Angiotensin II and Potassium Regulate Human CYP11B2 Transcription through Common *cis*-Elements

Colin D. Clyne, Yin Zhang, Liliya Slutsker, J. Michael Mathis, Perrin C. White, and William E. Rainey

Department of Obstetrics & Gynecology (C.D.C., Y.Z., J.M.M., W.E.R.)
and
Department of Pediatrics (L.S., P.C.W.)
University of Texas Southwestern Medical Center
Dallas, Texas 75235-9032

Aldosterone synthase is a mitochondrial enzyme that catalyzes the conversion of 11-deoxycorticosterone to the potent mineralocorticoid aldosterone. The gene encoding aldosterone synthase, CYP11B2, is expressed in the zona glomerulosa of the adrenal cortex. Although the major physiological regulators of aldosterone production are angiotensin II (ANG II) and potassium (K^+), the mechanisms by which these compounds regulate CYP11B2 transcription are unknown. Therefore we analyzed the human CYP11B2 5′-flanking region using a transient transfection expression system in the H295R human adrenocortical cell line. ANG II and K^+ increased expression of a luciferase reporter construct containing 2015 bp of human CYP11B2 5′-flanking DNA. This response was mimicked by treatment with the calcium channel activator BAYK8644, whereas activation of the protein kinase C pathway with 12-o-tetradecanoylphorbol-13-acetate had no effect. Reporter gene activity was also increased after activation of cAMP-dependent pathways by $(Bu)_2$cAMP. Deletion, mutation, and deoxyribonuclease I footprinting analyses of the CYP11B2 5′-flanking region identified two distinct elements at positions −71/−64 (TGACGTGA) and −129/−114 (CTCCAGCCTTGACCTT) that were both required for full basal reporter gene activity and for maximal induction by either cAMP or calcium-signaling pathways. The −71/−64 element, which resembles a consensus cAMP response element (CRE), bound CRE-binding proteins from H295R cell nuclear extracts as determined by electrophoretic mobility shift analysis. Analysis of the −129/−114 element using electrophoretic mobility shift analysis demonstrated binding of the orphan nuclear receptors steroidogenic factor 1 and chicken ovalbumin upstream promoter transcription factor. These data demonstrate that ANG II, K^+, and cAMP-signaling pathways utilize the same SF-1 and CRE-like *cis*-elements to regulate human CYP11B2 expression. (Molecular Endocrinology 11: 638–649, 1997)

0888-8809/97/$3.00/0
Molecular Endocrinology

INTRODUCTION

The renin-angiotensin system (RAS) is a major regulator of intravascular volume and blood pressure. Dysregulation of the RAS is associated with several forms of human hypertension (1, 2). To completely understand the pathogenesis of such diseases associated with hypertension, it is necessary to fully delineate the mechanisms regulating each of the components within this system. A key effector of the RAS is aldosterone, the primary human mineralocorticoid, which acts on the distal nephron to regulate sodium resorption, potassium excretion, and intravascular volume (3). Aldosterone is produced exclusively in the adrenal zona glomerulosa, and its secretion is regulated primarily by serum levels of angiotensin II (ANG II) and potassium (K^+) (4, 5). Temporally, the regulation of aldosterone production can be divided into two phases: an acute phase that occurs within minutes and reflects cholesterol transfer to mitochondrial side-chain cleavage enzyme and a chronic phase that requires several hours and reflects increased expression of aldosterone synthase (CYP11B2) (6). CYP11B2 is normally expressed only in the adrenal glomerulosa in contrast to the isozyme 11β-hydroxylase (CYP11B1), which is expressed in the adrenal fasciculata (7, 8). CYP11B2 catalyzes the three successive reactions that lead to the conversion of deoxycorticosterone to aldosterone (9–13). Dysregulated expression of CYP11B2, as seen in glucocorticoid-suppressible hyperaldosteronism, can lead to elevated circulating aldosterone levels and hypertension (14). A detailed analysis of the normal

mechanisms regulating human CYP11B2 expression would thus be of significant interest.

In the only published attempt to analyze the transcriptional regulatory region of the human CYP11B2 gene, chloramphenicol acetyltransferase (CAT) reporter constructs were transfected into mouse Y1 adrenocortical tumor cells, but levels of basal and stimulated expression were too low for the identification of transcriptional regulatory elements (15). In contrast, considerable progress has been made in defining the mechanisms by which cAMP regulates transcription of rat (16) and mouse (17–19) CYP11B2, as well as the one isozyme expressed in the bovine adrenal, CYP11B (20–22). Cyclic AMP is the second messenger for ACTH, the major hormonal regulator of glucocorticoid biosynthesis and expression of CYP11B1 in the adrenal fasciculata. Common elements in the 5′-flanking region mediating basal and cAMP-induced expression were identified in each of these genes, including a cAMP-response element (CRE) and an element binding an orphan nuclear receptor, steroidogenic factor-1 (SF-1).

The physiological relevance of ACTH in CYP11B2 transcription and mineralocorticoid production is unclear, however, because chronic treatment with ACTH decreases both plasma aldosterone levels (23, 24) and adrenal CYP11B2 expression (25). Thus, the regulated expression of CYP11B2 cannot be explained through cAMP-dependent mechanisms alone. Indeed, the principle physiological regulators of CYP11B2 expression, ANG II and K^+, do not increase cAMP levels in adrenal glomerulosa cells but instead increase the intracellular concentration of calcium ($[Ca^{2+}]i$) and activate protein kinase C (5, 26). To date, there have been no studies regarding the effects of ANG II, K^+, or the calcium-signaling pathway on CYP11B2 transcription.

One difficulty in studying CYP11B2 transcription has been the lack of an *in vitro* adrenocortical model system that retains the abilities to produce aldosterone and respond to ANG II and K^+. We recently described the human adrenocortical H295R cell line as a model for studying CYP11B2 regulation (27–30). These cells respond to ANG II and K^+ by increasing both aldosterone production and CYP11B2 expression. The current study was undertaken to analyze the 5′-flanking DNA of the human CYP11B2 gene and to define the *cis*-regulating elements and *trans*-acting factors that are necessary for ANG II and K^+ induction of CYP11B2 transcription. The results indicate that maximal induction of CYP11B2 transcription by ANG II or K^+ requires two key *cis*-elements, one of which binds cAMP-response element (CRE) binding proteins, and the other SF-1 and a second orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF). These two elements are also required for maximal cAMP-induced expression, suggesting that the Ca^{2+} and cAMP-signaling systems use the same *cis*-elements to regulate CYP11B2 transcription.

RESULTS

Multiple Signaling Pathways Regulate CYP11B2 Reporter Gene Expression in H295R Cells

H295R cells were transiently transfected with a CYP11B2-luciferase reporter construct containing 2015 bp of 5′-flanking sequence (pB2–2015) and treated with experimental agents for 8 h. As shown in Fig. 1, both ANG II and K^+ increased transcription of this reporter construct in a concentration-dependent manner, with significant induction occurring at 0.1 nM ANG II and 12 mM K^+, respectively. These results demonstrate that the first 2015 bp of 5′-flanking region contains sufficient sequence information to direct reporter gene expression in response to physiological regulators of human CYP11B2.

Previous studies have established that although the primary effect of K^+ on adrenal glomerulosa cells is to increase $[Ca^{2+}]i$, treatment with ANG II increases both $[Ca^{2+}]i$ and protein kinase C activity (5, 26). In contrast, ACTH increases cAMP levels through activation of adenylyl cyclase. To assess the relative contributions of these signaling pathways to the induction of reporter gene expression, H295R cells were treated with BAYK8644 (an L-type Ca^{2+} channel agonist), 12-o-tetradecanoylphorbol-3-acetate (TPA; an activator of protein kinase C) or $(Bu)_2$cAMP (an activator of protein kinase A). Both BAYK8644 and $(Bu)_2$cAMP increased reporter gene expression in a concentration-dependent manner, whereas TPA was completely ineffective (Fig. 1). To confirm that the observed effects of BAYK8644 and $(Bu)_2$cAMP were indeed mediated by Ca^{2+} and cAMP-dependent pathways, respectively, similar experiments were performed using the Ca^{2+} ionophore ionomycin (1 μM) and the adenylyl cyclase activator forskolin (10 μM). Both of these agents also increased expression of the reporter construct (data not shown). Thus, cAMP- and Ca^{2+}-dependent pathways increase CYP11B2 reporter gene expression in H295R cells, whereas protein kinase C-dependent pathways do not appear to play a role in this response. The time course of reporter gene induction was rapid. As shown in Fig. 2, luciferase activity increased dramatically within 60 min of agonist treatment to reach a maximum at 6 h. The induction of reporter gene expression, however, decreased in response to each agonist after 6 h.

Deletion Analysis of the Human CYP11B2 Promoter

To identify the *cis*-regulatory elements that mediate transcriptional activation by ANG II and K^+, a series of deletion constructs containing progressively shorter fragments of human CYP11B2 5′-flanking DNA (extending from position +2 to −2015, −1521, −864, −413, −221, and −65 bp, respectively) was prepared. These constructs were transiently transfected into H295R cells. Figure 3 shows luciferase activity of

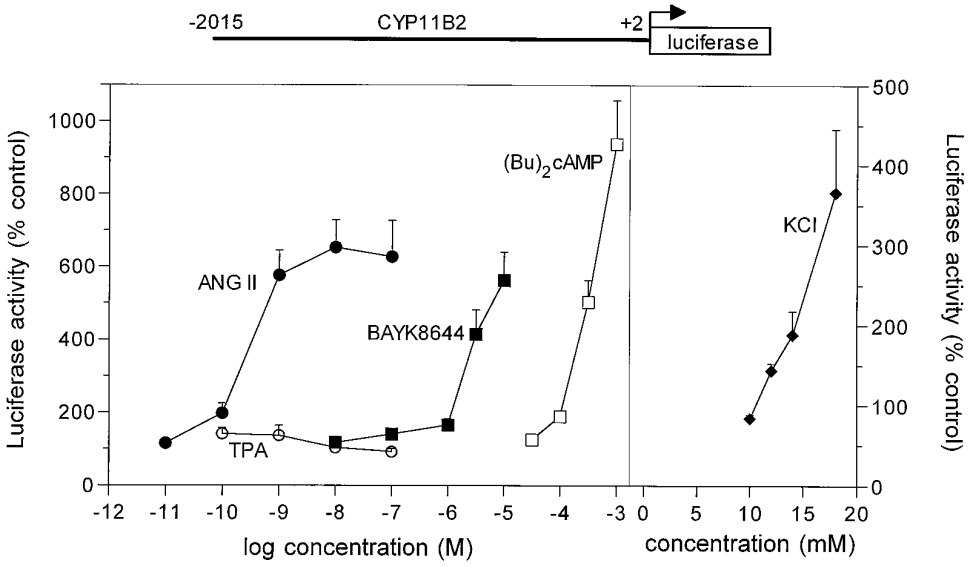

Fig. 1. Dose-Dependent Effects of Various Factors on CYP11B2 Reporter Gene Activity

H295R cells were transiently transfected with 1.0 μg pGL2 luciferase reporter vector containing 2015 bp human CYP11B2 5′-flanking DNA (−2015/+2). Forty eight hours after transfection, cells were treated with ANG II (●), KCl (♦), BAYK8644 (■), $(Bu)_2$cAMP (□), or TPA (○) at the concentrations indicated for 8 h before being lysed and assayed for luciferase activity. Results are expressed as a percentage of the control (untreated) activity and represent the mean ± SEM of determinations from three to six independent experiments, each performed in triplicate.

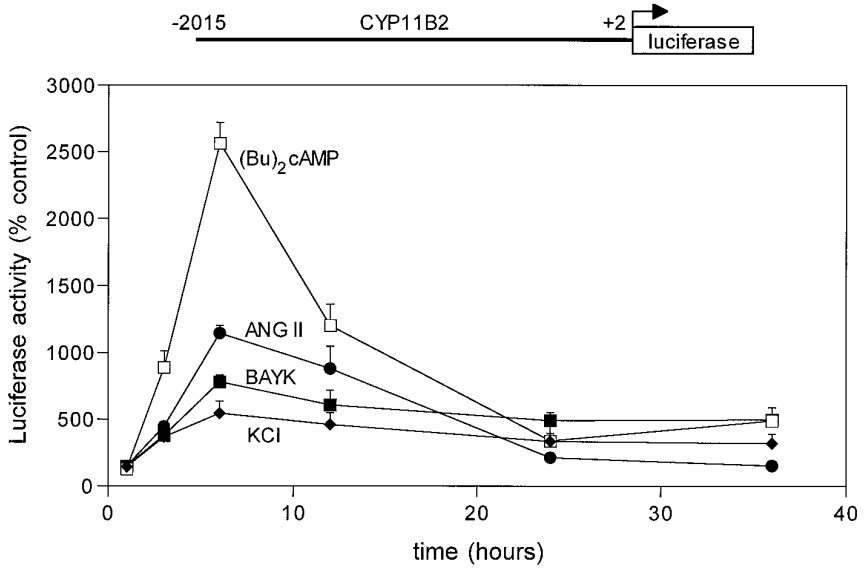

Fig. 2. Time Course of CYP11B2 Reporter Gene Induction

H295R cells were transiently transfected with 1.0 μg pGL2 luciferase reporter vector containing 2015 bp human CYP11B2 5′-flanking DNA (−2015/+2). After recovery, cells were treated with ANG II (10 nM) (●), KCl (16 mM) (♦), BAYK8644 (10 μM) (■), or $(Bu)_2$cAMP (1 mM) (□) for the times indicated before being lysed and assayed for luciferase activity. Results are expressed as a percentage of the control (untreated) activity, and represent the mean ± SEM of determinations from three independent experiments, each performed in triplicate.

these constructs under basal conditions or after treatment with different agonists for 6 h. Basal luciferase activity of each construct containing 5′-flanking sequences from −2015 to −221 bp was similar. Deletion to −65 bp, however, decreased luciferase activity by 85%. This result indicates that DNA sequences located between −65 and −221 bp are essential for basal gene expression.

ANG II and K^+ treatment resulted in 5.5- and 4.5-fold increases in luciferase activity, respectively

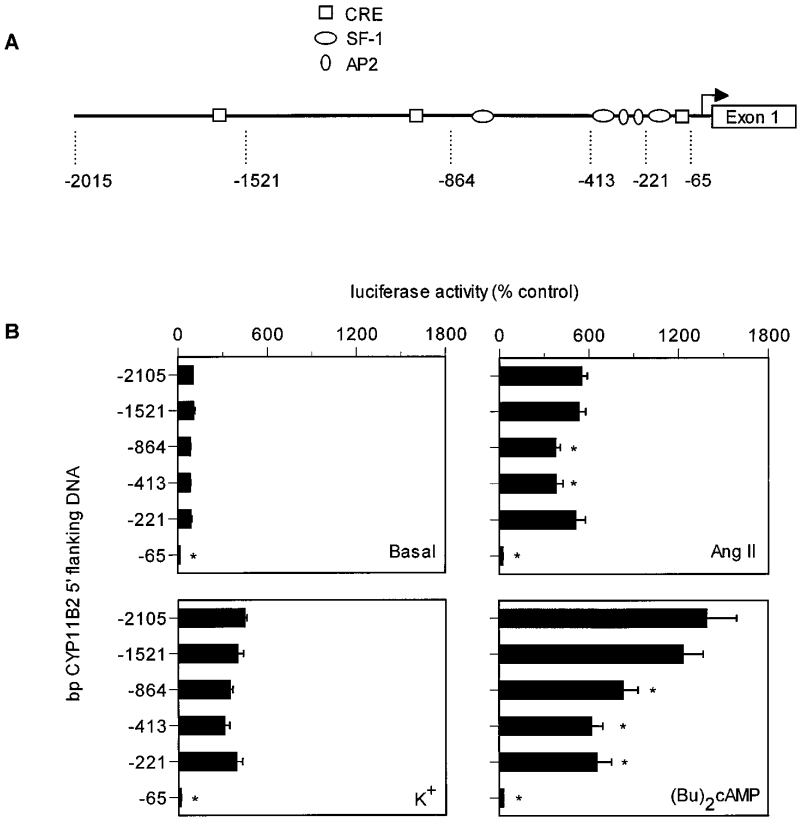

Fig. 3. Deletion Analysis of the 5′-Flanking Region of the Human CYP11B2 Gene

A series of CYP11B2 5′-deletion mutants was transiently transfected into H295R cells. Panel A shows the positions of putative transcription factor-binding sites, as identified by sequence comparison, and the relative lengths of the deletion constructs. B, Luciferase activities of lysates from control cells or cells treated with ANG II (10 nM), KCl (20 mM), and $(Bu)_2$cAMP (1 mM). Results are expressed as a percentage of the basal activity of the longest construct (pB2–2015) and represent the mean ± SEM of determinations from three to five independent experiments, each performed in triplicate. *, $P < 0.05$ *vs* pB2–2015.

(Fig. 3). In the series of 5′-deletion constructs, induction of luciferase activity in response to either agonist was maintained in cells transfected with plasmids containing up to −221 bp of 5′-flanking sequence. Deletion to −65 bp, however, completely abolished reporter gene induction in response to ANG II and K^+. The effect of $(Bu)_2$cAMP on luciferase activity in H295R cells transfected with each deletion construct was determined in a similar manner. Luciferase activity was stimulated 13-fold by $(Bu)_2$cAMP in cells transfected with pB2–2015. Reporter activity induced by $(Bu)_2$cAMP decreased by 50–60% upon deletion to −864 bp, and was completely abolished after deletion to −65 bp. These results indicate that the region of human CYP11B2 between −221 and −65 bp contains positive regulatory sequences critical for basal, cAMP, and Ca^{2+}-induced transcription.

DNase I Footprinting Analysis of the Proximal CYP11B2 5′-Flanking Region

To identify protein-binding sites within this proximal region, DNase I footprinting analysis was performed using H295R cell nuclear extracts. As shown in Fig. 4, two regions of protection were identified at position −129/−114 (CTCCAGCCTTGACCTT) and at position −81/−63 (AGTTCTCCCATGACGTGAT). Sequence analysis indicates that the −129/−114 region contains a nuclear receptor half-site that resembles the consensus binding site for SF-1 (TGACCT), whereas the −81/−63 region contains an element (TGACGTGA) sharing seven of eight bp homology with the consensus CRE.

The CYP11B2 −129/−114 Element Binds SF-1 and COUP-TF and Is Required for Basal and Agonist-Induced Reporter Activity

To begin characterization of nuclear proteins that bind the −129/−114 bp element, a synthetic oligonucleotide probe encompassing this sequence was prepared and used in electrophoretic mobility shift assays (EMSA). In the presence of H295R cell nuclear extracts, three specific protein-DNA complexes were formed (Fig. 5A). Formation of each of these complexes was abolished by the addition of a 10- to 100-fold molar excess of nonradiolabeled wild type competitor. In the presence of antibody directed against

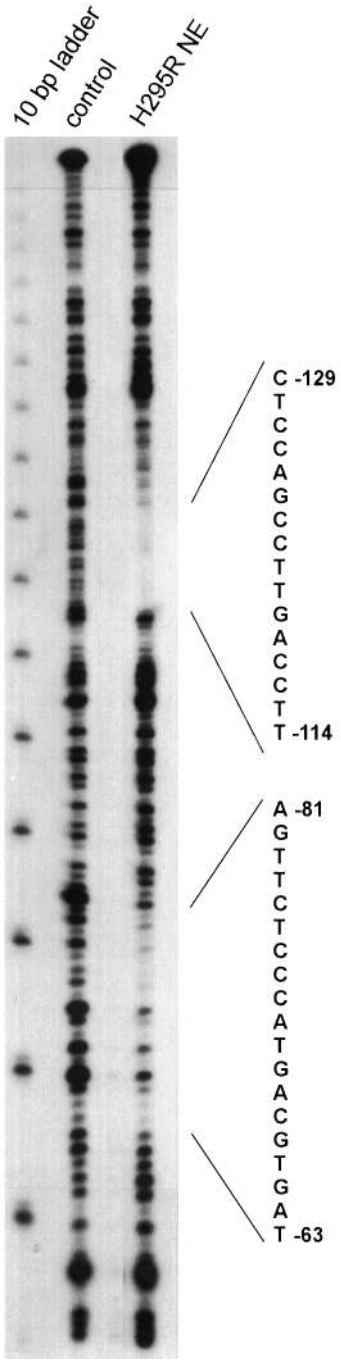

Fig. 4. DNase I Footprinting Analysis of the Proximal CYP11B2 Promoter

A 231-bp probe (50,000 cpm) corresponding to CYP11B2 position −229/+2 was digested with DNase I in the absence (control) or presence of H295R cell nuclear extract (NE, 20 μg) and subjected to denaturing gel electrophoresis. The protected regions are indicated on the *right* of the figure.

SF-1 protein, complex 3 was displaced (Fig. 5B). Because COUP-TF is known to bind to some SF-1 elements, we determined whether complexes 1 and 2 might represent COUP-TF protein binding. As shown in Fig. 5B, the addition of an antibody directed against COUP-TF caused these upper two bands to be displaced.

To determine whether this element is necessary for reporter gene expression, constructs were prepared containing a deletion of the −129/−114 element. A reporter construct (pB2–131), which begins immediately upstream of the protected region, exhibited basal and agonist-stimulated activities similar to those of the longer constructs described above. Deletion of the −129/−114 element resulted in a reduction of basal activity by approximately 80% compared with the wild type reporter plasmid (Fig. 6). In H295R cells transfected with pB2–131, luciferase activity was increased 4.9-, 4.2-, or 7.0-fold above basal after treatment with ANG II, K^+, or $(Bu)_2$cAMP, respectively. Deletion of the −129/−114 element (pB2–106) reduced reporter gene responses to ANG II (3.0-fold), to K^+ (2.8-fold), and to $(Bu)_2$cAMP (3.7-fold). These results indicate that the −129/−114 region of human CYP11B2 is essential both for full basal, as well as maximal Ca^{2+} and cAMP-induced transcription.

The CYP11B2 −71/−64 Element Binds CREB and Is Necessary for Calcium Induction

To begin characterization of nuclear proteins that bind the −71/−64 bp element, a synthetic oligonucleotide probe encompassing this sequence was prepared and used in EMSA. In the presence of H295R cell nuclear extracts, at least three specific protein-DNA complexes were formed (Fig. 7A). Formation of each of the three major complexes was abolished by the addition of a nonradiolabeled wild type competitor but not by a competitor containing a mutation in the CRE-like sequence (TtctagaA). A fourth faster migrating complex was observed that was also competed, to some extent, by the wild type oligonucleotide. However, this complex likely represents nonspecific interactions since its formation was also inhibited in the presence of the mutated CRE sequence. With the addition of monoclonal antibodies that recognized either CRE-binding (CREB) protein or which cross-reacted with CREB, CRE modulator, or ATF proteins, binding to the upper complexes 1 and 2 was abolished while complex 3 was not affected (Fig. 7B).

To determine whether this element was necessary for reporter gene activity, constructs were prepared containing the same mutation used in EMSA. Treatment of H295R cells transfected with the wild type −71/−64 element (pB2–354) increased luciferase activity in response to ANG II (4.9-fold), K^+ (3.6-fold), and $(Bu)_2$cAMP (5.7-fold). Mutation of the −71/−64 element (TGACGTGA to TtctagaA) reduced basal activity by approximately 50% and drastically reduced the transcriptional responses to ANG II (1.7-fold), K^+ (1.3-fold), and $(Bu)_2$cAMP (1.8-fold) (Fig. 8). These data suggest that the CRE is required not only for cAMP but also for Ca^{2+} induction of reporter gene activity. However, this element is not in itself sufficient because a reporter construct that retains the −71/−64 element

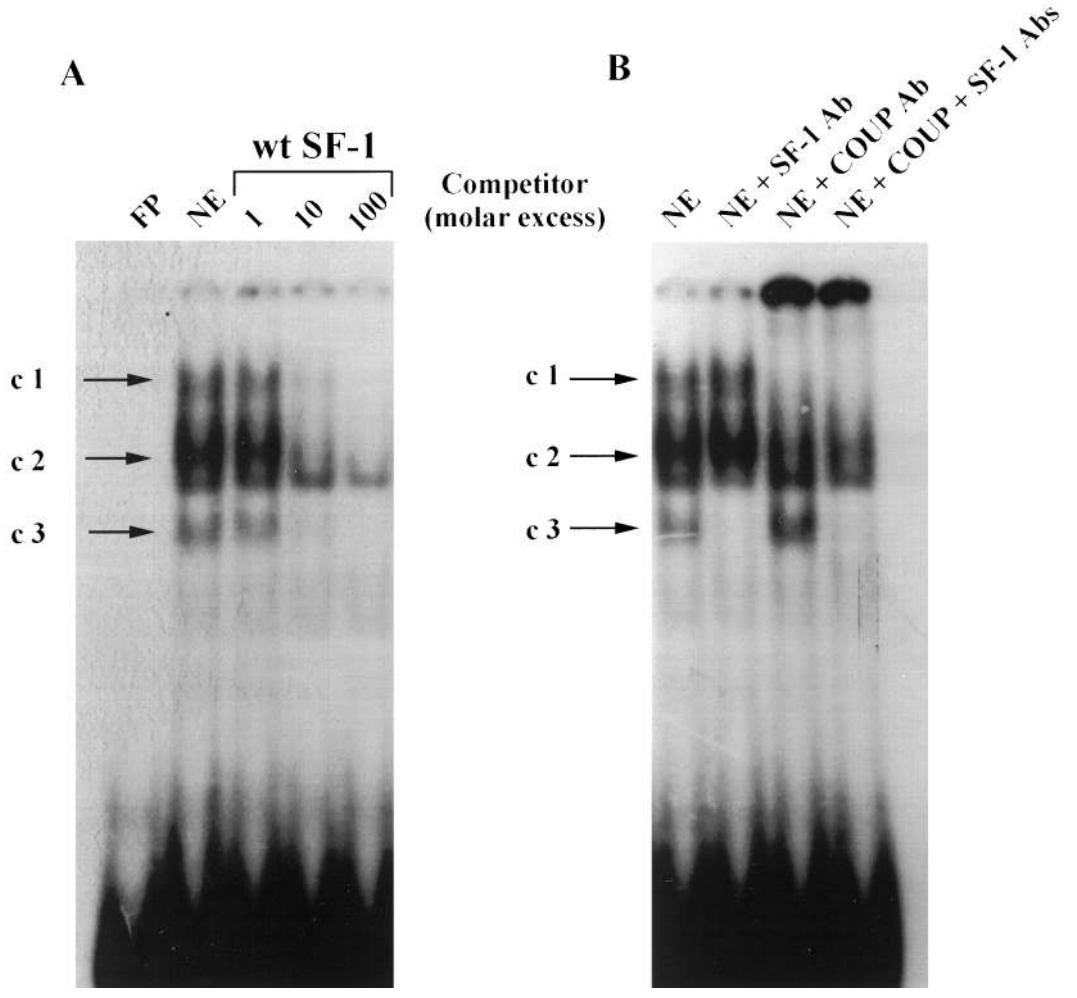

Fig. 5. Binding of H295R Nuclear Proteins to the −129/−114 Element

H295R nuclear extracts (NE, 5 μg) were incubated with radiolabeled probe encompassing the −128/−114 element (20,000 cpm) in the presence of (A) nonradiolabeled probe (wt) or (B) antibodies directed against SF-1 (SF-1 Ab) or COUP TF (COUP Ab). DNA/protein complexes (labeled C1, C2, and C3) were separated from free probe (FP) by gel electrophoresis and visualized by autoradiography.

but lacks the upstream −129/−114 element exhibited a decreased basal activity and markedly reduced agonist induction (pB2-106; Fig. 6). Taken together, these data suggest that the CRE (−71/−64) and SF-1 (−129/−114) sequences interact to promote full transcriptional activity in response to each signaling pathway.

DISCUSSION

Signaling Pathways Regulating CYP11B2

The regulation of CYP11B2 expression is the primary step in determining the capacity of the adrenal gland to produce aldosterone. Although ANG II and K^+ are the main physiological regulators of CYP11B2 expression, there have been no studies to define the molecular mechanisms by which these agonists control CYP11B2 transcription. In addition, the *cis*-regulatory elements involved in transcriptional regulation of the human CYP11B2 gene have not been determined. Herein, we identified two 5′-flanking *cis*-elements within the human CYP11B2 gene necessary not only for ANG II and K^+-induced expression, but also for transcription induced by cAMP.

In adrenal glomerulosa cells, ANG II increases $[Ca^{2+}]i$ and protein kinase C activity by activating phospholipase C, whereas K^+ increases $[Ca^{2+}]i$ through activation of voltage-sensitive Ca^{2+} channels (5, 26). Our data demonstrate that increases in $[Ca^{2+}]i$ and cAMP can independently increase CYP11B2 transcription. However, the protein kinase C pathway does not appear to play a role in regulating human CYP11B2 transcription. We have recently shown that CYP11B2 mRNA levels are increased after treatment with K^+ or ANG II in a concentration- and time-dependent manner (27–30). Because the effects of these

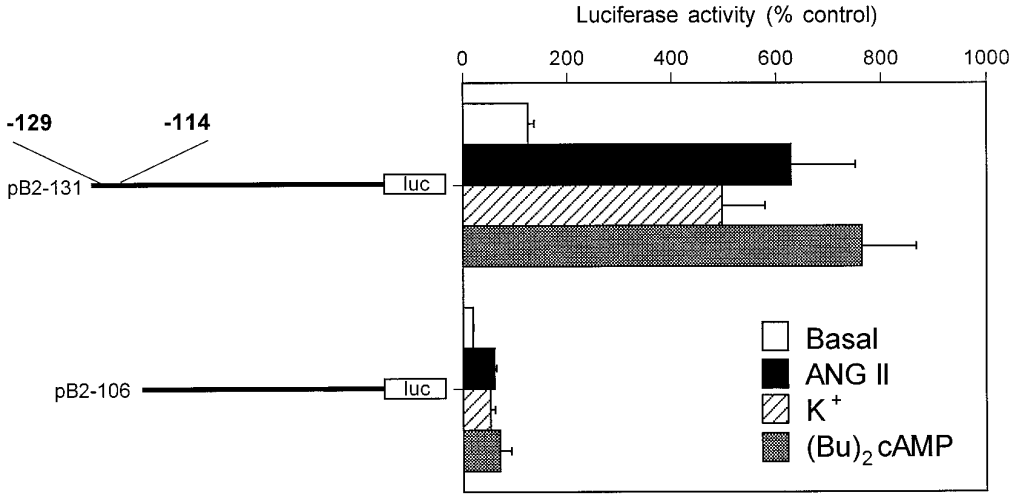

Fig. 6. Deletion Analysis of the 129/−114 Element

CYP11B2 5′-deletion constructs beginning immediately upstream (pB2–131) or downstream (pB2–106) of the −129 element were transiently transfected into H295R cells. After recovery, cells were treated with: ANG II (10 nM), KCl (20 mM), and $(Bu)_2$cAMP (1 mM). Luciferase activities are expressed as a percentage of the basal activity of pB2–2015 and represent the mean ± SEM of determinations from four independent experiments, each performed in triplicate.

agonists on CYP11B2 reporter gene expression and mRNA levels are similar, it is likely that changes in CYP11B2 mRNA levels correspond to changes in transcription of the gene.

CYP11B2 genes of the rat and mouse, when studied as chimeric reporter constructs in Y-1 adrenocortical cells, are regulated by cAMP (16, 17). Our data demonstrate the ability of cAMP to similarly increase transcription of the human gene. However, the role of ACTH and the cAMP-signaling pathway in long-term regulation of CYP11B2 expression have been questioned. First, although chronic administration of ACTH to humans produces a sustained increase in plasma cortisol levels, aldosterone increases only transiently, then falls to below baseline within 48 h (24). Second, targeted ablation of pituitary corticotropes, a maneuver that decreases plasma ACTH to undetectable levels, does not affect adrenal CYP11B2 expression (31). Thus, *in vivo* CYP11B2 expression is not positively regulated by circulating ACTH. Studies have shown that ACTH treatment increases CYP11B1 but not CYP11B2 mRNA in rat and human adrenal glomerulosa cells in primary culture (12, 25). Using the H295R adrenal cell line we have shown a preferential induction of CYP11B1 by cAMP over that observed for CYP11B2 (30). Although the physiological role of ACTH and cAMP in regulation of CYP11B2 expression is unclear, CRE-like motifs are present in the 5′-flanking region of CYP11B2 in all species thus far examined. Indeed, using the H295R and the Y-1 adrenal cell we have demonstrated that the cAMP-signaling pathway can effectively induce reporter gene expression driven by the CYP11B2 5′-flanking region (32). The mechanisms by which elevated ACTH levels decrease expression of CYP11B2 and aldosterone production *in vivo* will require further study.

Elements Required for CYP11B2 Expression

Agonist induction of the human CYP11B2 reporter constructs was dependent on a CRE-like sequence located at position −71. This proximal CRE is completely conserved between the CYP11B1 and CYP11B2 genes in human, rat, mouse, hamster, and the CYP11B gene of the cow (9–13, 21). Previous studies have shown that mutation of this element within the mouse gene leads to loss of cAMP induction (17). We find that mutation of this sequence in the human CYP11B2 flanking DNA caused a loss of induction not only by cAMP, but also by ANG II and K^+, suggesting that this element is necessary for both Ca^{2+}- and cAMP-induced transcription. It is unclear at present whether this CRE directly mediates Ca^{2+}-induced transcription or instead plays a permissive role for other Ca^{2+}-sensitive elements. There is, however, increasing evidence that the calcium-signaling pathway can directly utilize CREs to increase transcription (33, 34). One potential mechanism for this cross-talk involves the calcium/calmodulin-dependent protein kinases (CaMK). *In vitro*, CaMK I, II, and IV can phosphorylate CREB (35–37). CaMK are expressed in adrenocortical cells and appear to be involved in the acute stimulation of aldosterone production (38–41). Therefore, the CaMK are likely candidates linking intracellular Ca^{2+} signals to CYP11B2 transcription.

The proximal CRE was not sufficient to support human CYP11B2 expression, suggesting that other sequences are required to enhance transcription. Previous studies have shown that cAMP induction of the mouse *cyp11b2* gene also requires the presence of an element (AAGGTCTT) that binds SF-1, also referred to as Ad4BP (42, 43). Mutation of the mouse SF-1-binding site markedly impaired transcription (18), consis-

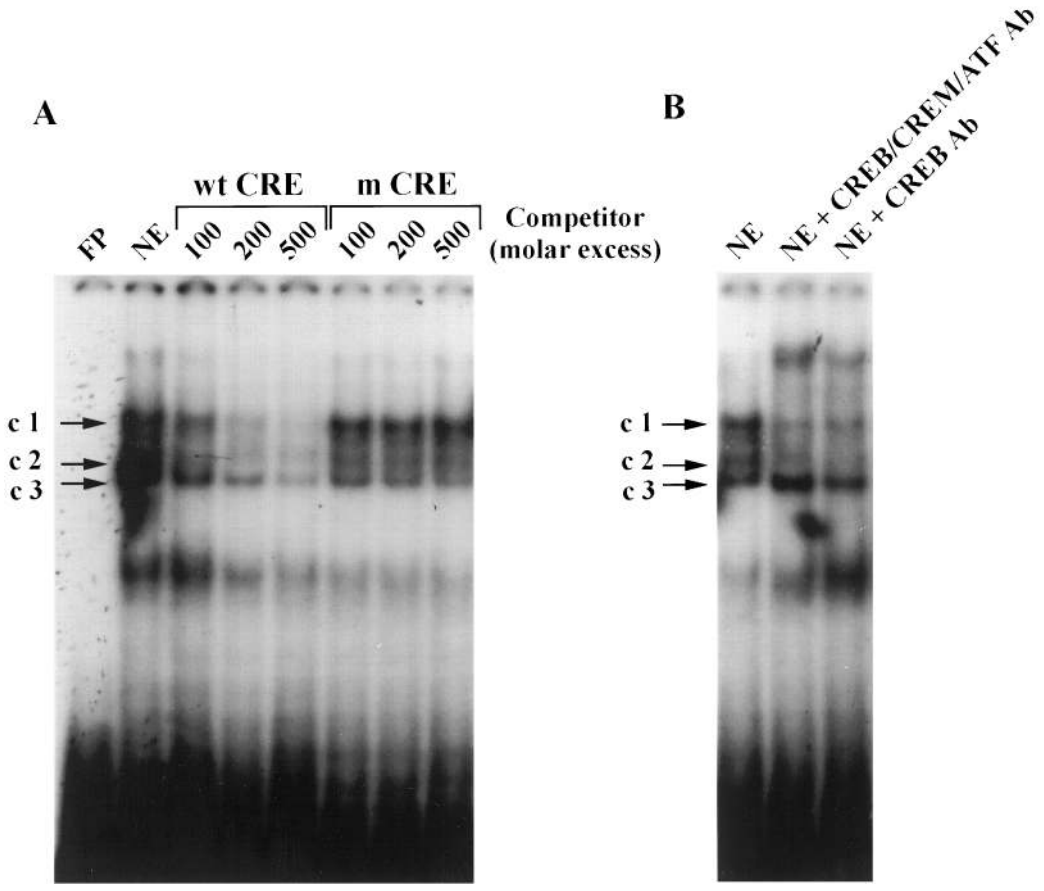

Fig. 7. Binding of H295R Cell Nuclear Proteins to the −71/−64 Element

Panel A, H295R nuclear extracts (NE, 5 μg) were incubated with radiolabeled probe encompassing the −71/−64 element (20,000 cpm) in the presence or absence of either nonradiolabeled probe (wt CRE) or mutated nonradiolabeled probe (m CRE) at the concentrations indicated. DNA/protein complexes (labeled C1, C2, and C3) were separated from free probe (FP) by gel electrophoresis and visualized by autoradiography. Panel B, H295R nuclear extracts were incubated on ice for 20 min in the presence or absence of antisera directed against either CREM/CREB/ATF, or CREB specifically.

tent with the established role of SF-1 in directing the tissue-specific expression of steroidogenic enzymes (44). Based on sequence alignments of the CYP11B genes of several mammalian species, the critical SF-1 site in both mouse CYP11B2 and cow CYP11B corresponds in human CYP11B2 to a conserved SF-1-like sequence at position −351/−343 (AAGGCTCC). However, the results of the deletion studies shown in Fig. 3 do not support a role for the −351/−343 element in transcription of the human gene, even though this sequence strongly binds SF-1 from H295R nuclear extracts in EMSA (data not shown)

In contrast to the mouse and cow CYP11B genes, human CYP11B2 transcription required the presence of an element located at −129/−114 (CTCCAGCCTTGACCT). This element shares 12 of 15 nucleotides with a region previously identified in the bovine CYP11B gene (CTCCAACCCTGACCC) termed Ad5 (Adrenal-5, Ref. 20). Although Ad5 was originally identified as an element binding bovine adrenal nuclear proteins, deletion of this element did not affect the ability of bovine CYP11B reporter constructs to be induced by cAMP when transfected in Y-1 cells (21). However, deletion of the Ad5 element from the human CYP11B2 5′-flanking region drastically impaired basal levels of transcription as well as preventing maximal induction by cAMP and Ca^{2+}-signaling pathways. Using EMSA, we demonstrated binding of SF-1 from H295R cell nuclear extracts to this element. Sequence analysis revealed an SF-1-like sequence on the noncoding strand of the element. The Ad5 element is not completely conserved in the CYP11B2 genes of various species (Fig. 9). In mouse *cyp11b2* there are 4-bp substitutions that disrupt the SF-1 site, possibly explaining why the Ad5 region neither binds nuclear proteins (18) nor enhances transcription of the mouse gene (17–19).

Two additional Ad5 protein complexes, which were recognized by COUP-TF antibody, were observed when H295R cell nuclear proteins were used in EMSA. Binding of SF-1 and COUP-TF to a common site has been described in the mouse 21-hydroxylase (CYP21) and bovine 17α-hydroxylase (CYP17) promoters (45, 46). Moreover, the binding of COUP-TF appears to

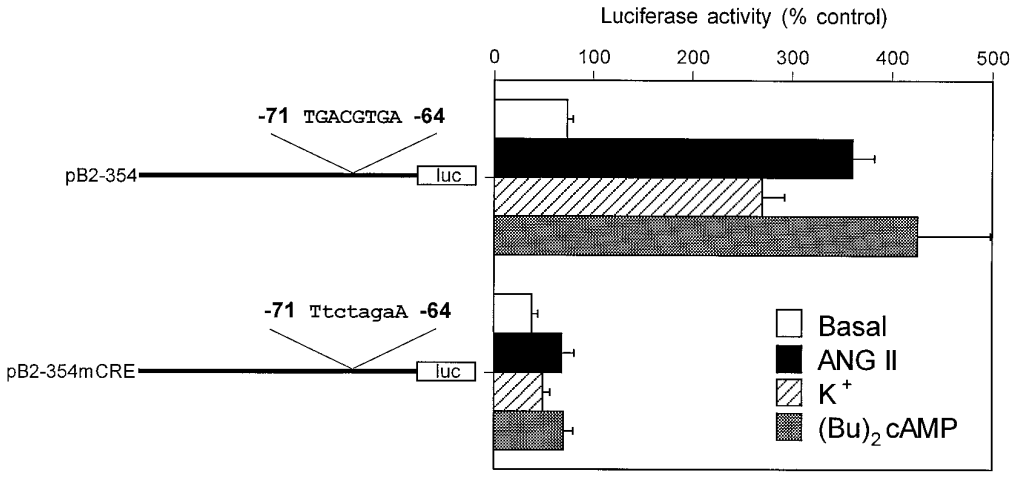

Fig. 8. Analysis of Putative CRE Element within the CYP11B2 Promoter

H295R cells were transiently transfected with a wild type CYP11B2 construct (pB2–354) or one containing a mutation in the core CRE-like sequence (pB2–354 mCRE). After recovery, cells were treated with: ANG II (10 nM), KCl (20 mM), and $(Bu)_2$cAMP (1 mM). Luciferase activities are expressed as a percentage of the basal activity of pB2–2015 and represent the mean ± SEM of determinations from three to seven independent experiments, each performed in triplicate.

5'	-129 CTCCAGCCTTGACCTT	-114	3'	Human CYP11B2
5'	-121 C**c**CCA**a**CC**a**TGACC**ag**	-106	3'	Rat CYP11B2
5'	-112 C**c**CCAG**t**C**a**TGACC**ag**	-97	3'	Mouse *cyp11b2*
5'	-218 CTCCA**a**CC**c**TGACC**ca**	-203	3'	Bovine CYP11B

Fig. 9. Alignment of the Ad5 DNA Sequences of Human, Rat, and Mouse CYP11B2 and Bovine CYP11B Genes

The nucleotide sequences of the Ad5 regions of the human, rat, and mouse CYP11B2 and bovine CYP11B are shown. The nucleotide sequences are numbered from the major transcriptional start site. Sequence differences with the human Ad5 element are in *lowercase bold*.

occur in competition with SF-1 for the element in the bovine CYP17 promoter (46). Overexpression of COUP-TF in steroidogenic and nonsteroidogenic cells suggest a role for this orphan receptor in repression of transcription through its competition with SF-1 (46). It will therefore be important to determine whether COUP-TF and SF-1 play synergistic or antagonistic roles in transcription of the human CYP11B2 gene.

Comparison to Previous Studies

In the only previously published analysis of human CYP11B2 transcription, CAT reporter constructs were transfected into mouse Y-1 cells, but no expression was detected unless almost the entire 5′-flanking region (to −64) was deleted (15). In that case, expression remained very low compared with the corresponding constructs made with the human CYP11B1 gene. It is possible that these discrepant results arise through the use of different cell models. For example, Y-1 cells do not respond to ANG II or K^+, suggesting that the phenotypic characteristics of the Y-1 cell more closely resemble cells of the zona fasciculata (47, 48). However the Y-1 adrenal cell has proven to be useful in the analysis of transcriptional regulation of the mouse and bovine CYP11B genes. In addition, we have obtained good basal and cAMP-induced expression in Y-1 cells using human CYP11B2 reporter constructs (32).

In summary, ANG II, K^+, and cAMP increase reporter gene expression driven by the 5′-flanking region of human CYP11B2. Two *cis*-elements have been identified, both of which are necessary for maximal induction of CYP11B2 by either Ca^{2+}- or cAMP-signaling pathways. The mechanism by which these independent pathways converge to enhance transcription of CYP11B2 will need to be defined. In addition, the potential interactions between CRE-binding proteins and the orphan nuclear receptors SF-1 and COUP-TF will need to be investigated.

MATERIALS AND METHODS

Plasmids

A transient expression system using the luciferase reporter gene was used to characterize the CYP11B2 promoter. The 5′-end of the CYP11B2 gene was amplified from a bacteriophage λ clone carrying CYP11B2 (13) and a 2017-bp fragment extending from position +2 [relative to the transcription start site, (13)] to the *Eco*RI site at −2015 bp was cloned into the promoterless pGL2-Basic (Promega, Madison, WI) luciferase reporter plasmid to create pB2–2015. Several 5′-dele-

tion constructs were prepared using available restriction endonuclease sites (*Sma*I, position −1521; *Xba*I, position −864; and *Pst*I, position −413). Smaller deletion constructs were prepared by PCR, introducing appropriate restriction sites (5′, *Kpn*I; 3′, *Xho*I) or desired mutations. PCR fidelity was confirmed by sequencing (Sequenase II: USB, Cleveland, OH) and the PCR products cloned into *Kpn*I/*Xho*I-digested pGL2. These deletion fragments corresponded to the following positions: −354, −221, −131, −106, and −65 bp. The promoterless vector (pGL2-Basic) and vector containing the SV40 early promoter (pGL2-Control, Promega) were used as controls.

Cell Culture and Transient Transfection

H295R adrenocortical cells were cultured as previously described (27, 28), using 2.0% Ultroser G (BioSepra SA, Villeneuve la Garenne Cedex, France) instead of Ultroser SF. Transient transfection was carried out using Lipofectamine reagent (GIBCO/BRL, Gaithersburg, MD) following the manufacturer's instructions. Cells were seeded onto 12-well plates to 30–40% confluency and used 48 h later. Transfection was carried out for 6 h at 37 C in a final volume of 0.5 ml DMEM/Ham's F12 medium (DMEM/F12, 1:1) (GIBCO/BRL) containing 5.0 μg Lipofectamine and 190 fmol plasmid DNA. After transfection, cells were incubated for 14 h to allow recovery and expression of foreign DNA. Cells were then incubated with 2.0 ml low serum medium (DMEM/F12 containing 0.1% Ultroser G) for a further 24 h before being rinsed and treated with test substances for the times indicated. Cells were then rinsed twice with PBS and lysed. Luciferase activity of the cell lysates was measured using the Luciferase Assay System (Promega). Luciferase activities were expressed as a percentage of the basal activity observed for the longest construct (pB2–2015), which allowed data from multiple experiments to be pooled for analysis. In addition, at least two separate plasmid DNA preparations were used for each reporter construct. Statistical significance of transformed data was determined using Mann-Whitney U test with a value of $P < 0.05$ considered significant.

Electrophoretic Mobility Shift Assay

Nuclear extracts from cultured H295R cells were prepared by the method of Dignam *et al.* (49). Double-stranded oligonucleotides were end-labeled using [α-^{32}P]dCTP and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and incubated (20,000 cpm) with 4 μg nuclear extract and 2.0 μg poly(deoxyinosinic-deoxycytidylic)acid (as nonspecific competitor) in a final volume of 20 μl for 20 min at 25 C. Where antibodies were included in the reaction, nuclear extract and antibody were preincubated on ice for 20 min before addition of probe. The following antibodies were used: mouse monoclonal anti-CREB, mouse monoclonal anti-CREB/CREM/ATF (both provided by Dr. James P. Hoeffler, Invitrogen Corp., San Diego, CA), rabbit polycolonal anti-COUP-TF (provided by Dr. Ming-Jer Tsai, Baylor College of Medicine, Houston Texas), and rabbit polyclonal anti-SF-1 (provided by Dr. Ken-ichirou Morohashi, Kyushu University, Fukuoka, Japan). The COUP-TF antibody does not discriminate between COUP-TF I and COUP-TF II (50) and appears to bind at least two proteins on Western analysis (51). For competition analysis, reaction mixtures contained various amounts of nonradiolabeled oligonucleotide added simultaneously with probe. The resulting DNA/protein complexes were separated from free probe by electrophoresis using a 5.4% polyacrylamide gel and 0.5 × TBE (final concentrations 44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) as running buffer for 2 h at 200 V. Gels were dried and radioactive complexes visualized after autoradiography at −70C for 24 h. Each figure is representative of a minimum of four independent analyses.

Dnase 1 Footprinting Assay

A 231-bp fragment of CYP11B2 5′-flanking DNA (−229/+2) was amplified by PCR and labeled using MMLV reverse transcriptase and [α^{32}P]-dCTP. Assays were performed using the HotFoot Footprinting kit (Stratagene, La Jolla, CA), following the manufacturer's instructions, using 50,000 cpm probe and 20 μg nuclear extract. Naked probe was digested with 0.2 U DNase I and, in the presence of H295R cell nuclear extracts, 3.0 U DNase I (2 min at 25 C). Digested fragments were separated by denaturing electrophoresis using an 8% polyacrylamide 7 M urea gel and 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) as running buffer. The positions of the protected regions were confirmed in three independent footprinting analyses.

Acknowledgments

The authors wish to thank Dr. Ken-ichirou Morohashi (Kyushu University, Fukuoka, Japan) for providing the SF-1 antiserum, Dr. James Hoeffler (Invitrogen Corp., San Diego, CA) for kindly providing the CREB/CREM/ATF and CREB antibodies, and Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX) for providing the COUP-TF antiserum.

Received November 14, 1996. Revision received January 21, 1997. Accepted January 28, 1997.

Address requests for reprints to: William E. Rainey, Ph.D., Department of Obstetrics & Gynecology, University of Texas Southwestern Medical School, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9032.

This work was supported by awards from the American Heart Association (95010570) and the NIH (DK-43140) (to W.E.R.), from the American Heart Association (Texas Affiliate 94G-086) (to J.M.M.), and from the NIH (DK37867 & DK42169) (to P.C.W.).

REFERENCES

1. Lifton RP 1995 Genetic determinants of human hypertension. Proc Natl Acad Sci USA 92:8545–8451
2. White PC 1994 Disorders of aldosterone biosynthesis and action. N Engl J Med 331:250–258
3. Hall JE (1991) The renin-angiotensin system: renal actions and blood pressure regulation. Compr Ther 17:8–17
4. Quinn SJ, Williams GH 1992 Regulation of aldosterone secretion. In: James VHT (ed) The Adrenal Gland. Raven Press, New York, pp 159–189
5. Aguilera G 1993 Factors controlling steroid biosynthesis in the zona glomerulosa of the adrenal. J Steroid Biochem Mol Biol 45:147–151
6. Quinn SJ, Williams GH 1988 Regulation of aldosterone secretion. Annu Rev Physiol 50:409–426
7. Ogishima T, Suzuki H, Hata J, Mitani F, Ishimura Y 1992 Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-450 11β in rat adrenal cortex: histochemical basis for the functional zonation. Endocrinology 130:2971–2977
8. Yabu M, Senda T, Nonaka Y, Matsukawa N, Okamoto M, Fujita H 1991 Localization of the gene transcripts of 11β-hydroxylase and aldosterone synthase in the rat adrenal cortex by *in situ* hybridization. Histochemistry 96:391–394
9. Domalik LJ, Chaplin DD, Kirkman MS, Wu RC, Liu W, Howard TA, Seldin MF, Parker KL 1991 Different isozymes of mouse 11β-hydroxylase produce mineralocorticoids and glucocorticoids. Mol Endocrinol

5:1853–1861
10. Matsukawa N, Nonaka Y, Ying Z, Higaki J, Ogihara T, Okamoto M 1990 Molecular cloning and expression of cDNAs encoding rat aldosterone synthase: variants of cytochrome P-450$_{11\beta}$. Biochem Biophys Res Commun 169:245–252
11. Lehoux J, Mason JI, Bernard H, Ducharme L, Lehoux J, Véronneau S, Lefebvre A 1994 The presence of two cytochrome P450 aldosterone synthase mRNAs in the hamster adrenal. J Steroid Biochem Mol Biol 49:131–137
12. Curnow KM, Tusie-Luns M, Pascoe L, Natajaran R, Gu J, Nadler JL, White PC 1991 The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. Mol Endocrinol 5:1513–1522
13. Mornet E, Dupont J, Vitek A, White PC 1989 Characterization of two genes encoding human steroid 11β-hydroxylase (P-450$_{11\beta}$). J Biol Chem 264:20961–20967
14. White PC, Curnow KM, Pascoe L 1994 Disorders of steroid 11 beta-hydroxylase isozymes. Endocr Rev 15:421–438
15. Kawamoto T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S, Ohnishi T, Ichikawa Y, Nakao K, Imura H, Ulick S, Shizuta,Y 1992 Role of steroid 11β-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. Proc Natl Acad Sci USA 89:1458–1462
16. Mukai K, Imai M, Shimada H, Ishimura Y 1993 Isolation and characterization of rat CYP11B genes involved in late steps of mineralo- and glucocorticoid syntheses. J Biol Chem 268:9130–9137
17. Rice DA, Aitkan LD, Vandedbark GR, Mouw AR, Franklin A, Schimmer BP, Parker KL 1989 A cAMP-responsive element regulates expression of the mouse steroid 11β-hydroxylase gene. J Biol Chem 264:14011–14015
18. Bogerd AM, Franklin A, Rice DA, Schimmer BP, Parker KL 1990 Identification and characterization of two upstream elements that regulate adrenocortical expression of steroid 11β-hydroxylase. Mol Endocrinol 4:845–850
19. Mouw AR, Rice DA, Meade JC, Chua SC, White PC, Schimmer BP, Parker KL 1989 Structural and functional analysis of the promoter region of the gene encoding mouse steroid 11β-hydroxylase. J Biol Chem 264:1305–1309
20. Kirita S, Hashimoto T, Kitajima M, Honda S, Morohashi K, Omura T 1990 Structural analysis of multiple bovine P-450(11β) genes and their promoter activities. J Biochem 108:1030–1041
21. Honda S, Morohashi K, Omura T 1990 Novel cAMP regulatory elements in the promoter region of bovine P450(11β) gene. J Biochem 108:1042–1049
22. Hashimoto T, Morohashi K, Takayama K, Honda S, Wada T, Handa H, Omura T (1992) Cooperative transcriptional activation between Ad1, a CRE-like element, and other elements in the CYP11B gene promoter. J Biochem 112:573–575
23. Abayasekara DR, Vazir H, Whitehouse BJ, Price GM, Hinson JP, Vinson GP 1989 Studies on the mechanisms of ACTH-induced inhibition of aldosterone biosynthesis in the rat adrenal cortex. J Endocrinol 122:625–632
24. Fuchs-Hammoser R, Schweigwe M, Oelkers W 1980 The effect of chronic low-dose infusion of ACTH (1–24) on renin, renin substrate, aldosterone and other corticosteroids in sodium replete and deplete man. Acta Endocrinol (Copenh) 95:198–206
25. Oertle M, Müller J 1993 Two types of cytochrome P-450$_{11\beta}$ in rat adrenals: separate regulation of gene expression. Mol Cell Endocrinol 91:201–209
26. Bird IM, Walker SW, Williams BC 1990 Agonist-stimulated turnover of the phosphoinositides and the regulation of the adrenocortical steroidogenesis. J Mol Endocrinol 5:191–209
27. Bird IM, Hanley NA, Word RA, Mathis JM, McCarthy JL, Mason JI, Rainey WE 1994 Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin II-responsive aldosterone secretion. Endocrinology 133: 1555–1561
28. Holland OB, Mathis JM, Bird IM, Rainey WE 1993 Angiotensin increases aldosterone synthase mRNA levels in human NCI-H295 cells. Mol Cell Endocrinol 94:R9–R13
29. Bird IM, Mathis JM, Mason JI, Rainey WE 1995 Ca^{2+}-regulated expression of steroid hydroxylases in H295R human adrenocortical cells. Endocrinology 136:5677–5684
30. Denner K, Rainey WE, Pezzi V, Bird IM, Bernhardt R, Mathis JM 1996 Differential regulation of 11β-hydroxylase and aldosterone synthase in human adrenocortical H295R cells. Mol Cell Endocrinol 121:87–91
31. Allen RG, Carey C, Parker JD, Mortrud MT, Mellon SH, Low MJ 1995 Targeted ablation of pituitary pre-opiomelanocortin cells by herpes simplex virus-1 thymidine kinase differentially regulates mRNAs encoding the adrenocorticotropin receptor and aldosterone synthase in the mouse adrenal gland. Mol Endocrinol 9:1005–1016
32. Clyne CD, White, PC, Rainey WE 1996 Calcium regulates human CYP11B2 transcription. Endocr Res 22:485–492
33. Schwaninger M, Lux G, Blume R, Oetjen E, Hikada H, Knepel W 1993 Membrane depolarization and calcium influx induce glucagon gene transcription in pancreatic islet cells through the cyclic AMP-responsive element. J Biol Chem 268:5168–5177
34. Eckert B, Schwaninger M, Knepel W 1993 Calcium-mobilizing insulin secretagogues stimulate transcription that is directed by the cyclic adenosine 3′,5′-monophosphate/calcium response element in a pancreatic islet beta-cell line. Endocrinology 137:225–233
35. Gonzalez GA, Yamamoto KK, Fischer WH, Montminy MR 1989 A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337:749–752
36. Lee CQ, Yun Y, Hoeffler JP, Habener JF 1990 Cyclic AMP responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. EMBO J 9:4455–4465
37. Sun P, Lou L, Maurer RA 1996 Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca^{2+}/calmodulin-dependent protein kinases type I, II, and IV. J Biol Chem 271:3066–3073
38. Papadopoulos V, Brown AS, Hall PF 1990 Calcium-calmodulin-dependent phosphorylation of cytoskeletal proteins from adrenal cells. Mol Cell Endocrinol 74:109–123
39. Fern RJ, Hahm MS, Lu HK, Liu LP, Gorelick FS, Barrett PQ 1995 Ca^{2+} calmodulin-dependent protein kinase II activation and regulation of adrenal glomerulosa Ca^{2+} signaling. Am J Physiol 38:F751–F760
40. Clyne CD, Nguyen A, Rainey WE 1995 The effects of KN62, a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, on adrenocortical cell aldosterone production. Endocr Res 21:259–295
41. Pezzi V, Clark BJ, Ando S, Stocco DM, Rainey WE 1996 Role of Calmodulin-dependent protein kinase II in acute adrenal aldosterone production. J Steroid Biochem Mol Biol 58:417–424
42. Lala DS, Rice DA, Parker KL 1992 Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor 1. Mol Endocrinol 6:1249–1258
43. Morohashi K, Honda S, Inomata Y, Handa H, Omura T 1992 A common *trans*-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J Biol Chem 267:17913–17919
44. Parker KL, Schimmer BP 1993 Transcriptional regulation of the adrenal steroidogenic enzymes. Trends Endocrinol Metab 4:46–50
45. Rice DA, Mouw AR, Bogerd AM, Parker KL 1991 A shared promoter element regulates the expression of three steroidogenic enzymes. Mol Endocrinol 5:1552–1561

46. Lund J, Bakke M 1995 Mutually exclusive interactions of two orphan nuclear receptors determine activity of a cyclic adenosine 3′,5′-monophosphate-responsive sequence in the bovine *CYP17* gene. Mol Endocrinol 9:327–339
47. Langlois D, Saez JM, Begeot M 1990 Effects of angiotensin II on inositol phosphate accumulation and calcium influx in bovine adrenal or Y-1 tumor adrenal cells. Endocr Res 16:31–49
48. Es-souni Routhier M, Bournot P, Ramirez LC 1995 Aldosterone synthase activity in the Y-1 adrenal cell line. J Steroid Biochem Mol Biol 52:581–585
49. Dignam JD, Lebozitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
50. Mietus SM, Sladek FM, Ginsburg GS, Kuo CF, Ladias JA, Darnell JJ, Karathanasis SK 1992 Antagonism between apolipoprotein AI, AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol Cell Biol 12:1708–1718
51. Wang L-H, Tsai SY, Cook RG, Beattie WG, Tsai M-J, O'Malley BW 1989 COUP transcription factor is a member of the steroid receptor superfamily. Nature 340:163–166