Steroidogenic Factor-1 Interacts with cAMP Response Element-Binding Protein to Mediate cAMP Stimulation of CYP1B1 via a Far Upstream Enhancer

Wenchao Zheng and Colin R. Jefcoate
Department of Pharmacology, Medical Science Center, University of Wisconsin, Madison, Wisconsin
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ABSTRACT
CYP1B1 activates polycyclic aromatic hydrocarbon carcinogens in cAMP-regulated tissues such as the adrenal, ovary, and testis. A 27-fold cAMP stimulation of the CYP1B1-luciferase reporter in Y-1 adrenal cells depends entirely on a far upstream enhancer region (FUER; −5298 to −5110). Cooperative participation of multiple steroidogenic factor 1 (SF-1) elements with the downstream cAMP response element (CRE) in FUER is essential for both basal and cAMP-stimulated activities of FUER. Basal and induced activities were similarly lowered by DAX-1, an SF-1 suppressor, and raised by steroid receptor coactivator 1, an SF-1 coactivator. cAMP response element-binding protein (CREB)-binding protein (CBP) that interacts preferentially with the phosphorylated-CREB increased the cAMP-induced FUER. 10T1/2 cells and human embryonic kidney (HEK)293 cells do not express SF-1. Introduction of exogenous SF-1 generated cAMP stimulation of the FUER in 10T1/2 fibroblasts. The same transfection only increased basal activity of FUER in HEK293 cells, despite presence of active CREB in cells. HEK293 cells therefore remain deficient in additional factor(s) critical to the cAMP stimulation of CYP1B1. Mutations of the protein kinase A (PKA) and the mitogen-activated protein kinase phosphorylation sites (Ser-430 and Ser-203) on SF-1 had no effect on the SF-1-dependent FUER stimulation in Y-1 and 10T1/2 cells. This contrasts with loss of activity with mutation of CREB at PKA phosphorylation site (Ser-133). SF-1 phosphorylation at these sites is therefore not essential for the cAMP stimulation and the cooperation with CREB. cAMP-enhanced activation protein 1 (AP-1) and stimulatory protein 1 (Sp1) complexes in the proximal promoter region contributed substantially to both basal and cAMP-stimulated FUER activity. Chromatin immunoprecipitation from primary rat adrenal cells demonstrated cAMP stimulation of histone acetylation proximal to, respectively, the FUER and AP-1 sites of CYP1B1.

CYP1B1 was first identified and purified from mouse embryonic fibroblasts (Pottenger and Jefcoate, 1990) and from rat adrenals (Otto et al., 1991). The CYP1B1 gene is unusual among vertebrate P450 cytochromes in exhibiting both cAMP and aryl hydrocarbon receptor (AhR)-regulated expression. AhR activation induces CYP1B1 in mouse embryonic fibroblasts and many extrahepatic tissues (Meyer et al., 2000). CYP1B1 is expressed constitutively in steroidogenic tissues like the adrenal, ovary, and testes where AhR levels are low.

For these tissues, regulation is primarily determined by hormones that elevate cAMP (Otto et al., 1991, 1992; Brake and Jefcoate, 1995). CYP1B1 is elevated in many important cancer-producing epithelia relative to the surrounding normal epithelia (Murray et al., 1997). Substantial attenuation of carcinogenesis initiated by polycyclic aromatic hydrocarbons is seen in CYP1B1-null mice. This is particularly evident in granulosa cell tumors of the ovary, a tissue in which CYP1B1 is regulated by hormones that elevate cAMP (Otto et al., 1992). A developmental function of CYP1B1 has been confirmed by the finding that functionally deficient mutations in CYP1B1 result in primary congenital glaucoma in humans.

Steroidogenic P450 cytochromes exhibit transcriptional regulation via a variety of nuclear transcriptional factors,

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; SF-1, steroidogenic factor 1; CREB, cAMP response element-binding protein; PKA, protein kinase A; FUER, far upstream enhancer region; CRE, cAMP response element; StAR, steroidogenic acute regulatory; SRC-1, steroid receptor coactivator 1; P/CAF, p300/CREB-binding protein-associated factor; PPR, proximal promoter region; AP-1, activation protein 1; Sp1, stimulatory protein 1; 8-Br-cAMP, 8-bromo-cAMP; HEK, human embryonic kidney; CBP, cAMP response element-binding protein binding protein; CMV, cytomegalovirus; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ChiP, chromatin immunoprecipitation; kb, kilobase; MAPK, mitogen-activated protein kinase; HAT, histone acetyltransferase.
including the orphan nuclear receptor steroidogenic factor 1 (SF-1) (Jacob and Lund, 1998) and CREB (Doi et al., 2002), a nuclear regulator that is directly phosphorylated by protein kinase A (PKA). In our previous work, we have identified a 189-base pair far upstream enhancer region (FUER; −5298 to −5110) of CYP1B1, which mediates cAMP-stimulation of CYP1B1 reporters in adrenal Y1 cells and testis MA-10 cells as well as in rat adrenal cells (Zheng et al., 2003). cAMP induction by FUER is totally dependent on the cAMP response element (CRE) located at the 3′ end (Zheng et al., 2003). We have also characterized a 230-base pair enhancer region (−1026 to −797) that regulates 2,3,7,8-tetrachlorodibenzo-p-dioxin induction through the AhR (Zhang et al., 1998). These two regions function essentially independently. Dissection of the FUER shows that the 5′ half is also essential for activity, suggesting cooperation of upstream complexes with the 3′ CREB complex. The sequence of FUER contains four SF1-binding elements, including one adjacent to the critical CRE. In this article, we show that SF1 is an essential partner of CREB in the FUER.

SF1 is a member of the nuclear receptor superfamily that contains a characteristic zinc finger DNA binding domain and a putative ligand binding/dimerization domain but has no known ligand activator. Potential PKA phosphorylation sites are present in SF1, and in vitro studies have directly demonstrated a PKA-dependent phosphorylation of SF1 (Zhang and Mellon, 1996; Carlone and Richards, 1997a; Jacob and Lund, 1998). Phosphorylation of SF1 via the mitogen-activated protein kinase pathway does contribute to transcriptional activity (Hammer et al., 1999). SF1 is expressed in the adrenal cortex, testis, ovary, pituitary gonadotrope cells, and hypothalamic (Lala et al., 1992; Honda et al., 1993) and plays an essential role in the development of these tissues (Lala et al., 1992; Honda et al., 1993; Luo et al., 1994). SF1 functions as a transcriptional regulator for a variety of CAMP-dependent target genes, including aromatase (Fitzpatrick and Richards, 1993), cholesterol side chain cleavage enzyme, steroidogenic acute regulatory (StAR) protein, and luteinizing hormone β. DAX1 is also regulated by SF1 (Yu et al., 1998) and functions as a repressor of SF1 activity. In several genes such as STAR and aromatase, SF1 functions cooperatively with CREB.

Post-translational modifications of histones, such as acetylation/deacetylation, methylation, or phosphorylation, provide the primary mechanism for controlling the access of genes in native chromatin to nuclear regulatory factor (Struhl, 1998). Coactivators, such as steroid receptor coactivator-1 (SRC-1), bind to AP2 recognition sequences that link to the putative ligand binding domains of the nuclear receptor superfamily, including SF1, AhR, and CREB (Chrivita et al., 1993), where binding is increased after CREB phosphorylation. CBP also associates with the histone acetyltransferase p300/CBP-associated factor (P/CAF) (Ogryzko et al., 1996). These coactivators predominantly function through histone acetyltransferase activity.

The proximal promoter region of CYP1B1 gene contains activation protein 1 (AP-1) and stimulatory protein 1 (Sp1) binding sites. In this report, we provide evidence for an essential cooperation between multiple SF1 and CREB complexes to provide FUER enhancer activity that is then facilitated by the AP-1 complex in the proximal promoter region. We show that the FUER and proximal promoter region are sites for histone acetylation during transcriptional stimulation of CYP1B1 in primary adrenal cells.

**Materials and Methods**

**Chemicals.** 8-Br-cAMP was obtained from Sigma-Aldrich (St Louis, MO).

**Cell Culture.** Y1 mouse adrenal cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in nutrient mixture F-10 supplemented with 2.5% fetal bovine serum, 15% donor horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. C3H10T1/2 (10T1/2) cells were cultured in Dulbecco’s modified Eagle’s/F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells were purchased from American Type Culture Collection and were cultured in minimal essential medium (Eagle’s) with 2 mM l-glutamine and Earle’s balanced salt solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 10% heat-inactivated horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Primary rat adrenocortical cells were isolated and cultured as described previously (Brake and Jefcoate, 1995).

**Plasmid Constructions and Cell Transfection.** Mouse SF1 (pBRCMV-SF1), DAX1 (pBRCMV-hDAX1), and human DAX1 (pBRCMV-mDAX1) expression vectors were provided by Larry J. Jameson (Northwestern University, Chicago, IL). Expression vectors for mutant SF1 (pSF1-S290A and pSF1-S430A) were provided by Takashi Yazawa (University of Fukui, Fukui, Japan) (Yazawa et al., 2003). Expression vector for P/CAP pCI-FLAG-PCAF was provided by Yoshihiro Nakatani. Mouse CBP expression vector pC-RSV-mCBP was provided by Richard H. Goodman (Oregon Health & Science University, Portland, OR). SRC-1 expression vector pSRC-1 was provided by Gary H. Perdew (The Pennsylvania State University, University Park, PA). pCMV-PKA was purchased from BD Biosciences Clontech (Palo Alto, CA). pCMV-PKA contains the gene encoding the catalytic subunit of PKA, which is driven by the human cytomegavirus (CMV) promoter.

DNA mutations were made with the ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s standard protocol, with the following sets of primers: 1) mutant SFa, 5’-tgTTGAGGCGTGTGATACAGACCA (forward), 5’-TGACCCGCGGA-GAGCCCAA (reverse); 2) mutant SFb, 5’-AAAGGCAAGGCGGACATCTTCGAC (forward), 5’-GTTGATGCCTGCTGATATAC (reverse); 3) mutant SFc, 5’-AACCACTTCAAGGCTACAC (forward), 5’-TCTCTTCGTTCCTGCTGCTG (reverse); 4) mutant SFd, 5’-CTGCCGTGGATGATGTTCA (forward), 5’-GAACTTTTGAGGCATAAAATGTT (reverse); 5) mutant SFa and mutant SFb, 5’-AAACAGGCAAGGGTACATCTTC (forward), 5’-TTAATGTTGATGTTGATGT (reverse); 6) mutant CRE2, 5’-CAACTTCTGACACAGCGGACT (forward), 5’-CTCTTCGTTCCTGCTGCTG (reverse); 7) mutant CRE2, 5’-CACTTGAGGGTCGGGTAC (forward), 5’-CAGGCTACCATGGGACTGC (reverse); 8) mutant AP1, 5’-GACGACACTGGTTGACCA (forward), 5’-GGAGGCGCGCTGTTGACCA (reverse); 9) mutant AP2, 5’-GGAGGCGCGCTGTTGACCA (forward), 5’-GACGACACTGGTTGACCA (reverse); 10) mutant AP3, 5’-AACCACTTCAAGGCTACAC (forward), 5’-TTAATGTTGATGTTGATGT (reverse); 11) mutant Sp1a, 5’-CGGCCCAACTCAGCACTTC (forward), 5’-GACGACACTGGTTGACCA (reverse); 12) mutant Sp1b, 5’-GGAGGCGCGCTGTTGACCA (forward), 5’-GACGACACTGGTTGACCA (reverse). These small letters in these primer sequences show mutant sequences. Transient transfections of luciferase reporters were performed by electroporation as described previously (Zheng et al., 2003).
Electrophoretic Mobility Shift Assay (EMSA). CREB EMSA was carried out as described previously (Zheng et al., 2003). Y-1 nuclear extracts were prepared from either untreated or 1 mM 8-Br-cAMP-treated (6 h) Y-1 cells. Oligonucleotide probes were end-labeled by T4 polynucleotide kinase. Binding reactions were performed in 10 μl of volume with 10 μg of Y-1 nuclear extract and 20,000 cpm of labeled probe in specific binding buffer. SF-1 binding buffer contains 20 mM HEPES, pH 7.9, 0.2 mM EDTA, 75 mM KCl, 0.5 mM dithiothreitol, and 10% (v/v) glycerol. AP-1 binding buffer contains 20 mM HEPES, pH 7.5, 0.2 mM EDTA, 50 mM NaCl, 0.25 mM dithiothreitol, and 10% (v/v) glycerol. Sp1 binding buffer contains 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 40 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, and 10% (v/v) glycerol. The binding reactions were resolved on a 6% non-denaturing polyacrylamide gel at 220 V for 2.5 h at 4°C. After electrophoresis, DNA-protein complexes were analyzed with a PhosphorImager. For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides or 3 μl of anti-AP-1 antibodies (Geneka Biotechnology, Inc., Montreal, QC, Canada), anti-SF-1 antibody, or anti-Sp1 antibody (Biotechnologies, Charlottesville, VA) was added to the nuclear extracts before addition of radiolabeled probe. Sequences of the oligonucleotides of SF-1 and CREB binding sites used in EMSA are shown in Fig. 1B. Sequences of other oligonucleotides used in EMSA are listed here: 1) consensus AP-1, 5'-CCTTGTGAGTCCGCGGAA; 2) mutant AP-1, 5'-CCTTGTGAGACCACGGGAA; 3) CYP1B1 AP-1, 5'-TGGACCCCATGCCTGGCTAACGTCGA; 4) mutant CYP1B1 AP-1, 5'-TGGACCCCATGCCTGGCTGACCGTCGA; 5) consensus Sp1, 5'-CCCTTTGCTGGAAGGCGCTGGTTGAG; 6) mutant Sp1, 5'-CCCTTTGCTGGAAGGCGCTGGTTGAG; 7) Sp1a, 5'-TGGACCCCATGCCTGGCTGACCGTCGA; 8) mutant Sp1a, 5'-TGGACCATGCCTGACCGTCA; 9) Sp1b, 5'-CCTACACGCCCACCTTAAACTG; and 10) mutant Sp1b, 5'-CCTACACGCCCAACCTTAAGGG. The small letters in the sequences show mutant bases.

Western Immunoblotting. Thirty μg/lane of total cellular protein was subjected to Western immunoblot analysis. Anti-CREB-1 antibody and anti-mouse SF-1 antibody were purchased from Geneka Biotechnology, Inc. and Upstate Biotechnology (Charlottesville, VA), respectively. Immune complexes were visualized using the enhanced chemiluminescence detection method (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol.

Chromatin Immunoprecipitation Assay. ChIP assay was performed using the acetyl-histone H3 immunoprecipitation assay kit (Upstate Biotechnology) per the manufacturer's recommendations. Cells (1 × 106) were cross-linked by addition of 1% formaldehyde to the medium for 10 min. Cell pellets were resuspended in 200 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), sonicated under conditions that reduced DNA length to between 200 and 400 base pairs, and debris was removed by centrifugation. The chromatin solution was diluted 10-fold in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and pre cleared for 30 min at 4°C with Salmon sperm DNA/protein A agarose-50% slurry. The chromatin solution was then incubated with 4 μl of anti-acetyl-H3 antibody (1 μg/1 μl) overnight at 4°C, and immune complexes were collected with Salmon sperm DNA/protein A agarose slurry. After washes and elution, cross-links were reversed by heating at 65°C for 4 h, and DNA was recovered by phenol extraction and ethanol precipitation.

Specific sequences in the immunoprecipitates were amplified and quantitated by real-time PCR. Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye on an ABI PRISM 7000 sequence detector System (Applied Biosystems, Foster City, CA). The primer sequences used include FUER primers targeting region –5233 to –5155 5'-GCAAGAAACCAAGGACAAGGT (forward), 5'-CTGTGCTGACGTCTCCCTAGT (reverse) and proximal promoter region primers targeting region –142 to –83 5'-CCTGGTGACCCACCTTGGCAGC (forward), 5'-ACTGCGAAAGTCGACCGGATC (reverse).

Results

SF-1 Is Essential for the cAMP Activation of FUER. In a previous report (Zheng et al., 2003), we showed that the cAMP regulation of CYP1B1 was completely dependent on the 189-base pair FUER (Fig. 1A). The activity of this region is dependent on a CRE sequence at the 3' end (CRE2) as well as a 111-base pair upstream sequence (~5298 to ~5188) that was inactive alone (Zheng et al., 2003). The FUER contains four SF-1 binding elements (SFa, SFb, SFc, and SFd) and two CRE elements (CRE1 and CRE2) (Fig. 1A). SFa overlaps CRE1, and SFb overlaps SFc, whereas SFd is just adjacent to the essential CRE2.

EMSA with nuclear extracts prepared from Y-1 cells (Fig. 1, B–E) indicated SF-1 binding at each site. Each complex was consistently stimulated by 8-Br-cAMP treatment (2.7 ± 0.3- and 2.3 ± 0.3-fold in experiment 1 and 3.1 ± 0.4-fold in experiment 2). SF-1 specificity was established at each element by complete removal of the complex after preincubation with an anti-SF-1 antibody. CREB binding to CRE1 was largely prevented by the binding of SF-1 to the overlapping SFa sequence. However, this CREB interaction dramatically increased when the SFa sequence was mutated (CRE1 and mSFa) (Fig. 1C). This weak CREB binding does not affect SF-1 binding, as evidenced by the lack of effect from either CRE1 or preincubation with anti-CREB antibody. The overlapping SFb and SFc sequences bind only a single SF-1 (Fig. 1D). When separate mutations were introduced in either SFb or SFc sequences, SF-1 preferentially binds the SFc element (a ratio of 3.5 ± 0.5- and 3-fold in experiment 1 and 4-fold in experiment 2) (Fig. 1D). Binding of SF-1 and CREB to, respectively, the SFd and CRE2 elements occurred independently, as evidenced by the effect of separate mutations (Fig. 1E).

The enhancer activity was quantified by transfection of reporter constructs formed by linkage of FUER to the 0.2-kb proximal promoter region of CYP1B1. Mutations were introduced into FUER to assess the functional contribution of each SF-1 regulatory element (Fig. 2A). The FUER contributed 3-fold more basal activity to the proximal promoter while further elevating cAMP-stimulation 27-fold. As previously reported, mutation of CRE2 removed all cAMP-simulated activity and most basal FUER activity. The cAMP-induced activities are somewhat less sensitive to mutations at SF-1 binding sites. Thus, SFa, SFb, and SFc mutations did not affect CAMP-induced activities, whereas SFd mutation adjacent to the key CRE2 element halved the induced activity. The single mutations each lowered basal activity (Fig. 2, A and B). The SFd mutation removed all basal stimulation to the proximal promoter contributed by the FUER. It is interesting that although mutation of either SFb or SFc maintained SF-1 binding in this region (Fig. 1D), mutation of each element appreciably and equally lowered FUER activity, indicating that they were not functionally redundant. Mutation of CRE1 had no effect except when SFa was also mutated (data not shown). The greater effects of single mutation on basal activities suggest that the cooperative effects of SF-1 complexes at each site are needed under basal conditions.
Mutations of SF-1 elements in pairs were appreciably more effective than single mutations. It is noteworthy that the double mutation of SFd and the more effective central element SFe removed 89% of the cAMP-induced FUER activity. The combined mutation of each of these four SF-1 binding sites abolished nearly all basal and induced enhancer activity of FUER.

Overall, these data showed the following key features of SF-1/CREB partnership: 1) FUER activity requires both SF-1 and CREB; 2) SF-1 optimally occupies the SFd element.

Fig. 1. A, sequence of FUER. SF-1 binding elements (SFa, SFb, SFc, and SFd) are shown underlined and cAMP response elements (CRE1 and CRE2) are shown in bold. B, probes used in electrophoretic mobility shift assays. Mutant sequences are shown in lowercase. C to E, electrophoretic mobility shift assays of SFa and CRE1 (C), SFb and SFc (D), and SFd and CRE2 (E). Electrophoretic mobility shift assays were carried out with nuclear extracts from untreated or 1 mM 8-Br-cAMP-treated (6-h) Y-1 cells. –, control; cA, 8-Br-cAMP-treated; α-CREB-1, anti-CREB-1 antibody; α-SF-1, anti-SF-1 antibody. EMSA data are shown from one of two independent experiments that provided similar cAMP-stimulated increase in SF-1 complexes.
adjacent to CRE2, but other SF-1 complexes at other SF-1 binding sites can also function as effective partners; and 3) multiple SF-1 complexes cooperate with CRE2 to give optimal activity.

**DAX-1 Suppresses Basal and Induced FUER Activity.** DAX-1 is a negative regulator of SF-1 function (Yu et al., 1998). To further test the involvement of SF-1 in FUER regulation, the FUER reporter was cotransfected with either a SF-1-expression vector or a DAX-1 expression vector (Fig. 3A). SF-1 increased basal activity and cAMP-stimulated activity similarly, whereas DAX-1 reduced both basal and cAMP-induced reporter activities by 3-fold (Fig. 3A). Equivalent results were obtained from the p7.0 CYP1B1 reporter, although induction levels were lower. This anomalous result was repeated in three separate transfection experiments with similar results. p7.0 carries 7.0 kb of rat CYP1B1 5’-flanking sequence linked directly to a luciferase reporter gene (Zheng et al., 2003). These DAX suppressions were greater than for mutation of any single SF-1 element, suggesting effects on multiple SF-1 complexes. The results further demonstrate that SF-1 is involved in FUER regulation of CYP1B1 in Y-1 cells. The data also provide evidence that SF-1 contributes equally to basal and cAMP-induced FUER activities.

Cotransfection of the catalytic subunit of protein kinase A stimulated the FUER reporter. This stimulation required both the CRE sites and the SF-1 sites (Fig. 3B). Mutation of the full combination of SF-1 binding elements or the CRE2 element each completely inactivated PKA-induced activity. Likewise, mutation at CRE2 completely removed the SF-1-mediated activation. These findings establish that cAMP stimulation and SF-1 stimulation each require the contributions from both SF-1 and CREB in Y-1 cells. SF-1 contains several putative phosphorylation sites for different protein kinases (Zhang and Mellon, 1996; Carlone and Richards, 1997a; Jacob and Lund, 1998; Hammer et al., 1999). Mut-
Fig. 3, A, effect of overexpression of SF-1 and DAX-1 on luciferase activities of CYP1B1 reporters in Y-1 cells. p0.2 (4.8 μg), pFUER/0.2 (5 μg), or p7.0 (11.1 μg) was cotransfected with 15 μg of empty vector, SF-1 expression vector (pBKCMV-SF-1), mouse DAX-1 expression vector (pBKCMV-mDAX-1), or human DAX-1 expression vector (pBKCMV-hDAX-1). Numbers above columns show fold-induction by 8-Br-cAMP. B, FUER activity requires both SF-1 elements and CRE2. Five micrograms of each luciferase reporter was cotransfected with 15 μg of empty vector, SF-1 expression vector (pBKCMV-SF-1), mouse DAX-1 expression vector (pBKCMV-mDAX-1), or human DAX-1 expression vector (pBKCMV-hDAX-1). Numbers above columns show fold-induction by 8-Br-cAMP. B, FUER activity requires both SF-1 elements and CRE2. C, mutations of the PKA and MAPK phosphorylation sites on SF-1 had no effect on the SF-1-dependent FUER stimulation in Y-1 cells. Five micrograms of each luciferase reporter was cotransfected with 15 μg of empty vector, wild or mutant SF-1 expression vectors. Twenty-four hours after transfection, cells were either untreated or treated with 1 mM 8-Br-cAMP for 12 h. Results represent the means ± S.D. of an experiment performed with triplicate transfections of separate cultures. The differences between mouse DAX-1 and human DAX-1 were replicated in three experiments.
tions of the PKA phosphorylation site (Ser-430) and the MAPK phosphorylation site (Ser-203) on SF-1 had no effect on the SF-1-dependent FUER stimulation in Y-1 cells (Fig. 3C). These results indicate that SF-1 phosphorylation is not essential for the cAMP stimulation and the cooperation with CREB.

SF-1/CREB Cooperation Requires Additional Cell-Selective Factors. This SF-1/CREB interaction was further supported with results from 10T1/2 cells that do not express SF-1 (Fig. 4A). CYP1B1 expression in 10T1/2 cells is regulated by AhR but not by cAMP (Zheng et al., 2003). Although the CRE tandem repeat reporter pCRE-luc responded 88-fold to 8-Br-cAMP (Zheng et al., 2003), 8-Br-cAMP failed to induce either the FUER reporter or a complete CYP1B1 reporter (Fig. 4B). Introduction of exogenous SF-1 generated basal and cAMP-dependent FUER reporter activity in 10T1/2

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** SF-1 restores cAMP-stimulation of FUER activity to 10T1/2 cells. A, SF-1 and CREB-1 expressions in Y-1 and 10T1/2 cells. Western blotting was done with 30 μg of total cellular protein each lane. Cells were either untreated or treated with 1 mM 8-Br-cAMP for 6 h. B, exogenous SF-1 restores cAMP-dependent FUER reporter activity to 10T1/2 cells. Five micrograms of each luciferase reporter was cotransfected with 15 μg of empty vector or pBKCMV-SF-1. pCRE-Luc carries luciferase gene with multiple copies of the CRE-binding sequence fused to HSV-TK-promoter. Twenty-four hours after transfection, cells were either untreated or treated with 1 mM 8-Br-cAMP for 6 h. C, dose-dependent effect of SF-1 on FUER activity in 10T1/2 cells. The FUER reporter (5 μg) was cotransfected with empty vector and increasing amounts of pBKCMV-SF-1 from 0 to 15 μg. D, mutations of the PKA and MAPK phosphorylation sites on SF-1 had no effect on the SF-1-dependent FUER stimulation in 10T1/2 cells. Five micrograms of each luciferase reporter was cotransfected with 15 μg of empty vector, wild or mutant SF-1 expression vectors. Twenty-four hours after transfection, cells were either untreated or treated with 1 mM 8-Br-cAMP for 6 h. Luciferase results represent the means ± S.D. for triplicate transfections of separate cultures. Similar results were obtained from three experiments.
cells (Fig. 4B). This reconstitution was exponentially dependent on the level of SF-1 vector to realize full cAMP-stimulation (Fig. 4C). This 21-fold stimulation was again abrogated by mutation of either the four SF-1 target sequences or the CRE2 site and by cotransfection of the DAX-1 expression vector. DAX-1 produced an equally effective suppression of the p7.0 CYP1B1 reporter. The data demonstrate that CREB and SF-1 can combine to confer cAMP-dependent responsiveness in 10T1/2 cells. Again, mutations of the PKA phosphorylation site (Ser-430) and the MAPK phosphorylation site (Ser-203) on SF-1 had no effect on the SF-1-dependent FUER stimulation in 10T1/2 cells (Fig. 4D).

HEK293 cells have been extensively used for studies of transcription factors. They express CREB at a 10-fold greater level than Y-1 cells and also do not express SF-1 (Fig. 5A). pCRE-Luc exhibited high basal activity and a 3.5-fold cAMP-dependent activation (Fig. 5B). Cotransfection of SF-1 substantially suppressed the p0.2 proximal promoter. This suppression was prevented by linkage of the FUER enhancer, but not the mutant FUER, with mutations in all SF-1 binding sites (Fig. 5C). Unlike other FUER activities, this effect did not depend on the CRE2 sequence. These effects of FUER indicate interactions between SF-1 and the FUER that overcome a 4-fold suppression effect of SF-1 on the proximal promoter. However, in spite of this response to SF-1 cotransfection and abundant CREB activity, the FUER reporter failed to respond to cAMP in HEK293 cells. These results suggest that transcriptional factors other than CREB and SF-1 are also critical for FUER induction by cAMP. These factors are present in Y-1 and 10T1/2 cells but not in HEK293 cells. This could be related to the suppression effect of SF-1 on the proximal promoters that are not seen in the other cells or to the lack of CREB/SF-1 cooperation.

**AP-1 Is Required for Optimal cAMP Stimulation of FUER Activity in Y-1 Cells.** The proximal promoter region may provide additional contributions to FUER activity. Sequence analysis of the CYP1B1 proximal promoter region identified a conserved potential AP-1 binding site (TGAG/CTCA) (Fig. 6A). AP-1 complexes, which are composed of either Fos-Jun or Jun-Jun dimmers, have been shown to mediate cAMP-dependent responses in other genes (Duval et al., 1999). EMSA with oligonucleotide duplexes encompassing the AP-1 site (−149 to −129) demonstrated the existence of a specific protein-DNA complex that was increased 3-fold in Br-cAMP-treated Y-1 cells (Fig. 6B). The specificity of complex formation was confirmed by competition with the consensus AP-1 and the sensitivity to anti-c-Fos and c-Jun antibodies.

Mutation of AP-1 reduced basal activity 1.7-fold and cAMP induction 2.4-fold when the FUER was linked to the proximal promoter (Fig. 6C) but did not affect the proximal promoter region activity. These results indicate that the effectiveness of the FUER in part depends on interaction with the AP-1 complex. The cAMP-induction factor was changed by the mutation from 26.7- to 20.8-fold. AP-1 mutation decreased the PKA stimulation of the FUER/0.2 reporter from 4.8- to 2.8-fold (Fig. 6D).

Two adjacent Sp1 binding sites of CYP1B1 proximal promoter for the human gene are essential to basal activity (Wo et al., 1997). These Sp1 sites are highly conserved among human, rat, and mouse CYP1B1 genes (Fig. 6A). Sp1 complexes at each site were formed with all Y-1 nuclear extracts and were also increased about 4.1-fold by 8-Br-cAMP treatment of the cells (Fig. 7A). Sp1 sites differed from AP-1 sites in that single or double mutations decreased p0.2 proximal promoter activity by 3- to 6-fold (Fig. 7B). Very similar decreases were seen for the 4.3-fold higher basal activity provided by linkage of FUER to p0.2 (Fig. 7B). The decrease in cAMP-stimulated activity (2-fold) for the FUER reporter was decreased less than the basal activity (3- to 4-fold) and again...
Fig. 6. A, alignment of proximal promoter sequences of rat, mouse and human CYP1B1 genes. AP-1 elements are shown in bold. Sp1 elements are shown underlined. B, electrophoretic mobility shift assay of AP-1 element in proximal promoter region. Electrophoretic mobility shift assay was carried out with nuclear extracts from either untreated or 1 mM 8-Br-cAMP-treated (6-h) Y-1 cells. CYP1B1 AP-1, CYP1B1 AP-1 oligonucleotide; mAP-1, mutant CYP1B1 AP-1 oligonucleotide; Con AP-1, consensus AP-1 oligonucleotide; Mut AP-1, mutant AP-1 oligonucleotide. −, control; cA, 8-Br-cAMP treated. α-c-Fos, anti-c-Fos antibody; α-c-Jun, anti-c-Jun antibody. This is representative of one of two experiments with similar results. C, AP-1 element functioned dependently on FUER. Numbers in right of columns show fold-induction by 8-Br-cAMP. D, mutation of the AP-1 element partially decreased PKA-mediated activity of FUER. Five micrograms of each luciferase reporter was cotransfected with 15 μg of empty vector or PKA catalytic subunit expression vector (pCMV-PKA). Numbers in right of columns show fold-induction by cotransfection of pCMV-PKA. C and D, 24 h after transfection, Y-1 cells were either untreated or treated with 1 mM 8-Br-cAMP for 12 h. Luciferase activity results represent the means ± S.D. for three separate experiments performed in triplicate.
similarly by single or double mutations. The stimulation by PKA transfection was also less susceptible to the mutation (1.7-fold decrease) (Fig. 7C). These results suggest that activation of FUER by cAMP partially offsets the loss of Sp1 activity.

Coactivators CBP and SRC-1 Enhance cAMP Stimulation of \textit{CYP1B1}. cAMP stimulation may be introduced through the binding selectivity and histone acetyl transferase activities of various coactivators. CBP is a coactivator for both SF-1 (Monte et al., 1998) and CREB (Chrivia et al., 1993). The interaction of CREB with CBP is enhanced by CREB phosphorylation by PKA (Chrivia et al., 1993). CBP contributes to histone acetylation activity either directly (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) or through the recruitment of other coactivators such as P/CAF (Blanco et al., 1998). SRC-1 interacts with AF2 domains, which are present in the C-terminal segment of many nuclear receptors, and also SF-1 (Ito et al., 1998). SRC-1 may

![Fig. 7. A, electrophoretic mobility shift assay of Sp1 elements in proximal promoter region. Electrophoretic mobility shift assay was carried out with nuclear extracts from either untreated or 1 mM 8-Br-cAMP-treated (6-h) Y-1 cells. Sp1a, Sp1a oligonucleotide; mSp1a, mutant Sp1a oligonucleotide; Sp1b, Sp1b oligonucleotide; mSp1b, mutant Sp1b oligonucleotide; Con Sp1, consensus Sp1 oligonucleotide; Mut Sp1, mutant Sp1 oligonucleotide. Control; cA, 8-Br-cAMP-treated. α-Sp1, anti-Sp1 antibody. This is representative of one of two experiments with similar results. B, Sp1 elements only affected basal activity of proximal promoter. Numbers in right of columns show fold-induction by 8-Br-cAMP. C, mutations of the Sp1 elements did not reduce PKA-mediated activity of FUER. Five micrograms of each luciferase reporter was cotransfected with 15 μg of empty vector or PKA catalytic subunit expression vector (pCMV-PKA). Numbers in right of columns show fold-induction by cotransfection of pCMV-PKA. B and C, 24 h after transfection, Y-1 cells were either untreated or treated with 1 mM 8-Br-cAMP for 12 h. Luciferase activity results represent the means ± S.D. for at least three separate experiments performed in triplicate.](/aspetjournals/692/508/508.jpg)
therefore selectively generate activity through SF-1 rather than CREB.

To test the involvement of these coactivators, CBP, P/CAF, or SRC-1 expression vectors were cotransfected with p0.2 and pFUER/0.2 in Y-1 cells (Fig. 8). CBP did not affect basal FUER activity, but it increased the cAMP-induced FUER activity. Constitutive CBP is therefore a potential mediator for the cAMP stimulation of FUER. SRC-1 produced a much larger increase in both basal and cAMP-mediated induction. This suggests that SRC-1 participates differently from CBP with respect to FUER activity. P/CAF had little or no effect on basal and cAMP-mediated transcriptional activation of the FUER reporter.

cAMP Activation Involves Histone Acetylation. ChIP assays provide the means to assess the extent of histone acetylation in the FUER and proximal promoter regions. In primary rat adrenal cells, cAMP substantially induces the normal CYP1B1 gene (Bhattacharyya et al., 1995; Brake and Jefcoate, 1995). Figure 9A shows that a 6-h cAMP stimulation of primary rat adrenal cells increases CYP1B1 transcription while producing a similar increase in transfected FUER (Zheng et al., 2003). We pooled cells from 20 pairs of rat adrenals to provide sufficient cells for triplicate controls and cAMP-stimulate assays. We used two sets of PCR primers targeting FUER and the proximal promoter for quantification of the histone acetylation on nucleosomes (Fig. 9B). Antibodies directed against acetylated histone H3 were used for immunoprecipitation of transcriptionally active chromatin. ChIP assays demonstrated a 2.4- to 3.6-fold increase by 8-Br-cAMP of H3 histone acetylation in both FUER and proximal promoter regions in three separate experiments.

**Discussion**

In a previous report, we showed that cAMP effectively stimulated CYP1B1 in adrenal cells through a 189-base pair FUER located 5 kb from the transcriptional start site (Zheng et al., 2003). This enhancer functions remarkably in transmitting a very strong stimulus to a variety of basal promoters and in the extent of cAMP stimulation (27-fold). It is interesting that a similar grouping of three SF-1 elements and CREs is found in the proximal promoter of the StAR gene, which exhibits a parallel rapid cAMP response in rat adrenal cells and a similar developmental profile in the neonatal rat adrenal (Manna et al., 2003). Here, we have shown that this FUER activity is dependent on the interactions between multiple SF-1 binding sites and a single CRE. The synergy between SF-1 complexes is shown by the contributions from upstream and downstream halves of the FUER, each of which contains sites that form SF-1 complexes. Thus, FUER activity is provided optimally from a combination of complexes at three sites: a combined SFd/CRE2 with modest activity at the 3’ end, and, respectively, a double SFb/SFc and an SFa/CRE1 site that function in a supplementary capacity in the upstream FUER. This multimeric involvement of SF-1 with a single CREB protein is seen in the StAR promoter and may therefore explain the similar regulation.

The FUER enhancer also produces 4.3-fold stimulation to proximal promoter activity in absence of cAMP. It is noteworthy that specific mutations of SF-1 elements singly and in combination indicate that cooperative interactions between these complexes contribute to both basal and cAMP-induced activities. Indeed, single SF-1 mutations produced more loss of basal activity, which is consistent with a need for all SF-1 complexes to adhere for optimal activity under basal conditions. Basal and induced FUER activities were also similarly inhibited by DAX-1, which is a negative regulator of SF-1 (Fig. 3A), and were similarly stimulated by the coactivator SRC-1, which interacts with the AF2 domain of SF-1 (Fig. 8) (Ito et al., 1998). We have previously shown that a CREB Ser/Ala-133 mutation, which prevents PKA phosphorylation,
inhibits FUER stimulation by cAMP (Zheng et al., 2003). SF-1 and CREB bind independently but in proximity at the 3’ end. Evidence has been provided that SF-1 and CREB can form direct complexes (Ito et al., 2000). This is not apparent in the EMSA complexes for SFd/CRE2 where binding of SF-1 and CREB were independent.

SF-1 and CREB may function synergistically when recruiting additional coactivators such as CBP. As measured by the EMSA of nuclear extracts, cAMP produced small increases in the levels of SF-1 and CREB complexes, even though SF-1 and CREB levels did not increase in cells. Transfection of SF-1 increases FUER activity, indicating that nuclear levels of this protein in Y-1 cells are not optimal for FUER reporter activity. The cooperation of multiple SF-1 and CREB complexes within the FUER unit is therefore likely to amplify the effect of these increases to produce a more substantial effect. However, the activity of the basal SF-1/CREB is probably more substantially enhanced by the phosphorylation of CREB. The similar contribution of multiple SF-1 proteins to basal and cAMP-stimulated activities is also shared by StAR (Manna et al., 2003).

Interestingly we show that CBP selectively increases cAMP stimulation relative to basal activity. CBP interacts with phosphorylated Ser-133 CREB and also binds to components of the basal transcription machinery (Bisotto et al., 1996). CBP also interacts directly with multiple nuclear hormone receptors, potentially including SF-1 (Monte et al., 1998), and seems to serve as a bridge between CREB and the transcriptional complex (Shaywitz and Greenberg, 1999). CBP may therefore serve as a signal integrator for these factors. CBP has inherent HAT activity and also recruits other proteins with HAT activity. This histone acetylation is associated with gene activation through alterations in chromatin structure (Struhl, 1998). The stimulation of histone H3 acetylation in the FUER and proximal promoter region of transcriptionally active CYP1B1 supports the hypothesis that recruitment of CBP and other HAT enzymes opens up both regions in parallel after cAMP activation of the CYP1B1 gene.

The FUER effects are transmitted to the transcription initiation complex through proximal AP-1 and Sp1 complexes and in parallel with activated histone acetylation during active cAMP-stimulated transcription of CYP1B1 (Fig. 9B). Differences between basal and cAMP-induced regulation indicate different roles for these complexes. Recent work on the induction of CYP1A1 via AhR shows transfer of enhancer components to the proximal promoter (Hestermann and Brown, 2003). AP-1 and Sp1 complexes in the proximal promoter region of CYP1B1 exhibit different roles in the FUER mechanism. The AP-1 complex located immediately upstream of the Sp1. The two Sp1 complexes are critical for proximal promoter activity and for basal FUER activity. However, cAMP-stimulation of FUER partially overcomes the lack of Sp1 activity. AP-1 is not required for proximal promoter activity but contributes a 2-fold stimulation to both basal and induced FUER effects.

Fig. 9. A, CYP1B1 mRNA expression in primary rat adrenocortical cells. Primary rat adrenocortical cells were prepared from 40 to 50 adrenal glands and cultured for 24 h. Cells were treated with 0.5 mM 8-Br-cAMP for 6 h. mRNA was isolated, separated, and hybridized as described previously (Zheng et al., 2003). B, cAMP induced acetylation of histone H3 in FUER and PPR regions. The chromatin immunoprecipitation assay was performed with primary rat adrenocortical cells untreated or treated with 1 mM 8-Br-cAMP for 6 h. Chromatin fragments were immunoprecipitated with anti-acetyl-histone H3 antibody, and FUER and PPR sequences were amplified and quantitated by real-time PCR using primers specific for FUER and PPR sequences. The bottom table shows the detailed ChIP assay data. Results represent the means ± S.D. for two separate experiments (EXP1, experiment 1; EXP2, experiment 2) performed in triplicate. Statistical measurements indicate statistically significant difference between the untreated and treated primary rat adrenocortical cells in ChIP assay (FUER, p = 0.0104; PPR, p = 0.0052).
This interaction between SF-1 and CREB has previously been reported for several cAMP-stimulated genes, including P450scc, which, like STAR, is presented in these same Y-1 cells (Monte et al., 1998; Manna et al., 2003). The interaction between SF-1 and CREB was first suggested by Parker and Schimmer (1995) for the cytochrome P450scc enzyme and has been observed with other genes associated with steroidogenesis (Carlone and Richards, 1997a). Ranging from additivity to synergy, a spectrum of interactions between SF-1 and CREB in modulation of promoter activity have been described previously (Ito et al., 2000). SF-1 is a key regulator of gonadal adenral embroyogenesis (Luo et al., 1994) and shows a pattern of expression generally specific to endocrine and neuronal tissues. The P450scc, like STAR and CYP1B1, shows very strong neonatal suppression in the rat, suggesting cooperative interaction of CREB at Ser-133 and recruitment of CBP may be sufficient for synergy, a spectrum of interactions between SF-1 and CREB. It is also notable that similar regulation is achieved by the androgen receptor in a cell-selective and after inductive stimulation through two rather distinct enhancer regions that function in a cell-selective and after inductive stimulation through two rather distinct enhancer regions that function in a cell-selective manner. It is also notable that similar regulation is achieved for STAR and CYP1B1, respectively, by conserved proximal interactions and by a remote unit. A regulatory advantage for the remote FUER is the possibility for separate cell-selective silencing commonly seen for alternative promoter that is found at similar distances, for example, in the cAMP regulation of aromatase in different cells (Shouz et al., 1998).

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References

Honda S, Morohashi K, Nomura M, Takeya H, Kitajima M, and Omura T (1993) Androgen regulation of CYP1B1 expression vector did not restore the cAMP-response of FUER in HEK293 cells, even though these cells exhibit an appreciable basal effect of SF-1 on the FUER and express active CREB (Fig. 5). These cells also express SRC-1 (Castro et al., 1999) and CBP (Xu et al., 2001) and did not show increased activity after co-transfection of these coactivators (data not shown). This indicates that transcriptional factors or coactivators other than these factors and coactivators, which are present in Y-1 and 10T1/2 cells but not in HEK293 cells, are also critical for FUER induction by cAMP.
Several mechanisms of SF-1 and CREB interaction have been proposed, including direct interactions between CREB and SF-1, and recruitment of coactivators by the orphan nuclear receptor (Ito et al., 2000; Jacob et al., 2001). Another possibility is that the cAMP pathway leads to the phosphorylation of SF-1 and increases its transcriptional activity. Although SF-1 is also phosphorylated by PKA in vitro (Zhang and Mellon, 1996; Carlone and Richards, 1997a), recent studies suggest that phosphorylation on Ser 203 is mediated by the MAPK pathway (Hammer et al., 1999). The data presented here show that SF-1 phosphorylation is not a key part of its cooperation with CREB. Phosphorylation may however account for small increases in DNA binding that exceed changes in SF-1 levels. Phosphorylation of CREB at Ser-133 and recruitment of CBP may be sufficient to provide the FUER stimulation.
We have now shown that CYP1B1 is regulated constitutively and after inductive stimulation through two rather similar enhancer regions that function in a cell-selective manner. It is also notable that similar regulation is achieved for STAR and CYP1B1, respectively, by conserved proximal interactions and by a remote unit. A regulatory advantage for the remote FUER is the possibility for separate cell-selective silencing commonly seen for alternative promoter that is found at similar distances, for example, in the cAMP regulation of aromatase in different cells (Shouz et al., 1998).

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Address correspondence to: Dr. Colin R. Jefcoate, Department of Pharmacology, University of Wisconsin, 1300 University Ave., Madison, WI 53706. E-mail: jefcoate@wisc.edu