Transactivation of the Human Renin Promoter by the Cyclic AMP/Protein Kinase A Pathway Is Mediated by Both cAMP-responsive Element Binding Protein-1 (CREB)-dependent and CREB-independent Mechanisms in Calu-6 Cells

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We examined the DNA sequence and transcription factor requirements for cAMP-induced transactivation of the human renin promoter using Calu-6 cells that express human renin mRNA endogenously. A series of constructs containing 896 base pairs of human renin 5′-flanking DNA fused to the luciferase gene and containing either the native, a consensus, or a nonfunctional cAMP response element (CRE) were used to assess DNA sequence requirements mediating the cAMP response. Expression vectors encoding the CREB-1 transcription factor, a dominant negative mutant form of CREB-1, and the catalytic subunit of protein kinase A (PKA) were used to assess transcription factor requirements mediating the cAMP response. Forskolin treatment alone only caused a 2–3-fold activation of the HREN promoter in Calu-6 cells, but nearly a 10-fold activation in JEG-3 cells, which do not express renin but are highly responsive to cAMP. Gel shift assays revealed the binding of five specific DNA-protein complexes consisting of the ATF-1 and CREB-1 transcription factors, one of which was an ATF-1-CREB-1 heterodimer suggesting the potential for regulation of CREB-1 activity by ATF-1. However, over-expression of CREB-1 did not significantly enhance forskolin-induced human renin transcriptional activity. Transfection of both Calu-6 and JEG-3 cells with a PKA expression vector resulted in a 10-fold induction of human renin transcriptional activity in constructs containing the native or consensus CRE and 5-fold activation in a construct containing a nonfunctional CRE. We confirmed that the PKA response has both a CREB-dependent and CREB-independent component by demonstrating that the PKA response was abolished by co-transfection of a dominant negative mutant form of CREB-1 into cells containing the native or consensus CRE construct but not in cells containing the nonfunctional CRE construct. We therefore conclude that the human renin promoter can be transcriptionally activated in a renin expressing cell line through the cAMP-PKA pathway and is mediated by both a CREB-dependent and CREB-independent mechanism.

Renin, the rate-limiting enzyme in the generation of angiotensin II is synthesized and released from renal juxtaglomerular (JG) cells. In kidney, renin synthesis and release is regulated by numerous physiological factors including sodium chloride sensed by the macula densa, arterial pressure sensed by baroreceptors present within the renal vasculature, sympathetic nerve activity via β-adrenergic receptor signaling mechanisms, and angiotensin II acting on high affinity angiotensin II receptors present on JG cells (reviewed in Ref. 1). Release of norepinephrine from sympathetic nerve terminals stimulates cell surface β-adrenergic receptors on JG cells and causes increased intracellular cyclic AMP (cAMP) through the classical mechanism (1). cAMP has been reported to be a major factor controlling renin synthesis and secretion (2, 3), and numerous reports have documented that cAMP can activate the renin promoter in both renin expressing (4, 5) and non-renin expressing (6, 7) cell lines.

Previous DNase I footprinting studies have revealed that sequences located between –374 and +16 of the human renin (HREN) 5′-flanking DNA region bind trans-acting factors present in choriodecidual cell nuclear extracts (5). These include proteins that bind to Ets, Pit-1, AGE-3, and ARP-1 like sequences. In addition, the sequence TAGCGTCA at position –225 to –218 of the HREN 5′-flanking DNA shares homology to the consensus cAMP-responsive element (CRE) binding site previously characterized in the somatostatin promoter (8). The CRE sequence has been reported to be required for the cAMP stimulation of HREN promoter-reporter constructs in primary cultures of choriodecidual cells (4, 5). In addition, a sequence present in the HREN 5′-flanking DNA which has homology to the pituitary-specific factor Pit-1 and binds members of the POU domain family of transcription factors has been reported to be required for the cAMP induction mediated through the HREN CRE (9, 10).

Although several studies have demonstrated that the CRE sequence is necessary for cAMP-mediated induction of the HREN promoter, the mechanistic details of this induction still
remain largely unexplored. Most investigators have assumed that HREN transcriptional induction caused by cAMP is due to the action of the CRE binding protein-1 (CREB-1) factor. Although purified CREB-1 can specifically bind to the HREN CRE (10), other members of the CRE/activating transcription factor (ATF) family of transcription factors can also bind to CRE-like elements and activate transcription in response to cAMP (11). Moreover, CREB/ATF transcription factors, as members of the leucine-zipper family of transcription factors (bZIP), can not only activate transcription in their homodimeric form but also regulate transcriptional activation by forming heterodimers. For example, ATF-1 has been reported to antagonize the transcriptional effects of CREB-1 on the somatostatin promoter by the formation of an ATF-1-CREB-1 heterodimer (12). Additionally, the fact that mutations in the CRED CRE do not totally abolish HREN promoter activity in response to cAMP (10) suggests that alternative mechanisms may also be active in renin expressing cells. The importance and magnitude of the CRE-independent mechanisms have yet to be explored.

One problem encountered by numerous investigators examining the mechanism of HREN regulation has been the absence of suitable renin expressing cell lines. Indeed numerous attempts to establish permanent cell lines that express HREN endogenously have been largely unsuccessful (13, 14). Although the HREN gene is expressed in many cell types, its expression is particularly high in JG (1) and choriocarcinoma cells (15), but both cell types lose their ability to express renin mRNA and produce renin when placed in culture (14, 15). We have reported that Calu-6 cells that are derived from a human pulmonary carcinoma express HREN mRNA endogenously (16). Moreover, the steady-state level of endogenous HREN mRNA is markedly increased in response to increased intra-cellular cAMP as a result of both transcriptional and post-transcriptional mechanisms (17). The expression of endogenous HREN mRNA in Calu-6 cells, along with the findings that 1) renin is expressed in human fetal lung (18), 2) in human pulmonary tumor tissue (19, 20), and 3) in the lung of transgenic mice and transgenic rats containing genomic HREN transgenes expressed from their own promoters (21, 22), provided evidence to propose that lung is a bona fide site of renin expression in humans and suggested that Calu-6 cells would be a novel tool to examine HREN gene regulation. Herein we use Calu-6 cells to examine the mechanism controlling the transcriptional activation of the HREN promoter by the cAMP pathway.

MATERIALS AND METHODS

Cell Culture—Calu-6 cells were grown in Eagle’s minimal essential media supplemented with 10% fetal bovine serum, sodium pyruvate, and non-essential amino acids as described previously (16). JEG-3 cells were grown in Dulbecco’s modified essential media supplemented with 10% calf serum. Cells were grown to 90% confluency in T75 tissue culture flasks and were split into 60-mm tissue culture plates the day before transfection. The cells were transfected when they reached 80% confluency (24, 25). A cytomegalovirus promoter-β-galactosidase fusion construct was used as an internal control to normalize for differences in transfection efficiency among flasks. All plasmid DNAs were purified by centrifugation on two cesium chloride density gradients prior to transfection. Plasmid DNA concentration was measured by absorbance at 260 nm and was confirmed by gel electrophoresis using known standards.

Transfection and Luciferase Assays—Calu-6 or JEG-3 cells, plated at 2 × 10^6 at 80% confluence, were transfected using calcium-phosphate precipitates containing 7.0 μg of test reporter plasmid DNA, 2.5 μg of either the wtCREB or KCREB expression vector, 2.5 μg of CFPA expression vector, and 0.25 μg of the CMV-β-galactosidase control. pcU19 vector DNA was used as carrier DNA in order to maintain a constant amount of nucleic acid (12 μg) per transfection. Approximately 10–20% of the cells are transfected using this protocol. Forskolin (10 μM) was added 18–24 h prior to performing the luciferase activity assay. Luciferase activity assays were performed using a commercially available kit (Promega, Madison, WI), following the directions recommended by the manufacturer, and activity was measured in a Monolight 2010 automatic luminometer. β-Gal activity was measured using the Galacto-light kit (Tropix, Bedford, MA) following the directions recommended by the manufacturer and as described previously by us (17). All luciferase and β-gal activity assays were performed in duplicate and the values averaged to obtain n = 1. Background luciferase activity was determined by performing the assay in untransfected cells. Importantly, there was no increase in β-gal activity in response to cAMP stimulation. The pGL-promoter (SV40 promoter) and pGL2-Basic luciferase vectors were included in each experiment to monitor the quality of transfection. Assay values were always at least 5–10 times greater than the promoterless construct. Relative luciferase activity was calculated as a percentage of the SV40 promoter after correction for transfection efficiency in order to eliminate day-to-day variation in transfection as described previously (17). The data represent the mean ± S.E. of six independent experiments performed in Calu-6 cells and four independent experiments performed in JEG-3 cells. The data were analyzed by one-way analysis of variance (ANOVA) (26) followed by Student’s modified t test with Bonferroni correction for multiple comparisons between means (27) using the modified mean error square term from the ANOVA or by unequal t test using the SigmaStat Software package (Jandel Scientific).

Nuclear Extracts and Gel Mobility Shift Assay—Crude nuclear extracts from either Calu-6 or JEG-3 cells were prepared as described previously (28) with the following modifications. Cells from 80% confluent monolayer cultures were harvested and washed in 1 x phosphate-buffered saline. Pelleted cells were resuspended in buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol, maintained on ice to swell, and lysed by being rapidly and repeatedly drawn through a 26-gauge needle. The crude nuclear pellet obtained by brief centrifugation was then incubated in 20 mM HEPES, pH 7.9, containing 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.0 μg/ml pepstatin, 2.0 μg/ml aprotinin, and 1.0 μg/ml leupeptin. Crude nuclear extract was dialyzed against 20 mM HEPES, pH 7.9, containing 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml pepstatin, 2.0 μg/ml aprotinin, and 1.0 μg/ml leupeptin, and 0.5 mM dithiothreitol. The dialyzed crude nuclear extract was snap-frozen in liquid nitrogen and stored at −80 °C in aliquots. The concentration of protein in the extracts was estimated according to the method of Bradford (29). The protein concentration of total nuclear protein determined by the Bradford method was found to be 630 nm in a microplate reader.

Oligonucleotides corresponded to the sense and antisense strands of the HREN 5′-flanking region surrounding the putative CRE. hRenCRE extended from −239 to −204 with respect to the transcription start site. hRenCRE* and hRenMut spanned the same sequences but were mutated to the consensus CRE or to the sequence which abolishes...
**Table I**

**Sequence of oligonucleotides used in the study**

The sequence (top strand only) of all oligonucleotides used for gel shift or as competitors are shown. All double-stranded oligonucleotides were blunt-ended so the bottom strand is the reverse-complement of what is shown. The CRE is underlined and the CRE mutants are shown in bold italics.

| Oligo name | Sequence |
|------------|----------|
| hRenCRE | GAGGGCTCTGACGGCTGACGCCAGAAATGTTTTTT |
| hRenCRE* | GAGGGCTCTGACGGCTGACGCCAGAAATGTTTTTT |
| hRenMut | GAGGGCTCTGACGGCTGACGCCAGAAATGTTTTTT |
| SomCRE | GCTCCCTGCTGGGACTCTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
CREB/ATF family of transcription factors. In order to identify the nuclear factor(s) that bind to the HREN CRE, we performed gel mobility shift assays using nuclear extracts from Calu-6 cells and labeled double-stranded oligonucleotides hRenCRE (Fig. 3A), hRenCRE* (Fig. 3C), and somatostatin CRE (SomCRE, Fig. 3B). In preliminary experiments, the shift products (labeled A–E in Figs. 3–5) migrated as a diffuse set of DNA-protein complexes close to a major nonspecific DNA-protein complex (labeled NS in Figs. 3–5). In order to resolve these complexes we ran our GMSA on sequencing length gels. In each experiment the double-stranded oligonucleotide was present in high excess but migrated four times further than the lowest point shown in each figure. Five specific shift products (and one nonspecific product) were observed with all three probes (Fig. 3). These band shift products were shown to be specifically competed with excess unlabeled hRenCRE, SomCRE, and hRenCRE* but were not competed with the same CRE mutant employed in the 900MUT construct (hRenMut oligo) or a nonspecific competitor encoding the binding site for nuclear factor Y. Since AP-2 and AP-1 can interact with CREs to mediate a cAMP response (32) and there is an AP-2 consensus homology residing adjacent to the HREN CRE on the opposite strand (33), two different double-stranded competitor oligonucleotides were generated that contain the AP2 sequence. The first contains the AP-2 consensus binding site from the human metallothionein IIA promoter (34) and the second contains the HREN AP-2 site and overlapping CRE that incorporated the hRenMut mutation (AP2Mut). No competition was observed when either oligonucleotide was added. However, we observed some competition for hRenCRE when an AP-1 site from the human collagenase gene (34) was used as a competitor.

Previous studies have demonstrated that ATF-1 can inhibit CREB-1-mediated activity of the somatostatin promoter when present as a CREB-1-ATF-1 heterodimer (12), and the GMSA results described above indicate that Calu-6 cells contain the CREB-1 transcription factor in a heterodimeric complex with ATF-1. We therefore asked whether HREN promoter activity could be transactivated by co-transfection of the luciferase constructs with an expression vector encoding CREB-1. Calu-6 and JEG-3 cells cotransfected with the luciferase and CREB-1 expression vectors were either left untreated or were treated with forskolin. In Calu-6 cells, forskolin caused a significant induction of reporter gene activity in cells containing 900CRE plus hRenCRE* (Fig. 4A), and hRenCRE* (Fig. 4B) oligonucleotides. No supershifted products were formed with three different nonspecific antibodies, demonstrating the specificity of the binding (Fig. 4C).

To identify specifically which of the transcription factors belonging to the ATF/CREB family contribute to the DNA-protein complexes observed in Calu-6 nuclear extracts, we performed a gel mobility supershift assay using ATF-1 and CREB-1-specific monoclonal antisera (Fig. 4). The ATF-1 antiserum was able to supershift products B, C, and D (and possibly E which forms a weak complex) to a position comigrating with product A. Only band B was supershifted with CREB-1 antiserum. These results suggest that products B–D contain ATF-1 and that product B is a CREB-1-ATF-1 heterodimer. Identical results were obtained with the SomCRE (Fig. 4A) and hRenCRE (Fig. 4B) oligonucleotides. No supershifted products were formed with three different nonspecific antibodies, demonstrating the specificity of the binding (Fig. 4C).
CREB-1 but only a small induction in cells containing 900L or 900MUT plus CREB-1 (Fig. 6A). Interestingly, the increase in 900L and 900CRE transcriptional activity induced by forskolin in cells containing the CREB-1 expression vector was only slightly greater than in cells without CREB-1 (compare the filled bars to the diagonal cross-hatched bars, Fig. 6A). Similarly, only a 2-fold induction in 900L and 900CRE activity was obtained after forskolin treatment of Calu-6/WT-11 cells, a subline of Calu-6 stably transfected with the CREB-1 expression vector and containing abundant CREB-1 mRNA (data not shown). In JEG-3 cells (Fig. 6B), there was a significant induction in 900CRE and 900L promoter activity in forskolin-treated cells co-transfected with CREB-1. The effects of forskolin treatment were greatest in cells containing 900CRE, intermediate in cells containing 900L, but not significant in cells containing 900MUT.

The transactivation of genes through a CRE is proposed to occur by binding of phosphorylated ATF/CREB transcription factor(s) to the CRE with recruitment of CREB binding protein (CREBP), and phosphorylation of CREB/ATF family members is essential for their trans-activation activity. In order to determine if the HREN promoter could be transactivated by phosphorylation of endogenous CREB/ATF proteins present in Calu-6 cells, cotransfection experiments were performed using the test luciferase plasmids and an expression vector encoding the catalytic subunit of PKA (cPKA). Overexpression of cPKA in Calu-6 cells significantly increased HREN promoter activity of 900L by 10.3-fold, 900CRE by 9.1-fold, and 900MUT by 5.7-fold (Fig. 7A). Similar results were obtained in JEG-3 cells (Fig. 7B). Interestingly, the cPKA induction in JEG-3 cells mirrored the response to forskolin (compare filled bars to cross-hatched bars, Fig. 7B). These data demonstrate that the HREN promoter can be transactivated in Calu-6 cells by PKA-mediated phosphorylation of transcription factors including, presumably, CREB-1. Moreover, these results suggest the possibility that either the basal activity of cPKA is low in Calu-6 cells, resulting in a low level of phosphorylated CREB-1 in response to forskolin, or that CREB-1 is rapidly dephosphorylated after an initial burst of activity. We also cannot rule out the possibility that the cPKA response is due to the activation of other transcription factors besides CREB-1, since a cPKA-mediated increase in HREN transactivation activity was observed in the 900MUT construct (see below).

In order to confirm that the luciferase activity correlated with transcription initiation from the HREN start site, total RNA was isolated from Calu-6 or JEG-3 cells transfected with the 900L construct and analyzed by RNase protection assay (see strategy in Fig. 1). In order to boost expression to detectable levels by RNase protection assay, we treated transfected JEG-3 cells with forskolin and co-transfected Calu-6 cells with the cPKA expression vector. A 150-base fragment indicating
Correct transcriptional initiation was clearly evident in the transfected cells, whereas no protected fragment was detected in nontransfected control cells (Fig. 8). A 127-base protected fragment encoding human β-actin was detected in all samples indicating equal loading of RNAs in each lane. The results conclusively indicate that the luciferase activity measured in our experiments was derived from transcripts initiating faithfully from the HREN promoter in both Calu-6 and JEG-3 cells.

In order to assess whether the HREN transcriptional response to PKA was mediated via a CREB-1-dependent mechanism, we introduced a dominant negative mutant form of CREB-1 (KCREB) into Calu-6 and JEG-3 cells and repeated the transient expression analyses. In these experiments, luciferase expression vectors were co-transfected with the constructs shown in Fig. 1. Open bars, luciferase vectors alone; cross-hatched bars, luciferase vectors plus cPKA expression vector. *, p < 0.001 untreated versus cPKA by t test; †, p < 0.05 untreated versus cPKA by ANOVA.

Discussion

Transcriptional activation by CREB serves as the final step in the signal transduction cascade initiated at cell surface receptors to activate adenylyl cyclase, increase intracellular...
Moreover, CREB-1 binds DNA as a dimer at the CRE, and in some cases, the level of induction that can be directed by the promoter activity to a small degree as previously reported (4, 5). In this study, we have shown that phosphorylation of CREB by PKA is essential for its activating functions (35). In the present study, we have shown that CREB-1 for binding to the hRenCRE* sequence exceeded that in addition to the CRE core sequence the composition of the flanking sequences is also a strong determinant of the strength of the transcriptional response observed (40). In this regard, it is interesting to note that the affinity of ATF-CREB factors in vitro was much higher with hRenCRE* (consensus) than with the native hRenCRE (Fig. 5), the level of transcriptional activation observed with 900CRE (consensus) and 900L (native) in vivo was similar once CREB-1 was phosphorylated by PKA (Fig. 7).

The similar level of activity of 900L and 900CRE in fully induced (cPKA-transfected) cells may be partially explained by the sequences flanking the HREN CRE, as it has been shown that in addition to the CRE core sequence the composition of the flanking sequences is also a strong determinant of the affinity of transcription factor binding and can influence the strength of the transcriptional response observed (40). In this regard, it is interesting to note that the affinity of ATF-1 and CREB-1 for binding to the hRenCRE* sequence exceeded that of the somatostatin CRE and that the hRenCRE sequence was more effective in competing with SomCRE than hRenCRE* for binding to these factors (Fig. 5).

**Mechanism of HREN Promoter Transactivation**—The mutant form of CREB (KCREB) we used in this study has a single nucleotide change from arginine to leucine in the DNA binding domain and acts as a dominant repressor of wild-type CREB. KCREB was previously shown to block the ability of CREB to mediate induction of a somatostatin-promoter reporter construct in differentiated F9 cells (23). Our results demonstrate that KCREB attenuated the PKA-mediated induction of transcriptional activity of constructs containing the native HREN CRE or consensus CRE, but not of constructs containing a nonfunctional CRE mutant. The observation that PKA induction of 900MUT transcriptional activity is not attenuated by KCREB suggests that the induction in HREN promoter activity has a CREB-dependent and CREB-independent component and that the CREB-dependent component requires the HREN CRE. It remains unclear what other factors may mediate the CREB-independent portion of the PKA response. In this regard, an AP-2 homology lies adjacent to the HREN CRE (on the opposite strand) (4). Although our GMSA competition results show that the AP-2 sequence could not compete for ATF-1 and CREB-1 binding to the CRE, we cannot rule out the possibility that AP-2 plays some role in regulation of the HREN promoter by cAMP. An AP-2 recognition site mediates the cAMP induction of the metallothionein IIA, growth hormone, prolactin, and...
proenkephalin genes (reviewed in Ref. 41). In addition, a Pit-1 sequence between coordinates −82 to −58, which binds the pituitary-specific transcription factor Pit-1 and can bind members of the POU domain family of transcription factors, has also been reported to be an essential component of the HREN cAMP response (10, 42, 43). HREN promoter constructs containing the Pit-1 site in the absence of the CRE are still induced 1.7-fold by cAMP (10). Therefore, both the AP-2 and Pit-1 sites, along with their cognate factors, may play a role in the CREB-1-independent portion of the HREN transcriptional response to cAMP.

Like CREB-1, ATF-1 exists as both a homodimer and ATF-1-CREB-1 heterodimer in a variety of cell types (44). ATF-1 was reported to be capable of antagonizing CREB-1-dependent activation of the somatostatin promoter by limiting the amount of CREB-1 that can form homodimers (12). Our band shift assays indicate that there were several DNA-protein complexes consisting of ATF-1, one of which was an ATF-1-CREB-1 heterodimer, but no detectable CREB-1 homodimers. In addition to CREB-1 and ATF-1, other Calu-6 factors may have the ability to form DNA-protein complexes with the CRE, and it remains unclear what other transcription factors make up the ATF-1-CRE complexes observed in our band shift experiments. These results are consistent with previous observations that the majority of CREB-1 in HeLa cells is associated with ATF-1 (44–46) and suggests that ATF-1 may suppress cAMP responsiveness by displacing or preventing binding of CREB-1 to the CRE or by preventing the interaction between CREB-1 and other members of the transcription complex. Overexpression of wild-type CREB-1 in Calu-6 cells was not sufficient, however, to induce HREN promoter activity, even when treated with forskolin. Although we are not ruling out a role for ATF-1 in regulating CREB-1 activity in Calu-6 cells, the data suggest that the inactivity of CREB-1 in response to forskolin may be due to another mechanism, such as low basal levels of PKA activity in Calu-6 cells. It is interesting to note in this regard that the levels of phosphorylated ATF-1 are approximately equal under basal or forskolin-stimulated conditions (Fig. 10). This suggests the possibility that phosphorylation of CREB and ATF-1 is differentially regulated in these cells. This is likely since both CREB and ATF-1 are targets of multiple kinases (see below).

Alternatively, it is possible that high level activity of phosphatases may dephosphorylate CREB after forskolin stimulation in Calu-6 cells. Indeed, as phosphorylation of CREB-1 by PKA on Ser-133 is associated with increased transcriptional activity (35, 47, 48), the rate of CREB-dependent transcription has been shown to be diminished by phosphorylase treatment (49, 50). Consistent with this possibility is the observation that phosphorylated CREB-1 is detectable after 3 h of forskolin treatment (Fig. 10), but not after 24 h of treatment (data not shown) unless the cells contain the cPKA expression vector. Since our luciferase measurements were performed 24 h after forskolin treatment, it is possible that the absence of significant induction may be due to dephosphorylation of CREB-1. Rapid phosphorylation and dephosphorylation of CREB-1 in renin expressing cells may provide a mechanism to tightly regulate the transcriptional activity of the HREN promoter in response to a changing environment. Clearly, it is necessary to establish whether similar mechanisms are operating in renin expressing cells in vivo.

cAMP is thought to be the major stimulatory second messenger responsible for causing increased renin secretion in response to sympathetic nerve stimulation via the β-adrenergic receptor mechanism, whereas the suppression of renin secretion is thought to involve increases in intracellular calcium (51). Angiotensin II causes increased calcium mobilization and decreased renin release as a result of angiotensin II type 1 receptor activation (52–54). Of the members of the CREB/ATF family, both CREB-1 and ATF-1 have been shown to be responsive to both the cAMP and calcium pathways. Both cAMP, by acting through cAMP-dependent protein kinase A, and calcium, by acting through Ca2+/calmodulin-dependent protein kinases, are capable of phosphorylating CREB-1 on Ser-133 and thus can regulate CREB-1-mediated transcription of target genes (47, 55). Transient expression studies have also shown that ATF-1 can be activated by increases in either cAMP or calcium (11, 56). Different members of the Ca2+/calmodulin-dependent protein kinase family can either activate or inhibit the activation of CREB-1, suggesting the calcium pathway can either antagonize or act jointly with the cAMP pathway for activation of CREB-1. Such convergent regulation of CREB activity by cAMP and calcium may be used to integrate multiple extracellular signals in renin-producing cells in vivo. Such a mechanism could be fundamental to the control of HREN promoter activity by intracellular signal transduction pathways that either increase transcription by PKA-mediated phosphorylation of CREB-1 or suppress transcription by Ca2+/calmodulin-mediated inhibition of CREB-1 activity, perhaps via ATF-1.

Conclusions—In summary, we have demonstrated that the HREN promoter can be transactivated by the cAMP pathway by both CREB-dependent and CREB-independent mechanisms in both renin expressing and non-renin expressing cell lines. Although the transcriptional response to forskolin alone did not require a functional CRE, the CRE-dependent portion of the response to elevated PKA required the CRE. Our results suggest that either the basal levels of PKA in Calu-6 cells may be low or, alternatively, that CREB is rapidly dephosphorylated after forskolin treatment, and experiments to differentiate between these possibilities are in progress. It is now critical to determine whether these mechanisms can be extended to those cells in vivo which express and release renin to response to physiological inputs. Accordingly, it is interesting to note that HREN promoter activity in transfected primary cultures of renin expressing choriodecidual cells was recently reported to be induced by forskolin only 2.4-fold in a construct containing an intact CRE and only 1.7-fold in a construct without a functional CRE (10) suggesting that similar mechanisms may be operating in this cell type as well.

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