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

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To delineate the *cis*-acting elements of the proximal promoter responsible for cyclic AMP (cAMP)-induced human renin gene transcription, 5'-flanking regions of the human renin gene were fused to a luciferase reporter gene and transfected in chorionic cells. Forskolin treatment induced the expression of luciferase by 2.4-fold when the reporter plasmid contained the promoter region (–582 to +16). Mutation or deletion of the cAMP response element (CRE) diminished (1.7-fold) but did not abolish cAMP-induced transcription, demonstrating that the (–582 to –145) region containing the CRE and the region (–145 to –38) containing a Pit-1 (pituitary-specific *trans*-acting factor) site were

both necessary for cAMP maximal induction. To study the molecular events mediating the cAMP induction, DNase I footprinting and electromobility shift assays (EMSAs) were performed with renin-producing chorionic cell and kidney cortex cell nuclear extracts, showing that the CRE-binding protein (CREB) interacts with the CRE and that tissue-specific factors, distinct from Pit-1, specifically bind the renin Pit-1 motif. Taken together, these results demonstrate that the cAMP response of the human renin gene may involve CREB binding the CRE and tissue-specific factors, different from Pit-1, that interact with the Pit-1 response DNA elements.

INTRODUCTION

The renin–angiotensin system plays a major role in blood pressure regulation and electrolyte homeostasis through the action of angiotensin II [1]. The first and rate-limiting step in the production of angiotensin II, i.e. the conversion of angiotensinogen into angiotensin I, is catalysed by the aspartyl protease renin (EC 3.4.23.15) which is synthesized in renal juxtaglomerular (JG) cells. Human JG cells are difficult to isolate and lose their ability to produce renin after the first passage in cell culture [2]. Attempts to immortalize tumoral JG cells using different Simian virus 40 (SV40) mutants resulted in the limited expression of the human renin gene [3]. Studies to identify factors influencing renin production have been performed mainly in the rat using isolated perfused kidney, kidney slices, or JG cell cultures where it has been shown that cyclic AMP (cAMP) is a major mediator of renin biosynthesis and secretion [4].

Although the renin gene is expressed in many cell types, it is particularly prevalent in extra-renal sites, e.g. chorio-decidual tissue [5–8], which has become a useful model to study human renin gene regulation. Caroff et al. [9] showed that forskolin and phorbol 12-myristate 13-acetate (PMA) act synergistically to increase both renin mRNA levels and renin production in a dose-dependent manner. In addition, we have recently mapped the proximal *cis*-regulatory elements involved in basal gene expression in chorionic cells by DNase I footprinting and functional assays [10]. Two negative *cis*-acting elements, (–374 to –273) and (–273 to –137), and the pituitary-specific *trans*-acting factor (Pit-1) motif located at position (–79 to –60) of the 5'-flanking region of the gene were shown to be important for basal transcription. Previous studies in chorionic cells by us and others

have suggested that human renin gene transcription is positively modulated by cAMP [10,11]. Moreover, recent studies showed that the human renin gene promoter basal or cAMP-stimulated activity in gonadocorticotrope (GC) cells, a rat pituitary lactotrope precursor cell line, is dependent upon the presence of the Pit-1-binding site (–79 to –64) [12,13]. However, the cAMP response element (CRE) of the human renin gene has not been identified precisely in cells which express the human renin gene. cAMP induction of many eukaryotic genes is mediated by members of the AP-2 [14] or CRE-binding protein (CREB)/activating transcription factor (ATF) family [15] which bind to the cAMP response element (CRE), consisting of CGTCA motifs of the palindromic consensus sequence TGACGTCA [16–18]. The CREB/ATF family belongs to the basic region/leucine zipper (bZIP) class of DNA-binding proteins of which CREB [19], cAMP response element modulator (CREM) [20], Jun D [21] and ATF-1 [22] are the best characterized.

In the present study, we show that the CRE sequence (–226 to –219) is functional and partially responsible for cAMP induction of transcription of the human renin gene promoter. In addition, binding of monomeric and homodimeric CREB derived from chorionic cells and kidney cortex cells to the human renin CRE is shown. The Pit-1 consensus binding site (–77 to –67) was also found to be required and acts in co-ordination with the CRE to confer a full cAMP responsiveness. Electromobility shift assays (EMSAs) performed with nuclear factors from chorionic cells, kidney cortex and GH₃B6 cells (which contain high amounts of Pit-1) enabled us to demonstrate that different proteins from chorionic and renal origin, distinct from Pit-1, specifically bind the Pit-1 consensus binding site of the human renin gene proximal promoter. Therefore, we suggest that *trans*-acting factors, present

Abbreviations used: cAMP, cyclic AMP; CRE, cAMP response element; CREB, CRE-binding protein; CREM, cAMP response element modulator; JG, juxtaglomerular; Pit-1, pituitary-specific *trans*-acting factor; PMA, phorbol 12-myristate 13-acetate; ATF-1, activating transcription factor-1; EMSA, electromobility shift assay; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; bZIP, basic region/leucine zipper.

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in renin-producing chorionic and kidney cortex cells, bind the Pit-1 consensus site of the human renin gene and act together with CREB to mediate transcriptional control of the human renin gene by cAMP.

MATERIALS AND METHODS

Plasmid constructions and site-directed mutagenesis

The plasmid pRSVL, designated here as pRSV-luciferase, in which luciferase transcription is driven by the Rous sarcoma virus (RSV) long terminal promoter, was provided by Dr. Swesh Subramani (version LpJD201) [23] and was used as an internal control of transfection. The plasmid pGL₂582+ was constructed as follows: a 598 bp fragment of the human renin gene (−582 to +16) was isolated by *Hind*III digestion of phrCAT06, kindly provided by Dr. Fukamizu (University of Tsukuba, Japan), derived from the plasmid subclone λHRn88 [24] and subcloned into the *Hind*III site of pGL₂basic (Promega). The resulting plasmid pGL₂582+ contained nucleotides (−582 to +16) of the renin gene followed by the luciferase coding sequence. The 161 bp fragment of the human renin gene (−145 to +16) was then isolated by *Hind*III/*Kpn*I digestion of pGL₂582+ and subcloned into the pGL₂basic polylinker creating plasmid pGL₂145+. pGL₂38+ was constructed as follows: a fragment (−145 to −38) of pGL₂145+ was deleted by *Kpn*I/*Nsi*I digestion, blunt-ended with T4 DNA polymerase and self-ligated with T4 DNA ligase. All constructions were verified by the dideoxy sequencing method (Sequenase Version 2.0 DNA Sequencing Kit, USB). For site-directed mutagenesis, the *Hind*III fragment (−582 to +16) was inserted into the polylinker of the M13mp18 vector (Boehringer Mannheim) to produce single-stranded DNA. CRE-MUT oligonucleotide replaced the consensus CRE CGTCA by *tcgga*, and Pit-1-Δ1 replaced the Pit-1-binding site (−77 to −67) core sequence TAATAAATCAG by the Δ1 mutation, TAATgggcCgG. *In vitro* mutagenesis was performed with a Muta-Gene D kit (Bio-Rad), and the mutated sequences were confirmed by sequencing on both strands. The mutated fragments were then subcloned into pGL₂basic creating plasmid pGL₂582MUT in which the CRE was mutated, pGL₂582Δ1 and pGL₂145Δ1 both containing the mutated Pit-1-like site, and pGL₂582Δ1MUT containing both mutations (CRE and Pit-1).

Cell culture and transfections

Primary and secondary cultures of human chorionic cells were prepared as described previously [8]. Transient DNA transfections of chorionic cells were performed by calcium phosphate precipitation on 21 µg of DNA/flask consisting of 1 µg of RSV-CAT (as an internal control for plate-to-plate transfection efficiency) and 20 µg of renin luciferase reporter plasmid. After overnight incubation, the cells were treated for 2 min with a 15% (w/v) glycerol shock and the medium replaced with serum-free defined medium as described previously [10]. The cells were then incubated for 24 h in the presence or absence of 10 µM forskolin.

Reporter assays

Transfected cells were washed three times with PBS, lysed by adding 150 µl of 1% Triton, 10 mM MgCl₂, 1 mM EDTA, 25 mM Tris/phosphate, pH 7.8, 15% (w/v) glycerol, 1 mM dithiothreitol and 0.2 mM PMSF, and harvested by scraping according to the protocol of N'Guyen et al. [25]. The lysates were centrifuged and the supernatant used immediately for the lu-

ciferase assay. Luciferase activity was estimated by measuring luminescence for 10 s with a Bio.Orbit 1250 luminometer, 30 s after addition of 50 µl of luciferase reporter buffer (Promega).

Quantitative determination of chloramphenicol acetyltransferase (CAT) was performed by a sandwich immunoassay (Boehringer Mannheim).

Statistical analysis

All results are given as means of three independent transfection experiments performed in triplicate 25 cm² flasks ± S.E.M. Levels of significance were calculated by Student's *t* test; *P* < 0.05 were considered significant. N.S. non significant.

Preparation of nuclear extracts and DNase I footprinting assays

The source of human kidney was ischaemic kidneys removed because of reno-vascular hypertension. These kidneys are considerably enriched in renin, as previously shown [26]. The cortex which contains most of the JG cells was carefully saved and the cell nuclear extracts were prepared according to a modified protocol of Gorski et al. [27]. A sample (15 g) of kidney tissue was homogenized by 10–12 strokes using a motor-driven pestle in 30 ml of modified Schibler homogenization buffer (SHB; 10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM EDTA, 2.4 M sucrose). The homogenate was filtered through four layers of gauze up to a volume of 85 ml. An aliquot (28 ml) of homogenate was layered on to 10 ml of Cushion solution (2 M sucrose, 10% bidistilled glycerol) and centrifuged for 45 min at 25000 rev./min in an SW 27 rotor at 0 °C. Nuclear pellets were resuspended in 54 ml of SHB/water in a 9:1 (v/v) ratio. An aliquot (27 ml) of the nuclear suspension was layered on 10 ml of Cushion solution and centrifuged under the same conditions. Nuclear extracts from the pelleted nuclei and human chorionic cell nuclear extracts were prepared by the method of Shapiro et al. [28]. DNase I footprinting assays were carried out as previously described [10].

EMSAs

Double-stranded oligonucleotides (CRE-REN) (5'-GAGGGC-TGCTAGCGTCACTGGACACAAGATTGCTTT-3') and (Pit-1-REN) (5'-AGGGTAATAAATCAGGGCAGAG-3') respectively corresponding to the (−234 to −199) and (−82 to −54) regions of the renin human renin promoter, Pit-1 GH corresponding to the (−12 to +7) region of the rat growth hormone gene containing the Pit-1 motif (Pit-1-GH) (5'-TCCGTATACATTTATTCATGGCTGGA-3') [29], and the (−65 to −33) positive 5' rat Pit-1 gene autoregulatory site (Pit-1-RAT) (5'-AACTATTAACATGTATAAATGGATTTCCTCAGAG-3') [30] were synthesized on a PCR-mate (Applied Biosystems). They were end-labelled with [γ -³²P]ATP and T4 polynucleotide kinase.

Each binding reaction was performed with 7 µg of human chorionic or human kidney cortex cell nuclear extracts or with 80 ng of purified CREB kindly provided by Dr. Marc Montminy (The Clayton Foundation Laboratories for Peptide Biology, La Jolla, CA, U.S.A.). These were incubated with 20000 c.p.m. of end-labelled double-stranded CRE-REN oligonucleotide for 15 min at 4 °C in 18 µl of buffer containing 10 mM Hepes (pH 7.8), 1 mM Na₂HPO₄ (pH 7.2), 0.1 mM EDTA, 50 mM KCl, 4 mM MgCl₂, 4 mM spermidine, 2.5% glycerol, 2 µg of poly(dI-dC) and 1 µg of sonicated salmon sperm DNA. Separation was carried out by electrophoresis in non-denaturing 6% polyacrylamide gels in 22 mM Tris/borate/0.5 mM EDTA. For CRE-REN competition experiments, the following unlabelled

oligonucleotides were used as competitors: CRE-REN, CRE-SOM containing the rat somatostatin (−68 to −32) consensus CRE [17] (5′-CTGGGGGCGCCTCCTTGCTGACGTCA-GAGAGAGAG-3′) and CRE-MUT where the consensus CRE (CGTCA) of the human renin gene was mutated to *tcgga* (5′-GAGGGCTGCTAG*tcgga*CTGGACACAAGATTGCTTT-3′) [31]; they were used at 25- to 200-fold molar excess.

With the Pit-1-REN, Pit-1-GH and Pit-1-RAT probes, human kidney cortex cell nuclear extracts were incubated with 20000 c.p.m. of end-labelled double-stranded probe for 2 h on ice in 18 μ l of buffer containing 10 mM Hepes (pH 7.8), 1 mM Na₂HPO₄ (pH 7.2), 0.1 mM EDTA, 50 mM KCl, 12 mM MgCl₂, 4 mM spermidine, 2.5 % glycerol, 1 μ g of poly(dI.dC) and 250 ng of sonicated salmon sperm DNA. Separation was carried out by electrophoresis in non-denaturing 7.5 % polyacrylamide gels in 22 mM Tris/borate/0.5 mM EDTA at 5 °C.

For Pit-1-REN competition experiments with chorionic cell nuclear extracts, the unlabelled oligonucleotide Pit-1-REN, Pit-1- Δ 1 where the Pit-1 binding site TAATAAATCAG was mutated to the Δ 1 mutation as described previously by Sun et al. [12] (5′-GGGTAAT*gggc*CcGGGCAGAG-3′), Pit-1-GH and Pit-1-RAT were used at 100-fold molar excess. With kidney cortex cell nuclear extracts, unlabelled Pit-1-REN, Pit-1-GH, Pit-1- Δ 1 and Pit-1-RAT were used at 50- to 100-fold molar excess. With GH₃B6 cell nuclear extracts (2.5 μ g per lane), kindly provided by E. Passequé (CNRS URA 1115, Collège de France, Paris, France), unlabelled Pit-1-REN, Pit-1- Δ 1, Pit-1-GH and Pit-1-RAT were used at 100-fold molar excess in the conditions described by Ngô et al. [32].

Electromobility supershift assays

The electrophoretic mobility supershift assays were performed using an anti-CREB serum 244 [33] raised against a synthetic peptide 126–162 of the CREB protein, kindly provided by Dr. Marc Montminy (The Clayton Foundation Laboratories for Peptide Biology, La Jolla, CA, U.S.A.). One μ l of antiserum 244 was added to the EMSA reaction mixture and then incubated for 1 h at 4 °C before electrophoresis was performed under the same conditions as described above for EMSA.

RESULTS

The CRE and the (−145 to −38) region are necessary for the cAMP stimulation of human renin gene transcription in chorionic cells

To identify precisely the 5′-flanking sequences of the human renin gene required for cAMP stimulation in chorionic cells, a series of chimeric genes were constructed in which different lengths of the 5′-flanking region were fused to a luciferase reporter gene. These constructs were then transfected into human chorionic cells which express the renin gene. Transfection efficiency was controlled by co-transfection with the pRSV-CAT plasmid. The effect of cAMP on renin promoter-luciferase reporter gene expression was then determined by treating chorionic cells with 10 μ M forskolin for 24 h. The results show that forskolin induced a 2.4-fold increase in transcription of the pGL₂582+ construct containing the (−582 to +16) fragment of the renin gene (Figure 1). In order to ascertain whether the CRE located at (−226 to −219) conferred the response to cAMP, the renin CRE (CGTCA) of pGL₂582+ was mutated to *tcgga*, creating the plasmid pGL₂582MUT, a mutation which has been shown to abolish the ability to confer cAMP responsiveness to a promoter-CAT construct [31]. The results show that the promoter activity of pGL₂582MUT is weaker compared with pGL₂582+

Renin promoter/luciferase constructs	Basal Activity	10 μ M Forskolin Treatment
pGL ₂ basic	0 $\pm$ 0.1 *	0 $\pm$ 0.1 (x 1) N.S.
pGL ₂ 38+	65 $\pm$ 3 †	63 $\pm$ 3 (x 1) N.S.
pGL ₂ 145+	151 $\pm$ 25 ‡	252 $\pm$ 21 (x 1.7)
pGL ₂ 145 Δ 1	73 $\pm$ 9 †	74 $\pm$ 7 (x 1) N.S.
pGL ₂ 582+	100 §	238 $\pm$ 4 (x 2.4) ¶
pGL ₂ 582MUT	73 $\pm$ 5 †	124 $\pm$ 13 (x 1.7)
pGL ₂ 582 Δ 1	67 $\pm$ 5 †	94 $\pm$ 8 (x 1.4) #
pGL ₂ 582 Δ 1MUT	49 $\pm$ 5 †	49 $\pm$ 4 (x 1) N.S.

Figure 1 Normalized luciferase activity of different renin promoter/luciferase constructs transfected into chorionic cells

Luciferase activity was normalized to co-transfected RSV-CAT activity. Each value represents the result of three independent transfection experiments performed in triplicate flasks. Values are means $\pm$ S.E.M. Levels of significance were calculated by Student's *t* test. Fold inductions (indicated in parentheses) were calculated as the ratio of normalized luciferase activity in forskolin-treated cells versus that in untreated cells. Groups that are significantly different ($P < 0.05$) from each other are indicated by the various location keys (||, ¶, #). Abbreviation: N.S., non significant.

at the basal level but is still partially stimulated by forskolin (1.7-fold). Furthermore, whereas the plasmid pGL₂145+ has the highest basal activity, the same level of activation of transcription (1.7-fold) as pGL₂582MUT was observed where the CRE is deleted (Figure 1). These results establish that the human renin gene CRE confers partial cAMP sensitivity and provide evidence for a multifactorial interaction between the CRE and other downstream elements. Among these the (−145 to −38) fragment contains the Pit-1 motif (−77 to −67) which could be involved in cAMP responsiveness. The respective roles of the CRE and Pit-1 sites in cAMP-stimulated transcription of the human renin gene were evaluated by mutating each site, either individually or together, to sites that no longer bind transcription factors. All the mutated constructs retained a significant luciferase activity compared with pGL₂basic. Forskolin-activated transcription of the luciferase reporter gene was completely abolished in transfections performed with pGL₂145 Δ 1 where the consensus Pit-1 binding site was replaced by the Δ 1 mutation (Figure 1). In addition, as previously described by us and others, when transfected into non-treated cells, pGL₂145 Δ 1 basal activity was strongly reduced as compared with the pGL₂145 plasmid [10,12]. Therefore, it would appear that the renin Pit-1 motif is also essential, together with the CRE, to confer cAMP responsiveness. The mutation of this site into pGL₂582+, creating pGL₂582 Δ 1, resulted in a strong reduction of the reporter gene cAMP-induced expression (1.4-fold), whereas its basal activity was similar to that of pGL₂582MUT. To confirm these results, a mutant plasmid pGL₂582 Δ 1MUT was constructed, where both cAMP and Pit-1 responsive elements were mutated. pGL₂582 Δ 1MUT retained the same basal activity as pGL₂582MUT and pGL₂582 Δ 1, although activation of transcription by cAMP was completely abolished (Figure 1), as also observed with pGL₂basic and pGL₂38+.

Figure 2 Gel mobility shift analysis of kidney cortex cell nuclear extracts (a) and purified CREB (b) with the human renin promoter (CRE-REN: —234 GAGGGCTGCTAGCGTCACTGGACACAAGATTGCTTT —199)

A double-stranded oligonucleotide containing the renin CRE was used as probe. Competitions were performed with homologous DNA (CRE-REN), with the somatostatin oligonucleotide (CRE-SOM: —68 CTGGGGGCGCCTCCTGGCTGACGTCAGAGAGAG —32) only for kidney cortex cell nuclear extracts or with the modified oligonucleotide (CRE-MUT: —234 GAGGGCTGCTAGCGTCACTGGACACAAGATTGCTTT —199). Arrows indicate the specific DNA/protein complexes. The fold molar excess of competitor is indicated for each competitor sequence. With purified CREB, competitions were performed with a 200-fold molar excess of homologous DNA (CRE-REN) or modified oligonucleotide (CRE-MUT).

Binding of renal and chorionic CREB at the human renin CRE

To characterize further the proteins involved in binding the human renin CRE, nuclear extracts from renin-rich human kidney cortex were therefore prepared. EMSAs were performed using a labelled oligonucleotide, CRE-REN (—234 to —199)

containing the human renin CRE and showed that two specific high-affinity DNA/protein complexes were formed between CRE-REN and kidney extracted proteins (Figure 2a). These two upper complexes, similar to those observed with chorionic cell nuclear extracts [10], represent sequence-specific interactions as shown by competition experiments using an excess of unlabelled CRE-REN. To define further the DNA sequence involved in binding, competition experiments were performed with an oligonucleotide representing the (—68 to —32) region of the rat somatostatin promoter containing the consensus CRE (CRE-SOM) [17]. This consensus CRE also competes against binding of the renal factor to CRE-REN (Figure 2a). When CRE-SOM was used as a probe, two shifted complexes migrated at the same position, suggesting that a similar or an identical factor interacted with CRE-REN and CRE-SOM (results not shown). Competition experiments were performed with modified CRE-MUT oligonucleotide. As shown in Figure 2(a), no competition was observed when a 25- to 100-fold molar excess of unlabelled CRE-MUT was used as competitor, whereas a slight competition could be observed with a 200-fold molar excess. In addition, the failure of this mutant CRE (CRE-MUT) to effectively compete for binding was also observed with human chorionic cell nuclear extracts (results not shown). Finally, the use of CRE-MUT as a probe showed no shifted bands with both extracts (results not shown). Purified CREB exhibited two shifted complexes by EMSA with CRE-REN, which were both competed for by a homologous probe, but not by the mutated CRE-MUT probe (Figure 2b).

Supershift experiments were performed to characterize further the CREBs present in chorionic cell or kidney cortex cell nuclear extracts interacting with the human renin CRE. The addition of anti-CREB serum 244 to the electromobility shift reaction mixture yielded a major complex which migrated more slowly both with chorionic cell nuclear extracts (Figure 3a) and kidney cortex cell nuclear extracts (Figure 3b). The same supershift was observed with purified CREB (Figure 3c). Experiments conducted with antisera directed against CREM and ATF-1, which belong to the same family of bZIP transcription factors showed no supershift with chorionic cell nuclear extracts or kidney cortex cell nuclear extracts (results not shown). These results,

Figure 3 Gel mobility supershift analysis of human chorionic cell nuclear extracts (a), human kidney cortex cells nuclear extracts (b) and purified CREB (c) with the human renin promoter (—234 to —199) and anti-CREB serum 244 raised against a synthetic peptide 126–162 of the CREB protein

Specific DNA/protein interactions are indicated by arrows. The dilution of the antiserum is indicated for each experiment.

Figure 4 DNase I footprinting assay of the (–90 to –46) region of the human renin gene

Footprint analysis was performed with human kidney cortex cell extracts. G,A,T,C represent the sequencing ladder; lanes 1 to 3 represent the reaction performed with 30 μ g of nuclear extracts and decreasing amounts of DNase I (0.1 U, 0.2 U, 0.4 U respectively); lanes 4 to 6 represent the reaction performed in the absence of nuclear extracts with 0.0125 U, 0.025 U and 0.05 U of DNase I, respectively. The footprint of the Pit-1 site is indicated on the right of the Figure.

combined with competition experiments, demonstrate that CREB effectively binds to the CGTCA element of the human renin gene promoter.

Demonstration of the binding of a transcription factor to the (–77 to –63) of the human renin gene promoter by DNase I footprinting and EMSA

To characterize further the Pit-1 consensus site (–79 to –60) of the human renin gene promoter, a DNase I footprinting assay was performed with kidney cortex cell nuclear extracts. Figure 4 shows that the renin Pit-1 site (–77 to –63) is protected by renal protein(s), as has been shown previously by us for chorionic cells [10]. In order to characterize the nucleotides involved in protein binding, nuclear extracts from chorionic and kidney cortex cells were mixed with a labelled oligonucleotide (–82 to –61) corresponding to the renin Pit-1 site (Pit-1-REN) and assayed by EMSA. In the presence of kidney cortex cell nuclear extracts, several bands were observed. A 25- to 100-fold molar excess of unlabelled homologous probe (Pit-1-REN) attenuated the signal corresponding to the two upper complexes, thus indicating the specificity of these interactions (Figure 5a). Mutations of the Pit-1-REN oligonucleotide to Pit-1- Δ 1 eliminated its ability to compete for these complexes formation, showing that the motif TAATAAATCAG of the Pit-1 consensus sequence is necessary for binding. EMSAs performed with chorionic cell nuclear extracts showed a different migration pattern, demonstrating that distinct specific complexes are shifted with labelled Pit-1-REN probe (Figure 5b). To characterize further the *trans*-acting factors extracted from chorionic and kidney cortex cells, competition experiments were performed with consensus Pit-1 sites of both the rat growth hormone gene (PIT-1-GH) [29] and the rat Pit-1 gene (PIT-1-RAT) [30]. Our results demonstrate that Pit-1-

Figure 5 Gel mobility shift analysis of human kidney cortex cell nuclear extracts (a) or human chorionic cell nuclear extracts (b) with the human renin promoter (Pit-1-REN: –82 AGGGTAATAAATCAGGGCAGAG –61)

A double-stranded labelled oligonucleotide containing the renin Pit-1-like site was used as a probe (Pit-1-REN). Competitions were performed with a range of 25- to 100-fold molar excess (a) or with 100-fold molar excess (b) of homologous DNA (Pit-1-REN), mutated oligonucleotide Pit-1- Δ 1 (–82 GGGTAATgggcCgGGCAGAG –61), or with the (–12 to +7) region of the rat growth hormone gene containing the Pit-1 motif (Pit-1-GH: –12 TCCGTATACATTTATTCATGGCTGGA +7) and the (–65 to –33) positive 5' rat Pit-1 gene autoregulatory site (Pit-1-RAT: –65 AACTATTAACATGTATAAATGGATTTCCTCAGAG –33). The specific DNA/protein complexes are indicated by arrows. n.s. represents the non-specific interactions.

Figure 6 Gel mobility shift analysis of human kidney cortex cell nuclear extracts with Pit-1-GH (–12 TCCGTATACATTTATTCATGGCTGGA +7) (a) or with Pit-1-RAT (–65 AACTATTAACATGTATAAATGGATTTCCTCAGAG –33) (b) as a probe

Competitions were performed with a 50- and 100-fold molar excess of Pit-1-GH, Pit-1- Δ 1 (–82 GGGTAATgggcCgGGCAGAG –61), Pit-1-RAT, and Pit-1-REN (–82 AGGGTAATAAATCAGGGCAGAG –61) for both Figures. The specific DNA/protein complexes are indicated by arrows. n.s. represents the non-specific interactions.

RAT and Pit-1-GH are competitors for the binding of the Pit-1-REN probe to kidney cortex cell nuclear extracts (Figure 5a) but do not compete for the binding to chorionic cell nuclear extracts (Figure 5b). Furthermore, no complex was detected when using chorionic cell nuclear extracts with PIT-1-RAT and PIT-1-GH (results not shown), whereas these probes bind kidney cortex cell nuclear extracts. Pit-1-GH exhibits two specific

Figure 7 Gel mobility shift analysis of GH₃B6 nuclear extracts with Pit-1-REN (–82 AGGGTAATAAATCAGGGCAGAG –61), Pit-1-GH (–12 TCCGTATACATTTATTCATGGCTGGA +7) and Pit-1-RAT (–65 AACTATTAACATGTATAAATGGATTTCCTCAGAG –33)

Competitions were performed with a 100-fold molar excess of Pit-1-REN, Pit-1-Δ1 (–82 GGGTAATgggcCcGGGCAGAG –61), Pit-1-GH and Pit-1-RAT oligonucleotides (see the Materials and methods section).

complexes whose signal is attenuated by adding a molar excess of unlabelled consensus Pit-1 binding site-containing oligonucleotides, Pit-1-GH and Pit-1-RAT but not by Pit-1-Δ1 (Figure 6a). The same results were obtained when labelling Pit-1-RAT (Figure 6b). A 50- to 200-fold molar excess of unlabelled Pit-1-REN also competed, although less efficiently, for the binding of kidney cortex cell nuclear extracts to Pit-1-GH and Pit-1-RAT, thereby suggesting that the same or a closely related factor strongly binds to Pit-1-GH and Pit-1-RAT and binds to Pit-1-REN with a lower affinity. Altogether, these EMSA results suggest that distinct factors are present in both cell types and specifically bind the renin Pit-1 motif.

Nevertheless, to discriminate further whether Pit-1 binds the renin Pit-1 site, EMSAs were performed with GH₃B6 cell nuclear extracts containing large amounts of Pit-1 [32]. In the presence of GH₃B6 nuclear extracts, no complex was observed with the Pit-1-REN probe (Figure 7). On the other hand, two major specific complexes are formed with the PIT-1-GH and the PIT-1-RAT probes (Figure 7). These complexes could correspond to the two previously observed complexes on the 1P Pit-1 site of the human prolactin gene (C1, the less retarded one and C2 migrating slower) [32,34], respectively corresponding to monomer of Pit-1 and heterodimer of Pit-1 and Oct-1. Whereas competition with a 100-fold molar excess of PIT-1-REN and PIT-1-Δ1 had no effect on the ability of PIT-1-GH and PIT-1-RAT to establish complexes with GH₃B6 cell nuclear extracts, a 100-fold molar excess of PIT-1-GH and PIT-1-RAT reduced the signal corresponding to these two complexes. Taken together, these results demonstrate that Pit-1 does not interact with the Pit-1 motif of the human renin gene.

DISCUSSION

The aims of this study were (1) to study whether the CRE (–226 to –219) could confer full cAMP responsiveness to cAMP-induced renin gene expression in renin-producing cells, (2) to determine whether the same transcription factor from chorionic

and renal origin is involved in binding to the human renin CRE, and (3) to identify the proteins binding the *cis*-acting sites involved in transcriptional regulation of the human renin gene by cAMP. By DNase I footprint analysis, using human chorionic cell nuclear extracts, we have previously shown a footprint corresponding to the CRE region of the human renin promoter, whereas previous studies showed that the first 100 bp of the human renin 5'-flanking region contained the major cAMP responsive region although there was no significant homology with a CRE consensus sequence [11]. Therefore, the CRE role in regulating the induction of the human renin gene expression by cAMP was tested by transient DNA transfections of various renin/luciferase constructs in secondary cultures of renin-producing chorionic cells. In this system, 10 μM forskolin produced a highly reproducible 2.4-fold increase in expression of a plasmid containing a fragment (–582 to +16) of the human renin gene promoter region (pGL₂582+) similar to that observed previously by us [10] and others [11]. The same level of stimulation of luciferase activity was observed using forskolin and PMA compared with forskolin alone (F. Pinet, unpublished work), thus we characterized the CRE using stimulation by forskolin alone. To assess the role of this CRE, site-directed mutagenesis of the renin CRE was performed by replacing the consensus CGTCA with *tcgga*. cAMP-induced expression of the reporter gene was still observed, but because it was of a lower magnitude (1.7-fold), it was apparent that the CRE motif was necessary, but not sufficient, to confer full cAMP stimulation.

As chorionic cells are extra-renal cells, it was important to verify whether similar or different transcription factors present in the kidney, which is the main site of renin synthesis, are involved in binding to the CRE. Electromobility shift experiments were performed to identify the protein factor binding to the renin CRE region and to study the specificity of these interactions. Nuclear extracts from renin-enriched human infarcted kidney cortex were therefore prepared and although they contained a heterogeneous cell population, the migration pattern of the DNA/protein interactions (Figure 2) were shown to be similar to those observed with human chorionic cell nuclear extracts [10]. On the basis of competition experiments, these results showed that the human renin CRE seemed to be a binding site for CREB. However, in addition to CREB, likely candidates for components of the binding complex are ATF-1 or CREM. Only the anti-CREB serum produced supershifted DNA/protein complexes: the two supershifted complexes suggest that CREB binds as a monomer and homodimer to the human renin CRE. Altogether these results clearly establish that CREB monomers and homodimers extracted from both chorionic and renin-rich human kidney cortex nuclei bind the renin CRE which is partially responsible for cAMP induction of the human renin gene transcription.

The ability of the Pit-1-binding sequence (–77 to –67) to confer cAMP induction of the renin gene in renin-producing chorionic cells was therefore investigated. The pGL₂145+ plasmid, in which the CRE has been deleted but which contains the Pit-1 site, is still activated by cAMP (1.7-fold) to a level similar to that in which the CRE is mutated. The direct involvement of the Pit-1 site in cAMP stimulation was implicated when site-directed mutagenesis of this site to a Δ1 site was found to result in a complete abolition of cAMP responsiveness with the pGL₂145Δ1 plasmid (Figure 1). As shown by the double mutation of these regions, the results demonstrate that the full activation of transcription is due to interactions of *trans*-activating factors on these two binding sequences, the CRE and the Pit-1 motif.

In an attempt to characterize the transcription factors binding to the human renin Pit-1-binding site, DNase I footprinting was

performed showing the binding of protein factors extracted from kidney cortex cells covering the Pit-1 site of the human renin promoter (−77 to −63). Based on competition experiments, EMSAs demonstrated that distinct nuclear proteins extracted from both kidney cortex cells and chorionic cells specifically bind the renin Pit-1 element. To further demonstrate whether Pit-1 binds the human renin Pit-1 site, EMSAs were performed with GH₃B6 nuclear extracts. GH₃B6 is a rat pituitary cell line exhibiting high Pit-1 levels [32] which has been shown to bind the 1P responsive element (−65 to −38) region of the human prolactin gene [35]. Our results demonstrate that Pit-1 present in GH₃B6 nuclear extracts specifically binds the Pit-1-GH probe and the Pit-1-RAT probe but does not establish interactions with the human Pit-1-REN probe. These EMSA results demonstrate that the human renin Pit-1 site is not a binding site for Pit-1 but binds tissue-specific factors expressed in the chorion and the kidney. This extends previous data on the mouse REN-1C gene where Tamura et al. [36] demonstrated that a nuclear factor present in human embryonal kidney 293 cells, a Pit-1-deficient cell line [37], interacts with the RP-2 element bearing high sequence similarity to the Pit-1-binding site.

We would like to emphasize that the results of transfection experiments clearly demonstrate that cAMP activation of the human renin gene transcription occurs partially via the CRE (−226 to −219) in human renin-producing cells. In contrast, Gilbert et al. [13] showed that, in rat pituitary GC cells, the CRE deletion had no effect on cAMP responsiveness of the human renin gene. It seems therefore apparent that the CRE is required for the forskolin response in renin-producing chorionic cells but not in pituitary cells because of different interactions at the Pit-1 site. Effectively, in GC cells, Pit-1-binding sites of the human prolactin proximal region are able to confer cAMP responsiveness to an heterologous thymidine kinase promoter (25-fold) [38]. In addition, Okimura et al. [39] demonstrated that transcriptional regulation in response to cAMP occurs in the absence of Pit-1 phosphorylation, thereby raising the hypothesis that cAMP effects on the prolactin promoter may involve another protein that either binds to Pit-1-binding sites or that interacts with Pit-1.

Taken together, these results strongly suggest that the CRE is necessary for full cAMP activation of the human renin gene transcription in renin-expressing chorionic cells. In addition, the Pit-1 response element is also involved in cAMP induction of the human renin gene expression. EMSA demonstrated that CREB extracted from chorionic cells and kidney cortex cells effectively binds the renin CRE and that factors from chorionic and renal origin, different from Pit-1, bind the Pit-1 site of the human renin gene proximal promoter. Overall, the present finding suggests that renin-producing chorionic cells and kidney cortex cells express tissue-specific factors which bind to the Pit-1 site of the human renin gene promoter. Cloning and characterization of this homologous factor present in chorionic and kidney cortex cells should provide important data for understanding *in vivo* human renin gene regulation.

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