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# Transcriptional Regulation of Human 11β-Hydroxylase (*hCYP11B1*)

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**ABSTRACT**

Steroid 11β-hydroxylase is a mitochondrial enzyme that catalyzes the conversion of deoxycortisol to cortisol. The gene encoding human 11β-hydroxylase (*hCYP11B1*) is expressed in the adrenal cortex under the control of circulating levels of ACTH. The current study was undertaken to define the *cis*-regulatory elements and transacting factors that regulate *hCYP11B1* transcription. The *hCYP11B1* 5′-flanking DNA was studied using transient transfection of luciferase reporter constructs in NCI-H295R human adrenocortical cells. A cAMP analogue ($(Bu)_2$cAMP) increased expression of a construct containing −1102 bp of *hCYP11B1* 5′-flanking DNA (pB1–1102). An element at position −71/−64 (TGACGTGA, previously termed Ad1) resembling a consensus cAMP response element (CRE) was required for maximal induction by cAMP. The Ad1 element bound several transcriptional factors in electrophoretic mobility shift assays, including CRE-binding protein, activating transcription factor-1 (ATF-1), and ATF-2, but only the ATF-2 complex migrated similarly to a complex seen using H295R nuclear extract. In addition, Western analysis of H295R and adrenal lysates demonstrated expression of high levels of ATF-2 and ATF-1. CRE-binding protein levels varied among the strains of H295R cells tested. Transcription of *CYP11B1* also appeared to be regulated by steroidogenic factor-1 (SF-1). Luciferase reporter gene activity was increased after cotransfection with expression vectors containing SF-1. An element in *hCYP11B1* at positions −242/−234 (CCAAGGCTC), previously termed Ad4, was required for maximal induction by SF-1 and was found to bind SF-1 in electrophoretic mobility shift assays. The key role for SF-1 in *hCYP11B1* transcription is in contrast to its lack of an effect on expression of the *hCYP11B2* (aldosterone synthase) isozyme. The differential effects of SF-1 on transcription of *hCYP11B1* and *hCYP11B2* may be one of the mechanisms controlling differential expression of these isozymes within the zonae fasciculata and glomerulosa of the human adrenal cortex. (*Endocrinology* **141:** 3587–3594, 2000)

THE ADRENAL cortex is responsible for the production of both mineralocorticoids and glucocorticoids. Mineralocorticoids are synthesized in the zona glomerulosa, whereas glucocorticoids are produced within the zona fasciculata. The zonal production of these steroids is in part the result of tightly controlled transcription of the CYP11B genes (1, 2). Aldosterone synthase is responsible for the conversion of deoxycorticosterone to aldosterone, is expressed only within the zona glomerulosa, and is mainly under the control of circulating angiotensin II and potassium levels. Steroid 11β-hydroxylase is responsible for the conversion of deoxycortisol to cortisol, is localized to the zona fasciculata, at least in rodents (2, 3), and is under the control of circulating ACTH levels.

The expression of each isozyme appears to be controlled at the level of gene transcription. The 5′-flanking region of *CYP11B2*, the gene encoding aldosterone synthase, has been analyzed in detail for the mouse (4–7), hamster (8, 9), and human genes (10, 11). However, the mechanisms regulating transcription of *CYP11B1*, the gene encoding 11β-hydroxylase, are less completely understood. Although the bovine CYP11B (*bCYP11B*) gene has been studied in some detail (12–16), cattle have only one active *bCYP11B* gene that is expressed in both the glomerulosa and fasciculata of the adrenal (17). Therefore, the regulatory elements in bovine *bCYP11B* 5′-flanking DNA may differ from those that control the zone-specific expression of the multiple CYP11B genes observed in rodents and humans.

One difficulty in studying the CYP11B genes has been the availability of an appropriate *in vitro* model system. Previous studies have used the mouse Y-1 adrenal cell line for a preliminary analysis of *hCYP11B1* 5′-flanking DNA (18, 19). More recently, we and others have characterized a human adrenocortical carcinoma cell line (H295R) that produces both aldosterone and cortisol (20, 21). These cells also express both *hCYP11B1* and *hCYP11B2* transcripts, which are under the differential control of cAMP and angiotensin II signaling pathways, respectively (22–24). The current study was undertaken to analyze the 5′-flanking DNA of the *hCYP11B1* gene and to define the *cis*-regulatory elements and transacting factors that are necessary for cAMP induction of transcription. The results suggest that ACTH induction of *hCYP11B1* transcription requires primarily two *cis*-regulatory elements, one (termed Ad1) resembling a consensus ccAMP response element (CRE), and the other (termed Ad4) that binds steroidogenic factor-1 (SF-1).

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## Materials and Methods

### *Preparation of reporter constructs with serial deletions of hCYP11B1 5′-flanking DNA*

A transient expression system using the luciferase reporter gene was used to characterize the *hCYP11B1* promoter. A 1102-bp fragment (pB1–

1102) extending from position −2 (relative to the transcriptional start site) to −1102 was cloned into the promoterless pGL3-Basic luciferase reporter plasmid (Promega Corp., Madison, WI). Several 5′-deletion plasmids were constructed using available restriction endonuclease sites (*Xba*I, pB1–924; *Nhe*I, pB1–777; *Xmn*I, pB1–514; *Dra*I, pB1–330; *Apa*I, pB1–214). The pB1–65 construct was prepared using PCR to introduce a unique *Kpn*I restriction site.

### *Preparation of mutant constructs*

Deoxyribonuclease I footprint analysis of the *bCYP11B* 5′-flanking DNA demonstrated several regions that bind nuclear proteins (12). These protected regions were termed Ad-1 to Ad-6. Similar sequences were observed in the *hCYP11B1* gene; therefore, this terminology will be used to discuss the regulatory elements. For the Ad1/CRE mutants, the sequence 5′-TGACGTGA-3′ (−71/−64) was changed to 5′-gGtaccGA-3′, which included a *Kpn*I site (*underlined*). To do so, PCR was performed on pB1–1102 using a set of primers, 1 and 2 (Table 1). The Ad4 sequence 5′-ATCCAAGGCTCT-3′ (−244/−233) was changed to 5′-ATCgAAttCTCT-3′, and the Ad5 sequence 5′-CCTGACCTCT-3′ (−121/−112) was changed to 5′-CCTGAatTCT-3′, each of which included an *Eco*RI site (*underlined*). The pB1–1102 construct was used as the template for PCR reactions using primers 3 and 4 for the Ad4 mutant and primers 5 and 6 for the Ad5 mutation (Table 1).

### *Cell culture and transfections*

H295R adrenocortical cells (25) were cultured in DMEM/F-12 (Life Technologies, Inc./BRL, Gaithersburg, MD) supplemented with 2% Ultroser G (BioSepra SA, Villeneuve la Garenne, France), 1% ITS Plus (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, and 5.35 μg/ml linoleic acid; Collaborative Research, Bedford, MA) and antibiotics. Mouse Y-1 adrenal cells were obtained from the American Type Culture Collection (Manassas, VA) and grown under the same conditions as the H295R cells. For transfection experiments, cells were subcultured onto 12-well culture dishes (150,000 cells/well) and were used 24 h later. Transfection was carried out using 2.0 μl Fugene (Roche Molecular Biochemicals, Indianapolis, IN) and 1.0 μg reporter plasmid DNA in DMEM/F12 medium (1.1 ml) for 6 h at 37 C. For cotransfection experiments, various amounts of expression plasmids were included in the transfection reaction, and the total amount of DNA was kept constant by the addition of carrier DNA (empty expression vector). After transfection, cells were incubated with 2.0 ml low serum medium (DMEM/F12 medium containing 0.1% Ultroser G) for 24 h before being treated with agonists for 6 h. Cells were then lysed and assayed for activity using a luciferase assay (Promega Corp.).

### *Electrophoretic mobility shift assay (EMSA)*

Nuclear extracts were prepared from H295R cells by the method of Schreiber *et al.* (26). Double stranded oligonucleotides (25 pmol) were labeled with 30 μCi [$\gamma^{-32}$P]ATP and 10 U T4 polynucleotide kinase at 37 C for 30 min. Nuclear extract and each radiolabeled probe (40,000 dpm) were incubated at room temperature for 20 min in 20 μl reaction mixture [20 mM HEPES (pH 8.0), 80 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mg/ml BSA, and 0.025 mg/ml poly(dI-dC) as nonspecific competitor]. When antibodies were included in the reaction, nuclear extract and antibody were preincubated on ice for 20 min before the addition of probe and reaction mixture. For competition analysis, reaction mixtures containing various amounts of nonradiolabeled oligonucleotide were added simultaneously with probe. The resulting DNA/protein complexes were separated from free probe by electrophoresis using a 4% high ionic strength native polyacrylamide gel with 1 × Tris-glycine running buffer (2). The gel was dried and visualized after autoradiography at −70 C for 24 h. The following double stranded DNA probes used were Ad4 (−250/−227; sequence 7 in Table 1), Ad4-mutant (sequence 8 in Table 1), Ad1 (−79/−56; sequence 9 in Table 1), and Ad1-mutant (sequence 10 in Table 1). Rabbit polyclonal anti-SF-1 (Ad4BP) was provided by Dr. Ken-ichirou Morohashi, Kyushu University (Fukuoka, Japan). Human SF-1, CRE-binding protein (CREB), activating transcription factor-1 (ATF-1), and ATF-2 were prepared using a Promega Corp. *in vitro* transcription/translation system.

**TABLE 1.** Oligonucleotide sequences used as primers or as probes for electrophoretic mobility shift assays

| | |
|---|---|
| 1 | 5′-GGGCCAGTTCTCCCAgGtaccGATCCCTCCCGAAGGC-3′ (sense) |
| 2 | 5′-GCCTTCGGGAGGGATCggtaCcTGGGAGAACTGGCCCTGG-3′ (anti-sense) |
| 3 | 5′-GATGAATAATCgAAttCTCTTGGATAAGATAAGGGCCCC-3′ (sense) |
| 4 | 5′-CTTATCCAAGAGaaTTcGATTATTCATCTCCTTGCAAGG-3′ (anti-sense) |
| 5 | 5′-CTGCCTCCAGCCCTGAatTCTGCCCTCGGTCTC-3′ (sense) |
| 6 | 5′-GAGACCGAGGGCAGAatTCAGGGCTGGAGGCAG-3′ (anti-sense) |
| 7 | (5′-TGAATAATCCAAGGCTCTTGGATA-3′) |
| 8 | (5′-TGAATAATCgAAttCTCTTGGATA-3′) |
| 9 | (5′-TTCTCCCATGACGTGATCCCTCCC-3′) |
| 10 | (5′-TTCTCCCAgGtaccGATCCCTCCC-3′) |

*Lowercase letters* indicate bases that were mutated.

### *Protein immunoblotting analysis*

Cultured H295R cells were used to prepare nuclear extract as previously described (26). Two groups of H295R cells were used to isolate nuclear extracts that were used for the Western analysis. One group of H295R cells was representative of the cells used for the transfection EMSA and studies (Fig. 3, H295 NE-1). The second population of H295R cells was maintained in culture for extended periods (>1 yr). Adult adrenal glands were obtained and homogenized in lysis buffer (27). HeLa cell nuclear extract, which was used as a positive control, was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). In addition, CREB, ATF-1, and ATF-2 were prepared using a Promega Corp. *in vitro* transcription/translation system. PAGE was carried out on the samples using a precast Novex gel electrophoresis system with 4–12% bis-Tris NuPage gels (Novex, San Diego, CA). Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes by wet transfer for 1 h at 25 V. After transfer the membranes were incubated with ATF-2 (1:1500 dilution), CREB (1:2000 dilution), or ATF-1 (1:800 dilution) antibodies overnight at 4 C. The ATF-2 and CREB antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). The ATF-1 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). In addition to the manufacturer's characterization, antibodies were tested for specificity using purified or *in vitro* translated CREB, ATF-1, or ATF-2. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized using enhanced chemiluminescence Western blotting detection reagents from Amersham Pharmacia Biotech (Piscataway, NJ).

## Results

### *Deletion analysis of the hCYP11B1 promoter*

To identify the *cis*-elements that regulate basal and cAMP-regulated transcription, a series of deletion constructs was prepared containing progressively shorter fragments of *hCYP11B1* 5′-flanking DNA (pB1–1102, pB1–924, pB1–777, pB1–514, pB1–330, pB1–214, and pB1–65). These constructs were transiently transfected into H295R cells. Figure 1 illustrates the reporter activity of constructs under basal conditions or after treatment with $(Bu)_2$cAMP (1 mM) for 6 h. Basal luciferase activities of the constructs containing 5′-flanking sequences from −1102 to −214 bp were similar. Further deletion to −65 bp decreased luciferase activity to that observed for the pGL3 Basic empty vector, indicating that DNA between −214 and −65 is essential for basal gene expression. $(Bu)_2$cAMP treatment of H295R cells transfected with pB1–1102 construct resulted in a 5-fold increase in luciferase activity. Deletion to −214 bp did not significantly alter $(Bu)_2$cAMP stimulation of luciferase activity. Deletion to

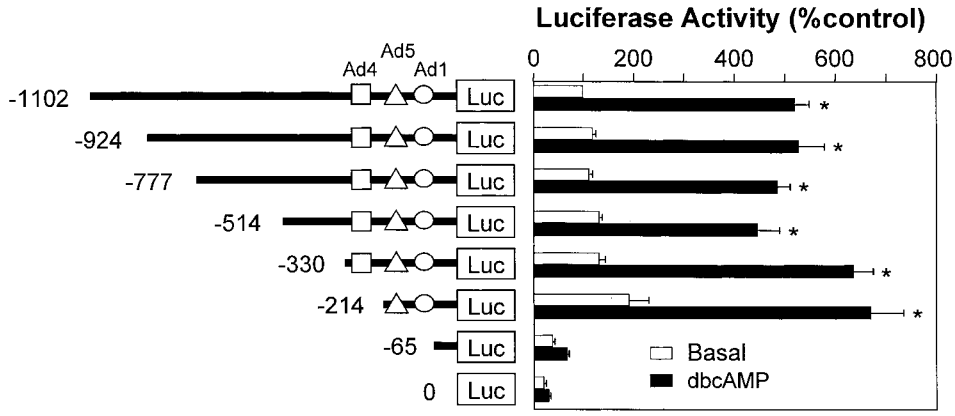


FIG. 1. Deletion analysis of the *hCYP11B1* 5′-flanking DNA to define basal and $(Bu)_2$cAMP regulatory regions. H295R cells were transiently transfected with luciferase reporter constructs containing serial deletions of *hCYP11B1* 5′-flanking DNA (1 μg/well). After recovery for 24 h, cells were treated with or without $(Bu)_2$cAMP (dbcAmp) (1 mM) for 6 h. Cells were then lysed, and luciferase activity was measured. Results are expressed as a percentage of the basal reporter activity of pB1–1102 and represent the mean ± SEM of data from four independent experiments (*, $P < 0.0001$ compared with the basal level).

−65, however, abolished $(Bu)_2$cAMP induction of reporter gene expression, indicating that DNA between −214 and −65 contains the minimally essential regions for both basal and cAMP-regulated transcription of *hCYP11B1*.

*Role of the Ad1 cis-element in hCYP11B1 transcription*

A near-consensus CRE, previously termed Ad1, was shown to play a critical role in transcription of *bCYP11B* as well as mouse and human *CYP11B2* (5, 11). To determine whether the corresponding sequence in *hCYP11B1* (TGACGTGA) is important for transcription, it was mutated (gGtaccGA) in pB1–1102. Mutation of Ad1 decreased both basal and $(Bu)_2$cAMP induction, suggesting that this element is needed for transcription of *hCYP11B1* (Table 2).

To begin characterization of nuclear proteins that bind the Ad1 element, a synthetic oligonucleotide probe encompassing this sequence was prepared and used in EMSA. In the presence of H295R nuclear extract, at least two specific protein-DNA complexes were formed (Fig. 2). Formation of these complexes was abolished by the addition of nonradiolabeled competitor. Because the Ad1 element is almost identical to a consensus CRE, transcription factors know to interact with this sequence were examined using EMSA. *In vitro* prepared CREB, ATF-1, and ATF-2 formed complexes with the Ad1 element, giving rise to complexes that migrated differently. ATF-2, but not CREB or ATF-1, formed a radiolabeled complex that comigrated with a complex observed using H295R cell nuclear extract (complex 1). Antibody directed against ATF-2 supershifted complex 1, suggesting that it represents a complex containing ATF-2. Antibodies directed against CREB or ATF-1 did not significantly modify

**TABLE 2.** Effect of the CRE at −71/−64 on transcription of *hCYP11B1*

| Plasmid | Relative luciferase expression[a] | |
|---|---|---|
| | −dbcAMP | +dbcAMP |
| *hCYP11B1* (pB1-1102) | 100 | 293 ± 76 |
| CRE mutant (pB1-1102 CRE) | 47 ± 8 | 64 ± 17 |

[a] The luciferase activity for *hCYP11B1* in the absence of dbcAMP is taken as 100%. Transfection experiments were repeated in three independent experiments in triplicate within each experiment. The values presented represent the mean ± SEM of the data combined from the three independent experiments.

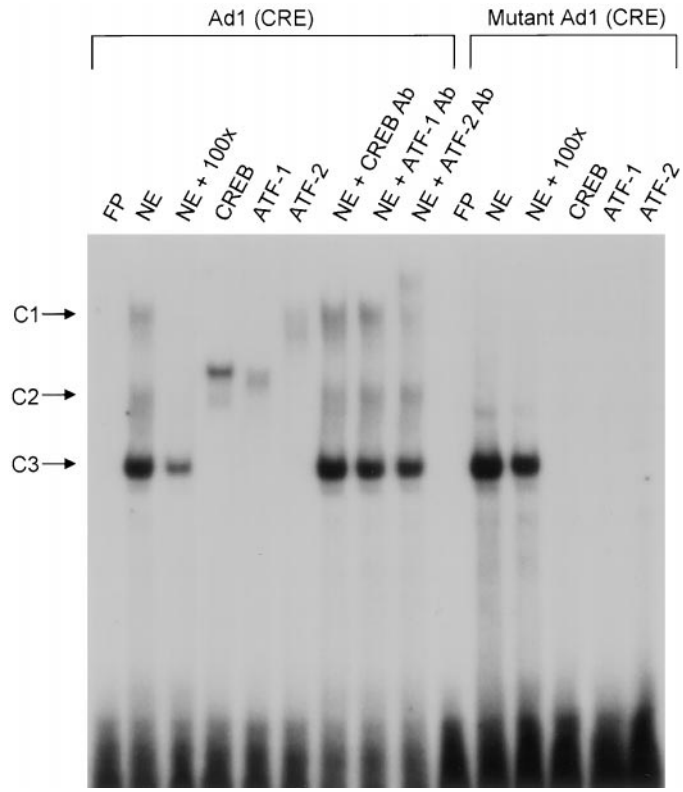


FIG. 2. Binding of specific transcription factors to *hCYP11B1* Ad1/CRE (−71/−64). EMSA was performed using a $^{32}$P-labeled oligonucleotide probe corresponding to *hCYP11B1* −71/−64. Radiolabeled probe alone is shown in lane 1 (FP), and probe with H295R nuclear extract (NE; 5.0 μg) is shown in lane 2. Protein/DNA complexes (designated 1, 2, and 3) were separated from free probe by electrophoresis. Nonradiolabeled self-competitor DNA was added at a 100-fold molar excess (lane 3, NE + 100×) to identify specific protein/DNA interactions. Lanes 4–6 show binding activity in the presence of *in vitro* prepared CREB, ATF-1, or ATF-2, respectively. Lanes 7–9 show the effects of incubation of probe with nuclear extract in the presence of antibodies directed against CREB, ATF-1, or ATF-2, as indicated. Lanes 10–15 examine the mutated Ad1/CRE oligonucleotide using EMSA. Radiolabeled probe only is shown in lane 10 (FP), and probe with H295R nuclear extract is shown in lane 11 (NE). Nonradiolabeled self-competitor DNA was added at a 100-fold molar excess, as shown in lane 12 (NE + 100×). Lanes 13–15 examine complex formation with *in vitro* prepared CREB, ATF-1, and ATF-2. Each oligonucleotide set was tested on a minimum of three H295R nuclear extracts.

any of the three major complexes. Oligonucleotides that included a mutated Ad1 sequence (gGtaaGA) did not form complexes with *in vitro* prepared CREB, ATF-1, or ATF-2 (Fig. 2).

As the adrenal expression of CRE-binding transcription factors has not previously been completely characterized, we carried out Western analysis using antibodies directed against CREB, ATF-1, and ATF-2 (Fig. 3). Nuclear extracts from H295R and HeLa cells as well as homogenates of human adrenal cortex expressed readily detectable levels of ATF-2 and varying amounts of CREB and ATF-1. CREB protein was detectable in HeLa nuclear extract, early passage H295R nuclear extract (H295 NE-1), as well as adrenal lysate. ATF-1 expression appeared higher in the H295R adrenal cell than adrenal lysate. Interestingly, CREB expression appeared to be lost in H295R cells cultured for long periods (Fig. 3, H295 NE-2).

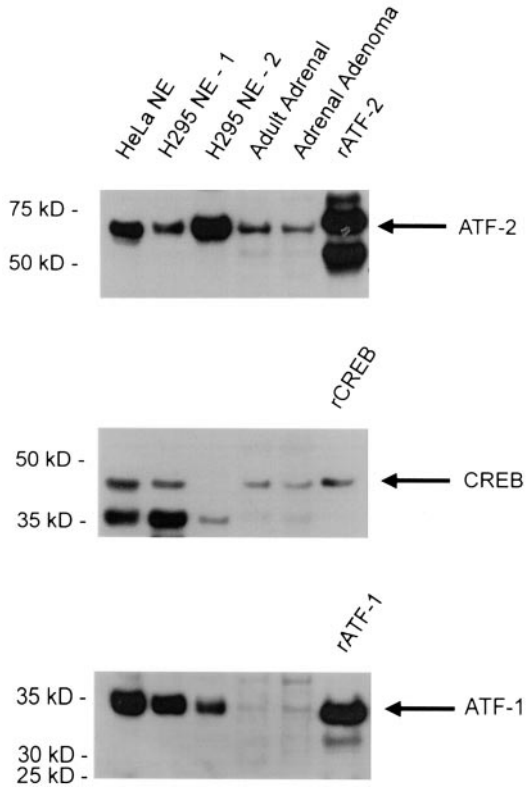


FIG. 3. Adrenal expression of CRE-binding proteins. Protein immunoblot analysis was used to examine expression of CREB, ATF-1, and ATF-2 in adrenal lysates and H295R nuclear extracts. Homogenates of adrenal gland (20 $\mu$g) and aldosterone-producing adrenal adenoma (20 $\mu$g) were compared with nuclear extracts from two strains of H295R cells (4.5 $\mu$g). The H295R NE-1 was isolated from cells that were similar to those used for the transfection and EMSA. H295R NE-2 was isolated from cells that had been in culture longer than 1 yr. These nuclear extracts were representative of three extracts tested. HeLa cell nuclear extract (5 $\mu$g) and recombinant ATF-1, CREB, or ATF-1 were used as a positive control for members of the CREB/ATF family of proteins. The antibody used to visualize CREB has previously been shown to also recognize ATF-1, as can be seen by the presence of a band of approximately 35 kDa.

*Role of the Ad4 and Ad5 elements in hCYP11B1 transcription*

Interestingly, as shown in Fig. 1, deletion of the Ad4 site (−242/−234) from *hCYP11B1* in pB1–214 did not affect basal or cAMP induction of reporter gene expression. Because cotransfection of expression vectors containing SF-1 enhanced reporter gene activity in constructs using 5′-flanking DNA from the bovine CYP11A (15), human CYP19 (28), mouse CYP21 (29), bovine CYP17 (30), and *bCYP11B* (31) genes, we carried out similar experiments in which H295R cells were transfected with the *hCYP11B1* serial deletion constructs and human SF-1 expression vector (Fig. 4). Reporter gene expression was similar using constructs containing −1102 to −330 bp of *hCYP11B1* 5′-flanking DNA. However, in cells cotransfected with SF-1 expression plasmid, deletion to −214 bp decreased luciferase activity to 53% of that seen in cells transfected with the −330 bp construct (pB1–330). This observation suggests that the Ad4 site is needed for maximal induction of transcription by SF-1.

Sequence analysis revealed two potential SF-1-binding sites that have previously been observed in the *bCYP11B* gene and were designated Ad4 and Ad5 (12). Previous work from our laboratory demonstrated an important role for the Ad5 element in *hCYP11B2* transcription (11), whereas the Ad4 element (through SF-1 interaction) plays a critical role in *bCYP11B* transcription (31). To define the relative contributions of the Ad4 (−242/−234) and Ad5 (−120/−112) *cis*-elements in regulating *hCYP11B1* transcription, they were each mutated using the pB1–1102 plasmid of *hCYP11B1* 5′-flanking DNA (Fig. 5). Mutation of the Ad4 site had little effect on basal activity, but decreased the stimulatory effect of SF-1 coexpression to 27% of the stimulation seen using the native pB1–1102 construct. In contrast, mutation of the Ad5 did not significantly affect luciferase activity. Constructs with mutations of both elements had basal and SF-1-stimulated levels of reporter activity near that observed with pGL3 Basic empty vector (not shown).

To determine whether induction by cAMP could enhance the effects of SF-1, H295R cells were transfected with native pB1–1102 or the Ad4, Ad5, or Ad4/Ad5 mutant constructs, either alone or with the SF-1 expression vector. Transfected cells were subsequently treated with $(Bu)_2$cAMP for 6 h, whereas control cells received no treatment (Fig. 5). When cells were transfected with pB1–1102 only and treated with $(Bu)_2$cAMP, activity increased 5-fold over basal levels. Additionally, cotransfection with SF-1 in the absence of $(Bu)_2$cAMP caused a 6-fold increase in reporter activity above basal levels. When cells were cotransfected with pB1–1102 and the SF-1 expression plasmid and treated with $(Bu)_2$cAMP, there was a synergistic effect, leading to a 40-fold increase above basal levels. Treatment with $(Bu)_2$cAMP also enhanced the effects of SF-1 on the three mutant constructs tested. However, only the $(Bu)_2$cAMP-treated cells cotransfected with the Ad5 mutant and SF-1 displayed levels of activity similar to that of the native pB1–1102 construct.

To determine whether the Ad4 element could bind SF-1, a synthetic oligonucleotide probe encompassing this sequence was prepared and used in EMSA (Fig. 6). In the presence of H295R nuclear extracts, two major DNA/protein

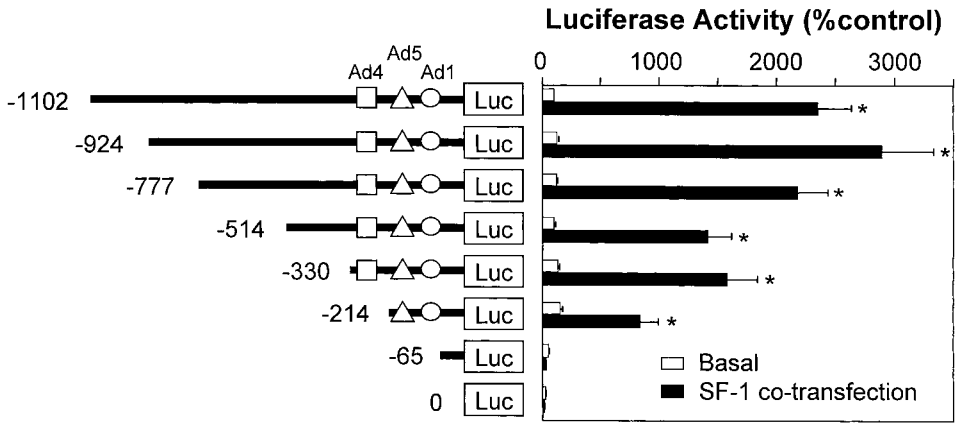


FIG. 4. Deletion analysis of the *hCYP11B1* 5′-flanking DNA to determine SF-1 sensitive regions. H295R cells were transiently transfected with luciferase reporter constructs containing serial deletions of *hCYP11B1* 5′-flanking DNA. Transfection of reporter constructs was performed with either empty pcDNA3 expression vector (1 μg/well) or expression vector containing the coding sequence for bovine SF-1 (1 μg/well). After recovery for 24 h, cells were lysed, and luciferase activity was measured. Results are expressed as a percentage of the basal reporter activity of the pB1–1102 and represent the mean ± SEM of data from five independent experiments (*, $P < 0.0001$ compared with the basal level).

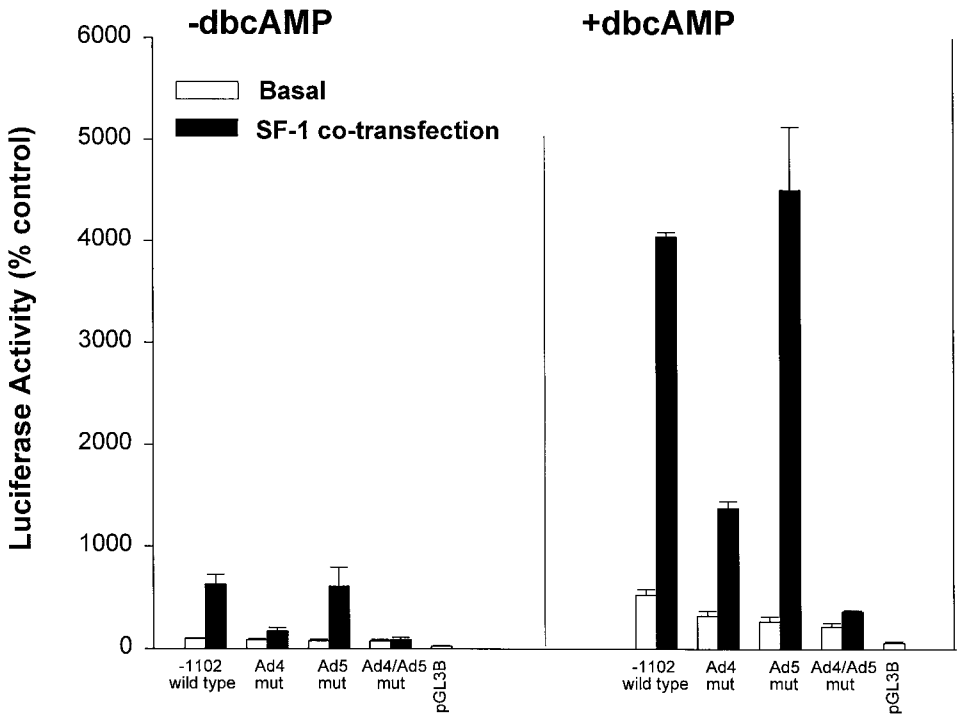


FIG. 5. Mutational analysis of the Ad4 and Ad5 elements. H295R cells were transiently transfected with a luciferase reporter vector driven by pB1–1102 containing the wild-type Ad4 and Ad5 sequences (−1102 wild-type), the mutated Ad4 sequence (−1102 Ad4 mutant), the mutated Ad5 sequence (−1102 Ad5 mutant), or the Ad4/Ad5 double mutant sequence (−1102 Ad4/Ad5 mutant). Cells were transfected with reporter construct and either pcDNA3 empty expression (1 μg/well) vector or with expression vector containing coding sequence for bovine SF-1 (1 μg/well). Where indicated, cells were treated with $(Bu)_2$cAMP (dbcAmp) (1 mM) for 6 h, followed by lysis and luciferase assay. Results are expressed as a percentage of the wild-type driven reporter activity and represent the mean ± SEM of an average of determinations from two to four independent experiments.

complexes were formed, only one of which could be completely displaced by nonradiolabeled oligonucleotide. *In vitro* prepared human SF-1 caused formation of a complex that migrated in parallel to that observed using H295R nuclear extract. In addition, antibody directed against SF-1 abolished the complex observed using H295R nuclear extract (data not shown). Oligonucleotides that included a mutated Ad4 element did not form complexes with SF-1 (Fig. 6).

### *hCYP11B1 constructs in Y-1 adrenal cells*

The only previous analysis of the 5′-flanking DNA from *hCYP11B1* was performed using the Y-1 mouse adrenal cell line and a chloramphenicol acetyltransferase reporter system (18, 19). In these studies, deletion of the region between −1093 and −505 bp of 5′-flanking DNA significantly increased reporter activity. As this is different from what we observed in H295R cells, we tested our pGL3 reporter constructs in Y-1 adrenal cells (Fig. 7). Representative constructs (pB1–924, pB1–330, pB1–214, and pB1–65 bp) from *hCYP11B1* 5′-flanking DNA were transfected with empty pcDNA3 expression vector or with the SF-1-containing expression vector. The results were very similar to those obtained using H295R cells. The basal level of reporter expression was not effected by deletion to −330 bp. Deletion to −214 increased basal activity, whereas further deletion to −65 bp decreased basal expression. Coexpression with SF-1 caused a 2.6-fold increase in reporter gene activity. This effect was significantly decreased by deletion to −214 bp and abolished when the pB1–65 construct was tested.

## Discussion

ACTH stimulation of steroid hormone production and expression of steroid-metabolizing enzymes is controlled though the cAMP signaling pathway. Interestingly, of the

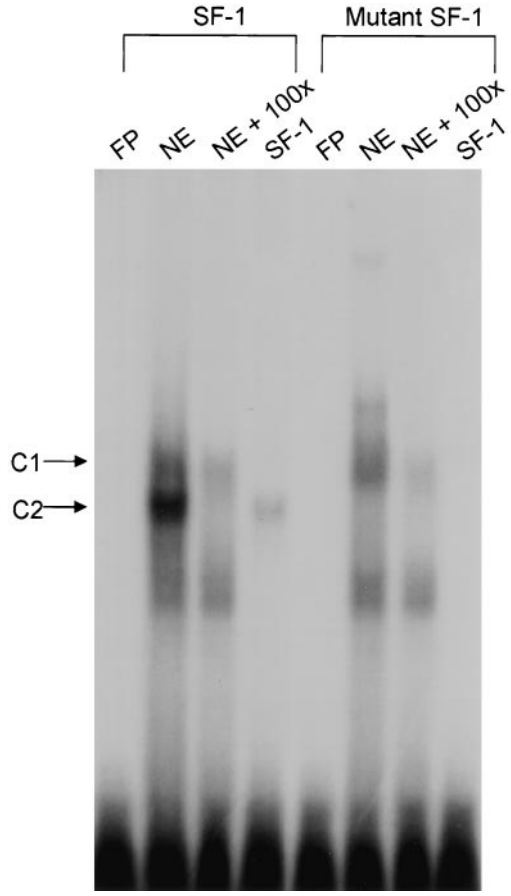


FIG. 6. Binding of SF-1 to *hCYP11B1* Ad4 element (−244/−233). EMSA was performed using a $^{32}$P-labeled oligonucleotide probe that included the *hCYP11B1* Ad4 element −244/−233. Radiolabeled probe alone is shown in lane 1 (FP), and probe with H295R nuclear extract (NE; 5.0 $\mu$g) is shown in lane 2. Protein/DNA complexes (shown by *arrows*) were separated from free probe by electrophoresis. Nonradiolabeled competitor DNA was added at a 100-fold molar excess ((NE + 100×) to identify specific protein/DNA interactions. Lane 4 corresponds to labeled probe with *in vitro* translated human SF-1. Lanes 5–8 demonstrate that the mutated Ad4/SF-1 oligonucleotide no longer binds *in vitro* prepared SF-1 proteins. EMSA analysis was performed using H295R nuclear extract from a minimum of three isolations.

steroid-metabolizing genes to date studied, only the *hCYP11B1* and *hCYP11B2* genes have a near-consensus CRE, whereas different *cis*-elements mediate cAMP responsiveness in other steroid-metabolizing genes (12). A critical role for the Ad1/CRE has been shown for mouse and human *CYP11B2* using deletion and mutational analysis, but previous studies of the 5′-flanking DNA of rat and human *CYP11B1* did not examine the role of the CRE in transcriptional regulation (5, 11, 18, 32, 33).

However, there has been considerable work directed toward defining the mechanisms that regulate transcription of the *bCYP11B* gene (12, 14, 16, 31, 34). Cattle, unlike humans and rodents, perform 11$\beta$-hydroxylation, 18-hydroxylation, and 18-oxidation though the action of a single 11$\beta$-hydroxylase isozyme (CYP11B) (35) that is expressed in both the glomerulosa and fasciculata. Omura and colleagues using DNase I footprint analysis defined protected regions in the *bCYP11B* proximal DNA termed adrenal (Ad) 1–6 (12). Analyses of the role of each of the putative *cis*-elements demonstrated a need for the Ad1 element in basal and cAMP-stimulated expression of reporter activity. The Ad1 element closely resembled a consensus CRE and bound similar proteins to the consensus CRE using nuclear extracts from steroidogenic and nonsteroidogenic cells. However, the nature of these proteins was not determined. Mutation of the Ad1 element blocked cAMP induction, thus furthering the idea that the Ad1 site is most likely the key element in ACTH-regulated induction of transcription of *bCYP11B*. Our observations on the *hCYP11B1* gene are very similar to those observed for the bovine gene in that mutation of the human CRE/Ad1 is critical for both basal and cAMP-dependent transcription.

All CYP11B genes studied to date are positively regulated by the cAMP signaling pathway. It was thought that both CYP11B genes would make use of the Ad1/CRE site for regulation; however, there is a loss of sequence identity in the mouse and rat *CYP11B1* gene (5′-AGTGACaTtATCA-3′) (36) compared with the human Ad1 sequence (5′-CATGACGT-GATCC-3′). The divergence in the rat *CYP11B1* sequence may explain why Ishimura and colleagues observed that an AP-1 sequence in the rat *CYP11B1* Ad1/CRE flanking DNA was important in cAMP induction of transcription of this gene (37, 38). In support of this premise, the researchers went on to demonstrate that nuclei in the rat adrenal fasciculata expressed much higher levels of AP-1-binding proteins (Jun and Fos) than were observed in the glomerulosa. This contrasts with the *hCYP11B1* gene, in which sequence similarity at the Ad1 site to a consensus CRE has been maintained while there is base substitution (compared with the rat AP-1) decreasing the sequence similarly to a consensus AP-1 site. The rationale for specific differences for the differential regulation of *CYP11B1* is not clear. However, the CYP11B genes appear to have had considerable evolutionary divergence, particularly when one considers that bovine, sheep, and pig have only one CYP11B gene. Further, study of the rodent *CYP11B1* genes will be needed to confirm that the CRE (Ad1) variations in sequence lead to transcriptional differences.

To better define the *trans*-acting factors that interact with the Ad1/CRE we used *in vitro* prepared CRE-binding proteins and H295R nuclear extract. CREB, ATF-1, and ATF-2 were able to form complexes with the Ad1/CRE element. However, when compared with nuclear proteins from H295R cells only the ATF-2/CRE complex migrated in a similar manner (complex 1; Fig. 2). The ability of an ATF-2 antibody to supershift complex 1 further supports the idea that ATF-2, within these cells, binds the *hCYP11B1* Ad1/CRE site. The nature of complex 2 remains to be determined. Although it did not migrate with *in vitro* prepared ATF-1 or CREB, the possibility of heterodimers of these proteins with other factors cannot be ruled out at this time. To determine whether members of the CREB/ATF family of proteins are expressed in H295R cells and adrenal cortex, we used Western analysis. H295R nuclear extracts and adrenal lysates expressed ATF-2, supporting the EMSA observations. Adrenal lysate also expressed detectable levels of CREB and ATF-2 with very low levels of ATF-1. A recent study of H295R cell expression of members of the CREB/ATF family found no

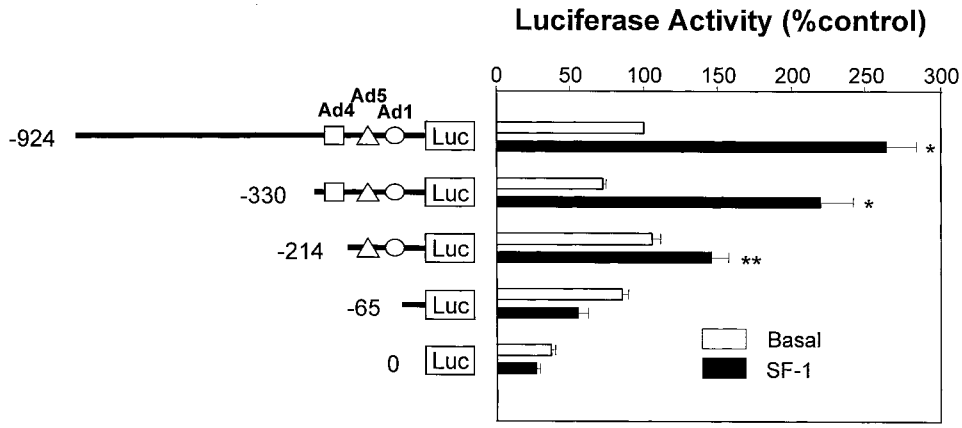


FIG. 7. Deletion analysis of the *hCYP11B1* 5′-flanking DNA to determine SF-1-sensitive regions in Y-1 mouse adrenocortical cells. Y-1 mouse adrenal cells were transiently transfected with luciferase reporter constructs containing serial deletions of *hCYP11B1* 5′-flanking DNA (1 $\mu$g/well). Transfection of reporter constructs was performed with either empty pcDNA3 expression vector (1 $\mu$g/well) or expression vector containing the coding sequence for bovine SF-1 (1 $\mu$g/well). After recovery for 24 h, cells were lysed, and luciferase activity was measured. Results are expressed as a percentage of the basal reporter activity of pB1–924 and represent the mean ± SEM of data from three independent experiments [*, $P < 0.0001$; **, $P < 0.01$ (compared with the basal level)].

expression of CREB (39). Our data suggest that certain strains (but not all) of the H295R cells have lost CREB expression. The mechanism causing the loss of CREB expression is not clear. However, Groussin and colleagues (39) demonstrate an increase in the level CREM expression that they suggest compensates for the loss of CREB. The expression of ATF-2 in adrenal cells and tissue has not previously been described. Activation of ATF-2 can occur through a number of signaling pathways used by certain hormones and growth factors, including alterations in intracellular calcium. Further experiments will be needed to determine which members of the CRE-binding protein family can enhance the transcription of the *hCYP11B1* gene.

In addition to the Ad1/CRE, *hCYP11B1* gene transcription relied on the Ad4 element previously characterized in *bCYP11B*. The *trans*-acting protein that binds the Ad4 sequence was termed Ad4-binding protein (Ad4BP) and was subsequently cloned from a bovine adrenal complementary DNA (cDNA) library (31). Ad4BP is equivalent to SF-1 and is expressed in other steroidogenic cells (29, 40). This protein is considered a key transcription factor in the regulation of a number of steroid hydroxylases as well as the steroidogenic acute regulatory (StAR) protein and 3$\beta$-hydroxysteroid dehydrogenase genes (41–43). Herein, we demonstrated that the Ad4 site in the *hCYP11B1* gene was important for transcription; however, its role was only apparent when cells (either Y-1 or H295R) were cotransfected with an SF-1 expression vector. The rationale for a need to overexpress SF-1 to induce *hCYP11B1* reporter activity via the Ad4 element is not clear, as by EMSA (Fig. 6) and immunoblot analysis (data not shown) the H295R and Y-1 cells express SF-1. Nevertheless, overexpression of SF-1 has also been shown to greatly increase reporter gene expression for constructs prepared from the CYP17, 3$\beta$HSD, CYP11A, and CYP19 genes (15, 28–31). The data thus support the requirement for SF-1 for maximal basal and cAMP-stimulated transcription of *hCYP11B1*. This need for SF-1 sharply differs from the effects of SF-1 on *hCYP11B2* (aldosterone synthase) transcription (11). *CYP11B2* transcription is not increased by SF-1 coexpression and mutation of the Ad4 element does not affect reporter gene expression. In the case of *hCYP11B2*, we have previously shown an important role for the Ad5 site in basal and stimulated expression of reporter genes. Thus, these two isozymes have diverged in the key *cis*-elements regulating gene transcription.

In summary, the regulation of the CYP11B genes appears to have evolved species-specific *cis*-regulatory elements to control transcription. In humans, *hCYP11B1* and *hCYP11B2* rely on the proximal Ad1/CRE site for transcription. This may differ in mouse and rat *CYP11B1*, where the Ad1 site has lost identity with the consensus CRE. Second, the requirement for SF-1 appears to have diverged between *hCYP11B1* and *hCYP11B2*. For *hCYP11B1* the Ad4 *cis*-element and expression of SF-1 are both required for maximal transcription. However, this is not the case for *hCYP11B2*, where the Ad4 element does not appear necessary for transcription (11). Such differences between the *CYP11B1* and *CYP11B2* genes may play an important role in the differential expression of these isozymes within the adrenocortical zones.

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