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Steroidogenic factor 1 differentially regulates basal and inducible steroidogenic gene expression and steroid synthesis in human adrenocortical H295R cells

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Abstract

The significance of steroidogenic factor 1 (SF-1) in adrenal steroidogenesis was studied using adrenocortical cell lines transformed with a dominant negative mutant of SF-1. Constitutive expression of the mutant did not only impair the activity of endogenous SF-1 but also diminish its own expression, suggesting that SF-1 was under autoregulation. Inhibition of the endogenous SF-1 activity significantly reduced basal and inducible transcription of *CYP17*, *CYP21B* and *CYP11B1*, but exhibited little effects on *StAR* and *CYP11A1* expression. Stimulating the transformed cells with potassium and cAMP freed *CYP11B2* from the mutant-caused transcriptional inhibition, whereas the transformation abolished induction of *CYP17* by both stimulants. Consistent with the transcriptional changes of steroidogenic genes, basal and inducible synthesis of cortisol and androgens drastically declined in the transformed cell lines. The relief of *CYP11B2* repression following the potassium and cAMP stimulation removed the restraint the mutant exerted on aldosterone synthesis, and resulted in aldosterone overproduction in the stimulated transformed cells. SF-1 also plays a role in regulating the adrenocorticotrophic hormone (ACTH) responsiveness of the adrenocortical cells. Inhibition of SF-1 activity significantly decreased basal expression of ACTH receptor and its induction by potassium and cAMP.

Keywords: SF-1; Dominant negative mutant; Adrenal steroidogenesis; Transcriptional regulation; cAMP; Potassium

1. Introduction

Adrenal cortex is a primary steroidogenic site where mineralocorticoids, glucocorticoids and androgens are synthesized. Although these three types of steroids are mainly produced in different zones of the adrenal cortex, their biosynthesis is not independent of one another. All the adrenal steroids are converted from cholesterol via sequential reactions catalyzed by cytochrome P450 steroid hydroxylases and 3β-hydroxysteroid dehydrogenase [1]. StAR, a protein that mobilizes cholesterol across the outer mitochondrial membrane, controls the availability of the precursor for the first conversion reaction occurring in the inner mitochondrial membrane [2,3]. Promoter analysis of genes encoding these steroidogenic proteins has found that an orphan nuclear receptor steroidogenic factor 1 (SF-1) positively regulates transcriptional expression of StAR, cytochrome P450 steroid hydroxylases and 3β-hydroxysteroid dehydrogenase [1].

The concentrations of circulating potassium ion and adrenocorticotrophic hormone (ACTH) influence the capacity of adrenal steroidogenesis. While potassium is classically regarded as a major regulator of mineralocorticoid synthesis, ACTH secreted from the anterior pituitary primarily regulates glucocorticoid and androgen production. ACTH mediates its effects by binding to the receptor on the adrenocortical cell surface and subsequently inducing the formation of the secondary messenger cAMP [4]. It has been reported that SF-1 is required for both basal and cAMP-stimulated transcriptional expression of ACTH receptor [5,6]. In addition, SF-1 has been suggested to be involved in cAMP-regulated gene induction of several P450 steroid hydroxylases [7–11].

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SF-1 is expressed cell-specifically along the endocrine axis of hypothalamus-pituitary-adrenal/gonad [12–15]. Homozygous disruption of the SF-1 gene in mice causes regression of adrenal and gonad [16]. Recently a point mutation in the DNA-binding domain of SF-1 has been documented to cause congenital lipoid adrenal hyperplasia and XY sex reversal in human [17]. All the mouse and human studies evidence that SF-1 plays a critical role in regulating adrenal and gonadal development. However, the physiological importance of SF-1 in steroidogenesis is still unclear because the morphological development of adrenal and gonad occurs before the inception of steroid biosynthesis.

The present study was aimed to explore the role of SF-1 in steroidogenic gene regulation by constitutive inhibition of the endogenous SF-1 activity of the human adrenocortical H295R cell line with a dominant negative mutant. H295R is a pluripotent cell line that expresses genes required for synthesis of all types of adrenal steroids, thus providing a simple system excellent for studying adrenal steroidogenesis [18]. In this study, we assessed the effects of the stable transfection of the mutant on basal steroidogenic gene expression as well as potassium- and cAMP-regulated expression. We also correlated the transcriptional changes to the production of a number of steroids.

2. Materials and methods

2.1. Cell culture and transient transfection

Human adrenocortical H295R cell line and its derivatives were cultured in DMEM/F12 medium (GibCO BRL, Gaithersburg, MD, USA) plus 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37 °C in the presence of 5% CO_2. Transient transfection analysis was performed following the calcium phosphate precipitation method (Fig. 1B) [19] or the lipofectamine method (Fig. 2) (GibCO BRL, Gaithersburg, MD, USA). Either CMV-βgal plasmid (Fig. 1B) or pβgal-control (Fig. 2) (Clontech Laboratories, Palo Alto, CA, USA) was cotransfected with the indicated reporter plasmids as a control. The activation levels of the reporters were determined based on their enzymatic activity after normalization to the cotransfected β-galactosidase (βgal) activity. The activities of CAT, luciferase (Luc) and βgal were measured according to the established methods [20–22].

2.2. Bpu-del transformation of the H295R cells

To construct a Bpu-del expressing cell line from H295R, cDNA encoding the Bpu-del mutant was cloned behind the CMV promoter of the puromycin-selectable vector pUHD10-3-puro and subsequently transfected into the H295R cells with the help of lipofectamine. The transfected cells were selected by culture in the medium supplemented with 7.5 μg/ml puromycin (Sigma–Aldrich, St. Louis, MO, USA). The survived cell colonies were isolated and grown

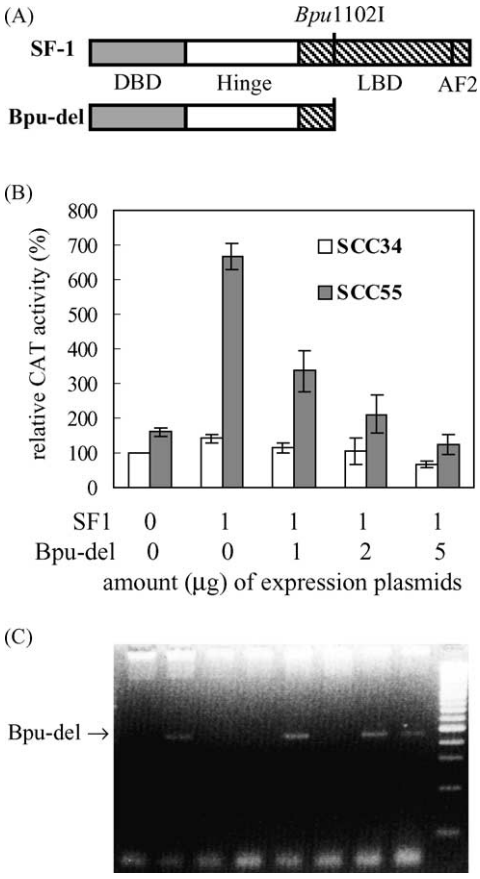


Fig. 1. Transformation of H295R cells with a dominant negative mutant of SF-1. (A) Structure of wild type SF-1 and mutant Bpu-del. DBD: DNA-binding domain, LBD: ligand-binding domain, AF2: activation function 2 domain. (B) Dominant negative effects of Bpu-del mutant on SF-1 transactivation activity. SF-1 and Bpu-del expression plasmids were cotransfected into H295R cells with either SCC34-CAT (3 μg) or SCC55-CAT (3 μg), the latter containing an upstream SF-1 binding site. Meanwhile, CMV-βgal (0.4 μg) was added as an internal control. The total DNA for each transfection was equalized by addition of the vector DNA of expression plasmid. Each bar represents the mean of two independent transfections. (C) PCR analysis of the genomic DNA of the putative transformed clones using Bpu-del-specific primers.

in the selective medium for several passages to eliminate the contaminated non-transformed cells. After the antibiotic selection, the genomic DNA of these cell clones was extracted using the TRIzol reagent (GibCO BRL, Gaithersburg, MD, USA) for PCR analysis to verify the implantation of Bpu-del. Once the Bpu-del transformation was confirmed, the established cell lines were cultured in the regular medium.

2.3. RNA isolation and RT-PCR analysis

RNA was isolated using the REzol reagent (PROtech technology, Tainan, Taiwan, ROC) following the supplemented protocol except that RNA was digested with nuclease-free DNase I (TaKaRa Biomedicals, Otsu, Shiga, Japan) and extracted with phenol and chloroform before isopropanol

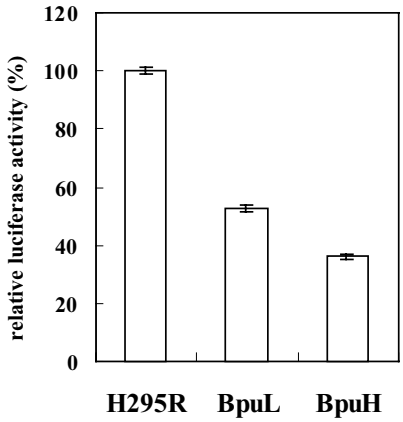


Fig. 2. Impairment of the endogenous SF-1 activity by Bpu-del transformation. The endogenous SF-1 activity of H295R and two Bpu-del transformed cell lines was measured by transient transfection ($n = 5$) of the cells with 3× SF-1-TATA-Luc (3 μg). Plasmid pβgal-control (0.5 μg) was cotransfected in order to normalize transfection efficiency.

precipitation. The mRNA abundance of each examined gene was measured in pair with β-actin by two-step RT-PCR. RNA was first reverse transcribed using an oligo-dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA), and then analyzed by real-time PCR using the LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostics GmbH, Mannheim, German). To eliminate the interference of primer dimers and improve the detection of cDNA amplification at the end of each cycle, an additional denaturation step was programmed following the amplification step in each cycle. The temperature of the second denaturation step was determined based on the melting curve of each PCR reaction. Gene-specific primer pairs were designed using the software programs Primer3 [23] and Probe Design (Roche Diagnostics GmbH, Mannheim, German). The PCR product of each primer pair was verified by sequencing.

2.4. Western blot analysis

SF-1 and Bpu-del proteins were detected by Western immunoblotting [24] of nuclear extracts using the antibodies raised against recombinant human SF-1 protein [25]. Nuclear extracts were prepared as described previously [26].

2.5. Steroid analysis and protein determination

Cell cultures of 70–80% confluence were incubated in the serum-free medium at 37 °C under 5% CO_2 for 24 h in the absence and presence of 14 mM KCl or 1 mM 8-Br-cAMP. The medium was then harvested for steroid analysis after removal of the cell debris by a 10 min spin at 3600 rpm using the Kubota 2100 table centrifuge. Progesterone and aldosterone were measured by radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX, USA), while 17-OH-progesterone, cortisol, dehydroepiandrosterone (DHEA) and androstenedione (A4) were analyzed using PE200 HPLC (Perkin-Elmer, CT, USA) followed by API3000 tandem mass spectrometry (PE-SCIEX, Ont., Canada). 17-OH-progesterone and cortisol were separated using an ODS-80A column (150 mm × 2.1 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) with a gradient mobile phase of 20–80% acetonitrile containing 0.1% formic acid at a flow rate of 0.2 ml/min for 18 min. The column was re-equilibrated with 20% acetonitrile–0.1% formic acid for 3 min before injection of the next sample. The eluent was ionized to $(M + H)^+$ ions by the turboionspray source at 300 °C. Each protonated steroid and its major cationic derivative after collision were scanned sequentially with MS–MS ($m/z = 331.3 \pm 97.2$ for 17-OH-progesterone, and 363.2 ± 121.2 for cortisol). DHEA and A4 were measured following the protocol described previously [27]. In addition, 10 ng/ml of progesterone-2,2,4,6,6,17,21,21,21-d9 (C/D/N Isotopes Inc., Que., Canada) was added to each sample or standard as an internal control in the LC–MS–MS analysis ($m/z = 324.3 \pm 100.2$). The levels of steroids in each sample were calculated by normalization of the steroid data to the cellular protein content. The cellular protein content was determined against bovine serum albumin standards by the Micro BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) after sonication lysis of the cells.

2.6. Statistical analysis

All data are presented as mean ± S.E. The n numbers of experiments are indicated in the corresponding figure or table legends. The impacts of the Bpu-del transformation and drug treatments were analyzed using Student's t-test with significance at the 0.05 level.

3. Results

3.1. Transformation of adrenocortical H295R cells with a dominant negative mutant of SF-1

In order to delineate the role of SF-1 in adrenal steroidogenic regulation, the SF-1 activity of human adrenocortical H29R cells was impaired by stable transfection of the cells with a dominant negative mutant Bpu-del. The Bpu-del mutant contains the intact DNA-binding domain of SF-1, but loses most of the ligand-binding domain, including the AF2 region (Fig. 1A). This deletion truncates the transactivation function of the mutant, but rewards a dominant negative effect on wild type SF-1 [25]. Transient transfection experiments demonstrated that SF-1 specifically activated the reporter activity of SCC55-CAT via interaction with its binding sequence situated within the 55-bp *CYP11A1* promoter (SCC55) in the H295R cells. Elimination of this SF-1 binding site from the reporter construct (SCC34-CAT) abolished the CAT activation. Cotransfection of Bpu-del into the

H295R cells resulted in a significant dose-dependent decline of the SF-1-activated CAT activity (Fig. 1B).

We cloned the Bpu-del mutant into a puromycin-selectable mammalian expressing plasmid (pUHD10-3-puro) and transfected this plasmid into the H295R cells. After several passages of puromycin selection, four cell clones were identified harboring the Bpu-del mutant in the genome by PCR analysis (Fig. 1C).

3.2. Suppression of SF-1 transactivation activity in the transformed cells

To verify the inhibitory effects of the Bpu-del transformation on the endogenous SF-1 activity, a luciferase reporter that contains three copies of the SF-1 binding sequence in front of the TATA box (3× SF-1-TATA-Luc) was transiently transfected into two transformed cell lines as well as the parental H295R cell line (Fig. 2). In contrast to the near background levels of luciferase activity observed in the TATA-Luc transfection (data not shown), the cells transfected with 3× SF-1-TATA-Luc exhibited substantial luciferase activation. The SF-1-dependent luciferase activity detected in the two transformed cell lines was 47.2 ± 1.1 and $63.7 \pm 0.9\%$ lower than in H295R ($P < 0.05$), indicating that the Bpu-del stable transfection successfully blocked a considerable fraction of the endogenous SF-1 activity. The transformed cell line exhibiting lower inhibition was named BpuL, while BpuH was designated for the higher inhibitory capability of the second transformed cell line.

3.3. Bpu-del and SF-1 expression in the parental and transformed adrenocortical cells

We subsequently examined mRNA and protein expression of the Bpu-del mutant in BpuL, BpuH and H295R (the latter as a negative control; data not shown). The levels of mRNA expression were quantified using real-time RT-PCR with the house keeping β-actin gene as a control (Fig. 3A upper panel), while protein levels were analyzed by Western blot (Fig. 3A lower panel). As expected, Bpu-del mRNA and protein were detected only in the transformed cell lines, and the mRNA and protein abundances of Bpu-del were higher in the BpuH cell line than in the BpuL cell line. The Bpu-del mRNA and protein levels in BpuL were not affected by a 24 h treatment with 14 mM KCl, but elevated more than two-fold by a similar treatment with 1 mM 8-Br-cAMP. Basal mRNA expression of the mutant in BpuH was 4.6 ± 0.2 times of that in BpuL. The 24 h KCl and cAMP treatments further raised Bpu-del mRNA expression to 13.6 ± 0.9 and 28.7 ± 0.6-fold of the basal level of BpuL, respectively. However, the protein abundance of the mutant in the BpuH nuclear extracts did not increase parallel with mRNA expression. The maximum nuclear level of the Bpu-del protein in the stimulated BpuH cells was around six times of the basal expression of BpuL based on densitometrical analysis.

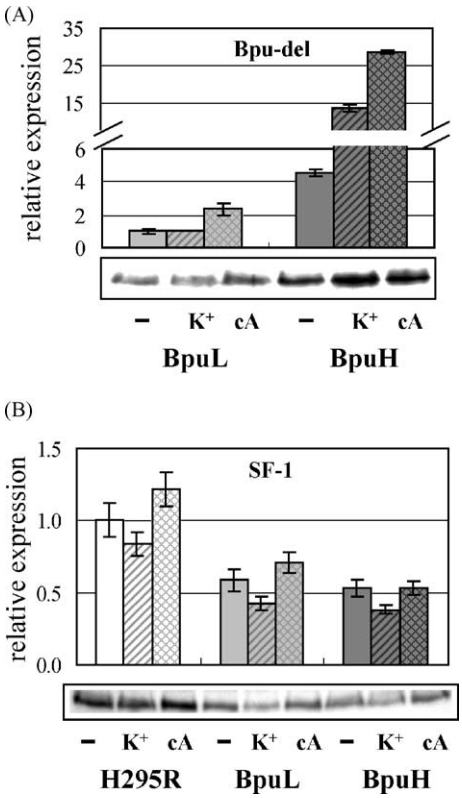
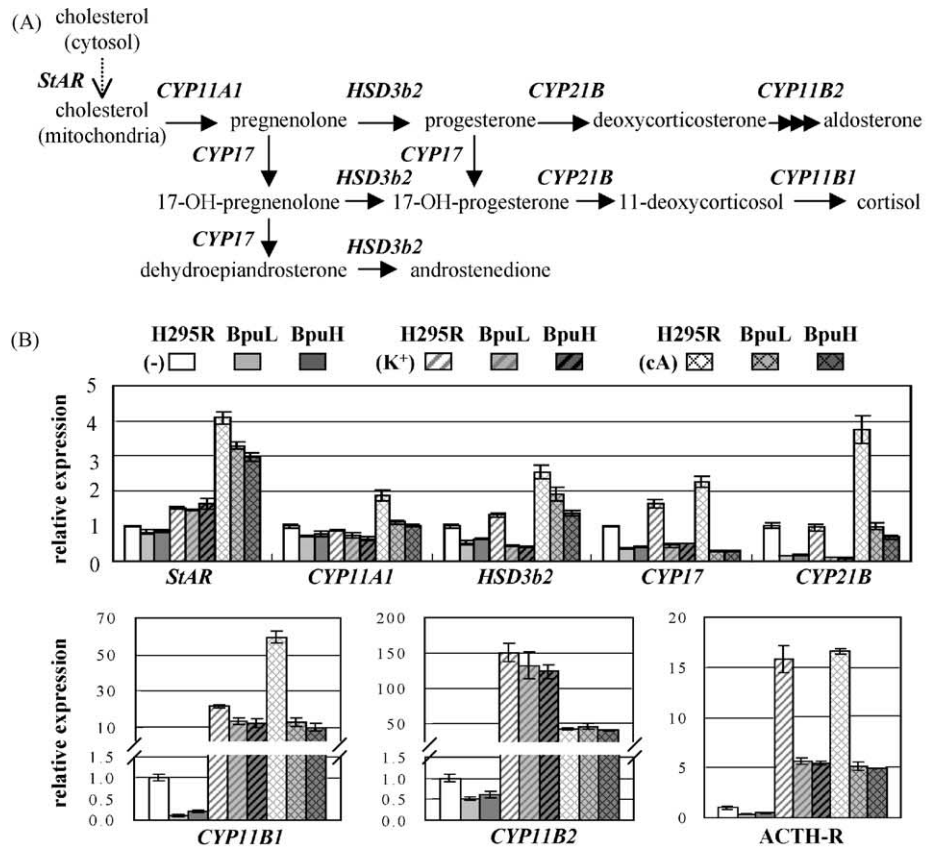


Fig. 3. Expression of Bpu-del and SF-1 in the absence (–) and presence of a 24 h treatment with 14 mM KCl (K^+) or 1 mM 8-Br-cAMP (cA). The mRNA abundance was determined by real-time RT-PCR analysis of three independent RNA samples using β-actin expression as an internal normalization control (A). Protein expression was analyzed by Western immunoblotting of nuclear extracts (120 μg protein each) with antibodies raised against human recombinant SF-1 (B).

The Bpu-del mutant appeared to inhibit expression of wild type SF-1. Fig. 3B showed that the average amount of SF-1 mRNA in BpuL and BpuH was 58.7 ± 7.7 and $53.1 \pm 6.3\%$ of the level in H295R, respectively ($P < 0.05$). KCl and cAMP had no statistically significant effects on SF-1 mRNA expression. The SF-1 mRNA abundance in both transformed cell lines remained around half of the amount detected in H295R after the 24 h KCl and cAMP treatments. Similar levels of inhibition were observed in nuclear protein expression.

3.4. Effects of Bpu-del on steroidogenic gene expression

The impacts of the Bpu-del transformation on genes responsible for aldosterone, cortisol and androstenedione synthesis were examined by real-time RT-PCR. As shown in Fig. 4, basal expression of the early-step steroidogenic genes *StAR* and *CYP11A1* was negligibly lower in the transformed BpuL and BpuH cells than in the parental H295R cells. Although treating the cells with 14 mM KCl for 24 h slightly increased *StAR* expression, these three cell lines exhibited similar transcript levels for either early-step gene under the KCl treatment. The 24 h treatment with 1 mM 8-Br-cAMP



(C) **Summary of real-time RT-PCR analysis**

		StAR	*CYP11A1*	*HSD3b2*	*CYP17*	*CYP21B*	*CYP11B1*	*CYP11B2*	ACTH-R
–	Bpu inhibition*	±	±	+	++	+++	+++	+	++
KCl	Bpu inhibition	–	–~±	++	++	+++	+	–	++
	Induction blockage≠	U	–	B	B	–	U	U	U
cAMP	Bpu inhibition	±	+	±~+	+++	++~+++	+++	–	++
	Induction blockage	U	U	U	B	U	U	U	U

* Bpu-del-caused transcriptional inhibition: "–" no inhibition, "±" < 30% inhibition, "+" 30~50% inhibition, "++" 50~80% inhibition, "+++" > 80% inhibition

≠ Bpu-del-caused induction blockage: "B" induction blocked, "U" induction unblocked, "–" no induction

Fig. 4. Effects of Bpu-del transformation on steroidogenic gene expression. (A) Adrenal steroidogenic pathways. Genes responsible for the steroid conversion reactions are listed beside the solid arrows in italic boldface. The StAR-mediated translocation of cholesterol from the cytosol to the mitochondria is indicated in a dashed arrow. (B) Real-time RT-PCR analysis. RNA was extracted from cells untreated (−) or treated with 14 mM KCl (K^+) or 1 mM 8-Br-cAMP (cA) for 24 h. Expression levels of each indicated gene in the H295R, BpuL and BpuH cell lines were quantified relatively to the basal expression in H295R after normalization to the cognate β-actin expression ($n = 3$). (C) Summary of real-time RT-PCR analysis. Transcriptional inhibition caused by the Bpu-del transformation, termed Bpu inhibition, was determined by comparison of expression levels between H295R and the transformed cells ($P < 0.05$). Induction blockage indicates the capability of the Bpu-del mutant to block KCl- or cAMP-elicited transcriptional induction. No induction means that expression of a given gene in H295R lacks statistically significant difference in the absence and presence of treatment.

elicited stronger stimulation on *StAR* and *CYP11A1*. The Bpu-del transformation did not block induction of these two early-step genes by cAMP. However, the scale of cAMP induction was reduced in the transformed cell lines.

The Bpu-del mutant imposed increasing inhibition on *HSD3b2*, *CYP17* and *CYP21B* under the basal condition. Treating the transformed BpuL and BpuH cells with KCl did not alter the transcript levels of these three genes, despite the fact that the KCl treatment induced *HSD3b2* and *CYP17* expression in the H295R cells. The Bpu-del transformation also eradicated the stimulatory effects of cAMP on the *CYP17* gene, but not those on *HSD3b2* and *CYP21B*.

Although cAMP-induced *HSD3b2* and *CYP21B* expression was considerably lower in BpuL and BpuH, the induction power of cAMP on these two genes was stronger in the transformed cell lines. The cAMP treatment apparently relaxed the restraint of Bpu-del on *HSD3b2* and *CYP21B*.

CYP11B1 is responsible for the final conversion of cortisol. The Bpu-del transformation significantly reduced basal and inducible expression of *CYP11B1*, but did not block transcriptional induction of the gene by either KCl or cAMP. Moreover, the KCl treatment attenuated the negative effects of the mutant on *CYP11B1*. The Bpu-del-elicited transcriptional inhibition was declined from more than 80% repression under the basal condition to about 40% repression after the KCl stimulation. On the other hand, the KCl and cAMP treatments completely relieved the mutant-caused repression on *CYP11B2*, the gene responsible for the final conversion of aldosterone. Similar levels of *CYP11B2* mRNA were detected in stimulated H295R and transformed cell lines.

ACTH tightly regulates adrenal steroidogenesis via interaction with the receptor on the adrenocortical cell membrane [28]. Therefore, the expression level of ACTH receptor is a fundamental factor determining the responsiveness of adrenocortical cells to the trophic hormone. Our RT-PCR analysis showed that basal mRNA expression of ACTH

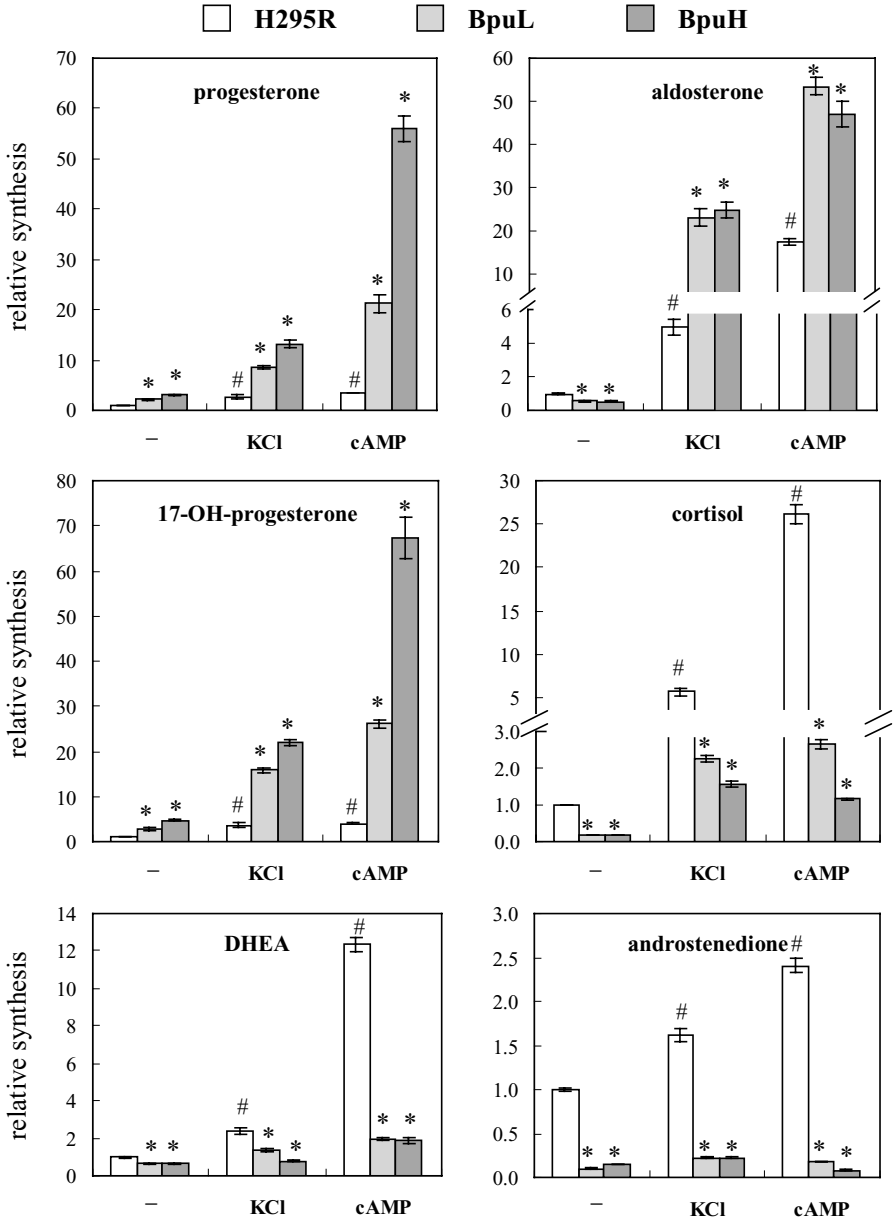


Fig. 5. Steroid production by H295R, BpuL and BpuH during a 24 h incubation with 14 mM KCl, 1 mM 8-Br-cAMP, or serum-free medium only (−). The amounts of steroids secreted during the incubation were normalized to the cellular protein contents ($n = 6$). $^{\#}P < 0.05$ vs. the basal condition. $^{*}P < 0.05$ vs. H295R under the same condition.

receptor in BpuL and BpuH was approximately one third of the level expressed by the parental H295R cell line. Stimulation with 14 mM KCl or 1 mM 8-Br-cAMP for 24 h elevated transcriptional expression of ACTH receptor around 16-fold in all the cell lines. Neither treatment changed the inhibition of Bpu-del on ACTH receptor expression (Fig. 4B and C).

3.5. Effects of Bpu-del on steroid production

Furthermore, we examined how the Bpu-del transformation altered steroid production (Fig. 5). We found that the Bpu-del transformation significantly diminished basal and inducible synthesis of cortisol, DHEA and androstenedione. A 24 h stimulation with 14 mM KCl reduced the gaps between the parental and transformed cell lines in cortisol production. The amounts of cortisol synthesized by the KCl-stimulated transformed cells were 30–40% of that produced by H295R, twice higher than the 16% production observed under the basal condition. In contrast, a 24 h stimulation with 1 mM 8-Br-cAMP widened the gaps. After the cAMP stimulation, cortisol synthesis of the transformed cell lines was down to 4–10% of that in H295R. Likewise, stimulation with KCl and cAMP intensified the inhibitory effects of the Bpu-del mutant on DHEA and androstenedione synthesis.

The Bpu-del transformation also lowered aldosterone synthesis under the basal condition. However, the transformed cell lines secreted approximately five and three times higher amounts of aldosterone than H295R when stimulated with KCl and cAMP, respectively. The transformed cell lines also produced larger quantities of progesterone and 17-OH-progesterone, especially when stimulated with KCl and cAMP. The amounts of progesterone and 17-OH-progesteron produced by the transformed cells seemed to be reversibly related to their cortisol and androgen synthesis.

3.6. Steroidogenic enzyme activities in the parental and transformed cell lines

Table 1 summarizes the product/precursor ratios that reflect relative activities of enzymes catalyzing the conversion reactions. Since the aldosterone/progesterone and cortisol/17-OH-progesterone ratios echo the activities of CYP11B2 and CYP11B1 in addition to CYP21B, respectively, the effects of Bpu-del on CYP11B1 and CYP11B2 activities can be assessed by comparison of these two ratios among the cell lines. The comparison suggested that the mutant exerted stronger inhibition on CYP11B1 activity than on CYP11B2 activity. Stimulation of the cells with KCl or cAMP elevated both activities in all the cell lines except the CYP11B1 activity in cAMP-treated BpuH. The KCl stimulation relieved the Bpu-del inhibition on CYP11B2 activity, but not CYP11B1 activity. The cAMP stimulation attenuated the negative effects of the mutant on CYP11B2 activity, whereas the treatment intensified the inhibition on CYP11B1 activity.

CYP17 contains two enzymatic activities: 17α-hydroxylase activity and 17,20-lyase activity. Different from the rodent enzymes, human CYP17 cannot efficiently convert 17-OH-progesterone into androstenedione [29]. Instead, the human enzyme facilitates androstenedione synthesis by catalyzing the formation of DHEA via 17-OH-pregnenolone. 3β-Hydroxysteroid dehydrogenase (3βHSD, the gene product of *HSD3b2*) converts DHEA into androstenedione as well as 17-OH-pregnenolone into 17-OH-progesterone (Fig. 4A). Therefore, the 17-OH-progesterone/progesterone and androstenedione/17-OH-progesterone ratios still effectively represent the 17α-hydroxylase and 17,20-lyase activities of CYP17, respectively. The ratios indicated that the Bpu-del mutant differentially influenced these two CYP17 activities. Similar levels of 17α-hydroxylase activity were observed among the parental and transformed

Table 1
Product/precursor ratios

Fold	Aldo/P4	Cort/17OHP4	17OHP4/P4	A4/17OHP4	A4/DHEA
Basal					
H295R	1.00 ± 0.11	1.000 ± 0.040	1.00 ± 0.06	1.000 ± 0.037	1.00 ± 0.06
BpuL	0.25 ± 0.01	0.058 ± 0.005	1.21 ± 0.07[a]	0.034 ± 0.003	0.16 ± 0.03
BpuH	0.17 ± 0.02	0.034 ± 0.002	1.66 ± 0.10	0.031 ± 0.001	0.22 ± 0.02
KCl					
H295R	1.98 ± 0.35	1.774 ± 0.057	1.44 ± 0.16	0.482 ± 0.011	0.68 ± 0.03
BpuL	2.71 ± 0.28[a]	0.144 ± 0.008	1.84 ± 0.11[a]	0.014 ± 0.001	0.17 ± 0.01
BpuH	1.88 ± 0.10[a]	0.071 ± 0.002	1.74 ± 0.07[a]	0.010 ± 0.000	0.27 ± 0.01
cAMP					
H295R	4.75 ± 0.24	6.496 ± 0.374	1.10 ± 0.07[b]	0.600 ± 0.034	0.19 ± 0.00
BpuL	2.54 ± 0.20	0.101 ± 0.002	1.25 ± 0.08[a]	0.007 ± 0.000	0.09 ± 0.01
BpuH	0.83 ± 0.04	0.018 ± 0.001	1.14 ± 0.03[a]	0.001 ± 0.000	0.05 ± 0.00

The production of precursor and product steroids during a 24 h incubation with serum-free medium only (basal), 14 mM KCl or 1 mM 8-Br-cAMP is compared. Data are expressed relatively to the basal H295R control in the form of mean ± S.E. ($n = 6$). Aldo: aldosterone, A4: androstenedione, Cort: cortisol, DHEA: dehydroepiandrosterone, P4: progesterone, 17OHP4: 17-OH-progesterone.

[a] Insignificant compared to H295R under the same condition ($P \geqq 0.05$).

[b] Insignificant compared to the basal condition ($P \geqq 0.05$).

cell lines except that BpuH exhibited a modestly increased 17α-hydroxylase activity under the basal condition. In contrast, the androstenedione/17-OH-progesterone ratio or the 17,20-lyase activity was significantly reduced by the Bpu-del transformation. Stimulation with KCl and cAMP did not only diminish the 17,20-lyase activity of H295R but also enhance the mutant-caused inhibition in BpuL and BpuH. The 3βHSD activity, reflected by the androstenedione/DHEA ratio, was also decreased in the transformed cells under all the tested conditions. However, the 3βHSD activity reduction caused by the mutant was less obvious after the KCl and cAMP treatments.

4. Discussion

All the steroidogenic genes required for adrenal steroidogenesis contain at least one copy of SF-1 binding sequence in the promoter region. SF-1 activates transcription from these promoters through these *cis*-acting sequences, implying that SF-1 may coordinately regulate steroidogenic gene expression [1]. However, this postulation is based on transient transfection experiments using promoters of limited length. The role of SF-1 as a steroidogenic coordinator is in question. Here we present a study demonstrating that the control of SF-1 over steroidogenic gene expression varies with genes and extracellular stimuli. Roughly speaking, SF-1 exhibits increasing impacts along the biosynthetic pathways (summary in Fig. 4C).

Inhibition of SF-1 activity abolished transcriptional induction of *CYP17* by either potassium or cAMP. Because the 17α-hydroxylase and 17,20-lyase activities of CYP17 determine the direction of steroid biosynthesis to the aldosterone, cortisol or androgen synthetic pathway, the control of SF-1 over *CYP17* transcription becomes fundamentally important in the regulation of steroid biosynthesis. The 17-OH-progesterone/progesterone and androstenedione/17-OH-progesterone ratios revealed that the Bpu-del transformation had little effects on the 17α-hydroxylase activity but severely inhibited the 17,20-lyase activity. The inhibition of the 17,20-lyase activity following *CYP17* repression seemed to be a key cause for the loss of basal and inducible androgen production in the BpuL and BpuH cell lines.

Although suppression of SF-1 activity did not block the *CYP11B1* gene being induced by potassium and cAMP, inducible *CYP11B1* expression was tremendously reduced in the transformed cells as basal expression. The significant decline of *CYP11B1* activation under either the basal condition or stimulation greatly diminished the conversion of cortisol from 17-OH-progesterone in the transformed cells. The decrease of cortisol and androgen formation caused that BpuL and BpuH secreted large amounts of biosynthetic intermediates progesterone and 17-OH-progesterone.

SF-1 had less control over the terminal steroidogenic gene *CYP11B2* of the aldosterone pathway as compared with its influence on the *CYP11B1* gene of the cortisol pathway and the *CYP17* gene of the androgen pathway. Stimulation with potassium or cAMP freed the *CYP11B2* gene from the Bpu-del inhibition and allowed steroid biosynthesis forward to the aldosterone pathway in BpuL and BpuH. This together with blockage of the other pathways drove the stimulated transformed cells to produce higher levels of aldosterone, a phenomenon similar to congenital adrenal hyperplasia in which deficiency of CYP11B1 or CYP17 causes aldosterone overproduction [30,31].

Full recovery of *CYP11B2* expression after the KCl and cAMP treatments suggested that SF-1 was not involved in the mechanisms underlying the potassium- and cAMP-regulated *CYP11B2* induction. Bassett et al. reported a similar finding that mutation of the SF-1 binding site in the *CYP11B2* promoter did not prevent activation of the promoter by agonists such as forskolin [32]. In the same report, the authors also proposed that SF-1 negatively regulated basal expression of *CYP11B2* because overexpression of exogenous SF-1 reduced the basal activity of a *CYP11B2*-reporter plasmid in H295R cells. However, when identical experiments performed in parallel in mouse adrenocortical Y1 cells, SF-1 acted as a positive regulator [32]. Our results agreed with the latter view by demonstrating that inhibition of SF-1 activity impaired basal *CYP11B2* expression in H295R. The contrasting results observed in the H295R cells presumably were due to the differences in the nature of the *CYP11B2* promoter. Our study was aimed to elucidate the influence of SF-1 on the native genes.

Our transcriptional analysis also provided evidence that potassium-induced steroidogenic regulation might involve a cross talk with the cAMP messenger system through enhancing the ACTH responsiveness of adrenocortical cells. The increase of ACTH receptor expression detected in the potassium- and cAMP-treated H295R cells might sensitize the adrenocortical cells to respond to lower concentrations of ACTH, and thus prolong the stimulatory action of each treatment. Impairing SF-1 activity diminished basal and inducible expression of ACTH receptor, and might consequently lower the acute and chronic responses of the adrenocortical cells to ACTH and potassium stimulation.

In addition, our study suggested that SF-1 regulated its own expression because the Bpu-del transformation diminished SF-1 mRNA and protein expression. Nomura et al. in a 1996 report proposed that SF-1 was under direct autoregulation. They located an SF-1 binding site in the first intron of the SF-1 gene. Mutation of this binding site decreased reporter expression from the intron I-containing promoter. On the other hand, cotransfection of exogenous SF-1 elevated expression of the reporter [33]. Our study supported this autoregulatory mechanism. The reduction of SF-1 activity in the Bpu-del transformed cells appeared to be the consequence of decreased SF-1 expression and mutant-mediated competitive inhibition.

In summary, our results confirmed that SF-1 played a critical role in the transcriptional regulation of *CYP17*, *CYP21B*, *CYP11B1*, ACTH receptor and SF-1. SF-1 also was involved in the mechanisms by which potassium and cAMP stimulated transcription of the *CYP17* gene. The negative effects of the Bpu-del mutant on inducible expression of *CYP21B*, *CYP11B1* and ACTH receptor only reflected the indispensability of SF-1 in transcriptional activation of these genes. In contrast, inhibition of SF-1 activity had little effects on potassium- and cAMP-induced *CYP11B2* expression. The differential SF-1 dependence of the genes crucial for distinct steroidogenic pathways suggested that SF-1 exerted a complex control on adrenal steroidogenic capacity in response to electrolyte status and hormonal signals.

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