CREM confers cAMP responsiveness in human steroidogenic acute regulatory protein expression in NCI-H295R cells rather than SF-1/Ad4BP

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Abstract

Steroidogenic acute regulatory (StAR) protein plays a critical role in steroid hormone synthesis. Tropic hormones induce human *StAR* gene expression by a cAMP-dependent pathway. Steroidogenic factor-1/adrenal-4-binding protein (SF-1/Ad4BP) plays an important role in the expression of human *StAR* gene. We investigated the mechanism of cAMP responsiveness in human *StAR* gene expression in NCI-H295R cells. The StAR promoter activity and protein levels in cells subjected to various treatments were examined. Anti-SF-1/Ad4BP IgG transfection treatment resulted in decreases in the basal StAR promoter activity and StAR protein levels, but did not affect cAMP-stimulated promoter activity and protein levels. The basal and cAMP-stimulated StAR promoter activity levels were reduced in SF-1/Ad4BP mutant (G35E)-transfected cells, but the cAMP induction of StAR promoter activity in response to 1 mM 8-Br-cAMP was not inhibited when G35E SF-1/Ad4BP mutant expression

vectors were co-transfected with cAMP-response element-binding (CREB) expression vectors. Although the basal StAR mRNA expression and protein levels were decreased by SF-1/Ad4BP-siRNA treatment, the cAMP-stimulated StAR mRNA expression and protein levels did not change. The basal StAR promoter activity level was not decreased by cAMP-response element modulator (CREM)-siRNA treatment, but the cAMP-stimulated StAR promoter activity level, the magnitude of cAMP induction of StAR promoter, and the cAMP-stimulated StAR protein level were decreased. The cAMP induction of StAR promoter activity in cells was inhibited when S117A CREM mutant expression vectors were transfected. We conclude that inhibition of the function of SF-1/Ad4BP does not reduce the cAMP induction of StAR promoter activity and protein level. CREM is needed to confer cAMP responsiveness in human StAR protein expression.

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Introduction

The first step in the biosynthesis of steroid hormones is the conversion of cholesterol into pregnenolone. Mitochondria are membrane-enclosed organelles in a cell, separated from the outside by lipid double lumens. The membranes contain phospholipids, and the inter-membrane space is an aqueous space. Cholesterol is hydrophobic and requires some factors to pass through the aqueous inter-membrane space. Steroidogenic acute regulatory (StAR) protein is a 30 kDa phosphorylated protein that binds to cholesterol and plays a key role in the movement of cholesterol from the outer mitochondrial membrane to the inner membrane, where P450_{scc} resides (Stocco & Clark 1996, Strauss *et al.* 1999, Christenson *et al.* 2000). StAR mutations cause congenital lipid adrenal hyperplasia, which impairs production of all steroid hormones (Lin *et al.* 1995, Bose *et al.* 1996). StAR-knockout mice have the same phenotype as humans with congenital lipid adrenal hyperplasia, and StAR protein has

been shown to mediate the true rate-limiting step in the synthesis of steroid hormones (Caron *et al.* 1997b).

StAR gene expression is present mainly in steroidogenic organs, such as adrenal gland, ovary, and testis (Clark *et al.* 1994, Sugawara *et al.* 1995). Production of steroid hormones in steroidogenic organs is rapidly increased by the stimulation of tropic hormones (adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH)). When the tropic hormones bind to their cognate receptors, intra-cellular cAMP levels increase via a G-protein-coupled mechanism and protein kinase A (PKA) is activated. The promoter activity of human *StAR* gene is increased by cAMP stimulation (Caron *et al.* 1997a, Sugawara *et al.* 1997b, Sandhoff *et al.* 1998, Clark & Combs 1999). The phosphorylation of StAR protein is also associated with PKA and increases steroid hormone production (Arakane *et al.* 1997). The human StAR promoter contains several response elements of transcription factors: steroidogenic factor-1/adrenal-4-binding protein (SF-1/Ad4BP), Sp1, CAAT/enhancer-binding

protein β (C/EBP β), sterol regulatory element-binding protein-1 α , and GATA-4 factors (Christenson *et al.* 1999, Sugawara *et al.* 2000, Tremblay *et al.* 2002). The transcription factors bind to the response elements of human StAR promoter and recruit co-activators or co-repressors and cooperate with other factors to control the human StAR promoter activity (Christenson *et al.* 1999, Sugawara *et al.* 2000, 2001). Although there is species difference in cAMP responsiveness, SF-1/Ad4BP is associated with basal and cAMP-stimulated human StAR promoter activities (Kiriakidou *et al.* 1996, Sugawara *et al.* 1996, LaVoie *et al.* 1999).

SF-1/Ad4BP is a nuclear receptor and combines with a specific DNA consensus site (Parker & Schimmer 1997). SF-1/Ad4BP expression is present in steroidogenic tissues and is essential for the development of steroidogenic tissues, where it plays a major role in the regulation of the expression of steroidogenic P450 enzymes, Müllerian-inhibiting substance and DAX-1 (Morohashi *et al.* 1993, Shen *et al.* 1994, Michael *et al.* 1995, Yu *et al.* 1998). Although the *StAR* gene expression level is increased by cAMP stimulation, SF-1/Ad4BP expression and protein levels are not increased by PKA induction (Nomura *et al.* 1998). SF-1/Ad4BP is phosphorylated by a PKA or mitogen-activated protein kinase (MAPK) and recruits nuclear receptor cofactor (Zhang & Mellon 1996, Hammer *et al.* 1999), but mutations of PKA and MAPK phosphorylation sites did not affect transactivity (Aesoy *et al.* 2002, Zheng & Jefcoate 2005). In patients, several mutations in SF-1/Ad4BP have been reported (Achermann *et al.* 1999, 2002, Biason-Lauber & Schoenle 2000, Correa *et al.* 2004, Mallet *et al.* 2004). SF-1/Ad4BP mutants of DNA binding are located in the P-box G35E and A-box R92Q. Patients had an XY sex-reversed female and adrenal insufficiency owing to a heterozygous mutation (G35E) and to a homozygous mutation (R92Q) in loss of function to SF-1/Ad4BP-binding sites.

The 1.3 kb human StAR promoter has three SF-1/Ad4BP-binding sites: a distal (−926 to −918), a middle (−105 to −96), and a proximal site (−43 to −36) (Sugawara *et al.* 1996, 1997a). The middle SF-1/Ad4BP-binding site contains a 5′-TGAC-3′ sequence. Although this sequence is not a canonical cAMP-response element (CRE; 5′-TGACGTCA-3′), it has recently been reported to be a CRE half-site and to have responsiveness to cAMP in the mouse StAR promoter (Manna *et al.* 2002, 2004). Although SF-1/Ad4BP is important in human StAR promoter activity, the mechanism of the cAMP response of SF-1/Ad4BP is not clear. In this study, we examined whether SF-1/Ad4BP and cAMP response element modulator (CREM) confer cAMP responsiveness in human *StAR* gene expression in NCI-H295R cells.

Materials and Methods

Plasmid constructs

A luciferase reporter gene for 1.3 kb human StAR promoter is the pGL2-1.3 kb StAR vector as previously described

(Sugawara *et al.* 1996). A 150 bp StAR promoter (pGL2-150StAR) was constructed by inserting a HindIII fragment prepared by PCR from the human 1.3 kb StAR promoter as a template into the pGL2 vector (Promega Corp., Madison, WI, USA). Mutations were produced using a Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). pGL2-150StAR/Mut1 has a mutation (5′-TATCCTTGAC-3′ to 5′-TATCCTCGAC-3′) in the sequence that encodes the SF-1/Ad4BP-binding site containing a 5′-TGAC-3′ sequence, which is a CRE-binding half-site. pGL2-150StAR/Mut2 has a mutation (5′-CAGCCTTC-3′ to 5′-CAGAATTC-3′) in the sequence of the SF-1/Ad4BP-binding site. pGL2-150StAR/Mut1/Mut2 has both the mutations in the sequence. Mutant StAR reporter constructs are depicted in Fig. 1A. Human SF-1/Ad4BP cDNA (pcDNA4BP/SF-1) was kindly provided by Dr Yanase, Graduate School of Medical Sciences, Kyushu University. SF-1/Ad4BP mutants (G35E in the P-box

Figure 1 cAMP response of the middle SF-1/Ad4BP site. (A) Schema of the human 150 bp StAR gene promoter. The human 150 bp StAR promoter has two SF-1/Ad4BP-binding sites (−105 to −96 and −43 to −36). One of the SF-1/Ad4BP-binding sites (−105 to −96) contains 5′-TGAC-3′ sequences. (B) The indicated plasmids and pCH110 were transfected into human adrenocortical carcinoma NCI-H295R cells. Cells were harvested after a 48-h culture period, and the cell lysate was subjected to a luciferase assay. Cells were treated with 8-Br-cAMP (1 mM) during the final 24-h culture (black bars) or not treated with 8-Br-cAMP (open bars). Promoter activity is expressed as a percentage of that of the 1.3 kb human StAR promoter. The results are presented as mean $\pm$ S.E.M. from three separate experiments with each treatment group consisting of three replicate cultures.

and R92Q in the A-box) were produced using a Transformer Site-Directed Mutagenesis Kit (Clontech). The β -galactosidase expression vector pCH110 was used for normalization of luciferase data, as previously described (Sugawara *et al.* 1996). RNA was isolated from human granulosa-like tumor KGN cells (Nishi *et al.* 2001). cDNA synthesis was carried out using 1 μ g total RNA and 200 units SuperScript II reverse transcriptase (Life Technologies, Inc./BRL, Washington, DC, USA). Human cAMP-response element binding (CREB) cDNA was generated by PCR reactions carried out with 1 μ l of reverse transcription reaction product using the following primers (sense, 5'-GCAGT-GACGGAGGAGCTTGATC-3'; antisense, 5'-TCTGAT-TTGTGGCAGTAAAG-3'). The PCR reaction was subjected to 30 cycles of denaturing at 94 °C for 45 s, annealing at 65 °C for 45 s, and extension at 72 °C for 1 min. Human CREM cDNA was prepared by PCRs with Marathon-Ready cDNA, 5'-stretched human testis cDNA (Clontech), using the following primers (sense, 5'-AC-TGGGCAAATTTCAATCCCTGC-3'; antisense, 5'-CAA-ACTTCCGGGCGATGCAGCATC-3'). PCR was performed according to the supplier's protocol. The PCR was subjected to 30 cycles of denaturation at 94 °C for 30 s and extension at 68 °C for 4 min. The PCR products were electrophoresed, and DNA fragments were cut from the gels. The fragments were ligated to PCR 2.1 vectors using a manual protocol (Invitrogen, Carlsbad, CA, USA). The PCR products were sequenced, and the sequences were compared with the previously published findings (Yoshimura *et al.* 1990, Ruppert *et al.* 1992, Masquillier *et al.* 1993, Gellersen *et al.* 1997). Human CREB expression vector (pCREB) was prepared by inserting an EcoRI fragment from human CREB cDNA into pSV.SPORT 1. Human CREM expression vector (pCREB) was prepared by inserting an EcoRI fragment from human CREM cDNA into pCMV5. Human CREM mutants (S117A in the P-box) were produced using a Transformer Site-Directed Mutagenesis Kit (Clontech). The plasmids were prepared for transfection studies using a Qiagen Maxiprep system (QIAGEN, Hilden, Germany).

Cell culture

Human adrenocortical carcinoma NCI-H295R cells were a gift from Dr Mitsuhiro Okamoto, Osaka University Medical School, Osaka, Japan. NCI-H295R cells were grown in DMEM/F12 containing 2% ULTROSER G (BioSeptra, Cergy-Pontoise, France) and 1% ITS Premix (Becton Dickinson and Co., Franklin Lakes, NJ, USA). Cultures of sub-confluent NCI-H295R cells were plated so that 35 mm tissue culture dishes received equal numbers of cells.

Transfection and luciferase assays

NCI-H295R cells were transfected with pGL₂ plasmids and pCH110 using 3 μ l FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany)/1 μ g DNA. Cultured cells

at 40–60% confluence were transfected with 0.5 μ g pGL₂ plasmid and 0.1 μ g PCH110. The cells were cultured for 48 h after transfection and then harvested. Some cells were co-transfected with a pGL₂ plasmid, pCH110, and various kinds of plasmids. Some dishes were treated with 8-Br-cAMP (1 mM) during the final 24-h culture. Luciferase assays were performed using a Luciferase Assay System (Promega). The assay results were normalized to β -galactosidase activity to compensate for variation in transfection efficiency as described previously (Sugawara *et al.* 2000). Each treatment group contained triplicate cultures, and each experiment was repeated three or four times.

Transfection of SF-1/Ad4BP antibody

NCI-H295R cells were plated in 35 mm tissue culture dishes and grown to 40–60% confluence. Cultured cells at 40–60% confluence were transfected with 0.5 μ g pGL₂ plasmid and 0.1 μ g pCH110. The cells were transfected on the next day with anti-mouse SF-1/Ad4BP rabbit IgG (Upstate Biotechnology, Lake Placid, NY, USA) using Chariot reagent (Active Motif, Carlsbad, CA, USA). Anti-SF-1/Ad4BP, 1 μ l was diluted with 100 μ l PBS, and 6 μ l Chariot reagent was diluted with water (1:1000). The diluted IgG and the diluted reagent were mixed and incubated at room temperature for 30 min. Growth medium was aspirated from the cells, and cells were washed with PBS. Transfection complex was added to the cells. The volume was adjusted to 600 μ l with serum-free medium, and the cells were incubated for 1 h. Cells were supplemented with 1 ml culture medium, incubated for 4 h, and then harvested. A luciferase assay and western blotting were performed using cell extracts.

Western blot analysis

NCI-H295R cell extracts were harvested with RIPA buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 1 $\times$ proteinase inhibitor) after transfection. Western blot analysis was performed as previously described (Sugawara *et al.* 2004). Ten micrograms of cell extract were subjected to 12% SDS-PAGE. After electrophoresis, the gels were transferred to nitrocellulose membranes for immunodetection with anti-StAR rabbit serum, a rabbit polyclonal anti-CREM IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and a mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (American Research Products, Inc., Belmont, MA, USA). Anti-human StAR protein serum was generously provided by Dr Jerome F Strauss III of the University of Pennsylvania, Philadelphia, PA, USA.

Small interfering RNA (siRNA)

Endogenous SF-1/Ad4BP mRNA was targeted in cells with transfection by the addition of a 23-nucleotide duplex (siRNA-SF-1/Ad4BP) (Dharmacon, Inc., Lafayette, CO,

USA). These duplex RNAs target 71 nucleotides downstream of the start codon of SF-1/Ad4BP. SiRNAs were constructed using the ribooligonucleotide pairs SF-1/AD4BP with the following sequences: 5'-CUACGGA-CUGCUCACGUGUGAdTdT-3' and 5'-UCACACGUG-AGCAGUCCGUAGdTdT-3'. As a control for the specificity of these duplexes, we used a scrambled ribooligonucleotide pair (siRNA-Scramble) with the following sequences: 5'-GCGCGCUUUGUAGGAUUCGdTdT-3' and 5'-CGAA-TCCTACAAAGCGCGCdTdT-3'. CREB mRNA was targeted in cells with transfection by the addition of a 23-nucleotide duplex. These duplex RNAs target 66 nucleotides downstream of the start codon of CREB. SiRNAs were constructed using the ribooligonucleotide pairs CREB with the following sequences: 5'-CAAAUGACAGUU-CAAGCCCAGdTdT-3' and 5'-CUGGGCUUGAACU-GUCAUUUGdTdT-3'. CREM mRNA was targeted in cells with transfection by the addition of a 21-nucleotide duplex. These duplex RNAs target 42 nucleotides downstream of the start codon of CREM. SiRNAs were constructed using the ribooligonucleotide pairs CREM with the following sequences: 5'-CAGCUUCUUUGACAGAGAGCAdTdT-3' and 5'-UGCUCUCUGUCAAAGAAGCUGdTdT-3'. The oligonucleotides were annealed according to the Dharmacon protocol. Three hundred picomoles of each duplex was introduced into cells using 15 µl metafectene (Biontex Laboratories GmbH, Munich, Germany) as recommended by the manufacturer. At 48 h after transfection, total RNA was extracted and reverse transcriptase (RT)-PCR was performed, as previously described (Sugawara *et al.* 2004), for SF-1/AD4BP (27 cycles) and for human StAR (27 cycles) with GAPDH (24 cycles) as a control using the primers 5'-GCGGACGCGGCGGGCATGGACTATT-3' (sense) and 5'-AACAGAGGCTCTCCCTCCTCCTGGTCTC-3' (antisense) for SF-1/Ad4BP and the primers 5'-GCAGC-AGCAGCGGCGGCAGCAG-3' (sense) and 5'-ATGAG-CGTGTGTACCACTG-3' (antisense) for StAR. RT-PCR for GAPDH as a control was performed using the primers 5'-TGCCGTCTAGAAAAACCTGC-3' (sense) and 5'-ACCCTGTTGCTGTAGCCAAA-3' (antisense). RT-PCR was also performed for human CREM (25 cycles) with GAPDH (25 cycles) as a control using the primers 5'-GACCATGGAAACAGTTGAATCCCA-3' (sense) and 5'-CGTCGACATTCTTTGGCAGC-3' (antisense) for human CREM (Groussin *et al.* 2000). In some experiments, cell extracts were harvested after transfection and then western blot analysis or luciferase assay was performed.

Data analysis

Values are presented as mean ± S.E.M. Significance between experimental values was determined by Student's unpaired *t*-test, and one-way ANOVA was used to test differences in repeated measures across experiments. Significant results from ANOVA were further analyzed by Tukey's *post hoc* test. *P* < 0.05 was considered significant.

Results

The human StAR promoter has three SF-1/Ad4BP-binding sites. Mutations were introduced into SF-1/Ad4BP-binding sites, two of the mutations being within 150 bp upstream of the transcriptions start site (−105 to −96 and −43 to −36). The indicated plasmids were transfected into human adrenocortical carcinoma NCI-H295R cells, and luciferase assays were performed. The basal and cAMP-stimulated promoter activity levels of pGL₂-150StAR were increased compared with the pGL₂-1.3 kb StAR promoter activity level. The mutated human StAR promoter activity level was decreased compared with that of pGL₂-150StAR. Basal and cAMP-stimulated promoter activity levels in cells transfected with pGL₂-150StAR/Mut1 were reduced to 8.6 and 4.2% respectively. Basal and cAMP-stimulated promoter activity levels in cells transfected with pGL₂-150StAR/Mut2 were reduced to 30 and 21.3% respectively. Mutations in both SF-1/Ad4BP-binding sites (pGL₂-150StAR/Mut1/Mut2) reduced promoter activity to nearly background levels (Fig. 1B). The cAMP induction with pGL₂-150StAR did not significantly differ from that of pGL₂-1.3 kb StAR. Although the cAMP induction with pGL₂-150StAR/Mut2 did not significantly differ from that with pGL₂-150StAR, the cAMP induction with pGL₂-150StAR/Mut1 and pGL₂-150/StARMut1/Mut2 were significantly (*P* < 0.05) different from that of pGL₂-150StAR (Table 1).

To inhibit SF-1/Ad4BP function in the nucleus, anti-SF-1/Ad4BP IgG was transfected into NCI-H295R cells using a reagent. The pGL₂-1.3 kb StAR plasmids were transfected into NCI-H295R cells and anti-SF-1/Ad4BP IgG was transfected. Although the cAMP-stimulated promoter activity level with anti-SF-1/Ad4BP IgG transfection did not differ from that of the control, the basal promoter activity level with anti-SF-1/Ad4BP IgG transfection was significantly decreased when compared with that of the control (Fig. 2A). The basal level of human StAR protein with anti-SF-1/Ad4BP IgG transfection was decreased compared with the control (Fig. 2B). The cAMP-stimulated StAR protein level with anti-SF-1/Ad4BP IgG transfection was not different from the control. The basal promoter activity and protein levels were decreased but cAMP-stimulated promoter activity and protein levels were not decreased by the IgG transfection treatment.

Table 1 StAR promoter induction by cAMP stimulation

	Fold
pGL ₂ -1.3 kb StAR	4.6 ± 0.6
pGL ₂ -150StAR	5.2 ± 0.5
pGL ₂ -150StAR/Mut1	2.5 ± 0.2*
pGL ₂ -150StAR/Mut2	3.7 ± 0.8
pGL ₂ -150StAR/Mut1/Mut2	1.9 ± 0.1*

Fold-induction is expressed as relative cAMP-stimulated StAR promoter activity to basal StAR promoter activity. *Significant difference for StAR promoter induction compared with pGL₂-150StAR. **P* < 0.05.

Figure 2 Transfection of an SF-1/Ad4BP antibody into NCI-H295R cells. (A) The indicated plasmids were transfected into NCI-H295R cells. The next day, cells were transfected with an SF-1/Ad4BP antibody using a reagent. Cells were treated with 8-Br-cAMP during the final 24-h culture or not treated with 8-Br-cAMP. Promoter activity is expressed as RLU (luciferase/β-galactosidase). *Significant difference for basal promoter activity compared with that of control; $*P < 0.05$. (B) The cells were collected from each dish by scraping. The cell extracts were subjected to 12% SDS-PAGE, and then western blot analysis was performed using anti-StAR.

Transfection of anti-SF-1/Ad4BP IgG affected the basal StAR promoter activity level and basal StAR protein level.

To investigate the effect of SF-1/Ad4BP on the human StAR promoter, a human wild-type SF-1/Ad4BP expression vector and SF-1/Ad4BP mutant expression vector were over-expressed in NCI-H295R cells, which have endogenous SF-1/Ad4BP expression (Fig. 3). The basal promoter activity level in human NCI-H295R cells with over-expression of human SF-1/Ad4BP was increased by 2.5-fold. The cAMP-stimulated promoter activity level also increased with SF-1/Ad4BP expression vectors. The basal and cAMP-stimulated promoter activity levels in cells with over-expression of the SF-1/Ad4BP mutant expression vector R92Q (A-box mutant) were increased by 3- and 1.5-fold respectively. The promoter activity with SF-1/Ad4BP R92Q mutant expression vectors did not differ from that with human wild-type SF-1/Ad4BP expression vectors. The basal StAR promoter activity and cAMP-induced promoter activity levels were reduced when SF-1/Ad4BP mutant (G35E) expression vectors were transfected into NCI-H295R cells. The magnitude of induction of StAR promoter activity by cAMP stimulation was reduced when an SF-1/Ad4BP mutant (G35E) expression vector was transfected.

To reduce SF-1/Ad4BP expression in cells, siRNA was transfected into NCI-H295R cells, and basal and cAMP-stimulated StAR mRNA expression and protein levels of StAR protein were examined. Basal StAR mRNA expression level was decreased by siRNA treatment. Although endogenous

Figure 3 Effect of SF-1/Ad4BP mutant on human StAR promoter. The pGL2-1.3 kb StAR vector (0.5 μg) and SF-1/Ad4BP (0.2 μg) or an SF-1/Ad4BP mutant (R92Q or G35E) expression vector (0.2 μg) were transfected into NCI-H295R cells. Cells were treated with 8-Br-cAMP during the final 24-h culture or not treated with 8-Br-cAMP. Values presented are the mean ± S.E.M. of the promoter activities, expressed as a percentage of that of the 1.3 kb human StAR promoter with an empty vector. *Significant difference for basal promoter activity compared with that of the empty vector. [†]Significant difference for cAMP-stimulated promoter activity compared with that of the empty vector. $*^{\dagger}P < 0.05$.

SF-1/Ad4BP expression was decreased by siRNA treatment, cAMP-stimulated StAR mRNA expression was not reduced in NCI-H295R cells (Fig. 4A). Cells were collected with RIPA buffer, and western blot analysis was performed. With siRNA treatment of SF-1/Ad4BP, the basal level of StAR protein was decreased but the cAMP-stimulated protein level was not decreased (Fig. 4B). Basal StAR mRNA and protein expression levels were decreased by SF-1/Ad4BP-siRNA treatment, but cAMP-stimulated StAR mRNA and protein expressions were not inhibited.

To examine the effect of CREB on cAMP response, CREB was over-expressed in NCI-H295R cells. StAR promoter plasmids and CREB expression vectors were over-expressed in NCI-H295R cells, which do not express endogenous CREB (Groussin *et al.* 2000). The basal and cAMP-stimulated promoter activity levels significantly increased with CREB over-expression compared with that without CREB expression in NCI-H295R cells (Fig. 5A). To investigate the interactions between CREB and SF-1/Ad4BP, CREB, SF-1/Ad4BP, and SF-1/Ad4BP mutant expression vectors were transfected into NCI-H295R cells. With SF-1/Ad4BP over-expression, both basal and cAMP-stimulated promoter activity levels of StAR did not differ from that without SF-1/Ad4BP over-expression. Although the promoter activity of StAR also did not differ with SF-1/Ad4BP mutant R92Q, the basal and cAMP-stimulated promoter activity levels of StAR with G35E SF-1/Ad4BP mutant expression were significantly decreased compared with those without SF-1/Ad4BP over-expression (Fig. 5B). G35E SF-1/Ad4BP mutant and CREB did not

Figure 4 Reduction in basal level of StAR protein by siRNA-SF-1/Ad4BP treatment. Endogenous *SF-1/Ad4BP* gene expression in NCI-H295R cells was eliminated by transfection of a 23-nucleotide RNA duplex (siRNA-SF-1/Ad4BP). As a control, scrambled sequences were introduced into the cells. The next day, 8-Br-cAMP (1 mM) was added to some of the culture media. After 48-h culture, RNA was extracted from the cells. (A) The effects of siRNA treatment were assayed by RT-PCR for *SF-1/Ad4BP* and *GAPDH* gene. (B) Some cells were collected from each dish by scraping. The cell extracts were subjected to 12% SDS-PAGE, and then western blot analysis was performed using anti-StAR.

confer responsiveness of human StAR promoter activity to cAMP stimulation.

To clarify cAMP induction, CREB and CREM transcription was inhibited in cells by siRNA treatment. The basal and cAMP-stimulated StAR promoter activity levels were not reduced by siRNA-CREB treatment. In contrast, the promoter activity of cAMP stimulation was reduced by siRNA-CREM treatment (Fig. 6A). The cAMP response of StAR promoter activity is associated with CREM expression. CREM isoforms, CREM $\tau_2\alpha$ and CREM α , are expressed in NCI-H295R cells (Groussin *et al.* 2000). The effects of siRNAs were assayed by RT-PCR for *CREM* and *GAPDH* gene. Endogenous CREM mRNA expression level was decreased by siRNA-CREM treatment in NCI-H295R cells in the basal and cAMP-stimulation states (Fig. 6B). To determine the effect on levels of StAR protein, we performed western blot analysis using siRNA treatment. The basal and cAMP-stimulated StAR protein levels were not affected by siRNA-CREB treatment. The basal and cAMP-stimulated StAR protein levels were decreased with siRNA-CREM (Fig. 6C). Phosphorylation of CREM at Ser117 is important for association with CREB-binding protein (CBP) (Laurance *et al.* 1997). To determine the specificity of the effect of CREM on cAMP responsive StAR transcription, we converted Ser117 into an alanine and examined the association with the phosphorylation mutant of CREM. The StAR promoter plasmids, the CREM expression vector, and the S117A CREM mutant expression vector were co-transfected into NCI-H295R cells. The cAMP-stimulated promoter

Figure 5 Effect of CREB protein on StAR promoter. (A) StAR promoter plasmids (0.5 μ g) and CREB expression vectors (0.5 μ g) were over-expressed in NCI-H295R cells. The cells were treated with 8-Br-cAMP (1 mM) during the final 24-h culture or not treated with 8-Br-cAMP. Promoter activity is expressed as RLU (luciferase/ β -galactosidase). *Significant difference for basal promoter activity compared with that of the empty vector. †Significant difference for cAMP-stimulated promoter activity compared with that of the empty vector. ** $P < 0.05$. (B) The pGL2-1.3 kb StAR vector (0.5 μ g), SF-1/Ad4BP (0.2 μ g) or an SF-1/Ad4BP mutant (R92Q or G35E) expression vector (0.2 μ g) and CREB expression vectors (0.5 μ g) were transfected into NCI-H295R cells. Cells were treated with 8-Br-cAMP (1 mM) during the final 24-h culture or not treated with 8-Br-cAMP. Values presented are the mean $\pm$ s.e.m. of the promoter activities, expressed as a percentage of that of pGL2-1.3 kb StAR with an empty vector. *Significant difference for basal promoter activity compared with that of the empty vector. †Significant difference for cAMP-stimulated promoter activity compared with that of the empty vector. ** $P < 0.05$.

activity level significantly decreased ($P < 0.05$) (Fig. 7). CREM conferred responsiveness of human StAR protein to cAMP stimulation.

Discussion

Transcriptional regulation of *StAR* gene expression is associated with many transcription factors. SF-1/Ad4BP, C/EBP β , GATA-4, and CREM have been reported to be regulators of cAMP-induced StAR transcription (Silverman *et al.* 1999, Manna *et al.* 2002, 2004, Tremblay *et al.* 2002). SF-1/Ad4BP is a key regulator of multiple genes, including

Figure 6 Effects of siRNA-CREM treatment on StAR promoter and protein levels. Endogenous *CREB* gene expression in NCI-H295R cells was eliminated by treatment with siRNA. As a control, scrambled sequences were introduced into the cells. The next day, 8-Br-cAMP (1 mM) was added to some of the culture media. (A) The effects of siRNAs were assayed by promoter activity. Promoter activity is expressed as RLU (luciferase/β-galactosidase). *Significant difference for cAMP-stimulated promoter activity compared with that of siRNA scramble. * $P < 0.05$. (B) The effects of siRNAs were assayed by RT-PCR for *CREM* and *GAPDH* gene. Direct sequences of PCR products amplified with *CREM* primers correspond to *CREM*τ₂α (1131 bp) and *CREM*α (942 bp) (Groussin *et al.* 2000). (C) Some cells were collected from each dish by scraping. The cell extracts were subjected to 12% SDS-PAGE, and then western blot analysis was performed using anti-StAR, anti-CREM, and anti-GAPDH.

genes responsible for gonadal and adrenal development, steroidogenesis, and reproduction (Lala *et al.* 1992, Honda *et al.* 1993, Parker & Schimmer 1997). SF-1/Ad4BP increases promoter activity of not only StAR but also steroidogenic genes, including *CYP17*, *CYP11A*, and *CYP19* genes

Figure 7 Effects of CREM on cAMP responsive StAR transcription. pGL2-1.3 kb StAR promoter plasmids (1 μg), pCH110 (0.5 μg) and CREM expression vectors (0.5 μg), or S117A CREM mutant expression vector (0.5 μg) were transfected into NCI-H295R cells. The cells were treated with 8-Br-cAMP (1 mM) during the final 24-h culture or not treated with 8-Br-cAMP. Values presented are the mean ± s.e.m. of the promoter activities, expressed as a percentage of that of pGL2-1.3 kb StAR with an empty vector. *†Significant difference from each other at $P < 0.05$.

(Morohashi *et al.* 1993, Michael *et al.* 1995, Sewer *et al.* 2002). SF-1/Ad4BP interacts with several proteins, including SRC-1, CBP, N-CoR, Sox 9, WT1, and MBF1, and controls gene expression (Crawford *et al.* 1998, De Santa Barbara *et al.* 1998, Ito *et al.* 1998, Kabe *et al.* 1999, Hossain & Saunders 2003). It is not known whether SF-1/Ad4BP activates transcription of only the human *StAR* gene or co-activates transcription of other genes with other factors. In this study, we analyzed cAMP induction of the human *StAR* promoter by inhibiting an SF-1/Ad4BP function in NCI-H295R cells.

Analysis of mutations of the SF-1/Ad4BP sites showed reductions of basal and cAMP-stimulated promoter activity levels. Of all the SF-1/Ad4BP-binding sites, the most important was the -105 to -95 site. The results obtained for NCI-H295R are consistent with those of human granulosa-lutein cells, which undergo terminal differentiation (luteinization) in response to human chorionic gonadotropin (Kiriakidou *et al.* 1996, Sugawara *et al.* 1997a). It has been reported that forskolin, which stimulates adenyl cyclase, could reorganize SF-1/Ad4BP from a diffuse distribution pattern to formation in the nucleus of granulosa tumor KGN cells (Fan *et al.* 2004). PKA stimulation modifies the interaction of SF-1/Ad4BP with its activator, transformation/transcription domain-associated protein (TRRAP), and suppressor, dosage-sensitive sex reversal (DAX-1) protein (Fan *et al.* 2004).

SF-1/Ad4BP is translated in the cytoplasm and enters the nuclei of cells, where it binds to a DNA consensus sequence. When an antibody is introduced into the cell nucleus by a protein transfection reagent, transcription activation is reduced (Cheriyath *et al.* 2002). Although the transfection reagent decreased the basal promoter activity and protein levels, the cAMP-stimulated promoter activity and protein

levels did not change. The affinity of the proximal SF-1/Ad4BP-binding sites (−105 to −95 and −42 to −35) is lower than that of the distal SF-1/Ad4BP-binding site (−926 to −918) (Sugawara *et al.* 1997a). One possibility is that IgG is not sufficient for formation of a complex with SF-1/Ad4BP, and a small amount of free SF-1/Ad4BP can respond to cAMP stimulation. Alternatively, SF-1/Ad4BP antibody might not completely obstruct the interaction between other transcription factors, co-activators and co-repressors, or other factors might compensate the dysfunction of SF-1/Ad4BP. Other factors, GATA-4, C/EBP β , or CREB, may compensate the loss of the effect of SF-1/Ad4BP with IgG transfection on cAMP-stimulated StAR promoter activity or LRH-1/NR5A2 transcription factor may induce cAMP-stimulated promoter activity. It has been reported that LRH-1 also binds SF-1/Ad4BP-response elements and controls promoter activity in steroidogenic cells, including NCI-H295R cells and granulosa cells (Wang *et al.* 2001, Kim *et al.* 2004).

Several mutations of SF-1/Ad4BP in patients, who have adrenal insufficiency and sex-reversal with retained Müllerian structures have been reported (Achermann *et al.* 1999, 2002, Biason-Laubier & Schoenle 2000). The R92Q A-box mutation is a homozygous mutation with an autosomal recessive mode of inheritance. The G35E P-box mutation is a heterozygous mutation. The P-box of SF-1/Ad4BP interacts directly with the half-site sequence of SF-1/Ad4BP-binding (PyCA AGGTCA) motif, which is located in the major groove of the DNA helix. On the other side, the A-box region interacts with the minor groove of the DNA helix (PyCA AGGTCA) and stabilizes the SF-1-DNA interaction. Mutation in the A-box region (R92Q) has little effect on DNA binding (Ito *et al.* 2000). Although P-box mutation (G35E) results in complete loss of SF-1/Ad4BP-binding activity, A-box mutation (R92Q) results only in partial impairment of binding (Achermann *et al.* 2002). P-box mutation resulted in reduction of cAMP-stimulated promoter activity and impairment of cAMP induction. The SF-1/Ad4BP-binding motif in the major groove of the DNA helix (P-box) seems to be associated with the induction of cAMP stimulation. Although a P-box mutant cannot bind DNA, the protein interaction domains, which bind other transcription factors, are intact. Other transcription factors may not compensate the promoter activity because the transcription factors may form a complex with mutant SF-1/Ad4BP and because there is supposed to be a shortage of co-activators.

Transfection with siRNA is a widely used method for reducing endogenous gene expression (Elbashir *et al.* 2001). When endogenous SF-1/Ad4BP expression level was decreased, basal StAR protein level was reduced but cAMP-stimulated StAR protein level did not change. This is because siRNA treatment cannot completely inhibit the transcription of SF-1/Ad4BP and a small amount of SF-1/Ad4BP protein might be sufficient to increase cAMP-stimulated StAR protein level. This result is consistent with the results for the R92Q A-box mutant. The mRNA and protein levels of SF-1/Ad4BP remain constant after an increase or decrease in the cAMP level

(Nomura *et al.* 1998). Since an association of SF-1/Ad4BP with the StAR promoter has been reported without an increase in SF-1/Ad4BP within 15 min after cAMP stimulation (Hiroi *et al.* 2004), cAMP-stimulated promoter activity is independent of SF-1/Ad4BP protein level. Other factors, such as GATA-4, have been reported to be phosphorylated in response to cAMP stimulation and cooperate to increase cAMP-stimulated StAR promoter activity level (Tremblay *et al.* 2002). Phosphorylated transcription factors might cooperate with SF-1/Ad4BP and recruit co-activators.

Transcriptional activation by cAMP is mediated through the interaction of the CREB protein in many target genes. Although the human *StAR* gene promoter regions lack the consensus CRE, CRE half-sites are found in SF-1/Ad4BP-binding sites. CREB over-expression affects the basal and cAMP-stimulated StAR promoter activity levels in NCI-H295R cells, which lack endogenous CREB expression. Mutant G35E with CREB over-expression had no transcription activity of human StAR. The CBP acts as a bridging protein with other transcription factors, including CREB, CREM, SF-1/Ad4BP, c-Fos, and c-Jun (Bannister & Kouzarides 1995, Bannister *et al.* 1995). In the mouse StAR promoter, CREM was found by EMSA using nuclear extracts from MA-10 mouse Leydig tumor cells to be the predominant protein binding to the cAMP responsive region (Manna *et al.* 2002). Using DNA-affinity chromatography, CREB has been found to interact with the mouse StAR promoter with dependence on AP-1-binding sites, which are present near SF-1/Ad4BP sites (Clem *et al.* 2005). Although the antibodies used in these two studies are different, the results show that both CREB and CREM bind to the mouse StAR promoter.

Phosphorylation of CREB by PKA permits binding of CBP, and then the CREB/CBP complex enhances DNA accessibility by HAT activity and binds to the CRE and the TATA box regions of the promoter (Parker *et al.* 1996, Wolf *et al.* 1999). (Bu)₂cAMP treatment has been reported to increase phosphorylated CREM and CBP interaction with the mouse StAR promoter (Clem *et al.* 2005). SiRNA-CREM treatment reduced the cAMP-stimulated promoter activity levels of human StAR. The human cAMP-stimulated promoter activity decreased with S117A CREM mutant transfection. In the human StAR promoter, as in the mouse StAR promoter, PKA may phosphorylate CREB/CREM and bind CBP to control transcription activity. Although CREB is not expressed in NCI-H295R cells, CREM, which is a CREB family member, is needed to confer cAMP responsiveness in human StAR protein expression.

Recently, phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol-phosphates) have been reported to be ligands of SF-1/Ad4BP and lipid binding has been shown to be required for maximum activity (Krylova *et al.* 2005, Li *et al.* 2005, Ortlund *et al.* 2005, Wang *et al.* 2005). To clarify the species difference in cAMP response to the StAR promoter, detailed analysis of the human StAR promoter taking into account the presence of SF-1/Ad4BP ligands is needed.

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References

- Achermann JC, Ito M, Hindmarsh PC & Jameson JL 1999 A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. *Nature Genetics* **22** 125–126.
- Achermann JC, Ozisik G, Ito M, Orun UA, Harmanci K, Gurakan B & Jameson JL 2002 Gonadal determination and adrenal development are regulated by the orphan nuclear receptor steroidogenic factor-1, in a dose-dependent manner. *Journal of Clinical Endocrinology and Metabolism* **87** 1829–1833.
- Aesoy R, Mellgren G, Morohashi K & Lund J 2002 Activation of cAMP-dependent protein kinase increases the protein level of steroidogenic factor-1. *Endocrinology* **143** 295–303.
- Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM & Strauss JF III 1997 Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *Journal of Biological Chemistry* **272** 32656–32662.
- Bannister AJ & Kouzarides T 1995 CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO Journal* **14** 4758–4762.
- Bannister AJ, Oehler T, Wilhelm D, Angel P & Kouzarides T 1995 Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation *in vivo* and CBP binding *in vitro*. *Oncogene* **11** 2509–2514.
- Biason-Lauber A & Schoenle EJ 2000 Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidogenic factor 1 (NR5A1/SF-1) and adrenocortical insufficiency. *American Journal of Human Genetics* **67** 1563–1568.
- Bose HS, Sugawara T, Strauss JF III & Miller WL 1996 The pathophysiology and genetics of congenital lipid adrenal hyperplasia. International congenital lipid adrenal hyperplasia consortium. *New England Journal of Medicine* **335** 1870–1878.
- Caron KM, Ikeda Y, Soo SC, Stocco DM, Parker KL & Clark BJ 1997a Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Molecular Endocrinology* **11** 138–147.
- Caron KM, Soo SC, Wetsel WC, Stocco DM, Clark BJ & Parker KL 1997b Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipid adrenal hyperplasia. *PNAS* **94** 11540–11545.
- Cheriyath V, Desgranges ZP & Roy AL 2002 c-Src-dependent transcriptional activation of TFII-I. *Journal of Biological Chemistry* **277** 22798–22805.
- Christenson LK, Johnson PF, McAllister JM & Strauss JF III 1999 CCAAT/enhancer-binding proteins regulate expression of the human steroidogenic acute regulatory protein (StAR) gene. *Journal of Biological Chemistry* **274** 26591–26598.
- Christenson LK, Strauss JF & Strauss JF III 2000 Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. *Biochimica et Biophysica Acta* **1529** 175–187.
- Clark BJ & Combs R 1999 Angiotensin II and cyclic adenosine 3', 5'-monophosphate induce human steroidogenic acute regulatory protein transcription through a common steroidogenic factor-1 element. *Endocrinology* **140** 4390–4398.
- Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269** 28314–28322.
- Clem BF, Hudson EA & Clark BJ 2005 Cyclic adenosine 3', 5'-monophosphate (cAMP) enhances cAMP-responsive element binding (CREB) protein phosphorylation and phospho-CREB interaction with the mouse steroidogenic acute regulatory protein gene promoter. *Endocrinology* **146** 1348–1356.
- Correa RV, Domenice S, Bingham NC, Billerbeck AE, Rainey WE, Parker KL & Mendonca BB 2004 A microdeletion in the ligand binding domain of human steroidogenic factor 1 causes XY sex reversal without adrenal insufficiency. *Journal of Clinical Endocrinology and Metabolism* **89** 1767–1772.
- Crawford PA, Dorn C, Sadovsky Y & Milbrandt J 1998 Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. *Molecular and Cellular Biology* **18** 2949–2956.
- De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, Scherer G, Poulart F & Berta P 1998 Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene. *Molecular and Cellular Biology* **18** 6653–6665.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K & Tuschl T 2001 Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411** 494–498.
- Fan W, Yanase T, Wu Y, Kawate H, Saitoh M, Oba K, Nomura M, Okabe T, Goto K, Yanagisawa J *et al.* 2004 Protein kinase A potentiates adrenal 4 binding protein/steroidogenic factor 1 transactivation by reintegrating the subcellular dynamic interactions of the nuclear receptor with its cofactors, general control nonderepressed-5/transformation/transcription domain-associated protein, and suppressor, dosage-sensitive sex reversal-1: a laser confocal imaging study in living KGN cells. *Molecular Endocrinology* **18** 127–141.
- Gellersen B, Kempf R & Telgmann R 1997 Human endometrial stromal cells express novel isoforms of the transcriptional modulator CREM and up-regulate ICER in the course of decidualization. *Molecular Endocrinology* **11** 97–113.
- Groussin L, Massias JF, Bertagna X & Bertherat J 2000 Loss of expression of the ubiquitous transcription factor cAMP response element-binding protein (CREB) and compensatory overexpression of the activator CREMtau in the human adrenocortical cancer cell line H295R. *Journal of Clinical Endocrinology and Metabolism* **85** 345–354.
- Hammer GD, Krylova I, Zhang Y, Darimont BD, Simpson K, Weigel NL & Ingraham HA 1999 Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. *Molecular Cell* **3** 521–526.
- Hiroi H, Christenson LK, Chang L, Sammel MD, Berger SL & Strauss JF III 2004 Temporal and spatial changes in transcription factor binding and histone modifications at the steroidogenic acute regulatory protein (StAR) locus associated with StAR transcription. *Molecular Endocrinology* **18** 791–806.
- Honda S, Morohashi K, Nomura M, Takeya H, Kitajima M & Omura T 1993 Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *Journal of Biological Chemistry* **268** 7494–7502.
- Hossain A & Saunders GF 2003 Role of Wilms tumor 1 (WT1) in the transcriptional regulation of the Müllerian-inhibiting substance promoter. *Biology of Reproduction* **69** 1808–1814.
- Ito M, Yu RN & Jameson JL 1998 Steroidogenic factor-1 contains a carboxy-terminal transcriptional activation domain that interacts with steroid receptor coactivator-1. *Molecular Endocrinology* **12** 290–301.
- Ito M, Achermann JC & Jameson JL 2000 A naturally occurring steroidogenic factor-1 mutation exhibits differential binding and activation of target genes. *Journal of Biological Chemistry* **275** 31708–31714.
- Kabe Y, Goto M, Shima D, Imai T, Wada T, Morohashi K, Shirakawa M, Hirose S & Handa H 1999 The role of human MBF1 as a transcriptional coactivator. *Journal of Biological Chemistry* **274** 34196–34202.
- Kim JW, Peng N, Rainey WE, Carr BR & Attia GR 2004 Liver receptor homolog-1 regulates the expression of steroidogenic acute regulatory protein in human granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **89** 3042–3047.
- Kiriakidou M, McAllister JM, Sugawara T & Strauss JF III 1996 Expression of steroidogenic acute regulatory protein (StAR) in the human ovary. *Journal of Clinical Endocrinology and Metabolism* **81** 4122–4128.

- Krylova IN, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, Juzumiene D, Bynum JM, Madauss K, Montana V *et al.* 2005 Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. *Cell* **120** 343–355.
- Lala DS, Rice DA & Parker KL 1992 Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Molecular Endocrinology* **6** 1249–1258.
- Laurance ME, Kwok RP, Huang MS, Richards JP, Lundblad JR & Goodman RH 1997 Differential activation of viral and cellular promoters by human T-cell lymphotropic virus-1 tax and cAMP-responsive element modulator isoforms. *Journal of Biological Chemistry* **272** 2646–2651.
- LaVoie HA, Garmey JC & Veldhuis JD 1999 Mechanisms of insulin-like growth factor I augmentation of follicle-stimulating hormone-induced porcine steroidogenic acute regulatory protein gene promoter activity in granulosa cells. *Endocrinology* **140** 146–153.
- Li Y, Choi M, Cavey G, Daugherty J, Suino K, Kovach A, Bingham NC, Kliewer SA & Xu HE 2005 Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. *Molecular Cell* **17** 491–502.
- Lin D, Sugawara T, Strauss JF III, Clark BJ, Saenger DM, Rogol A & Miller WL 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** 1828–1831 (see comments).
- Mallet D, Bretones P, Michel-Calemard L, Djoud F, David M & Morel Y 2004 Gonadal dysgenesis without adrenal insufficiency in a 46, XY patient heterozygous for the nonsense C16X mutation: a case of SF1 haploinsufficiency. *Journal of Clinical Endocrinology and Metabolism* **89** 4829–4832.
- Manna PR, Dyson MT, Eubank DW, Clark BJ, Lalli E, Sassone-Corsi P, Zeleznik AJ & Stocco DM 2002 Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Molecular Endocrinology* **16** 184–199.
- Manna PR, Eubank DW & Stocco DM 2004 Assessment of the role of activator protein-1 on transcription of the mouse steroidogenic acute regulatory protein gene. *Molecular Endocrinology* **18** 558–573.
- Masquillier D, Foulkes NS, Mattei MG & Sassone-Corsi P 1993 Human CREM gene: evolutionary conservation, chromosomal localization, and inducibility of the transcript. *Cell Growth and Differentiation* **4** 931–937.
- Michael MD, Kilgore MW, Morohashi K & Simpson ER 1995 Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (P1) of the human aromatase P450 (CYP19) gene in the ovary. *Journal of Biological Chemistry* **270** 13561–13566.
- Morohashi K, Zanger UM, Honda S, Hara M, Waterman MR & Omura T 1993 Activation of CYP11A and CYP11B gene promoters by the steroidogenic cell-specific transcription factor, Ad4BP. *Molecular Endocrinology* **7** 1196–1204.
- Nishi Y, Yanase T, Mu YM, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K *et al.* 2001 Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. *Endocrinology* **142** 437–445.
- Nomura M, Kawabe K, Matsushita S, Oka S, Hatano O, Harada N, Nawata H & Morohashi K 1998 Adrenocortical and gonadal expression of the mammalian *Fitz-F1* gene encoding Ad4BP/SF-1 is independent of pituitary control. *Journal of Biochemistry (Tokyo)* **124** 217–224.
- Ortlund EA, Lee Y, Solomon IH, Hager JM, Safi R, Choi Y, Guan Z, Tripathy A, Raetz CR, McDonnell DP *et al.* 2005 Modulation of human nuclear receptor LRH-1 activity by phospholipids and SHP. *Nature Structural and Molecular Biology* **12** 357–363.
- Parker KL & Schimmer BP 1997 Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocrine Reviews* **18** 361–377.
- Parker D, Ferreri K, Nakajima T, LaMorte VJ, Evans R, Koerber SC, Hoeger C & Montminy MR 1996 Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Molecular and Cellular Biology* **16** 694–703.
- Ruppert S, Cole TJ, Boshart M, Schmid E & Schutz G 1992 Multiple mRNA isoforms of the transcription activator protein CREB: generation by alternative splicing and specific expression in primary spermatocytes. *EMBO Journal* **11** 1503–1512.
- Sandhoff TW, Hales DB, Hales KH & McLean MP 1998 Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* **139** 4820–4831.
- Sewer MB, Nguyen VQ, Huang CJ, Tucker PW, Kagawa N & Waterman MR 2002 Transcriptional activation of human CYP17 in H295R adrenocortical cells depends on complex formation among p54(nrb)/NonO, protein-associated splicing factor, and SF-1, a complex that also participates in repression of transcription. *Endocrinology* **143** 1280–1290.
- Shen WH, Moore CC, Ikeda Y, Parker KL & Ingraham HA 1994 Nuclear receptor steroidogenic factor 1 regulates the Mullerian inhibiting substance gene: a link to the sex determination cascade. *Cell* **77** 651–661.
- Silverman E, Eimerl S & Orly J 1999 CCAAT enhancer-binding protein beta and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells. *Journal of Biological Chemistry* **274** 17987–17996.
- Stocco DM & Clark BJ 1996 Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17** 221–244.
- Strauss JF III, Kallen CB, Christenson LK, Watari H, Devoto L, Arakane F, Kiriakidou M & Sugawara T 1999 The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. *Recent Progress in Hormone Research* **54** 369–394.
- Sugawara T, Holt JA, Driscoll D, Strauss JF III, Lin D, Miller WL, Patterson D, Clancy KP, Hart IM, Clark BJ *et al.* 1995 Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. *PNAS* **92** 4778–4782.
- Sugawara T, Holt JA, Kiriakidou M & Strauss JF III 1996 Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. *Biochemistry* **35** 9052–9059.
- Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F & Strauss JF III 1997a Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor 1. *Steroids* **62** 5–9.
- Sugawara T, Kiriakidou M, McAllister JM, Kallen CB & Strauss JF III 1997b Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry* **36** 7249–7255.
- Sugawara T, Saito M & Fujimoto S 2000 Sp1 and SF-1 interact and cooperate in the regulation of human steroidogenic acute regulatory protein gene expression. *Endocrinology* **141** 2895–2903.
- Sugawara T, Abe S, Sakuragi N, Fujimoto Y, Nomura E, Fujieda K, Saito M & Fujimoto S 2001 RIP 140 modulates transcription of the steroidogenic acute regulatory protein gene through interactions with both SF-1 and DAX-1. *Endocrinology* **142** 3570–3577.
- Sugawara T, Nomura E & Fujimoto S 2004 Expression of enzyme associated with steroid hormone synthesis and local production of steroid hormone in endometrial carcinoma cells. *Journal of Endocrinology* **180** 135–144.
- Tremblay JJ, Hamel F & Viger RS 2002 Protein kinase A-dependent cooperation between GATA and CCAAT/enhancer-binding protein transcription factors regulates steroidogenic acute regulatory protein promoter activity. *Endocrinology* **143** 3935–3945.
- Wang ZN, Bassett M & Rainey WE 2001 Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11 beta-hydroxylase. *Journal of Molecular Endocrinology* **27** 255–258.
- Wang W, Zhang C, Marimuthu A, Krupka HI, Tabrizizad M, Shelloe R, Mehra U, Eng K, Nguyen H, Settachatgul C *et al.* 2005 The crystal structures of human steroidogenic factor-1 and liver receptor homologue-1. *PNAS* **102** 7505–7510.
- Wolf S, Martinez C & Majzoub JA 1999 Inducible binding of cyclic adenosine 3', 5'-monophosphate (cAMP)-responsive element binding protein (CREB) to a cAMP-responsive promoter *in vivo*. *Molecular Endocrinology* **13** 659–669.
- Yoshimura T, Fujisawa J & Yoshida M 1990 Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. *EMBO Journal* **9** 2537–2542.

- Yu RN, Ito M & Jameson JL 1998 The murine Dax-1 promoter is stimulated by SF-1 (steroidogenic factor-1) and inhibited by COUP-TF (chicken ovalbumin upstream promoter-transcription factor) via a composite nuclear receptor-regulatory element. *Molecular Endocrinology* **12** 1010–1022.
- Zhang P & Mellon SH 1996 The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3', 5'-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17 α -hydroxylase/c17-20 lyase). *Molecular Endocrinology* **10** 147–158.
- Zheng W & Jefcoate CR 2005 Steroidogenic factor-1 interacts with cAMP response element-binding protein to mediate cAMP stimulation of CYP11B1 via a far upstream enhancer. *Molecular Pharmacology* **67** 499–512.

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