Cooperation of SF-1, CREB and AP1 to Mediate cAMP-Regulated *CYP11A1* Transcription *in vitro* and *in vivo*

Ing-Cherng Guo

郭應誠

[1] Department of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

國立台灣大學獸醫學系

Please address all correspondence to:

Dr. Ing-Cherng Guo,
Department of Veterinary Medicine, National Taiwan University, Taipei, 106 Taiwan.
TEL: 886-2-2363 0231 ext 2761 ext 2202.
FAX: 886-2-2366 1475.
E-mail: iguo@ccms.ntu.edu.tw.

Abbreviated title: cAMP Responsive Sequence of *CYP11A1* Gene

Key words: *CYP11A1*, P450scc, cholesterol side-chain cleavage, steroidogenesis, SF-1, adrenal, NR5A1, cAMP, AP-1

This work was supported by Academia Sinica and by grants from National Science Council, Republic of China, NSC 90-2320-B-002-158.

ABSTRACT

Expression of the human *CYP11A1* gene, encoding cytochrome P450scc that catalyzes the first and rate-limiting step of steroid biosynthesis, is controlled by cAMP signaling in a tissue-specific manner. To investigate the tissue-selective cAMP responsiveness of the human *CYP11A1* gene, we characterized the functions of elements in the upstream cAMP-responsive sequences. The electrophoretic mobility shift assay demonstrated that two CRE/TRE-like elements formed stable complexes with Y1 and JEG-3 nuclear proteins of the Jun family in cAMP-dependent manner. One additional element was recognized by SF-1 present in Y1 but absent in JEG-3 cells. Although none of the binding elements alone was sufficient to mediate cAMP stimulation on the heterologous *tk* promoter, site-directed mutagenesis of these elements in the 2.3-kb *CYP11A1* promoter led to reduced response to cAMP stimulation in transient transfections. Impairment of *CYP11A1* promoter activity was also observed in transgenic mutant mice. Combined mutation of both CRE/TRE-like elements caused complete loss of hormonal responses in transgenic adrenal glands and testes. In conclusion, the cooperation of two CRE/TRE-like and SF-1-binding elements defined the cAMP-stimulated, tissue-specific *CYP11A1* promoter activity both *in vitro* and *in vivo*.

INTRODUCTION

Steroid hormones that control the balance of minerals, glucose and sexual characteristics are essential for the maintenance of life and for the continuation of a species (1). *CYP11A1* gene encodes cytochrome P450scc enzyme, which catalyzes the first and rate-limiting step of steroid biosynthesis by the conversion of cholesterol into pregnenolone (2). *CYP11A1* is expressed in all steroidogenic tissues such as adrenal glands, ovaries, testes, and placenta. *CYP11A1* expression is regulated by pituitary hormones, including ACTH (Adrenocorticotropic hormone) (3), LH (Luteinizing hormone) and FSH (Follicle-stimulating hormone) (4). These pituitary hormones act through a common intracellular second messenger cAMP (5). Recently *CYP11A1* was also detected in non-classical steroidogenic tissues such as embryonic hindgut (6), brain (7), skin (8), lymphocyte (9), uterine endometrium (10), and pancreas (11).

Investigations of the promoter function of the human *CYP11A1* gene revealed that the 5'-flanking region contained sequences conferring cAMP responsiveness (12-14). Transcription factor SF-1/Ad4BP (Steroidogenic factor-1/Ad4-binding protein; also named NR5A1) (15) plays a central role to mediate cAMP signal. (16). Previous studies suggested expression of *CYP11A1* gene was controlled by interaction of SF-1 with adjacent elements like Sp1 (Specific protein 1) (17-19), SCC2 (20), SF-3 (Steroidogenic factor-3) (21), and CRE (cAMP-responsive element) (22-24). Analyses of the human *CYP11A1* promoter activity in adrenal Y1 cells (25), adrenal NCI-H295 (26), testis MA10 cells (27), and placental JEG-3 cells (28) indicated that stimulation of gene expression by cAMP depended on the cell type.

We had identified an upstream cAMP-responsive sequence (*U-CRS*) spanning –1640

to –1500 from the transcription start site of the human *CYP11A1* gene (17, 29). A sequence identical to the SF-1-binding site, TCAAGGTCA, is at -1615 (17, 30). SF-1 was expected to function as an obligatory factor to bring the cAMP signal by coordinating with other elements. In addition, two CRE/TRE-like sequences located around –1630 and -1555 (31) are highly homologous to cAMP-responsive element (CRE, TGACGTCA) (32) and phorbol ester TPA-responsive element (TRE, TGAC/GTCA) (33). The CRE-binding protein mediated cAMP-protein kinase A signal to activate transcription (34). TRE is known to bind to AP1, which consisted of the Fos and Jun protein dimer to activate transcription (33).

The involvement of TRE in tissue-specific and cAMP-dependent expression of *CYP11B1* was observed (35, 36). *CYP11B1* encodes P450$_{11\beta}$ and belongs to the same family as *CYP11A1*. The presence of TRE or its variant in the 5'-flanking region of both *CYP11A1* and *CYP11B1* genes indicates that TRE may be an important regulatory element for the transcription of both genes. The function of CRE/TRE sequence in *CYP11A1* transcription, however, has not been carefully studied, although there were some initial reports (23, 37). The roles of SF-1 and CRE/TRE binding proteins and their interaction in cAMP signaling also need examination.

In the present paper, we have characterized protein-binding activities for CRE/TRE and SF-1 elements in *U-CRS*, and analyzed their functions and interactions on the heterologous or native *CYP11A1* promoter in transient transfection and transgenic mice to delineate the mechanism for tissue-selective cAMP stimulation. We demonstrated that SF-1 and CRE/TRE-binding proteins play important roles in cAMP-regulated *CYP11A1* promoter activity.

RESULTS

Two CRE/TRE-like and one SF-1-binding elements in the upstream cAMP-responsive sequence of the human *CYP11A1* gene

In our earlier reports, we identified a region named *U-CRS* (upstream cAMP-responsive sequence) at about 1.6 kb upstream from the transcription start site of the human *CYP11A1* gene that directed cAMP-stimulated expression in mouse adrenal Y1 cells (17), but did not function in nonsteroidogenic HeLa cells or in human choriocarcinoma cell line JEG-3 (24, 29) This 100-bp DNA fragment spanning –1645 to –1546 contains three sets of sequences, named *C1*, *U*, and *C2*, as illustrated in Fig. 1. The *C1* sequence (TGATGTCA) offered a near perfect consensus CRE or TRE except the middle T base. The *C2* element (TGACTGA) has one base mismatch to consensus CRE/TRE. The *U* site is identical to the SF-1-binding site and shares some homology with half CRE.

To confirm the protein-binding activities of these elements, three oligonucleotide probes corresponding to *C1*, *U*, and *C2*, respectively, were incubated with mouse adrenal Y1 and human choriocarcinoma JEG-3 nuclear extracts in electrophoretic mobility shift assays. Proteins in both Y1 and JEG-3 nuclear extracts interacted with *C1* probe to form two major complexes, the intensities of which were increased by 8-Br-cAMP treatment but decreased after competition with unlabeled *C1*, TRE, and CRE (Fig. 2A). Similar results were observed in the interaction of *C2* probe with Y1 or JEG-3 nuclear extracts. Treatment of cAMP enhanced the formation of *C2*-protein complexes, which were erased by excess *C2*, TRE, and CRE (Fig. 2B). These results suggested that the interactions of *C1* or *C2* with CRE/TRE-binding proteins in both Y1 and JEG-3 cells were stimulated by

cAMP signal.

The *U* probe and Y1 nuclear extract, but not JEG-3 extract, formed a specific DNA-protein complex (Fig. 2C). The observation that *U*-binding protein was expressed in Y1, but not in JEG-3, agreed with our earlier results of footprinting analysis (17).

SF-1 and AP-1 bound to the upstream regulatory region of the *CYP11A1* gene

Since *U* shares 5/6 homology with consensus CRE (30), we further examined if CRE-binding protein (CREB) binds to *U*. The complex formed with *U* and Y1 nuclear extract was competed by unlabeled *U* and SF-1 oligonucleotides (Fig. 3A, Lanes 1, 2, 4, 6), but not by non-specific competitor Sp1 (Fig 3A, Lane 9). Unlabeled CRE oligonucleotide also did not compete with *U* (Fig. 3A, Lane 3). The anti-SF-1 serum effectively blocked the complex formation (Fig. 3A, Lane 7 & 8), whereas anti-CREB serum had no effect (Fig. 3A, Lanes 5, 8). These results showed that the CREB did not bind to *U*.

The AP-1 family members consist of Fos/Jun proteins. We examined the *C1*- and *C2*-binding proteins with antibodies specific for subunits of AP-1. The super-shift assay showed that the both *C1*- and *C2*-binding proteins were recognized with antibodies against JunB, c-Jun, or Jun D, but not with anti-FosB antibodies (Fig. 3B). Therefore, the Jun proteins interacted with *C1* and *C2*.

Functions of putative CRE/TRE and SF-1-binding elements on the heterologous *tk* promoter activity

In order to test functions of CRE/TRE-like and SF-1-binding elements in *U-CRS*, we connected these elements with the *tk* promoter to drive *CAT* reporter gene expression, and then monitored *CAT* expression after transient transfection. In Y1 cells, p*SCC#9*, which

contains the entire *U-CRS* sequence, could be stimulated by cAMP by 12 folds. Other clones, including p*C1*, p*U*, p*C1/U* and p*C2*, like their parental p*tkCAT* vector, did not respond to cAMP induction (Fig. 4A). The plasmids, p*C1*, p*U*, and p*C2*, were constructed by inserting one single element corresponding to *C1*, *U*, and *C2*, respectively, in front of the *tk* promoter. Similarly, p*C1/U* contained both *C1* and U elements. All tested clones expressed basal activity comparable to p*tkCAT* (data not shown). These results suggested putative CRE/TRE and SF-1 elements alone or in combination of two could not enhance heterologous *tk* promoter activity in response to cAMP induction.

As shown in Fig. 2, JEG-3 nuclear extract did not contain *U*-binding protein. In JEG-3 cells, p*SCC#9* expressed low reporter *CAT* activity (Fig. 4B, Lanes 5 & 6). Cotransfection of SF-1 expression plasmid increased *CAT* activity of p*SCC#9* (Fig. 4B, Lanes 7 & 8). Cyclic AMP treatment did not further stimulate expression of p*SCC#9*. These results suggested that SF-1 interacted with *C1*- and *C2*-binding proteins for the function of *U-CRS*.

Sequences of CRE/TRE and SF-1 essential for binding

In addition to dissecting *U-CRS* in front of the heterologous *tk* promoter we also analyzed its activities in the 2.3-kb *CYP11A1* promoter by site-directed mutagenesis. Two mutant oligonucleotides with three base mutations, *C1mt* (T*T*A*A*G*G*CA) and *C2mt* (TG*C*C*G*G*C*), were synthesized (Table 1). These mutated sequences could not compete for binding to *C1* and *C2* as demonstrated in EMSA experiments (Fig. 5, Lanes 1-3&7-9). The *U* sequence is a typical SF-1-binding site, TCAAGGTCA. We also generated *Umt,* which has mutations at the 4th A and the 8th C (TCA*C*GGT*G*A, Table 1) of *U*. As expected, *Umt* could not compete with the *U* probe in the DNA-protein binding assay (Fig. 5, Lanes

4-6). These results indicated that the combination of these two bases (-1614 A and –1610 C) is essential for SF-1 binding.

Functions of CRE/TRE and SF-1 elements in cell culture

The binding-sites in *U-CRS* were mutated in the 2.3-kb *CYP11A1* promoter by site-directed mutagenesis to characterize their role. In total, seven mutants were produced, including three single mutants (*C1mt*, *Umt*, and *C2mt*), three double mutants (*C1*/*Umt*, *C1*/*C2mt*, and *U*/*C2mt*), and one triple mutant, *C1*/*U*/*C2mt* (Fig. 6A). After the DNA containing either the wildtype or mutated 2.3-kb *CYP11A1* promoter connected with reporter *LacZ* gene and internal control *RSVCAT* plasmid were together transfected into Y1 cells, the reporter β-galactosidase activity was measured and normalized against the internal control *CAT* activity. All tested clones expressed similar basal activities (data not shown). Their fold of cAMP stimulation, however, was reduced. After statistical analyses from many independent experiments, we found that mutation of *C1* or *C2* alone had no significant effect on cAMP response (Fig. 6B). *U* mutation alone and double *C1/C2* mutation resulted in somewhat reduced cAMP response ($p<0.05$). Double *C1/U* or *U/C2* mutations resulted in further reduction of cAMP response ($p<0.01$). Triple *C1/U/C2* mutation caused a significant reduction of fold of cAMP induction ($p<0.001$).

In this transfection experiment, our results differentiated the individual and cooperative functions of *C1*, *U*, and *C2* in cAMP-regulated *CYP11A1* transcription and suggested that the effect of the *U* appeared to be more important than *C1* and *C2*.

Functions of CRE/TRE and SF-1 elements in transgenic mice

To further study the functions of CRE/TRE-like and SF-1 elements *in vivo*, we generated transgenic mice harboring five mutant constructs. The adrenal homogenate was

used for β-galactosidase activity assay to monitor the tested promoter activity. Lots of transgenic wildtype lines (5 out of 11 tested lines) expressed significant level of β-galactosidase activity (Fig. 7). Higher than 1000 units of β-galactosidase activity was considered as positive expression (represented by filled circles). In comparison with expression rate of wildtype as 100%, the expression rates of single *U* mutant and single *C2* mutant lines were fairly reduced (40% and 51%). The worst expression rates were seen in both double *C1/C2* and *U/C2* mutants (27% and 20%). The double mutation with either two CRE/TRE elements or one CRE/TRE combined with one SF-1 elements further took 70~80% of *CYP11A1* promoter activity off. These *in vivo* results reflected the physiological importance of these upstream CRE/TRE and SF-1 elements to regulate transcription of *CYP11A1*.

Function of CRE/TRE elements in hormonal regulation of transgenic mice

In transfection experiment the combination of *C1* and *C2* elements appeared to be important in mediating cAMP signal on *CYP11A1* promoter activity (Fig. 6). To verify this point, the same mutant constructs were tested for their response to hormonal regulation in transgenic mouse lines. Although most of mutant lines expressed the reporter gene at very low levels, we selected the best one from each mutant line to receive hormone treatment. The β-galactosidase activity from adrenal lysate was monitored. Although both *C1mt* and *C2mt* lines still responded to ACTH somewhat, the *C1/C2mt* line completely could not respond to ACTH stimulation (Fig. 8). The corticosterone levels in sera from all tested mutant lines remained stimulated by ACTH, showing the tropic effect of ACTH on adrenal steroid hydroxylases.

The effect of hCG injection on reporter gene expression from the testis was also

assayed. Although serum testosterone was dramatically induced by hCG, the β-galactosidase activities of testis lysate from all hCG-injected mutant lines expressed as low as that from saline-injected mice (Fig. 9). These results demonstrated that the combined action of *C1* and *C2* elements were required in the response to both ACTH and hCG regulation of gene expression in the adrenal and testis.

We also tested whether the best *C1mt* and *C2mt* lines expressed the reporter gene in a tissue-specific manner. As shown in Fig. 10, the reporter gene driven by the mutant promoters were expressed selectively in adrenal and ovary, although this expression was much weaker than that of the wildtype. This is because the proximal SF-1-binding site is still intact in these mutant lines.

DISCUSSION

We have characterized the binding and functional activities of two putative CRE/TRE and one SF-1 elements in the upstream cAMP-responsive sequence (*U-CRS*) of human *CYP11A1* in detail. Although either of them alone or a combination of two elements was not sufficient to stimulate the heterologous *tk* promoter activity in response to cAMP in transfected cells, their combined mutations reduced cAMP-regulated activity of *CYP11A1* promoter. Furthermore, our transgenic mouse studies showed that combination of CRE/TRE elements were essential in response to tropic hormonal stimulation. This, in combination with our previous studies of mutating the upstream SF-1-binding site in transgenic mice (16), demonstrated that the upstream CRE/TRE and SF-1 elements play important roles in the cAMP-regulated transcription of human *CYP11A1*.

SF-1/Ad4BP is a common transcription factor for all steroidogenic genes (38, 39)

and a key determinant of endocrine development and function (40). The role of SF-1 in cAMP response was less certain. In mouse testicular MA-10 cells, SF-1 was required for constitutive expression of *Cyp11a1*, but did not play a direct role in cAMP induction (41). In adrenal Y1 cells, the *U-CRS*-harboring p*SCC#9* responded to cAMP stimulation but p*U* with a single SF-1 element did not (Fig. 4A). Moreover, exogenous SF-1 expression restored activity of *pSCC#9* in SF-1-deficient placental JEG-3 cells (Fig. 4B). These results indicated that SF-1 is essential but not sufficient to mediate cAMP signal. In 5'-flanking region of the human *CYP11A1* gene, there are two SF-1-binding sites, including upstream *U* and proximal *P*. *U* is important for mediating hormonal regulation and *P* is critical for basal *CYP11A1* transcription (16).

SF-1/Ad4BP and CREB/ATF (CRE-binding protein family) synergize for cAMP-dependent expression of the human *CYP11A1* gene (23). Furthermore, the physical interaction of a CREB-binding protein, CBP, with SF-1 mediates cAMP signal in a CRE-independent manner (42). In bovine *CYP11A*, the interaction between SF-1- and Sp1-binding sites was shown to be important for cAMP induction (18). These observations confirmed that SF-1 mediated cAMP signal through cooperation with other transcription factors.

Our DNA-protein interaction experiments demonstrated that both *C1* and *C2* elements bound to CRE/AP1-like proteins, which were induced after cells were treated with 8-Br-cAMP (Fig. 2) (43). CRE sequence alone is sufficient to mediate cAMP response of some genes (44). Since *C1* and *C2* bind to similar proteins as CRE, one would expect that they could also mediate cAMP response like CRE. Yet this is not the case. As shown in Fig. 4A, *C1* or *C2* alone failed to respond to cAMP. That could result from

weaker interaction between the imperfect CRE/TRE and CREB/AP1-like proteins. The cooperation with other transcription factor(s) to form a more stable DNA-protein complex was presumed to be required for transactivation. Since the locations of *U, C1* and *C2* elements are close to each other, the potential interactions between them are expected. We previously showed the interaction between AP1 and SF-1 in gene activation (45, 46), so that the interaction between *U, C1* and *C2* elements could activate *CYP11A1* promoter. Moreover, our super-shift results showed the binding of Jun proteins to *C1* and *C2* elements and SF-1 protein to *U* element (Fig. 3).

Point mutations of the regulatory elements have been used to study the functions of the promoter. Here, we generated mutants with single, double, or triple mutation at *C1, C2* and *U* elements in the context of the intact 2.3-kb *CYP11A1* promoter. It appeared that the triple mutation was most effective in reducing cAMP response (Fig. 6). Therefore the combination of all three elements appears to be required to achieve maximal cAMP stimulation.

The effects of *C1, C2*, and *U* elements were more pronounced in the transgenic mouse study. Double mutations at *C1/C2* or *U/C2* elements decreased the rate of reporter gene expression to 20~30% and the levels of reporter gene expression were also greatly reduced (Fig. 7). The hormonal stimulation test showed that combination of both *C1* and *C2* were required for fully mediating tropic signal (Fig. 8&9). Our previous observations in transgenic mice also indicated the requirement of *U* in hormonal regulation (16). Taken together, the CRE/TRE and SF-1 elements in *U-CRS* should play critical roles in regulating *CYP11A1* transcription hormonally. Our transgenic results are the first *in vivo* evidence to demonstrate the roles of *C1, C2* and *U* elements in *CYP11A1* transcription by

themselves and in combination.

In summary, cAMP regulation of *CYP11A1* expression could not be totally attributed to any single element. We demonstrated in this paper the importance of interactions among multiple elements including one SF-1 and two CRE/TRE-like elements in cAMP response of *CYP11A1* transcription.

MATERIALS AND METHODS

Cell culture and transfection

Mouse adrenocortical Y1 (47) and human choriocarcinoma JEG-3 (48) tumor cells were grown in F-10 medium supplemented with 10% horse serum and 2.5% fetal calf serum, 0.12% sodium bicarbonate, penicillin (100 U/ml), and streptomycin (100 μg/ml). Transfection with linear or circular DNA was carried out by lipofectAMINE (Life Technologies, Bethesda, USA) or the calcium phosphate procedure (49). Cells were split into two on the second day. One plate was treated with 1 mM 8-Br-cAMP for 24 h before analysis.

Analysis of transcription activity

CAT expression was measured by *CAT* activity assay (50) or by primer extension for RNA assay (51). The β-galactosidase activity was measured by chemiluminescent detection as previously described (16). Cells cotransfected with tested plasmid and internal control plasmid *RSV- gal* or *RSVCAT* were scraped in cold PBS solution, frozen in liquid nitrogen and thawed at 37 ^{0}C for three times to break down cell membranes, and their nuclei were pelleted down at 4 ^{0}C. Cell lysate was tested for the *CAT* assay or β-galactosidase activity assay. The reporter β-galactosidase activity was normalized with *CAT* activity of the internal control. For primer extension, cytoplasmic RNA extracted

from cells cotransfected with tested plasmid and internal control plasmid *RSVCAT* was annealed with 0.1 pmole ^{32}P-labeled *CAT* primer (5'-TTTAGCTTCCTTAGCTCCTGAA AATCT-3') in 50 µl hybridization buffer (0.04 M PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 0.2% NaDodSO4) at 60 ^{0}C overnight. The RNA-primer hybrid was isopropanol precipitated and the pellet was redissolved in 50 µl reverse transcription buffer (50 mM Tris-HCl pH 8.3, 74 mM KCl, 6 mM MgC1$_2$, 10 mM dithiothreitol, 0.5 mM each nucleotide triphosphate, 0.2 U of RNasin, 4 U of AMV reverse transcriptase), and incubated at 42 ^{0}C for 2 hr to allow reverse transcription. After extension, the RNA-cDNA hybrid was hydrolyzed with 100 µl 0.25 N NaOH at 55 ^{0}C for 30 min and the residual cDNA solution was neutralized with 40 µl 3 M sodium acetate pH5.2, extracted with phenol/chloroform, and precipitated with one volume of isopropanol in the presence of 5-10 µg tRNA as a carrier. The cDNA pellet was dissolved in 1 µl sequencing dye, electrophoresed in 5% sequencing gel and autoradiographed. The intensities of specific bands including cDNA products of tested plasmids and internal control *RSVCAT* were quantified by densitometric scanning or by a Phosphor-Imager (Molecular Dynamics, Sunnyvale, California) and normalized.

RNA isolation

Cytolasmic RNA was harvested by NP-40 lysis method (52). Briefly, transfected cells were scraped into 1 ml phosphate-buffered saline (PBS), centrifuged, and resuspended in 500 µl lysis buffer (0.14 M NaCl, 1.5 mM MgC1$_2$, 10 mM Tris-HC1 pH 8.6, 0.5% NP-40). After incubation on ice for 5 min and quick spin, the cytoplasmic RNA in the supernatant was collected and treated with 50 µl NaDodSO4 (10%) and 12.5 µl proteinase K (20 mg/ml) at 55 ^{0}C for 30 min, extracted with acidic phenol and chloroform

after the addition of 100 µl 10 M ammonium acetate, and precipitated with one volume of isopropanol at room temperature.

Preparation of nuclear extract for EMSA

To prepare the nuclear extracts, cells were treated with NP-40 lysis method described in RNA isolation in addition of proteinase inhibitors (0.5 mM DTT, 0.5 mM PMSF, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). The pelleted nuclei were extracted with 0.1 volume of 3 M KCl in one volume of nuclear extraction buffer (20 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 0.4 mM EDTA pH8.0, 20 % glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 µg/ml aprotinin, and 2 µg/ml leupeptin) by swirling at 4 ^{0}C for 30 min. The extracts were centrifuged at 10,000 x g at 4 ^{0}C for 30 min, aliquoted, and frozen in liquid N_2 and stored at –70 ^{0}C immediately.

Electrophoretic mobility shift assay (EMSA)

The procedure was described early (19). Briefly, two complementary strands of oligonucleotides were annealed as a probe after they were separately labeled with [γ-^{32}P]ATP by kinase. The probe was incubated with 5 µg nuclear proteins in 15 µl binding buffer (2 µg poly (dG-dC), 25 mM HEPES pH 7.9, 0.1 mM EDTA pH8.0, 0.5 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, and 10 % glycerol) on ice for 30 min in the presence or absence of double stranded competitor oligonucleotide. For the super-shift experiment, the nuclear extract was preincubated with 2 µg antibodies in binding buffer on ice for 15 min, followed by the addition of the probe and further incubation on ice for 30min. The DNA-protein complexes were separated in 5 % native polyacrylamide gel at 4 ^{0}C overnight before autoradiography.

Oligonucleotides

Sequences and the description of the sense strand of the oligonucleotides used in plasmid construction and electrophoretic mobility shift assay are listed in Table 1.

Construction of plasmids

Plasmid p*SCC#9*, *RSV- gal,* and *RSVCAT* were described before (24, 52). The plasmids *C1*, *U*, and *C2* were resulted from the insertion of double stranded *C1*, *U*, and *C2* oligonucleotides into p*tkCAT* at *Xba I* site. The fragment of *C1* connected with *U* was inserted into p*tkCAT* at *Xba I* site to generate p*C1*/*U*. Each plasmid contained a single copy of inserted element and was sequenced to confirm the junction points, the orientation, and the copy number of the insert.

Generation and genotyping of transgenic mouse lines

Injected DNA fragments composed of the wildtype or mutated *CYP11A1* promoter joined to *LacZ* and SV40 polyA. The transgene copy numbers in the genome of transgenic mice determined by Southern blotting varied between 1-22, but mostly below 4. All transgenic founders and their offspring were of the FVB strain. Genomic DNA was prepared from tails or placentae of embryos. Genotyping was performed by PCR amplification of *LacZ* with primers 5'-*Gal* (AGGCATTGGTCTGGACACCAGCAA) and 3'-*Gal* (GATGAAACGCCGAGTTAACGCCAT) to produce a 476-bp fragment. Mice were housed under standard specific pathogen free laboratory conditions.

***-galactosidase assays* of Tissues**

Tissues were homogenized in the smallest volume of lysis buffer (100 mM potassium phosphate pH7.8, 0.2 % Triton X-100, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride, and 5μg/ml leupeptin). After centrifugation, the

supernatant was incubated at 48 ^{0}C for 1 h to inactivate the endogenous β-galactosidase activity. Ten μl of tissue lysate was mixed with 50 μl reaction buffer. After incubation at room temperature for 1 h and reaction with Light Emission Accelerator, the light product was counted for 5 sec with a luminometer.

Hormonal stimulation test

For the adrenal function test, five to eight female transgenic mice were subcutaneously injected with 1 I.U. ACTH (Cortrosyn, Organon, Oss, Holland) once a day for 7 days. For the testicular function test, five to eight adult male transgenic mice were intraperitoneally injected with 10 I.U. hCG (Sigma Chemical Co., St. Louis, MO) twice a day for 7 days. Mice were anesthetized with ether 2 h after the last injection. Serum corticosterone and testosterone were measured using ^{125}I-labelled RIA kits (ICN Biomedicals, USA and DSLabs, USA). Adrenal and testicular lysate were assayed for β-galactosidase activity.

Experimental animals

All studies concerning the use of mice were conducted in accord with the rules established by the Animal Committee at the institute of Molecular Biology, Academia Sinica.

Statistical Analysis

The statistical differences between tested plasmids and control plasmid p*tkCAT* in *CAT* assay or between wildtype and mutant plasmids in β-galactosidase activity assay were evaluated by the ANOVA analysis. The p value <0.05 was considered statistically significant.

Acknowledgments

We would like to thank Ya-Hui Tsai and Shu-Jan Chou for excellent technical assistance and the Transgenic Core Facility at Academia Sinica for the generation of transgenic mouse lines.

.

REFERENCES

1. Guo I-C, Wu L-S, Lin J-S, Chung B-c 1994 Mechanism of trophic hormone action on the regulation of steroid biosynthesis. Cont Med Edu 4:414-423
2. Miller WL 1988 Molecular biology of steroid hormone synthesis. Endocr Rev 9: 295-318
3. Simpson ER, Waterman MR 1988 Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu Rev Physiol 50:427-440
4. Richards JS, Hedin L 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. Annu Rev Physiol 50:441-463
5. John ME, John MC, Boggaram V, Simpson ER, Waterman MR 1986 Transcriptional regulation of steroid hydroxylase genes by ACTH. Proc Natl Acad Sci USA 3:4715-4719
6. Keeney DS, Ikeda Y, Waterman MR, Parker KL 1995 Cholesterol side-chain cleavage cytochrome P450 gene expression in the primitive gut of the mouse embryo does not require steroidogenic factor 1. Mol Endocrinol 9:1091-1098
7. Zhang P, Rodriguez H, Mellon SH 1995 Transcriptional regulation of P450scc gene expression in neural and steroidogenic cells: implications for regulation of neurosteroidogenesis. Mol Endocrinol 9:1571-1582
8. Slominski A, Ermak G, Mihm M 1996 ACTH receptor, *CYP11A1*, *CYP17*, and *CYP21A2* genes are expressed in skin. J Clin Endocr Metab 81:2746-2749
9. Zhou Z, Shackleton CHL, Pahwa S, White PC, Speiser PW 1998 Prominent sex steroid metabolism in human lymphocytes. Mol Cell Endocrinol 138:61-69
10. Hukkanen J, Mantyla M, Kangas L, Wirta P, Hakkola J, Paakki P, Evisalmi S,

Pelkonen O, Raunio H 1998 Expression of cytochrome P450 genes encoding enzymes active in the metabolism of tamoxifen in human uterine endometrium. Pharmacol Toxicol 82:93-97

11. Morales A, Cuellar A, Ramirez J, Vilchis F, Diaz-Sanchez V 1999 Synthesis of steroids in pancreas: evidence of cytochrome P-450scc activity. Pancreas 19:39-44
12. Chung B-c, Hu M-C, Lai C-C, Lin C-H 1989 The 5'-region of the P450XIA1 (P450scc) gene contains a basal promoter and an adrenal-specific activating domain. Biochem Biophysl Res Commun 160:276-281
13. Inoue H, Higashi Y, Morohashi KI, Fujii-Kuriyama Y 1988 The 5'-flanking region of the human P-450(SCC) gene shows responsiveness to cAMP-dependent regulation in a transient gene-expression system of Y-1 adrenal tumor cells. Eur J Biochem 171:435-440
14. Hu M-C, Chou S-J, Huang Y-Y, Hsu N-C, Li Hung, Chung B-c 1999 Tissue-specific, hormonal, and developmental regulation of SCC-LacZ expression in transgenic mice leads to adrenocortical zone characterization. Endocrinology 140:5609-5618
15. Nuclear-Receptor-Nomenclature-Committee 1999 A unified nomenclature system for the nuclear receptor superfamily. Cell 97:161-163
16. Hu M-C, Hsu N-C, Pai C-I, Wang C-KL, Chung Bc 2001 Functions of the upstream and proximal steroidogenic factor 1 (SF-1)-binding sites in the *CYP11A1* promoter in basal transcription and hormonal response. Mol Endocrinol 5:813-818
17. Guo I-C, Tsai H-M, Chung B-c 1994 Actions of two different cAMP-responsive sequences an enhancer of human *CYP11A1* (P450scc) gene in adrenal Y1 and placental JEG-3 cells. J Biol Chem 269:6362-6369

18. Liu Z, Simpson ER 1997 Steroidogenic factor (SF-1) and Sp1 are required for regulation of bovine *CYP11A* gene expression in bovine luteal cells and adrenal Y1 cells. Mol Endocrinol 11:127-137

19. Guo I-C, Chung B-c 1999 Protein binding activity and cyclic AMP-responsiveness of a weak Sp1 site in proximal promoter of human *CYP11A1* gene. J Genet Mol Biol 10:9-18

20. Clemens JW, Lala DS, Parker KL, Richards JS 1994 Steroidogenic factor-1 binding and transcriptional activity of the cholesterol side-chain cleavage promoter in rat granulosa cells. Endocrinology 134:1499-1508

21. Rice DA, Kirkman MS, Aitken LD, Mouw AR, Schimmer BP, Parker KL 1990 Analysis of the promoter region of the gene encoding mouse cholesterol side-chain cleavage enzyme. J Biol Chem 266:11713-11720

22. Takayama K, Morohashi K, Honda S, Hara N, Omura T 1994 Contribution of Ad4BP, a steroidogenic cell-specific transcription factor, to regulation of the human *CYP11A* and bovine *CYP11B* genes through their distal promoter. J Biochem (Tokyo) 116:193-203

23. Watanabe N, Inoue H, Fujii-Kuriyama Y 1994 Regulatory mechanisms of cAMP-dependent and cell-specific expression of human steroidogenic cytochrome P450scc (*CYP11A1*) gene. Eur J Biochem 222:825-834

24. Chung B-c, Guo I-C, Chou S-J 1997 Transcriptional regulation of the *CYP11A1* and ferredoxin genes. Steroids 62:37-42

25. Moore CCD, Brentano ST, Miller WL 1990 Human P450scc gene transcription is induced by cyclic AMP and repressed by 12-*O*-tetradecanoylphorbol-13-acetate and

A23187 through independent cis-elements. Mol Cell Biol 10:6013-6023

26. Rodriguez H, Hum DW, Staels B, Miller WL 1997 Transcription of the human genes for cytochrome P450scc and P450c17 is regulated differently in human adrenal NCI-H295 cells than in a mouse adrenal Y1 cells. J Clin Endocr Metab 82:365-371
27. Hum DW, Staels B, Black SM, Miller WL 1993 Basal transcriptional activity and cAMP-responsiveness of the human P450scc promoter transfected into MA-10 Leydig cells. Endocrinology 132:546-552
28. Hum DW, Aza-Blan P, Miller WL 1995 Characterization of placental transcriptional activation of the human gene for P450scc. DNA Cell Biol 14:451-463
29. Guo I-C, Huang C, Chung B-c 1993 Differential regulation of the *CYP11A1* (P450scc) and ferredoxin genes in adrenal and placental cells. DNA Cell Biol 12:849-860
30. Inoue H, Watanabe N, Higashi Y, Fujii-Kuriyama Y 1991 Structure of regulatory regions in the human cytochrome P-450 (desmolase) gene. Eur J Biochem 195:563-569
31. Guo I-C 1999 Effect of cAMP on expression of cytochrome P450scc. J Chin Soc Vet Sci 25 [Suppl]:8-15
32. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675-680
33. Lee W, Mitchell P, Tjian R 1987 Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741-752
34. Sassone-Corsi P 1995 Transcription factors responsive to cAMP. Annu Rev Cell Dev Bi 11:355-377
35. Mukai K, Mitani F, Shimada H, Ishimura Y 1995 Involvement of an AP-1 complex in

zone-specific expression of the *CYP11B1* gene in the rat adrenal cortex. Mol Cell Biol 15:6003-6012

36. Muki et al Eur. J. Biochem. 256, 190-200, 1998 (please fill in the correct information)
37. Morohashi KI, Zanger UM, Honda SI, Hara M, Waterman MR, Omura T 1993 Activation of *CYP11A* and *CYP11B* gene promoters by the steroidogenic cell-specific transcription factor, Ad4BP. Mol Endocrinol 7:1196-1204
38. Morohashi KI, Honda SI, 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
39. 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
40. Parker KL, Schimmer BP 1997 Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 18:361-377
41. Chau YM, Crawford PA, Woodson KG, Polish JA, Olson LM, Sadovsky Y 1997 Role of steroidogenic factor-1 in basal and 3', 5'-cyclic adenosine monophosphate-mediated regulation of cytochrome P450 side-chain cleavage enzyme in the mouse. Biol Reprod 57:765-771
42. Monte D, Dewitte F, Hum DW 1998 Regulation of the human P450scc gene by steroidogenic factor 1 is mediated by CBP/p300. J Biol Chem 273:4585-4591
43. Chen C, Guo I-C 2000 Effect of cAMP on protein binding activities of three elements in upstream promoter of human *CYP11A1* gene. Life Sci 67:2045-2049
44. Rice DA, Aitken LD, Vandenbark 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

45. Li L-A, Lala D, Chung B-c 1998 Function of steroidogenic factor1 (SF-1) ligand-binding domain in gene activation and interaction with AP1. Biochem Biophy Re Commun 250:318-320
46. Li L-A, Chiang EF-L, Chen J-C, Hu N-C, Chen Y-J, Chung B-c 1999 Function of steroidogenic factor 1 domain in nuclear localization, transactivation, and interaction with TFIIB and c-Jun. Mol Endocrinol 13:1588-1598
47. Schimmer BP 1989 Adrenocortical Y1 cells. Method Enzymol 58:570-574
48. Kohler PO, Bridson WE 1971 Isolation of hormone-producing clonal lines of human choriocarcinoma. J Clin Endocrinol 32:683-687.
49. Gorman C 1985 High efficiency gene transfer into mammalian cells. In: Glover, DM, eds. DNA cloning: a practical approach. Vol II. UK, Oxford: IRL Press; 132-190
50. Gorman CM, Moffat LF, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044-1051
51. Chang C-Y, Huang C, Guo I-C, Tsai H-M, Wu D-A, Chung B-c 1992 Transcription of the human ferredoxin gene through a single promoter which contains the 3', 5'-cyclic adenosine monophosphate-responsive sequence and Sp1-binding site. Mol Endocrinol 6:1362-1370
52. Guo I-C, Chung B-c 1999 Cell-type specificity of human *CYP11A1* TATA box. J Steroid Biochem Mol Biol 69:329-334

Table 1 Sequences of the sense strands of the oligonucleotides synthesized for electrophoretic mobility shift assay and plasmid construction.

Name	[a]Position	[b]Sequences
C1	-1637/-1626	5'-ttcctTGGCTGATGTCAttccaggct-3'
C1mt	-1637/-1626	5'-ccttcctTGGCT*T*A*A*G*G*CAttccaggctca
U	-1617/-1609	5'-ctagacaggcTCAAGGTCAtcagtgagt-3'
Umt	-1617/-1609	5'-cattccaggcTCA*C*GGT*G*Atcagtgaggca-3'
C2	-1558/-1544	5'-ctagattcaTGACTGATGAGGTAGtggt-3'
C2mt	-1558/-1544	5'-cagaaggttcaTG*C*C*G*G*C*TGAGGTAGtggt
SF-1	-1616/-1609	5'-ctagaCAAGGTCAtcat-3'
Sp1	Consensus	5'-ctagagatcGGGGCGGGGCgatct-3'
CRE	Consensus	5'-ctagaccggcTGACGTCAtcaagct-3'
TRE	Consensus	5'-agcttggTGACTCAtccg-3'

a: Position indicates the correlated location of the presumed protein-binding site with capital letters in 5'-regulatory region of the human *CYP11A1* gene.

b: Oligonucleotides including *C1*, *U*, and *C2* were designed to expose sticky *XbaI* or *SalI* ends after annealing two complementary strands. The *italic* and underlined *capital letters* indicate the mutated nucleotides.

FIGURE LEGENDS

Fig. 1. Location and sequences of CRE/TRE-like and SF-1 elements from –1645 to –1546 of the 5'-flanking sequence of *CYP11A1*. (A) The CRE/TRE-like and SF-1 elements are boxed and named as *C1*, *U*, and *C2*. **(B)** The homology of *C1* or *C2* to CRE and TRE, and *U* to SF-1 and CRE.

Fig. 2. Protein-binding activities of CRE/TRE-like and SF-1 elements in electrophoretic mobility shift assays. The **(A)** *C1*, **(B)** *C2*, and **(C)** *U* oligonucleotides were end-labeled with ^{32}P and incubated with 5 μg of Y1 or JEG-3 nuclear extract in the presence or absence of 500-fold molar excess of cold competitors listed on the top. Nuclear extracts from Y1 or JEG-3 cells treated with (+) or without (-) 1 mM 8-Br-cAMP are indicated. The specific DNA-protein complexes are marked with arrowheads.

Fig. 3. SF-1 binds to the *U* element and Jun protein binds to *C1* or *C2* element. End-labeled **(A)** *U* **(B)** *C1* or *C2* oligonucleotide was incubated with 5 μg of cAMP-treated Y1 nuclear extract to form specific DNA-protein complexes. The complexes were preincubated with specific antibodies, or 100-fold molar excess of cold competitors as shown at the top of each lane.

Fig. 4. Functions of *C1, U,* and *C2* elements in transfection. (A) Each DNA element, as indicated in each column, was inserted in front of the *thymidine kinase* promoter driving the *CAT* reporter gene. These test plasmids together with internal control *RSVCAT* were transfected into Y1 cells and tested for cAMP response. The significant difference in comparison with parent vector p*tkCAT* is indicated as *** ($p<0.001$). **(B)** The JEG-3 cells were cotransfected with tested plasmid p*tkCAT* or p*SCC#9* together with internal control *RSV- gal* in the presence (+) of or in the absence (-) of SF-1 expression plasmid, then

treated with (+) or without (-) 1 mM 8-Br-cAMP. After normalization of internal control –galactosidase activities, the reporter *CAT* activities were assayed and the ^{14}C-labelled products were illustrated on the thin-layer chromatography. The substrate and products are marked with filled circle and arrowheads, respectively.

Fig. 5. Essential sequences of *C1, C2* and *U* element for specific binding. Nuclear extracts (5 μg) from Y1 cells were incubated with **(A)** ^{32}P-end labeled *C1*, **(B)** *U*, and **(C)** *C2*. Except the control group (-), the reactions were preincubated with 100-fold excess of competitors as indicated at top of each lane. Only protein-DNA complexes are shown.

Fig. 6. Functions of *C1, C2* and *U* elements on cAMP-regulated *CYP11A1* promoter in transfected Y1 cells. (A) Construction of seven mutant plasmids by site-directed mutagenesis at *C1, C2* and *U* elements of 2.3-kb *CYP11A1* promoter. The names and mutated sequences are indicated. **(B)** Transcriptional activity of wildtype and mutated *CYP11A1* promoter. Linear or circular DNA of wildtype and seven mutants were separately transfected into Y1 cells together with an internal control *RSVCAT* plasmid. The transfected cells were split into two plates, one of which was treated with 1 mM 8-Br-cAMP for 24 hours. The reporter β-galactosidase activity in each transfected cell lysate was measured, normalized against the internal control *CAT* activity, and calculated for cAMP induction fold. The significant difference between mutant and wildtype was indicated as * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$). The percentage of activity of each mutant compared to wildtype was shown in each column.

Fig. 7. Functions of *C1, C2,* and *U* elements in transgenic mice. Transgenic mouse lines carrying the wildtype or mutated human *CYP11A1* promoter linked to the *LacZ* gene were analyzed. β-Galactosidase activity from adrenal homogenates of each mouse line was

measured. Each circle represents an individual transgenic line. Open and filled circles represent the absence and presence of β-galactosidase, respectively.

Fig. 8. Lack of ACTH induction from mutated *CYP11A1* promoter. (A) ACTH-stimulated reporter β-galactosidase activities of adrenal homogenates from transgenic mice were compared with that from the saline-injected controls. **(B)** The levels of serum corticosterone from the same mice were measured.

Fig. 9. Lack of hCG induction from mutated *CYP11A1* promoter. (A) hCG-stimulated reporter β-galactosidase activities of testicular homogenates from transgenic mice were compared with that from the saline-injected controls. **(B)** The levels of serum testosterone from the same mice were measured.

Fig. 10. Tissue-selective expression of the *CYP11A1*-driven reporter gene. The tissue distribution of reporter β-galactosidase activities from mouse lines expressing wildtype or mutated *CYP11A1* promoter were detected and compared with that in non-transgenic mice. Tissues including adrenal (A), ovary (O), brain (B), heart (H), lung (L), liver (L), spleen (S) and kidney (K) were tested.