Differential regulation of aldosterone synthase and 11 β -hydroxylase transcription by steroidogenic factor-1

M H Bassett, Y Zhang, C Clyne, P C White¹ and W E Rainey

Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390–9032, USA

¹Department of Pediatrics, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390–9032, USA

(Requests for offprints should be addressed to W E Rainey; Email: braine@mednet.swmed.edu)

Abstract

11 β -Hydroxylase (hCYP11B1) and aldosterone synthase (hCYP11B2) are closely related isozymes with distinct roles in cortisol and aldosterone production respectively. Aldosterone synthase catalyzes the final step in aldosterone biosynthesis and is expressed only in the zona glomerulosa of the normal adrenal. 11 β -Hydroxylase catalyzes the final reaction in the production of cortisol and is expressed at higher levels in the zona fasciculata. The mechanisms causing differential expression of these genes are not well defined. Herein, we demonstrate contrasting roles for the orphan receptor steroidogenic factor-1 (SF-1) in the regulation of human (*h*) CYP11B1 and hCYP11B2. Human NCI-H295R (H295R) or mouse Y-1 cells were transiently transfected with luciferase reporter constructs containing 5'-flanking regions of hCYP11B1, hCYP11B2, human 17 α -hydroxylase (hCYP17), human cholesterol side-chain cleavage (hCYP11A1) or mouse (*m*) cyp11b2 (*mcyp11b2*). Co-transfection of vectors encoding SF-1 increased expression of hCYP11B1, hCYP11A1 and hCYP17 constructs, but inhibited hCYP11B2 reporter activity. Murine, bovine and human SF-1 were unable to increase transcription of hCYP11B2 in H295R cells. Both hCYP11B2 and *mcyp11b2* promoter constructs were inhibited similarly by human SF-1. In mouse Y-1 cells, reporter expression of hCYP11B2 and *mcyp11b2* was very low compared with hCYP11B1 constructs, suggesting that this adrenal cell model may not be appropriate for studies of CYP11B2. Electrophoretic mobility shift assay demonstrated that SF-1 interacted with an element from both hCYP11B1 and hCYP11B2. However, mutation of this element, termed Ad4, did not prevent agonist stimulation of hCYP11B2 by angiotensin II or forskolin but blocked activity of hCYP11B1. In some, but not all, reports of genetic linkage analysis, a naturally occurring single nucleotide polymorphism within the Ad4 element of hCYP11B2 (–344C/T) has been associated with cardiovascular disease. Herein, we have demonstrated that this polymorphism influenced binding of SF-1 in electrophoretic mobility shift assays, with the C allele binding SF-1 more strongly than the T allele. However, when hCYP11B2 constructs containing these alleles were transfected into H295R cells, there was no difference in agonist-stimulated expression or the response of either reporter construct to co-expression with human SF-1. Taken together, these data suggest that SF-1 and the Ad4 element are not major regulators of adrenal hCYP11B2 gene expression. Thus far, hCYP11B2 is the first steroid hydroxylase gene which is not positively regulated by SF-1.

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Introduction

The adrenal cortex is the site of synthesis of the potent mineralocorticoid, aldosterone, and the glucocorticoid, cortisol. CYP11B2 (aldosterone synthase), which is expressed only within the zona glomerulosa of the adrenal cortex, is responsible

for converting deoxycorticosterone to aldosterone (Kawamoto *et al.* 1990, Curnow *et al.* 1991, Domalik *et al.* 1991, Ogishima *et al.* 1992, LeHoux *et al.* 1995, Pascoe *et al.* 1995). On the other hand, CYP11B1 (11 β -hydroxylase) appears to be expressed predominantly in the fasciculata of the adrenal, where (in humans) it is responsible for the

conversion of deoxycortisol to cortisol (Kawamoto *et al.* 1990, Curnow *et al.* 1991, Erdmann *et al.* 1995). In humans and rodents, the capacity of the adrenal cortex to differentially produce mineralocorticoid and glucocorticoid relies to a large extent on the zonal expression of the human (h) CYP11B isozymes which, in turn, ultimately depends upon the various *cis*-elements and *trans*-acting factors that control gene transcription.

The *cis*-elements controlling zone-specific expression lie mainly in the 5'-flanking regions of these genes. This has been demonstrated by genetic studies of individuals with glucocorticoid-suppressible hyperaldosteronism (GSH), an inherited form of hypertension in which CYP11B2 is expressed in a manner similar to the normal pattern of expression of CYP11B1. In GSH, hCYP11B2 is regulated by adrenocorticotropin (ACTH) via cAMP-dependent signaling and it is expressed in the zona fasciculata. This disorder is caused by intergenic recombinations that juxtapose the 5'-flanking region and the first 2–4 exons of CYP11B1 with coding sequences of CYP11B2 (Lifton *et al.* 1992a,b, Pascoe *et al.* 1992).

Several conserved *cis*-elements have been identified within the 5'-flanking region of CYP11B genes of different species (Rainey 1999). DNase I footprint analysis of the bovine (*b*) CYP11B 5'-flanking DNA demonstrated several regions, termed Ad1 through Ad6, that bound nuclear proteins (Morohashi *et al.* 1992a, Takayama *et al.* 1994). Similar sequences have been observed in the hCYP11B1 (Wang *et al.* 2000) and hCYP11B2 (Clyne *et al.* 1997) genes. Of these elements, Ad1 and Ad4 are required for cAMP-induced and basal transcription respectively of hCYP11B1 (Wang *et al.* 2000). The Ad4 sequence (AAGGCT/CC) is a nuclear receptor half-site which binds steroidogenic factor-1 (SF-1, also called Ad4 BP) (Ikeda *et al.* 1995, Morohashi & Omura 1996), a monomeric orphan nuclear receptor that is required for the co-ordinated development and function of the adrenals and gonads. In the adult adrenal and gonad, SF-1 is essential for full expression of every steroid hydroxylase gene studied thus far, including cholesterol side-chain cleavage enzyme (CYP11A1) (Clemens *et al.* 1994, Liu & Simpson 1997), 11 β -hydroxylase (CYP11B1) (Wang *et al.* 2000), 21-hydroxylase (CYP21) (Ikeda *et al.* 1993), 17 α -hydroxylase/17,20 lyase (CYP17) (Givens *et al.* 1994, Bakke & Lund 1995) and aromatase (CYP19)

(Michael *et al.* 1995). It is also required for full expression of other key components of steroidogenesis such as steroidogenic acute regulatory protein (Sugawara *et al.* 1997), 3 β -hydroxysteroid dehydrogenase type II (Leers-Sucheta *et al.* 1997) and the ACTH receptor (Cammass *et al.* 1997, Marchal *et al.* 1998).

However, the role of SF-1 in expression of hCYP11B2 is uncertain. Studies of a series of reporter constructs with serial deletions of the 5'-flanking region of hCYP11B2 suggested that the Ad4 element was not essential for expression (Clyne *et al.* 1997). On the other hand, a naturally occurring single nucleotide polymorphism involving this site (-344C/T) has been implicated as affecting aldosterone levels, blood pressure, heart size and risk of myocardial infarction, although none of these associations has been consistently observed in all studies (White & Slutsker 1995, Brand *et al.* 1998, Hautanen *et al.* 1998, Kupari *et al.* 1998, Davies *et al.* 1999, Hautanen *et al.* 1999, Schunkert *et al.* 1999, Tamaki *et al.* 1999, Patel *et al.* 2000, Ylitalo *et al.* 2000).

The current study was undertaken to clearly define the role of SF-1, the Ad4 element and the Ad4 polymorphism in hCYP11B2 expression. Our results have demonstrated that, while SF-1 appears to play a critical role in hCYP11B1 transcription through interactions with the Ad4 element, this element and SF-1 do not play major roles in adrenal cell hCYP11B2 gene expression. Thus, hCYP11B2 is apparently the first of the human steroid hydroxylase genes which does not require SF-1 for full expression. The differences in the *cis*-elements that regulate hCYP11B1 and hCYP11B2 transcription may help to explain the differential expression of these isozymes within the adrenal cortex.

Materials and methods

Preparation of reporter constructs and expression vectors

A transient expression system using a luciferase reporter gene was used to characterize the Ad4/SF-1 binding site present in the hCYP11B1 (Wang *et al.* 2000), hCYP11B2 (Clyne *et al.* 1997) and mouse (*m*) *cyp11b2* (Bogerd *et al.* 1990) gene promoters. The hCYP11B1 (pB1-1102), hCYP11B2 (pB2-1521) and *mcyp11b2* (pmB2-1500) constructs

Table 1 Oligonucleotide sequences used as primers for site-specific mutagenesis or as probes for electrophoretic mobility shift assays (EMSA)

	Sequence												
<i>hCYP11B2</i> mAd4	5'	AAG	AAT	CCg	Aat	tCC	CCT	CTC	ATC	TCA	CG		3'
<i>hCYP11B1</i> wtAd1	5'	CCG	GTT	CTC	CCA	TGA	CGT	GAT	CCC	TCT	CGT	AC	3'
<i>hCYP11B2</i> wtAd1	5'	CCG	GTT	CTC	CCA	TGA	CGT	GAT	ATG	TTT	CGT	AC	3'
<i>hCYP11B1</i> wtAd4	5'	CCG	GTG	AAT	AAT	CCA	AGG	CTC	TTG	GAT	AGT	AC	3'
<i>hCYP11B2</i> wtAd4 (C)	5'	CCG	GAA	AAG	AAT	CCA	AGG	CCC	CCT	CTC	AGT	AC	3'
<i>hCYP11B2</i> wtAd4 (T)	5'	CCG	GAA	AAG	AAT	CCA	AGG	CTC	CCT	CTC	AGT	AC	3'

The *hCYP11B2* reporter construct with mutated (m) Ad4/SF-1 bases (lowercase letters) was produced using the indicated primer. EMSAs were performed using wild-type (wt)Ad1 and wtAd4 oligonucleotides corresponding to the indicated sequence in either *hCYP11B1* or *hCYP11B2*. The *hCYP11B2* Ad4 polymorphic oligonucleotides are indicated (C or T).

used in our study contained 1102, 1521 and 1500 bp 5'-flanking DNA respectively and were cloned into the promoterless pGL3-Basic luciferase reporter plasmid (Promega, Madison, WI, USA). Unless otherwise indicated, experiments involving the *CYP11B2* plasmids refer to constructs containing the C allele at position -344 relative to the translation start site. For generation of the *hCYP11B2* Ad4 mutant construct, the sequence 5'-CCAAGGCC-3' (-344/-336) was changed to 5'-CcgAattCC-3' by site-specific mutagenesis (Stratagene, La Jolla, CA, USA) using the primer shown in Table 1. The 5'-flanking region of pSCC-4400 (Hu *et al.* 1999) was subcloned into the pGL3-Basic vector to generate the *hCYP11A1* promoter plasmid, pA1-4400. The preparation of the *hCYP17* promoter construct (pC17-1088) has been described previously (Hanley *et al.* 2001).

The coding sequences of mouse and bovine SF-1 were kindly provided by Keith Parker (University of Texas Southwestern Medical School, Dallas, TX, USA) and Ken-ichirou Morohashi (National Institute for Basic Biology, Okazaki, Japan) respectively. Human SF-1 was provided by Meera Ramayya (University of Washington, Seattle, WA, USA). Coding sequences for all three SF-1 plasmids were excised from their vectors and subcloned into the pRc/RSV expression plasmid (Invitrogen, Carlsbad, CA, USA). Keith Parker also supplied the mouse pmB2-1500 construct.

Cell culture and transfection assay

NCI-H295R (H295R) human adrenocortical tumor cells or mouse Y-1 adrenal tumor cells were cultured in Dulbecco's modified Eagle's/

Ham's F12 (DME/F12) medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 2% Ultrosor G (BioSeptra SA, Villeneuve la Garenne Cedex, France), 1% ITS (Insulin Transferrin Selenium) Plus (Collaborative Research, Bedford, MA, USA) and antibiotics. We have previously described the use of the H295R and Y-1 cell lines to study the human *CYP11B1* and *CYP11B2* reporter constructs (Clyne *et al.* 1996, 1997, Wang *et al.* 2000). For transfection experiments, cells were subcultured onto 12-well culture dishes at a density of 300 000 cells/well (H295R) or 150 000 cells/well (Y-1) for use 36 h later. Transfection was carried out using 2.0 µl Fugene (Roche, Indianapolis, IN, USA) and 1.0 µg reporter plasmid DNA in DME/F12 medium (1.1 ml) for 6 h at 37 °C. For co-transfection experiments, various amounts of expression plasmids were included in the transfection reaction, and the total amount of DNA was kept constant by addition of carrier DNA (empty expression vector). Following transfection, cells were incubated with 2.0 ml low serum medium (DME/F12 medium containing 0.1% Ultrosor G and antibiotics) for 18–24 h to allow for recovery and expression of foreign DNA. Where indicated, transfected cells were treated with agonists for 6 h. Cells were then lysed and assayed for activity using the luciferase assay system (Promega).

Electrophoretic mobility shift assay (EMSA)

H295R nuclear extracts were prepared by the method of Schreiber *et al.* (1989). To prepare radiolabeled probe, double-stranded oligonucleotides (25 pmol) containing 5' protruding ends were labeled, by fill-in reaction, using [α -³²P]dCTP and

Moloney murine leukemia virus reverse transcriptase at 37 °C for 30 min (Gibco-BRL). Five micrograms of nuclear extract and each radiolabeled probe (40 000 d.p.m.) 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 bovine serum albumin and 0.075 mg/ml poly dI-dC as non-specific competitor). For competition analysis, reaction mixtures containing various amounts of non-radiolabeled oligonucleotide were added simultaneously with probe. The resulting DNA/protein complexes were then separated from free probe by electrophoresis using a 4% high ionic strength native polyacrylamide gel with 1 × Tris-glycine running buffer. The gel was dried and visualized following autoradiography at -70 °C for 24 h. Human SF-1 was prepared using a Promega *in vitro* transcription/translation system. The *hCYP11B1*- and *hCYP11B2*-specific Ad1 and Ad4 oligonucleotide sequences that were used for EMSA are listed in Table 1.

Statistical analysis

Statistical comparison of means of three or more samples was accomplished by ANOVA. Significance was accepted at the 0.05 level of probability.

Results

Comparison of the effects of SF-1 upon various steroidogenic enzymes

Several steroidogenic P450 genes, including *hCYP11B1* and *hCYP11B2*, contain consensus binding sites for SF-1 in their proximal 5'-flanking DNA. In order to examine the effects of SF-1 upon various steroidogenic enzymes, we co-transfected H295R cells with reporter constructs containing the 5'-flanking DNA of *hCYP11A1* (pA1-4400), *hCYP11B1* (pB1-1102), *hCYP11B2* (pB2-1521) or *hCYP17* (pC17-1088) with an expression plasmid containing the coding region of human SF-1. While *hCYP11A1*, *hCYP11B1* and *hCYP17* promoter activities increased significantly over basal levels (4-fold, 6-fold and 22-fold respectively), the activity of the *hCYP11B2* promoter decreased by greater than 50% when compared with basal levels of *hCYP11B2* gene expression (Fig. 1). These

Figure 1 Comparison of the effects of SF-1 on transcription of various steroidogenic enzymes. H295R adrenocortical cells were transfected with luciferase reporter constructs containing 5'-flanking DNA (1 µg/well) from *hCYP11A1* (pA1-4400), *hCYP11B1* (pB1-1102), *hCYP11B2* (pB2-1521) or *hCYP17* (pC17-1088). Cells were co-transfected with either empty pRc/RSV expression vector or the indicated amounts of human SF-1 expression plasmid (1 µg/ml) along with a β-galactosidase expression vector (0.05 µg/well). Twenty-four hours after transfection, cells were lysed and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase and expressed as a percentage of the basal reporter activity of pA1-4400, pB1-1102, pB2-1521 or pC17-1088. Results represent the means ± S.E.M. of data from at least three independent experiments each performed in triplicate. **P* < 0.0001.

data suggest that the regulation of *hCYP11B2* transcription is quite different from that of the other cytochrome P450 genes involved in steroid metabolism. The following experiments were designed to determine if indeed *hCYP11B2* represents the first steroidogenic cytochrome P450 gene that is not positively regulated by SF-1.

Concentration-dependent effects of SF-1 on *hCYP11B1* and *hCYP11B2* reporter gene activity

In order to clarify the role of SF-1 in the transcriptional regulation of the two *hCYP11B* genes, we co-transfected H295R cells with pB1-1102 or pB2-1521 and increasing concentrations of an expression vector containing the coding sequence for human SF-1. As shown in Fig. 2, the *hCYP11B1* promoter was activated by SF-1 in a concentration-dependent manner;

Figure 2 Concentration-dependent effects of SF-1 on *hCYP11B1* and *hCYP11B2* reporter gene activity. H295R adrenocortical cells were transfected with luciferase reporter constructs containing pB1–1102 or pB2–1521 5'-flanking DNA (1 µg/well). Cells were co-transfected with either empty pRc/RSV expression vector or the indicated amounts of human SF-1 expression plasmid along with a β-galactosidase expression vector (0.05 µg/well). Following recovery for 24 h, cells were lysed and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase and expressed as a percentage of the basal reporter activity of pB1–1102 or pB2–1521. Results represent the means ± S.E.M. of data from two independent experiments each performed in triplicate.

activity increased to 10-fold above basal levels when cells were co-transfected with 1 µg SF-1 plasmid. In contrast, we observed a reduction of luciferase activity by 44% for the *hCYP11B2* reporter at 1 µg SF-1 expression plasmid.

The response of *hCYP11B1* and *hCYP11B2* to mouse, bovine and human SF-1

There have been conflicting reports in the literature regarding the importance of the Ad4 element in the transcriptional regulation of the *CYP11B2* gene (Bogerd *et al.* 1990, Clyne *et al.* 1997, LeHoux & Lefebvre 1998). We therefore compared the ability of SF-1 from three different species to transactivate the human *CYP11B1* or *CYP11B2* gene promoters. As shown in Fig. 3, mouse, bovine and human SF-1 proteins were able to activate the *hCYP11B1* promoter plasmid but failed to stimulate *hCYP11B2*.

Figure 3 Comparison of the effects of mouse, human and bovine SF-1 on *hCYP11B1* and *hCYP11B2* transcription. H295R adrenocortical cells were transfected with luciferase reporter constructs containing pB1–1102 or pB2–1521 5'-flanking DNA (1 µg/well). Cells were co-transfected with either empty pRc/RSV expression vector or the indicated SF-1 expression plasmid (1 µg/ml). Following recovery for 24 h, cells were lysed and assayed for luciferase activity. Data are expressed as a percentage of the basal reporter activity of pB1–1102 or pB2–1521. The results in each panel represent the means ± S.E.M. of data from three independent experiments, each one done in triplicate. * $P < 0.0001$.

Comparison of the effects of SF-1 on human *CYP11B2* and mouse *cyp11b2* reporter constructs

Initial transfection studies of the mouse *cyp11b2* promoter in mouse Y-1 adrenal tumor cells suggested that the Ad4/SF-1 element was important for basal transcription (Bogerd *et al.* 1990). Therefore, we compared the ability of human SF-1 to stimulate human or mouse *cyp11b2* reporter constructs in both human H295R and Y-1 adrenal cells (Fig. 4). SF-1 failed to stimulate reporter activity for either the mouse or human *CYP11B2* promoter constructs in the human H295R cell line. SF-1 did slightly induce human (2-fold) and mouse (1.25-fold) *CYP11B2* reporter construct activity in mouse Y-1 cells. However, the basal activity of the *hCYP11B2* reporter constructs in Y-1 cells is only 3% of the basal activity observed with the *hCYP11B1* promoter vector, whereas in H295R cells the basal activity of the *hCYP11B2* reporter is generally 1.5- to 2-fold greater than that of *hCYP11B1* (data not shown).

Figure 4 Comparison of the effects of SF-1 on mouse and human *CYP11B2* reporter constructs in H295R and Y-1 adrenal cells. (A) Transcriptional regulation of *hCYP11B2* and *mcyp11b2* by SF-1 in human H295R cells. (B) Transcriptional regulation of *hCYP11B2* and *mcyp11b2* by SF-1 in mouse Y-1 cells. H295R and Y-1 adrenocortical cells were transfected with luciferase reporter constructs containing pB1–1102, pB2–1521 or pmB2–1500 5'-flanking DNA (1 µg/well). Cells were co-transfected with either empty pRC/RSV expression vector or human SF-1 expression plasmid (1 µg/ml). Following recovery for 24 h, cells were lysed and assayed for luciferase activity. Data are expressed as a percentage of the basal reporter activity of pB1–1102, pB2–1521 or pmB2–1500. The results in each panel represent the means $\pm$ S.E.M. of data from three independent experiments, each one done in triplicate. * $P < 0.0001$.

The role of the Ad4 element in regulating *hCYP11B1* and *hCYP11B2* transcription

There is good evidence suggesting an important role for the Ad4 element in *bCYP11B* and *hCYP11B1* transcription (Morohashi *et al.* 1992b, Wang *et al.* 2000). In order to determine if differences in transcriptional regulation of the *hCYP11B* genes are due to binding properties of SF-1 to the *hCYP11B1* and *hCYP11B2* Ad4 consensus sequence, EMSA was carried out using both Ad4 sequences (Fig. 5). In the presence of H295R nuclear extract and the specific Ad4 oligonucleotides for *hCYP11B1* or *hCYP11B2*, at least two major DNA/protein complexes were

Figure 5 EMSA of the Ad4 consensus SF-1 binding sequence in *hCYP11B1* and *hCYP11B2*. EMSA was performed using a 32 P-labeled oligonucleotide probe corresponding to *hCYP11B1* or *hCYP11B2* Ad4 (Table 1). Samples containing radiolabeled probe alone (FP) or probe incubated with 5 µg H295R nuclear extract (NE) are shown. Protein/DNA complexes (C1 and C2) were separated from free probe by gel electrophoresis. Non-radiolabeled self-competitor DNA (NE+100xAd4) or non-specific Ad1 oligonucleotide (NE+100xAd1) was added to the reaction mixture in a 100-fold molar excess in order to identify non-specific protein/DNA interactions. Lanes labeled SF-1 show binding activity for *hCYP11B1* and *hCYP11B2* in the presence of *in vitro* prepared SF-1.

detected. One of the complexes (C1) was displaced from both labeled Ad4 sequences by the addition of a 100-fold molar excess of non-radiolabeled competitor but failed to be displaced by a non-specific oligonucleotide (Ad1). These results indicated that the C1 complex represented a specific protein/DNA interaction. Additionally, *in vitro* translated SF-1 protein formed a complex with both of the Ad4 oligonucleotides; these SF-1/oligo complexes co-migrated with the C1 band present in both the *hCYP11B1* Ad4 and *hCYP11B2* Ad4 elements.

Recently, we reported that mutation of the Ad4 consensus sequence of *hCYP11B1* reduced basal,

Figure 6 Effects on SF-1 binding of the –344C/T single nucleotide polymorphism in *hCYP11B2*. EMSA was performed using 32 P-labeled oligonucleotide probes containing either the T allele (left panel) or C allele (right panel) of *hCYP11B2* (Table 1). Probes were incubated either in the absence of SF-1 (lanes 1 in each panel) or with increasing concentrations of *in vitro* synthesized SF-1 protein as indicated. The position of the band corresponding to SF-1 is indicated by an arrow to the left of the Figure.

dibutyryl cyclic AMP-stimulated and SF-1-transactivated reporter gene activity in H295R cells (Wang *et al.* 2000). In contrast, mutation of the Ad4 consensus sequence in the *hCYP11B2* luciferase reporter plasmid had little effect on basal, angiotensin II (Ang II)- or forskolin-induced, or SF-1-stimulated activity when transfected into these cells (data not shown). Thus, the Ad4 element does not appear to be important for transcriptional regulation of *hCYP11B2*. These results confirmed and extended our previous observations using reporter constructs carrying serial deletions of the 5'-flanking region of *hCYP11B2* (Clyne *et al.* 1997).

Analysis of the single nucleotide polymorphism located within the Ad4 element of *hCYP11B2*

Although these results demonstrated that the Ad4 element did not play a major role in regulating *hCYP11B2* expression in H295R cells, previous reports had suggested that a single nucleotide polymorphism in the Ad4 element located 344 nucleotides 5' to the translation initiation site of the gene might have important effects *in vivo* (White & Slutsker 1995, Brand *et al.* 1998, Hautanen *et al.* 1998, 1999, Kupari *et al.* 1998, Davies *et al.* 1999, Schunkert *et al.* 1999, Tamaki *et al.* 1999, Patel *et al.* 2000, Ylitalo *et al.* 2000). This polymorphism

(–344C/T) is located within the AAGGC(C/T)C core sequence of the Ad4/SF-1 binding site. In order to assess the relative binding affinities of the C and T alleles, synthetic oligonucleotides containing these alleles were radiolabeled and used in EMSA (Fig. 6). When incubated in the presence of increasing concentrations of *in vitro* prepared human SF-1, both elements bound SF-1 in a concentration-dependent manner. However, the –344C allele bound SF-1 approximately five times more strongly than did the –344T allele. To determine if this single nucleotide difference affected gene expression, luciferase reporter constructs were prepared containing either the C or T allele. When these constructs were transfected into H295R cells, their expression was equally repressed by co-transfection with increasing concentrations of the SF-1 expression plasmid (Fig. 7A). Moreover, no allelic differences were observed for reporter construct activity under basal or forskolin treatments (Fig. 7B). However, a small preferential increase (10%) in activity was observed for the –344C allele after treatment with Ang II.

Discussion

SF-1 plays a pivotal role in the development of the adrenals and gonads. In addition, the expression of

Figure 7 Effects on transcriptional activity of the -344C/T single nucleotide polymorphism of *hCYP11B2*. (A) SF-1 dose-response analysis of the T/C gene polymorphism using the pB2-347T (B2-T) and pB2-347C (B2-C) promoter constructs. H295R adrenocortical cells were transfected with reporter constructs and either empty pRc/RSV expression vector and/or the indicated amounts of SF-1 expression plasmid along with a β -galactosidase expression vector (0.05 $\mu\text{g/well}$). Following recovery for 24 h, cells were lysed and assayed for luciferase and β -galactosidase activity. Data were normalized to β -galactosidase and expressed as a percentage of the basal reporter activity of pB2-347T or pB2-347C. Results represent the means $\pm$ S.E.M. of data from four independent experiments each performed in triplicate. (B) Response of the T or C allele to treatment with agonist. Luciferase activities of lysates from control H295R cells or cells treated with Ang II (10 nM) or forskolin (FOR; 10 μM) were measured. Results are expressed as a percentage of the activity of the pGL3-Basic construct and represent the means $\pm$ S.E.M. of determinations from five independent experiments, each performed in triplicate. ** $P=0.0079$.

several of the enzymes involved in steroid hormone biosynthesis relies heavily on SF-1 for maximal gene transcription. However, the exact role of

SF-1 in the transcription of each of the individual cytochrome P450 genes that encode the steroid-metabolizing enzymes is less understood. Herein, we have used promoter constructs prepared using the 5'-flanking DNA from the human *CYP11A1*, *CYP17*, *CYP11B1* and *CYP11B2* genes. Our data suggest that co-expression of SF-1 is very effective at activating transcription of *CYP11A1*, *CYP17* and *CYP11B1*. However SF-1 does not appear to play an important role in the transcription of *hCYP11B2*.

Role of SF-1 in *hCYP11B2* expression

There is a relatively high degree of sequence similarity between the first 400 bases located in the 5'-flanking regions of *hCYP11B1* and *hCYP11B2*. Both promoters have identifiable consensus *cis*-elements that were first characterized in the *bCYP11B* gene (Morohashi *et al.* 1992a, Takayama *et al.* 1994, Rainey 1999). Within the Ad4 elements of *hCYP11B1* and *hCYP11B2*, 10 out of 11 nucleotide bases are identical. Since *hCYP11B1* is strongly up-regulated by SF-1 through the Ad4 element, the failure of SF-1 to stimulate *CYP11B2* transcription – instead of its inhibition of reporter expression – seems surprising. Because *CYP11B2* is unique among genes for steroidogenic enzymes in its negative response to SF-1, it is important to exclude trivial explanations for these observations. There could be a non-specific effect of the carrier vector on the promoter constructs. This is unlikely because the amount of DNA was kept constant in all transfections by the addition of empty expression vector. The inhibition could be a collateral effect of the specific expression vector used in our studies. This is also unlikely because the inhibition was observed when SF-1 was co-transfected in expression vectors driven by either RSV (pRc/RSV) or CMV (pcDNA 3.1) promoter (data not shown). The inhibition could be a squelching phenomenon related to the concentrations of SF-1 plasmid used in the study. While this is possible, the highest concentration used for co-transfection (1 $\mu\text{g/ml}$) was highly effective at stimulating *hCYP11B1* as well as *hCYP17* and *hCYP11A1* promoter constructs. Another possibility could be an effect related to the species of SF-1 used in the expression vector. We tested human, bovine and mouse SF-1 and saw similar effects in each case. It is also possible that these results might

apply only to the human *CYP11B2* gene, but we obtained similar results with both human and mouse reporter constructs. Finally, while the inhibitory effect of SF-1 on *hCYP11B2* reporter activity is surprising, there is a recent study suggesting that SF-1 does have a repressor domain (Ou *et al.* 2001). Further studies will be needed to determine if the inhibitory effect of SF-1 co-expression represents a physiologic effect that might occur *in vivo*.

The different results observed in H295R and Y-1 cells deserve comment. There were dramatic differences between the basal levels of reporter activity of *hCYP11B1* and *hCYP11B2* in transfected Y-1 cells; the activity of the *hCYP11B2* reporter was only 3% of the activity observed for *hCYP11B1*. In contrast, basal levels of *hCYP11B2* expression exceeded those of *hCYP11B1* in transfected H295R cells. These results suggest that the Y-1 cell line may have adopted a phenotype similar to fasciculata cells, which express only low levels of *hCYP11B2*. In contrast, we have previously shown that H295R cells produce aldosterone and also express *hCYP11B2* mRNA, which can be induced by Ang II and K⁺ treatment (Bird *et al.* 1993, Denner *et al.* 1996, Pezzi *et al.* 1997). In that regard, the H295R cells may be a more appropriate *in vitro* model system than mouse Y-1 cells for attempting to define the *cis*- and *trans*-acting factors that control the regulation of *hCYP11B1* and *hCYP11B2* gene expression. Presumably, the inhibition of *hCYP11B2* by SF-1 in H295R cells, and the slight stimulation of the same construct by SF-1 in Y-1 cells, are due to the differences in the type and concentration of specific binding proteins (co-repressors or co-activators) present or absent in each cell type. Alternatively, it is conceivable that transcription factors needed for SF-1 to activate *hCYP11B2* transcription are not present in the H295R cells. This concern is lessened by the fact that the H295R cells increase expression of *hCYP11B2* mRNA following treatment with Ang II and K⁺.

Role of the -344C/T polymorphism

Several reports have associated the -344C/T polymorphism with variations in aldosterone secretion, blood pressure and other cardiovascular parameters (White & Slutsker 1995, Brand *et al.* 1998, Hautanen *et al.* 1998, 1999, Kupari *et al.*

1998, Davies *et al.* 1999, Schunkert *et al.* 1999, Tamaki *et al.* 1999, Patel *et al.* 2000, Ylitalo *et al.* 2000), and we found clear allelic differences in the binding of SF-1 to the polymorphic Ad4 element. Thus, we felt it was possible that this polymorphism could modify the response of this gene to SF-1 and explain the previously noted associations. However, plasmids containing the two alleles were expressed at similar levels under both basal and forskolin-stimulated conditions, and these plasmids were equally repressed when co-transfected with SF-1 expression plasmid. The small allelic difference in stimulation by Ang II is of uncertain significance. It is possible that this polymorphism has additional effects *in vivo* that cannot be modeled by reporter constructs in H295R cells, either because these effects require additional, and as yet unidentified, *cis*-elements within *hCYP11B2*, or because H295R cells lack necessary transcriptional factors. Alternatively, the observed *in vivo* associations may reflect genetic linkage disequilibrium between the -344C/T polymorphism and other polymorphisms elsewhere in or near *hCYP11B2*. Indeed, the -344C/T polymorphism is in linkage disequilibrium with a gene conversion in intron 2 of the *hCYP11B2* gene (White & Slutsker 1995) and also with a missense mutation, R173K (Mulatero *et al.* 2000).

The genes that encode the *hCYP11B* isozymes have clearly evolved distinct mechanisms to control transcription as indicated by their differential expression within the adrenal cortex. However, few studies to date have directly compared the mechanisms regulating these promoters. Herein, we have demonstrated that these genes vary dramatically in their requirement for SF-1, a transcription factor that has been proposed to be necessary for expression of most, if not all, cytochrome P450s involved in steroidogenesis. It is tempting to hypothesize that the lack of expression of *hCYP11B2* in the zona fasciculata is, in part, due to the lack of sensitivity to SF-1. The contrasting requirements for SF-1 provide new insight into the differential regulation of these two closely related genes.

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