formamide dye, and electrophoresed on a 6% acrylamide/7 mol/L urea sequencing gel.

**Gel Mobility Shift Assays**

The following double-stranded synthetic oligonucleotide, cor-
responding to the −234 to −200 renin promoter (containing the CRE-like sequence, underlined), was used for gel shift analysis (only the + strand is shown): REN, 5′-GAGGGCT-
GCAGCCGTCACCTGACACAGATTGTTT-3′. The following double-stranded oligonucleotide of the rat somatostatin promoter containing the consensus CRE29 was used as a com-
petitor: SMS, 5′-CTGGGGGCCCCTCCTGCTGTCG-
GACGTCAAGAGAGAG-3′. The REN oligonucleotide was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Nu-
clear extract (7 μg) was incubated for 15 minutes at 4°C in 18 μL of a reaction mixture containing 10 mmol/L HEPES (pH 7.8), 1 mmol/L NaHPO₄ (pH 7.2), 0.1 mmol/L EDTA, 50 mmol/L KCl, 4 mmol/L MgCl₂, 4 mmol/L spermidine, 2.5% glycerol, 2 μg of double-stranded poly(dL-dC), 1 μg of salmon sperm DNA, and 20 000 cpm of labeled double-stranded renin oligonucleotide in the presence or absence of 2- to 50-fold excesses of competitor oligonucleotides. The protein-DNA complexes were analyzed by nondenaturing electrophoresis through 6% polyacrylamide gels run in 0.25× Tris borate EDTA.

**Statistical Analysis**

All results are given as mean±SEM. Levels of significance were calculated by Student’s t test; P<0.05 was considered significant.
Results
Human Renin Gene Expression in Transfected Cells

Human renin gene expression was evaluated by transient DNA transfections into two cell types derived from human chorionic tissue: chorionic cells in secondary culture, which expressed the renin gene to a high extent (renin content of the culture medium measured by ELISA reached 20 ng·mL⁻¹·24 hours⁻¹), and JEG-3 cells, which did not express it (no renin detected in the culture medium). Plasmids containing the 5'-flanking region of the human renin gene (up to +16 relative to the transcription start site) were fused to the luciferase reporter gene, which was preferred to the CAT gene because of its higher sensitivity. To normalize for plate-to-plate differences in transfection efficiency, a reporter plasmid RSV-CAT was cotransfected and assayed independently. Luciferase activity was normalized to the cotransfected internal control RSV-CAT and plotted as a percentage of the RSV luciferase signal for each transfection.

Fig 1 shows the basal activity of the renin promoter. In chorionic cells, there was a moderate but not significant decrease in luciferase activity with shorter plasmids from p2616+ to p582+. The basal luciferase activities of p582+ and p892+, and p2616+ were 4.65%, 7.26%, and 8.43% of RSV luc, respectively. This suggests that major cis-regulatory elements are located downstream -582 bp. Two controls were performed: (1) The antisense plasmid p582− did not express luciferase activity (0.1±0.1%, n=2) compared with the sense plasmid p582+ (4.65±0.08%, n=6). (2) In JEG-3 cells, the renin luciferase fusion reporter gene displayed very low activity (0.23%, 0.17%, and 0.04% of RSV luc for the same plasmids, respectively) compared with that in chorionic cells.

To locate cis-regulatory elements, sequential deletions of the 5'-flanking region of the p582+ plasmid were performed, and the resulting renin-luciferase constructs were transfected into chorionic cells. Results were expressed as percentage of the activity of the p582+ plasmid to normalize the results of different transfections. Fig 2 shows that progressive 5'-deletions extending to nucleotide -374 had no effect on promoter activity. In contrast, deletions extending to nucleotide -273 gave a twofold stimulation above that of the renin 5'-flanking region of 582 bp. Further deletion of sequences between -273 and -67 greatly increased promoter activity: the plasmids p137+, p110+, and p67+ demonstrated threefold to fivefold increases in luciferase activity compared with p582+ plasmid. The specificity of the renin promoter activity was confirmed by the very low luciferase activity observed with the p110− plasmid, where the renin DNA was inserted in the antisense orientation. The 5'-sequences within p110+ were sufficient to direct specific expression of this renin gene construct in the chorionic cells, since JEG-3 cells transfected with p110+ plasmid did not exhibit the same high level of expression as identically transfected chorionic cells (Table). The p67+ plasmid demonstrated a 1.6-fold increase in luciferase activity, suggesting that the 67 bp of renin promoter are not sufficient to direct specific expression of renin gene. Smith and Morris described that JEG-3 cells expressed no detectable renin mRNA by Northern blot.
analysis, and little renin mRNA detected by PCR compared with chorionic cells (Fig 3) confirmed these data. Moreover, by quantitative reverse transcriptase PCR, we showed that renin mRNA from JEG-3 cells represented 0.3% of renin mRNA from chorionic cells (data not shown). Finally, the exact site of transcription driven by the renin/luciferase fusion gene constructs was determined by primer extension. The p582+ and p110+ plasmids were transfected into chorionic cells, the RNA was extracted, and the transcriptional initiation site was mapped by primer extension experiments. One major fragment was obtained with both plasmids, confirming the correct initiation of transcription (data not shown).

**Footprint Analysis of the Human Renin Promoter**

Changes in the expression of the serial deleted renin/luciferase genes (p582+ to p67+) suggest that factors within chorionic cells interact with renin cis-acting sequences. To demonstrate that these regulatory regions involved in the expression of the renin gene are sites for contact with DNA binding proteins from chorionic cells, the promoter sequence spanning −336 to +16 was subjected to DNase I footprint analyses in the presence and absence of nuclear extracts from human chorionic cells. This study showed the presence of six footprints, designated A through F (Fig 4). Footprint A comprised sequences from −29 to −6 and contained a centrally located, purine-rich sequence (GGAA) shown to be a conserved recognition site for ets-domain proteins. Footprint B, which extended from −79 to −62, was homologous with the Pit-1 binding site consensus sequence A(A/T)TTANCAT. Footprint C (from −107 to −83) did not show any sequence homology with previously described regulatory elements. Footprint D, from −234 to −214, showed strong homology with the CRE, TGACGTCA. Footprint E extended from −259 to −245, and its sequence was similar to the binding site for the ARP-1. Footprint F, from −293 to −272, showed strong homology with the sequence AGE3 described recently by Tamura et al on the human angiotensinogen gene.

**Functionality of the CRE Element**

The functionality of the CRE in the renin promoter in chorionic cells was further assessed by two methods: (1) transient DNA transfections performed in the presence of forskolin and (2) gel mobility shift analysis using the renin CRE-like sequence and chorionic cell extracts. Transient DNA transfections were performed with the different constructs described above, and the cells were treated or not treated with \(10^{-5}\) mol/L forskolin for 24 hours. All transfections were performed in the absence of FCS to avoid nonspecific interference in renin gene expression. Fig 5 shows a clear twofold to threefold increase of promoter activity within the region −2616 to −273. With the p137+ and p110+ plasmids, which lack element D (−234 to −214), there was only modest stimulation of promoter activity (1.81±0.2, \(P<.05\), and 1.62±0.28, \(P=NS\), respectively). No significant stimulation was observed with the p67+ plasmid. The promoterless plasmid, BS luci, was not stimulated by forskolin (0.96 of unstimulated luciferase activity), demonstrating that the induction of luciferase activity in cells transfected with the renin/luciferase constructs was mediated only via the renin promoter sequence.

Finally, the specific interaction of chorionic cell nuclear factors with element D (containing the CRE-like sequence) was investigated using gel mobility shift analysis. Double-stranded oligonucleotide containing element D was used as a labeled probe. Two protein/DNA complexes (Fig 6, arrows) were observed that were specifically competed by an excess of either homologous unlabeled DNA (REN) or somatostatin (SMS) oligonucleotide containing the consensus CRE. Both REN and SMS probe were able to completely compete the chorionic cell nuclear binding protein. This suggested that a CRE binding protein (CREB)-like substance interacted with element D of renin promoter.

**Discussion**

Since the structure of the human renin gene was first determined, several studies have been carried out to identify the cis-regulatory elements that control its transcriptional activity. Because human renin gene expression very likely depends on transcriptional regulatory factors present only in renin-expressing cells, it is important to perform such studies in cell lines that express renin. Cells derived from chorionic tissue are a major extrarenal site of renin production after kidney tissue. Human chorionic cell cultures have been characterized by Pinet et al, who used electron microscopy to show that these cultures contained a single type of elongated cell. Immunofluorescence studies using specific renin antibodies have shown that all cells in culture were stained and therefore contained both renin and prorenin. Chorionic cells in culture produce predominantly prorenin, and they seem to have only the constitutive pathway. Nevertheless, even though the chorionic cell in culture does not process prorenin, this model could be used to study renin gene transcription.
Human chorionic cells are a suitable model to study the transcriptional regulatory elements involved in renin gene expression: (1) These cells synthesized large amounts of prorenin. Their renin mRNA was identical in size (1.6 kb) to that of kidney renin mRNA\(^\text{15,16}\) and was easily detectable by Northern blot analysis. In contrast, in non-renin-producing cells, JEG-3 cells, renin mRNA could be detected only slightly by PCR. (2) A highest luciferase activity (between 20- and 200-fold) of renin promoter/luciferase constructs was obtained with chorionic cells compared with JEG-3 cells by transient DNA transfection. This finding in JEG-3 cells is in agreement with the results of Smith and Morris\(^\text{18}\) who showed that, in JEG-3 cells, the human renin promoter was not able to direct CAT transcription in the absence of the herpes simplex virus tk promoter. The CAT activity detected when the renin promoter region was fused to the tk promoter\(^\text{17,18}\) might have been driven by the tk promoter, which is more potent than the renin promoter. In contrast, Duncan et al\(^\text{16}\) reported that human chorionic cells are capable of using the first 600 bp of the renin promoter to direct CAT expression. (3) A correct initiation of transcription was found with renin/luciferase fusion genes transfected within chorionic cells.
In the present study, no major basal regulatory elements were detected upstream of -582 up to -2616. In contrast, deletion analysis of the 5′-flanking region of the p582+ plasmid revealed that the first 110 bp of the renin promoter were sufficient for a specific and high expression in human chorionic cells (more than threefold higher than p582+) and could therefore correspond to the “basal promoter.” Indeed, this highest activity is not due to plasmid “read-through” artifact, since the activity of the shortest 5′-flanking DNA fragment (p110+ and p67+) was not increased when transfected into a non-renin-producing cell line, the JEG-3 cells. A negative regulatory element was found between the -374 and -273 bp by deletion analysis of the 582 bp of renin promoter. DNase I footprinting showed that this region of DNA binds trans-activating factors present in chorionic cellular nuclear extract. This footprint has strong homology with a sequence of the mouse angiotensinogen gene that binds the constitutive factor named AGF3 by the authors. They suggested that AGF3 could play important roles in the differentiation-dependent promoter activation of the mouse angiotensinogen gene in adipocytes. In the case of the human renin promoter, the present results suggested that an “AGF3-like” protein from chorionic cells could repress human renin expression. Further deletions of the 5′-region until -137 bp revealed the presence of another negative regulatory element. Interestingly, the DNase I protection assay revealed that this region contains a sequence, footprint E (-259 to -245), that binds nuclear extract from chorionic cells. This putative cis-element has strong homology with a sequence of the human apolipoprotein AI gene that binds the ubiquitous ARP-1, a member of the orphan steroid receptor superfamily that decreases apoAI gene expression. These results suggest that an ARP-1-like protein might decrease renin gene expression in chorionic cells. Both negative elements found in chorionic cells are not operative in JEG-3 cells, since the p110+ plasmid did not exhibit a highest activity, suggesting that these trans-acting factors, AGF3 and ARP-1, may not be present in these latter cells.

No difference in luciferase activity was found with the plasmids p137+ and p110+ in relation to the fact that no footprint was found between -137 and -110 bp of the human renin promoter. Further deletions until -67 bp showed an increase in luciferase activity. This DNA
region binds two factors: one named element C, which did not share any homology with previously described regulatory elements; the other, element B, is an adenine/thymine rich region very similar to the Pit-1 binding site consensus sequence. Epidemiologically, Sun et al.33 showed that this region of the human renin promoter binds nuclear extracts isolated from GC cells, a pituitary lactotrope precursor cell line. Furthermore, they showed that the activity of the renin promoter/luciferase constructs transfected into HeLa cells was greatly increased by cotransfection with a Pit-1 expression vector. Pit-1, a specific pituitary factor, is a member of the POU family of transcription factors, which plays a critical role in the proliferation of specific cells and in their expression of specific genes.34 In contrast to the results of Sun et al.35 in GC cells, deletion of this Pit-1-like binding site did not affect luciferase gene expression in choriocarcinoma. This suggests cell-specific use of this regulatory element. Such an observation, concerning the differential expression of a promoter in different cell lines, has been made by Pauwber et al.36 for the apolipoprotein B promoter in HepG2 (hepatic) and CaCo-2 (intestinal) cell lines. They showed that this difference was due to the relative amounts of nuclear factors that bind to specific sequences located within the promoter. Another explanation for this discrepancy could be the presence, in choriocarcinoma cells, of an nuclear factor binding the downstream element A (−29 to −6 bp), which contains the consensus GGAA ets motif.37 Many of the ets-domain proteins have been shown to be transcription activators in various tissues.38 Thus, the high luciferase activity of plasmid p67+ might result from the presence, in choriocarcinoma, of an ets-domain protein involved in the expression of the human renin gene. This domain might be not operative in GC cells.

The functionality of the renin promoter was further assessed by forskolin stimulation. Previous studies by Duncan et al.39 had shown that, in choriocarcinoma, plasmid constructs containing either the first 600 or 100 bp of the human renin promoter fused to CAT were markedly stimulated by 8 hours of incubation with 10−6 mol/L forskolin. In contrast, plasmids containing the 5′-flanking region of the renin gene (from −584 to −146 and from −146 to +11) fused to the tk promoter were not induced by forskolin.39 However, these experiments were performed in the presence of FCS, which is capable of stimulating the transcription of several genes via different serum response elements.39 cAMP might also activate serum response elements,40 and therefore, interactions between serum- and cAMP-induced stimulation could not be excluded. In JEG-3 cells, Burt et al.41 had also used plasmids containing the 5′-flanking region of the renin gene fused to the tk promoter and reported a modest 60% cAMP-induced stimulation. It was for these reasons that further studies on the effects of cAMP on renin gene expression were required.

In the present study, all stimulations were performed in the absence of FCS to avoid nonspecific interference. The results show that element D (−234 to −214) was required for forskolin to stimulate transcription twofold to threefold. This element contains a motif, TAGGCTCA, that shares a six-nucleotide homology with the consensus CRE octamer TGACGTCA.33 It contains the short motif CCGTA, which has been shown to be a binding site for CREB.41 The present results show that nuclear extracts isolated from choriocarcinoma cells bound to this sequence. Two specific DNA/protein complexes were found and could result from the formation of monomeric and dimeric DNA/protein complexes as previously described.42 In addition, this DNA/protein binding was displaced by the SMS oligonucleotide containing the consensus binding site for CREB.43 Taken together, these results are in favor of element D being a functional CRE site. However, the modest forskolin-stimulated increase in luciferase activity observed with constructs p137+ and p110+ (50%) cannot be explained by this CRE-like sequence. Interestingly, Peers et al.44 have shown that Pit-1 is involved in the cAMP stimulation of the human prolactin gene and that several Pit-1 binding sites fused to the tk promoter confer cAMP responsiveness to GC cells. It is therefore possible that the Pit-1-like binding site present on the renin gene is also involved in regulation by cAMP and that stimulation by cAMP is the result of multiple responses acting in combination to yield a measurable degree of stimulation. Further studies will be necessary to determine whether the Pit-1-like sequence is necessary to confer cAMP responsiveness on the renin gene.
Overall, these results clearly identify several functional cis-regulatory regions in the renin gene and potential trans-activating factors governing renin gene expression in human choriocarcinoma cells as represented schematically in Fig 7. No studies have yet been reported in human JG cell regulation of renin transcription because of the lack of suitable cell lines or cell culture models. However, like choriocarcinoma JG cells respond to forskolin by an increase in renin mRNA and renin release, and factors similar or identical to the CREB identified in the present study could be involved. Moreover, choriocarcinoma cells in culture respond to angiotensin II by an elevation of intracellular calcium and a decrease in renin production (unpublished data) like JG cells.

However, characterization of transcription factors that bind human renin promoter from kidney cortex or juxtaglomerular cells would permit determination of renin-producing cell-specific factors. We can speculate that some of the transcription factors characterized could be implied in the renin tissue-specific expression and in renin regulation in vivo.

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