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IDENTIFICATION OF CYCLIC AMP RESPONSE ELEMENT IN THE HUMAN RENIN GENE

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SUMMARY: **In order to identify the mechanism by which cyclic AMP stimulates expression of the human renin gene (*REN*), the effect of forskolin was tested in transient expression analyses of *REN* 5'-flanking DNA-chloramphenicol acetyltransferase (CAT) reporter gene constructs in secondary cultures of human chorio-decidual cells, a major site of renin synthesis. Forskolin induced a mean 5-fold stimulation which was localized to DNA in the region –249 to –162 with respect to the transcription start site (+1). Such DNA also mediated a response to forskolin in heterologous (HSV thymidine kinase) promoter constructs. Strong cAMP-response element (CRE) homology at –222 to –218 resembled the target for members of the CRE binding protein (CREB) family. Gel shift assays demonstrated similarly migrating nucleoprotein complexes for oligonucleotides containing the putative *REN* CRE as for a canonical CRE, in chorio-decidual, JEG-3 and HeLa nuclear extracts. Mutation of residues critical for CREB attachment reduced binding. In conclusion, a CRE was identified at –222 to –218 that appears critical for cAMP-induced human renin gene transcription.**

Renin (EC 3.4.23.15) is secreted into the bloodstream by the juxtaglomerular (JG) cells of the kidney in response to factors that increase second messenger cAMP in the cell [1, 2] and is the rate-limiting enzyme in generation of angiotensin II, an octapeptide having a crucial role in maintainence of extracellular fluid volume homeostasis and blood pressure, as well as stimulation of vascular smooth muscle cell growth [3]. Blockade of the renin-angiotensin system can, moreover, assist in the treatment of hypertension, congestive heart failure, and other cardiovascular and renal diseases [4]. The control of renin synthesis and secretion is thus of considerable importance. Although the sequence of the human renin gene (*REN*) and its 5'-flanking DNA was determined nearly a decade ago [5, 6], progress in deciphering *cis*-acting

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sequences involved in the control of *REN* expression has been hampered by the lack of availability of an immortalized renin-producing cell line for transient expression and DNA-protein binding studies. Decidua, which lines the human uterus, has high concentrations of renin mRNA [7] and cultured chorio-decidual cells respond to treatment with the adenylate cyclase agonist, forskolin, with increases in prorenin mRNA content and secretion [8] that are similar to the increases elicited in the kidney in response to cyclic adenosine monophosphate (cAMP) [9]. The results suggest the existence of DNA element(s) capable of mediating an increase in renin transcription in response to elevation in cAMP inside renin-synthesizing cells.

The purpose of the present investigation was to identify the forskolin response element in the human renin gene. To do this we performed transient expression analyses, in human chorio-decidual cells, of a series of constructs containing varying lengths of *REN* 5'-flanking DNA up to nucleotide –2595, fused to the chloramphenicol acetyltransferase (CAT) reporter gene. In addition, gel retardation analyses were conducted to determine the binding, to forskolin-responsive DNA, of factors present in nuclear extracts of not only cultured chorio-decidual cells, but also of cells known to contain cAMP response element (CRE) binding protein (CREB).

MATERIALS AND METHODS

Cell culture: Chorion laeve was dissected away from placenta obtained from vaginal deliveries and washed twice in Dulbecco's phosphate-buffered saline (DPBS). The maternal side of the chorion, to which some of the decidua is attached, was scraped, minced and digested in 0.1% collagenase and 0.1% DNAse I in DPBS for 90 min at 37°C. Suspensions were centrifuged and washed twice in DPBS. Aggregates of cells released by digestion were broken up by trituration through a 20 G hypodermic needle. Cells were plated at high density in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS) and antibiotics. Typically one chorion yielded thirty 100 mm dishes of cultured cells.

Plasmid constructs: Subcloning of *REN* 5'-flanking DNA and production of most of the CAT-fusion constructs tested have been described previously [10]. Additional constructs with finer 5'-deletions of *REN* nucleotides in the region –452 to –145 (numbering relative to the transcription start site, +1) were produced by digestion of plasmid pREN465CAT [10] with *Sac*I and *Psp*AI, followed by exonuclease III and mung bean nuclease treatment and religation. Deletion end-points were determined by dideoxy chain-termination sequencing. All plasmids were purified by centrifugation on two cesium chloride density gradients prior to transfection and purity was checked by agarose gel electrophoresis.

Transfection and CAT assays: Secondary cultures of ~6 x 10^6 chorio-decidual cells, harvested between days 10-16 of growth, which is the time of peak prorenin production [7], were co-transfected with 50 μg of each construct and 5 μg pCH110 (Pharmacia LKB, Piscataway, NJ), using an Invitrogen (San Diego, CA) Electroporator at 1000 F and 210 V, with a 0.4 cm electrode gap, in a total volume of 0.4 ml of DMEM/Ham's F12 medium (DMEM/F12; 1:1) containing 10% (vol/vol) NCS at room temperature. After electroporation cells were plated on to duplicate 65 mm culture dishes in DMEM/F12, 10% NCS containing either 10 mM forskolin or an equal volume (0.1%) of dimethyl sulfoxide vehicle. Cells were harvested 40 h later by scraping, were washed twice in DPBS and resuspended in 200 μl of a solution containing 0.25 M Tris-HCl, pH 7.9, and 0.2% Triton X-100. Cells were then lysed by 3 cycles of freezing and thawing. An aliquot of extract was heated at 65°C for 10 min to destroy endogenous acetyl coenzyme A-consuming activity and CAT activity was determined by a non-chromatographic method involving [^{14}C]-labeled acetyl CoA [11]. Each lysate was also assayed for β-galactosidase activity, using the method of Miller [12], except that 5 mM chlorophenol-β-D-galactoside was used as a substrate, and absorbance was read at 574 nm. Results were normalized by dividing CAT activity (cpm) by β-galactosidase activity (OD units) and were expressed relative to value for the promoterless control plasmid. Statistical analyses were performed by Student's *t*-test.

Mobility shift assays:Crude nuclear extracts from similarly-maintained 10-16 day cultures of chorio-decidual cells were prepared by the isotonic miniprep method [13]. The following oligonucleotides, corresponding to the sense and antisense strands of the –263 to –203 segment of *REN* 5'-flanking DNA (with CRE homology shown in bold), were synthesized: 5'-GATCTCCAGGGGTCACAGGGCCAAGCCAGATAGAGGGCTGC**TAGCGTCA**CTGGACA CAAGATTG-3', 3'-AGGTCCCCAGTGTCCCGGTTCGGTCTATCTCCCGACG**ATCGCAGT** GACCTGTGTTCTAACCTAG-5', and in their hybridized, double-stranded form were termed *REN*64. Other oligonucleotides were synthesized to correspond to *REN* nucleotides –239 to –204, either as wild-type (*REN*36), or with mutations (shown in lower case) within the CRE homology, as follows: 5'-AGATAGAGGGCTGC**aAGgGTCt**CTGGACACAAGATT-3', 3'-TCTATCTCCC GACG**tTCcCAGa**GACCTGTGTTCTAA-5' (*REN*36M1) and: 5'-GATCAGATAGAGGGCTGC **TAttcggA**CTGGACACAAGATT-3', 3'-TCTATCTCCCGACG**ATaagccT**GACCTGTGTTCTA ACTAG-5'(*REN*36M2). In addition, the following 28mer containing the rat somatostatin CRE (*SOM*CRE) was synthesized: 5'-GCCTCCTTGGC**TGACGTCA**GAGAGAGAG-3', 3'-CGGAGG AACCG**ACTGCAGT**CTCTCTCTC-5' [14]. One strand of each oligonucleotide pair was end-labeled with ^{32}P, using [γ-^{32}P]ATP (>5000 Ci/mmol; ICN Biomedicals, Irvine, CA) and T4 polynucleotide kinase (Promega, Madison, WI), and then annealed to a 2- to 3-fold excess of the complementary strand in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA by slow cooling from 70°C to room temperature. Purification involved electrophoresis on a 12% polyacrylamide gel, elution into 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 and ethanol precipitation at –20°C. Binding conditions for the mobility shift assays were as described previously [15], with slight modifications, as follows: 10 mM HEPES-KOH, pH 7.8, 1 mM EDTA, 5 mM $MgCl_2$ 100 mM KCl, 6% (vol/vol) glycerol, 2% Ficoll 400, 2 µg bovine serum albumin, 40 µg/ml poly(dI-dC).poly(dI-dC), and 10 µg of crude nuclear extract, in a total volume of 25 µl. The mixture was preincubated for 20 min at 25°C before initiation of binding by addition of 0.1 pmol of labeled double-stranded oligonucleotide. Incubation was then continued for a further 20 min at 25°C, before cooling on ice after addition of glycerol/loading dye mixture. Where appropriate, specific competitor double-stranded oligonucleotides were added, in at least 50-fold molar excess, at the preincubation step. Samples were then electrophoresed at 10 V/cm on a 5% non-denaturing polyacrylamide gel (29 : 1, acrylamide : *bis*-acrylamide) containing 2.5% (vol/vol) glycerol in 0.5 x TBE (0.045 M Tris borate, 0.1 mM EDTA, pH 8.3 at 25°C) at 4°C. Gels were dried overnight at 25°C between two layers of cellulose acetate film before autoradiography at –80°C.

RESULTS

Effect of forskolin on reporter gene expression from homologous promoter: Fig. 1 shows the effect of forskolin on CAT activity for a range of homologous *REN* promoter constructs containing different lengths of *REN* 5'-flanking DNA. The promoterless CAT vector, pPCAT [16] showed no increase in normalized CAT expression in the presence of 10 mM forskolin, suggesting that there were no nonspecific transcriptional effects originating in the plasmid sequences. However, for constructs containing the –2595 to +13 *REN* 5'-flanking DNA, forskolin induced an increase in CAT activity that was, on average, 5.5-fold that obtained with vehicle alone. Deletion constructs with 5'-end points at –1300, –895, –453, –375, –365, -274, and -249 gave a similar, average 5-fold induction of CAT activity in response to forskolin. However, those with 5'-end points at –162 and –145 showed CAT activities that were only 1.6- and 1.7-fold, respectively, above basal values. All of the constructs with 5'-end points from between –2595 and –249 had forskolin induction values that were significantly different from both the promoterless control (*P* values 0.0049 to 0.0000023) and the construct deleted to –162 (*P* values 0.0031 to 0.00033). For the two shorter deletions, difference from promoterless control was less significant (*P* = 0.00094 for pREN175CAT; *P* = 0.062 for pREN162CAT). Taken together, these data suggested that sequences in the –249 to –162 region of *REN* 5'-flanking DNA were necessary for

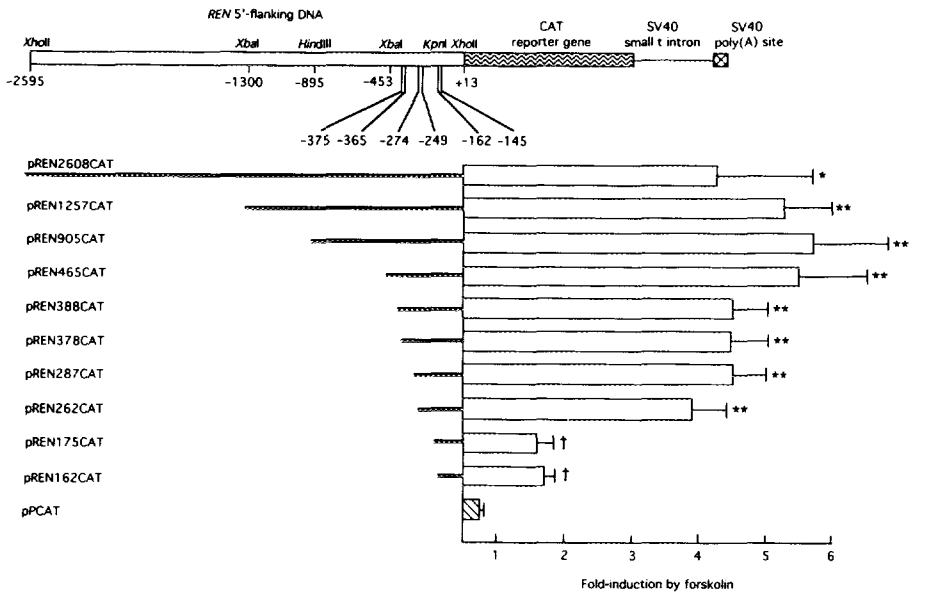

Fig. 1. Transient expression analyses in chorio-decidual cells of various fragments of the –2595 to +13 region of human renin 5'-flanking DNA which contained the homologous *REN* promoter. Shown is fold stimulation of normalized CAT activities (mean ± S.E. of 4-7 different transfections) for each construct for cells treated with 10 mM forskolin for 40 h post-transfection. The 5' deletion endpoints are indicated in the diagram at the top and the *REN* 5'-flanking DNA fragments tested are shown as solid bars to the left of the histogram of CAT results. pPCAT was the promoterless control. All DNA in the constructs had a 5'→3' orientation. ** $P<0.001$, * $P<0.005$ (compared with results for pPCAT and pREN175CAT), † $P<0.05$ (compared with results for pPCAT).

mediation of the full response to forskolin, and that the –162 to +13 sequences retained only minimal cAMP inducibility.

Effect of forskokin on reporter gene expression from heterologous promoter: Various segments of the –2595 to –145 *REN* DNA were fused to the heterologous *tk* promoter and similarly assayed for responsiveness to forskolin. Results, depicted in Fig. 2, show that CAT activity for the *tk* promoter alone was slightly, but not significantly ($P = 0.1$) increased, by 1.6 fold, by forskolin. Constructs containing *REN* 5'-flanking DNA sequences –2595 to –1300, –1300 to –895 and –895 to –453 showed CAT activites after treatment with forskolin that were similar to basal values and that seen for ptkCAT. In contrast, sequences containing the –453 to –145 region of DNA mediated an increase in transcription in response to forskolin. Moreover, all constructs lacking this segment were unable to mediate a response to forskolin. Thus, the –452 to –145 DNA was sufficient to confer cAMP inducibility on the *tk* promoter. The pattern observed was furthermore consistent with data in Fig. 1, thus supporting the localization of the responsive element to nucleotides –249 to –162. Furthermore, at least for DNA extending to –2595, the –453 to –145 segment was not only necessary for the response, but was also sufficient.

Protein-DNA interactions at a putative CRE: Inspection of the *REN* 5'-flanking sequence in the region which mediated the response to forskolin revealed, at nucleotides –225 to –218 inclusive, strong homology to the canonical cAMP responsive element [14]. No other homologies to the CRE, or closely related AP-1 binding site, were found in this region. In order to provide evidence

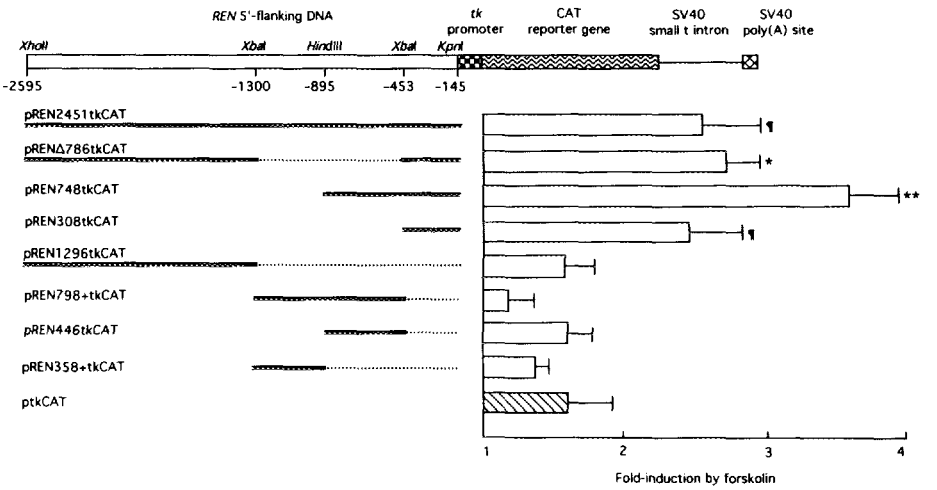

Fig. 2. Transient expression analyses in chorio-decidual cells, of constructs containing the –2595 to –145 region of human renin 5'-flanking DNA linked to a heterologous promoter (*tk*). Shown is fold stimulation of normalized CAT activities (mean ± S.E. of 3-4 separate transfection experiments) for each construct for cells treated with 10 mM forskolin. The position and nature of restriction sites in *REN* 5'-flanking DNA that were used in making the constructs are indicated in the diagram at the top. The *REN* 5'-flanking DNA fragments tested are shown as solid bars to the left of the histogram corresponding to CAT results for that fragment. ptkCAT was the promoterless control. $**P < 0.01$, $*P < 0.05$, $¶P < 0.1$ (compared with results for ptkCAT).

in support of a role for the putative CRE in the response, we tested the ability of DNA encompassing this sequence, and of specific mutants, to specifically bind nuclear protein(s). Double-stranded oligonucleotide corresponding to positions –263 to –203 (*REN*64) of *REN* 5'-flanking DNA was tested, as were the mutated sequences, *REN*36M1 and *REN*36M2, and the consensus CRE present in the somatostatin promoter (*SOM*CRE). These served, respectively, as negative and positive controls for CREB binding in experiments to identify binding proteins in nuclear extracts of chorio-decidual cells. Fig. 3 shows the results of a mobility shift assay which revealed that both the *REN*64 and *SOM*CRE oligonucleotides formed a complex of similar mobility (band 2). *REN*64 was also able to form three other specific complexes, labeled 3, 4 and 5, not formed with the *SOM*CRE. Unlabelled *REN*64 competed each band, as expected for binding that was specific. With *REN*64 as the labeled species, *SOM*CRE and the *REN*36M1 and *REN*36M2 mutants were unable to compete band 2 binding. However, with labeled *SOM*CRE, *REN*64, but not the mutants, showed some competition for band 2. A shorter oligonucleotide, *REN*36 (nucleotides –239 to –204), competed bands 4 and 5, and this competition was unchanged by the 36mer mutants. The only specific band seen with *REN*36 was band 5, which was competed efficiently by all renin oligonucleotides, except *REN*36M1. In all lanes, there was an additional band migrating between bands 3 and 4. Since this did not self-compete it was judged to be nonspecific. Labeled *REN*36M1 showed no retarded bands, apart from this nonspecific band, suggesting that the mutation had disrupted binding. Labelled *REN*36M2 retained binding of band 4 and showed the same competition pattern as seen for the wild-type sequence, indicating that this mutation did not alter binding of the *REN* sequences in this region. Since band 4 binding was

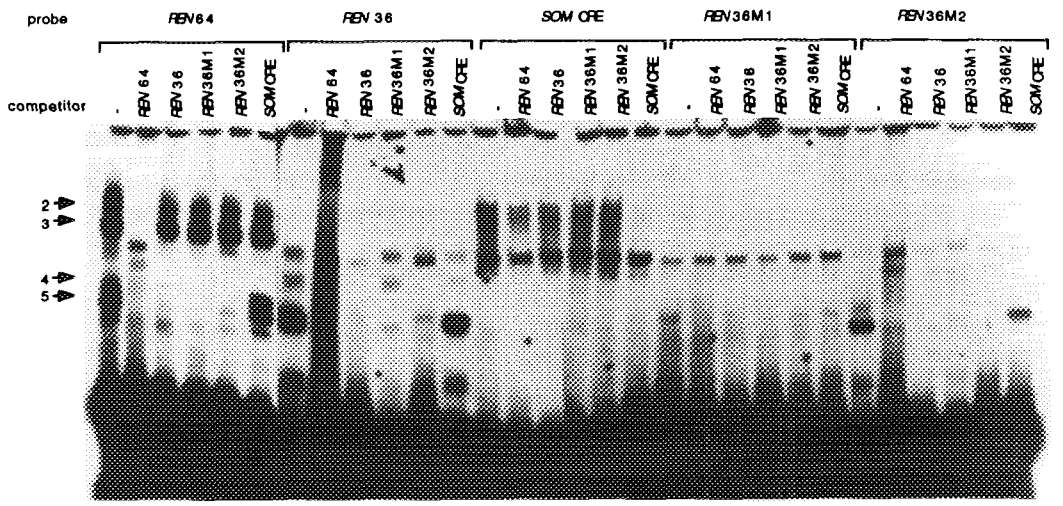

Fig. 3. Mobility shift analyses involving reciprocal probe and competition assay, in which 10 μg nuclear protein extract from chorio-decidual cells was mixed with 2 ng ^{32}P-labeled *REN*64 or *REN*36 oligonucleotide containing putative CRE in the *REN* promoter, canonical *SOM*CRE, or oligonucleotides with mutations in the putative *REN* CRE (*REN*36M1 and *REN*36M2). Specific, unlabeled competitors were added in 50-fold molar excess at the preincubation step; l, no competitor added. Specific bands are numbered 2-5. The band that migrated slightly faster than band 3 was non-specific binding. Note that band 2 was seen with both *REN*64 and *SOM*CRE probes.

weak and easily competed it probably required factor(s) that bound in that portion of the larger oligonucleotide not present in the 36mer, in order to stabilize its own binding. Band 4 did not moreover appear to be binding at the putative CRE. Since mutation M1, but not M2, obliterated band 5, the site of binding may immediately flank the putative CRE. Because band 2 was seen for both *REN*64 and *SOM*CRE, which had only the CRE in common, and was competed, it most likely arose as a result of the same protein that attached to the CRE. Since *REN*36 did not form a band 2 complex, band 2 may need to be stabilized by other protein(s) which bound to it. For band 3, which was also seen for *REN*64 but not *REN*36, binding characteristics suggested an assignment to –263 to –240.

These results prompted further studies to compare the oligonucleotide binding pattern with that for extracts of cells rich in CREB, viz. JEG-3 [17] and HeLa [18]. All bands were seen, but the intensity varied between cell types, with bands 2 and 3 being strongest in HeLa, followed by JEG-3, and bands 3 and 4 being stronger in chorio-decidual cells (Fig. 4). *REN*36M2 was unable to compete bands 2 or 3 in chorio-decidual or HeLa extracts, but showed weak competition for band 2 in JEG-3. In JEG-3 the *SOM*CRE competed completely all bands to which labeled *REN*64 bound. With labeled *SOM*CRE, the major complex was band 2. An extra band, band 1, was seen for this oligonucleotide in JEG-3 and HeLa extracts. Both bands 1 and 2 were specific, being competed with a 100-fold molar excess of unlabeled SOMCRE. Unlabeled *REN*64 strongly competed *SOM*CRE bands 1 and 2 in JEG-3 extracts, and less so for HeLa. The fact that all three nuclear extracts formed band 2 with *both SOM*CRE and *REN*64, and partially competed each other, is consistent with band 2 having arisen from binding of a similar or identical protein in each cell type. The differences observed in degree of competition between functional CREs from

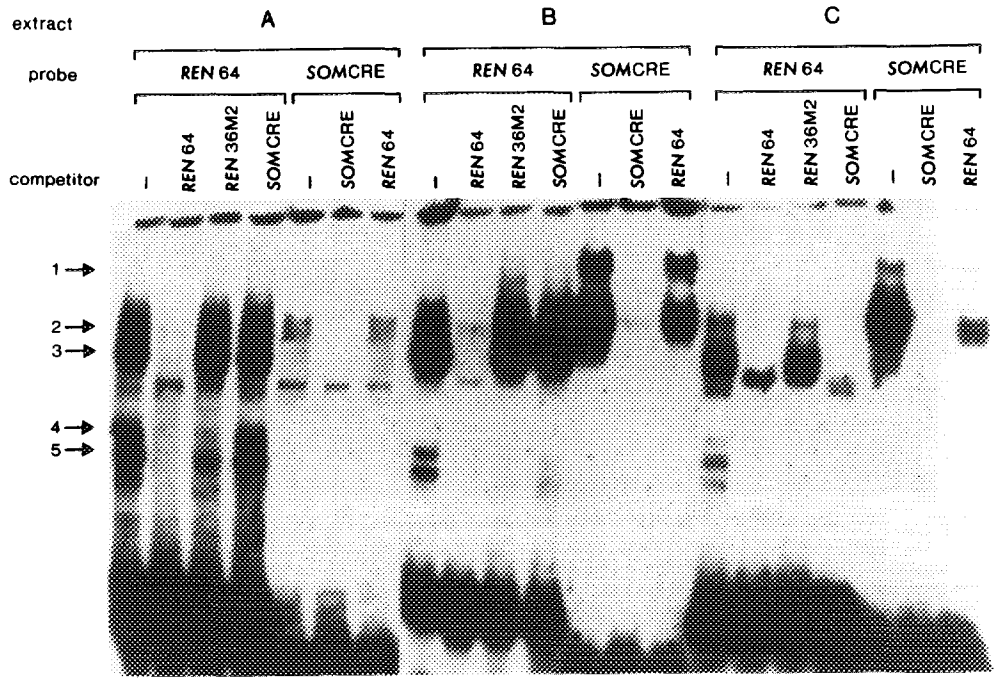

Fig. 4. Comparison of the *REN*64 and *SOM*CRE binding for nuclear extracts of different cells. Probe DNA, as indicated, was incubated with nuclear extracts of (A) chorio-decidual, (B) HeLa and (C) JEG-3 cells and competitor oligonucleotides were added in 100-fold molar excess, except for lanes marked -, where no competitor was added. Specific bands are labeled 1-5, bands 2-5 are equivalent to those in Fig. 3 and were formed with ^{32}P-labeled *REN*64 in all nuclear extracts. Band 2 was the only complex formed, for both *REN*64 and *SOM*CRE, in each of the nuclear extracts tested.

different promoters is, moreover, well documented [19-23], and results from effects of flanking bases on the strength of binding to the core site.

DISCUSSION

The present results have localized an important forskolin-responsive DNA site in the human renin promoter, residing within an 87 bp segment of DNA extending from nucleotides –249 to –162. We also observed a weak response for the –145 to +13 DNA. Previous, limited studies by others have only ever noted the weak effect and involved examination of the –149 to +13 [24] and –100 to +11 [8] segment of *REN* 5'-flanking DNA. In one of these studies [24] the constructs used all contained a heterologous (*tk*) promoter, and JEG-cells, that do not express the renin gene [25]. This proximal segment of *REN* 5'-flanking DNA does not, moreover, contain the well characterized 8-base palindrome [14] conserved in many genes whose expression is regulated by cAMP. The other study besides ours involved human placental membrane cell cultures, and tested just the –584 to +11 DNA [8]. In both this and the JEG-3 cell study [24], non-specific effects on CAT activity arising from the vector sequences could not be be distinguished, since results for 5'*REN*-CAT constructs were not compared with those for a promoterless control. It has,

moreover, been shown that some vector backbones harbor elements which respond to elevated cAMP [26], suggesting that the promoterless control, pPCAT, must be tested in order to account for any cryptic effects arising from sequences in the the vector used. In addition, the placental cell study was unable to show induction by forskolin of the –584 to –146 *REN* 5'-flanking DNA in constructs containing a heterologous (*tk*) promoter, nor of the –584 to –100 DNA in homologous promoter constructs. Unlike our experimental design, however, they used an 8 h treatment with forskolin, whereas we used 40 h, even though, like us [7], they demonstrated that prorenin in the medium reached peak levels at 40 h [8]. It is possible that differences in exposure time, other experimental conditions, or failure of this (and the other) study to correct for transfection efficiency by not cotransfecting with a plasmid like pCH110 may account for the different results.

Thus, in our experiments involving comparison with a promoterless control, we have demonstrated that, in relation to the –275 to +13 DNA, the element necessary for full induction by forskolin of the *REN* promoter is located between nucleotides –249 and –162. The –453 to –145 *REN* 5'-DNA, that contains the –249 to –162 region, was also sufficient for induction by forskolin of the heterologous *tk* promoter. Moreover, we found no evidence for other functional CREs in *REN* 5'-flanking DNA, at least up to nucleotide –2595. Since the *REN* 5'-flanking sequences involved in the forskolin response were able to function with another promoter, the response does not rely on any unique property exclusive to the *REN* promoter.

Inspection of the –249 to –162 DNA revealed the sequence TAGCGTCA at –225 to –218, which had strong identity (mismatches underlined) with the consensus CRE [14]. No other homologies to the CRE or the closely related AP-1 binding site (TGACTCA) were present in the –249 to –162 region. The homology observed contained, moreover, an intact pentameric half site, the minimum sequence necessary for function as a CRE [27]. The putative CRE at –225 to –218 fits the description of weak, or asymmetric, binding sites [27], which, unlike the canonical, symmetrical or strong binding site, contains only one copy of the overlapping dyad repeat pentamer, CGTCA, proposed as a monomer binding site [27]. Cyclic AMP stimulation enhances CREB binding to the 'weaker' CRE, but not the 'stronger' CRE, which thus potentially provides another regulatory step in cAMP control of CRE binding and, hence, activation of transcription. Another strong homology to the consensus CRE, at –594 to –587, [28] did not appear functional in our hands in constructs which contained this DNA, since no increase in CAT activity was seen with –1300 to –453 and –895 to –453 *REN* DNA in heterologous *tk* promoter constructs, nor for relevant fragments containing this sequence in homologous *REN* promoter constructs. Such inactivity in our hands was not altogether unexpected, as the sequence TGACCTCA, which although differing from the consensus by only one base (consensus CRE = TGACGTCA), does not contain a complete copy of the pentamer half-site sequence and would therefore not be expected to bind members of the CREB/ATF-family.

Evidence that the CRE homology was indeed a functional cAMP response element came from our findings that (i) a complex, with identical mobility to that produced by the somatostatin consensus CRE oligonucleotide, was formed using the oligonucleotide *REN*64 (–263 to –203), (ii) the latter *REN* oligonucleotide was competed by an oligonucleotide containing the somatostatin consensus CRE for formation of the putative CREB/DNA complex, and (iii) mutant

REN oligonucleotides, differing in residues that have been shown in studies of CREs in other genes to diminish binding, led to a reduction in binding and an inability to compete. Our finding that competition between the *REN*64 and *SOM*CRE oligonucleotides was not absolute has been a common finding in analogous studies of CREs within the differing sequence contexts of other cAMP-responsive promoters [19-23]. Such weak competition may be explained by the observation that the immediately adjacent (5 or 6 base pairs) flanking sequences can be important determinants for CREB binding and also influence the strength of transcriptional responses that result [20]. Furthermore, our finding of similar comigrating complexes from nuclear extracts prepared from three distinct cell lineages implies that the chorio-decidual factor is not a tissue-specific CREB/ATF family member. It should also be pointed out that the present findings may have little or no relevance to the control by cAMP of the renin promoter of other species, such as the mouse, in which DNA insertions and sequence differences may account for dissimilar binding and promoter control [29].

Also present in the –249 to –162 region were, at –232 to –225 and at –198 to –191, two regions in which 7/8 nucleotides matched the AP-2 consensus sequence, 5'-CCC$^{A}/_{C}$N$^{G}/_{C}$$^{G}/_{C}$ $^{G}/_{C}$-3' [30], raising the possibility that AP-2 alone, or in cooperation with CREB, could contribute to any effect of cAMP that involves this DNA. In the human proenkephalin promoter, an AP-2 site situated 5 bp downstream from the ENKCRE-2 site in the cAMP and phorbol ester inducible promoter has been shown to be necessary for responsiveness to these two agents [31]. Moreover, AP-2 protein protected the CRE half site in DNase I footprints. One of the two AP-2 homologies in *REN*64 is in the –263 to –240 segment that we found was necessary for band 2 formation (being absent from *REN*36, which did not form band 2). Further studies will, however, be required to show whether or not one of the other retarded complexes seen with *REN*64 could involve AP-2. We also noted in the –263 to –203 DNA homologies to binding sites for other factors not involved in cAMP responses. Those whose tissue distribution makes them candidates in binding to this region of *REN* 5'-flanking DNA include the ubiquitous factors NF-1 and Sp1 [30], as well as UBP-1 [32], PPAR [33] and HNF-4 [34].

In conclusion, we have provided evidence for a major cAMP-responsive *cis*-acting element in human renin 5'-flanking DNA whose effect on *REN* promoter activity may involve the binding of CREB to a CRE homology at –222 to –218. Whether the CRE is operative in other tissues, such as kidney, in which renin synthesis is also controlled by cAMP, remains to be determined. This DNA site may make a major contribution to control of human renin gene expression, although since cAMP increases mRNA stability, at least in cultured mouse JG cells [35], other levels of control could also be involved in stimulation of renin synthesis in response to cAMP.

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