Endocrine, *vol. 14, no. 2, 253–261, March 2001* 0969–711X/00/14:253–261/$12.25

Characterization of a Steroidogenic Factor-1-Binding Site Found in Promoter of Sterol Carrier Protein-2 Gene

Dayami Lopez, Wendy Shea-Eaton, and Mark P. McLean

Department of Obstetrics and Gynecology and Biochemistry and Molecular Biology, University of South Florida, Tampa, FL

Sterol carrier protein-2 (SCP2) is thought to mediate intracellular cholesterol transport in steroidogenic tissues. To elucidate the mechanism underlying the expression of this gene, a 300-bp fragment of the SCP2 promoter was cloned and analyzed for regulatory motifs. This promoter region contained a SF-1 binding motif, three activator protein-1 elements, an insulin response element, and a peroxisomal proliferator response element. The putative SF-1 binding region reacted with recombinant SF-1 DNA-binding domain in a mobility shift assay. The SCP2 promoter fragment was linked to a luciferase reporter gene and cotransfected in the presence or absence of SF-1 into HTB-9 cells. The results indicated that SF-1 was able to increase SCP2 promoter activity, an effect that was enhanced by cAMP. Similar results were obtained when the SCP2 promoter construct was cotransfected into Y1 cells. Cotransfection studies carried out in Kin 8 cells, a Y1 cell line with a mutation that prevents cAMP activation of PKA, revealed that a functional PKA is required for cAMP induction of SCP2 gene transcription. These results demonstrated that SF-1 confers cAMP responsiveness to the SCP2 promoter suggesting that SF-1 activation may be critical in regulation of this cholesterol transport protein.

Key Words: Steroidogenic factor-1; cyclic adenosine monophosphate; sterol carrier protein-2.

Introduction

Sterol carrier protein-2 (SCP2), also known as nonspecific lipid transport protein, is a 13.2-kDa protein that mediates intracellular transport and metabolism of cholesterol *(1).* In steroid hormone–producing tissues, one of the proposed functions of SCP2 is to enhance the transport of cholesterol to mitochondria, where the first step in steroid hormone synthesis takes place *(1–3).* Evidence for the role of SCP2 in steroid hormone production includes the ability of SCP2 to increase cholesterol transport from isolated lipid droplets to the mitochondria *(1)*, the ability of SCP2 to stimulate cholesterol side-chain cleavage by isolated mitochondria *(1)*, the ability of anti-SCP2 antibodies to reduce steroid secretion in rat adrenal cells *(4)*, and coexpression of SCP2 and cholesterol side-chain cleavage enzyme in COS cells resulting in enhanced synthesis of progestins *(5)*. Several studies have shown that SCP2 gene expression appears to be under the control of factors such as adrenocorticotropic hormone and gonadotropins via cyclic adenosine monophosphate (cAMP) activation, which, in turn, stimulates steroidogenesis *(6–9)*. These data suggest that SCP2 expression may be regulated similarly to other genes involved in steroid hormone production.

cAMP induction of steroidogenic enzymes has been shown to be essential for the proper maintenance of the steroid hormone biosynthetic pathway *(10,11).* One of the mechanisms by which cAMP activates steroid hormone synthesis is by inducing the expression of steroidogenic genes *(10,11)*, a process that appears to be mediated by the orphan nuclear receptor, steroidogenic factor-1 (SF-1) *(12).* Studies have shown that SF-1 regulates many steroidogenic genes including cholesterol side-chain cleavage cytochrome P450 (CYP11A) *(13–15)*, aromatase cytochrome P450 (CYP19) *(16–18),* 17α-hydroxylase/c17,20 lyase (CYP17) *(19),* 3β-hydroxysteroid dehydrogenase $\Delta^{5\rightarrow4}$-isomerase type II (3β HSD) *(20),* steroidogenic acute regulatory protein (StAR) *(21–24),* and the high-density lipoprotein receptor (HDL-R) *(25,26).* All of these studies have clearly shown that SF-1 can mediate cAMP responsiveness.

In view of the current data, we cloned the rat SCP2 promoter and examined the effects of cAMP on the regulation of this gene. We found that SF-1 is required for both basal and cAMP-induced regulation of the SCP2 gene.

Results

To determine the effect of cAMP on SCP2 mRNA levels in luteal cells, Northern blot analysis was performed. Luteal cells were prepared as described in Materials and Methods. Cells were treated for 6, 12, and 24 h with dibutyryl cAMP [$(Bu)_2$cAMP]. RNA preparation and Northern blot analysis were performed as described under Materials and Methods. A characteristic Northern blot is shown in Fig. 1A. As previously shown *(27),* the rat SCP2 cDNA probe hybrid-

Received September 11, 2000; Revised November 6, 2000; Accepted November 6, 2000.

Author to whom all correspondence and reprint requests should be addressed: Dr. Mark P. McLean, Department of Obstetrics and Gynecology, 4 Columbia Drive, Room 529, Tampa, FL 33706. E-mail: mmclean@com1.med.usf.edu

ized to a major transcript (SCP2) at approx 0.8–1 kb, and two minor transcripts at 1.8 and 2.2 kb, respectively. The 2.2-kb transcript corresponds to the SCP2-related protein, SCPx. Differences in loading were corrected using the β-actin signal. Increases in SCP2 mRNA levels in response to cAMP treatment were seen even at 6 h (Fig. 1A). Quantitative analysis of Northern blot autoradiograms demonstrated that $(Bu)_2$cAMP (1 m*M*) significantly increased SCP2 mRNA levels about twofold, 24 h after treatment ($n = 3$; $p < 0.05$) (Fig. 1B). These results suggest that cAMP may regulate SCP2 gene expression at the transcriptional level. Interestingly, SCPx mRNA levels also increased under these conditions (Fig. 1A).

To examine whether the SCP2 promoter contains a motif through which cAMP regulates this gene, cloning of the promoter was performed using the Advantage *Tth* polymerase mix and the PromoterFinder DNA Walking Kit (Clontech, Palo Alto, CA). Five separate adapter-ligated rat genomic libraries were screened by polymerase chain reaction (PCR). Two nested gene-specific primers were designed to the 5'-flanking region of the rat SCP2 cDNA *(28).* These primers were then used with adapter-specific primers in nested PCR reactions. A 0.4-kb DNA fragment was obtained in the library, from which 300 bp corresponded to regions in the SCP2 promoter. The nucleotide sequence of the rat SCP2 promoter is shown in Fig. 2. As previously reported for other species, the rat SCP2 promoter lacked a TATA box. This DNA segment contained four activator protein-1 (AP-1) sites, one insulin response element (IRE), one peroxisomal proliferator response element (PPRE), and one SF-1-binding motif. No putative cAMP-response element (CRE) was found in this region. The putative SF-1-binding site was located at position –37 (5'-GGCCTTGG-3') relative to the translation start site. This motif had 75% identity to the SF-1-binding sites reported for the rat steroidogenic acute regulatory protein (StAR) gene (5'-CACCTTGG-3') *(24),* the luteinizing hormone β (LHβ) subunit gene (5'-GACCTTGT-3') *(29, 30),* and the human StAR gene (5'-GACCTTGA-3') *(22).*

Because SF-1 has been shown to mediate cAMP responsiveness, we examined whether a similar mechanism was involved in the SCP2 regulation by cAMP. We performed cotransfection studies in human bladder carcinoma HTB9 cells. The SCP2 promoter was cloned into the pGL3-basic luciferase vector as described under Materials and Methods. This construct was then transfected into HTB9 cells in either the presence or absence of SF-1-pCMV. As shown in Fig. 3, the luciferase activity produced from the SCP2 promoter construct was increased twofold ($p < 0.05$) when cotransfected with the SF-1 plasmid. This activity was enhanced by $(Bu)_2$cAMP treatment (fourfold, $p < 0.01$). No induction by $(Bu)_2$cAMP was observed in the absence of SF-1 (Fig. 3). In addition, cotransfection of the SCP2 promoter construct with rSF-1, a plasmid containing SF-1 cDNA in the reverse orientation, showed no induction by $(Bu)_2$cAMP

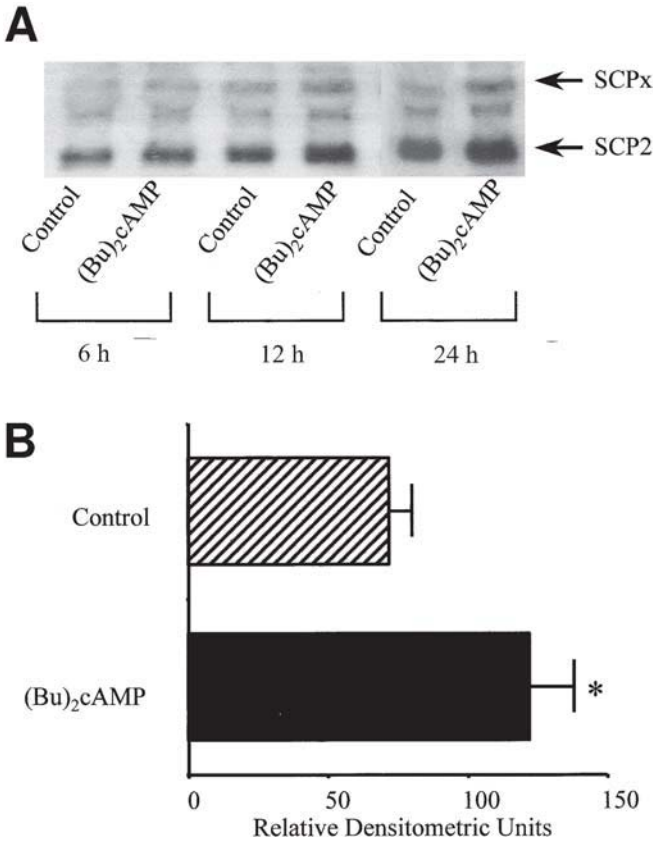

Fig. 1. Effect of $(Bu)_2$cAMP on SCP2 mRNA levels in luteal cells. Luteal cells were prepared and cultured as described under Materials and Methods. $(Bu)_2$cAMP (1 m*M*) was added to the medium of the cells 6, 12, and 24 h before lysing the cells. Total RNA isolation and Northern blot analysis were carried out as described under Materials and Methods. (**A**) Characteristic Northern blot autoradiogram is shown. (**B**) Northern blot results corresponding to the 24-h time point were analyzed using a Hoefer Scanning Densitometer. Data are presented as means ± SEM ($n = 3$). *$p < 0.05$.

(Fig. 3). These results suggest that SF-1 directly mediates cAMP activation of the SCP2 promoter.

To examine the effect of endogenously expressed SF-1 on the expression of the SCP2 gene, cotransfection studies were carried out in mouse adrenocortical Y1 cells. For these experiments, 8-bromo-cAMP (8-Br-cAMP) was used because this cAMP analog is more stable than $(Bu)_2$cAMP in Y1 cells. As shown in Fig. 4, the luciferase activity of the SCP2 promoter construct was induced fourfold in the presence of 1 m*M* 8-Br-cAMP ($p < 0.01$).

To determine whether SF-1 protein binds to the putative SF-1-binding sites found in the SCP2 promoter, mobility shift assays were performed. As shown in Fig. 5A, incubation of radiolabeled SCP2 SF-1-binding motif with purified glutathione-*S*-transferase SF-1 (GST-SF-1) fusion protein resulted in the production of a slower migrating DNA-protein complex (lane 5). When unlabeled binding motif was included in the binding reaction (lane 6), the DNA-protein complex was dramatically diminished (Fig. 5A). A

CCTCTGAAAG AACAGTGCAA ACCCTTAACC ACAGAGAGCT GTCTTGGTCC -261

CCAAATTATT GT TCTTATGA GGATACCAGT CACACTGGAT GAAGGTTCAC -211

AP-1

TCTTGTGGCT TCATT TTACG TCTTTAAAAA TCTT TCT TCC AAATACAACC -161

GCAATATAAA ATATTGAAAT TTCAGAAACA TCAACACATG GATTTGGGAG -111

GACGTTCAGT CAATAAGTTC ATGAGTGGGA AAGAGGCAGG TTCCCAGGAC -61

AP-1 IRE

AAAGGTGGTT CTGCAGCACA ATTCAGGCCT TGGAGGAGCT GTGGTAGTCA -11

PPRE AP-1 SF-1 AP-1

CCTCTACAGG **ATG**

Fig. 2. Nucleotide sequence of the rat SCP2 promoter. Nucleotide position +1 is assigned to the "A" of the ATG start codon (in boldface), and negative numbers refer to promoter sequences. Putative AP-1, SF-1, IRE, and PPRE motifs are indicated.

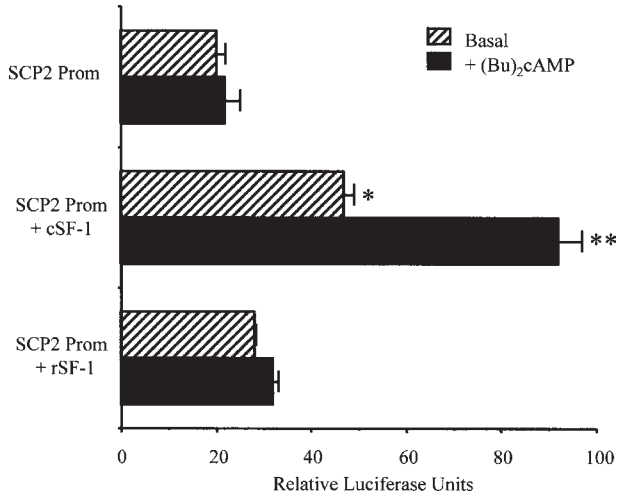

Fig. 3. Effect of SF-1 on the expression of luciferase activity under control of the SCP2 promoter in human bladder carcinoma HTB-9 cells. Cells were transfected with the SCP2 promoter construct in either the presence or absence of SF-1-pCMV (cSF-1) or the pCMV plasmid containing SF-1 cDNA in the reverse orientation (rSF-1). $(Bu)_2$cAMP (1 m*M*) was added to some of the plates 24 h before lysing the cells. Data are represented as relative luciferase units ± SEM and are from a typical experiment performed in triplicate. This experiment was repeated two times. $*p < 0.05$; $**p < 0.01$.

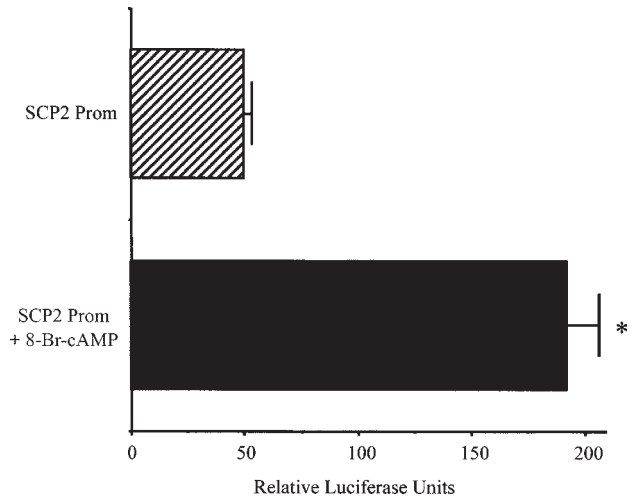

Fig. 4. Effects of endogenous SF-1 on the expression of luciferase activity under control of the SCP2 promoter in mouse adrenocortical Y1 cells. Cells were transfected with the SCP2 promoter construct as described under Materials and Methods. 8-Br-cAMP (1 m*M*) was added to some plates 24 h before lysing the cells. Data are represented as relative luciferase activity ± SEM and are from a typical experiment performed in triplicate. This experiment was repeated four times. $*p < 0.01$.

known SF-1-binding site from the rat HDL-R promoter *(26)* was used as a positive control (Fig. 5A, lanes 1–3). To examine the specificity of SF-1 binding to this binding motif, competition studies using unlabeled binding site were conducted (Fig. 5B, lanes 2–6). As shown in Fig. 5, the addition of increasing levels of unlabeled SCP2 SF-1-binding site gradually diminished the DNA-protein complex. A 200-fold molar excess of unlabeled SCP2 SF-1-binding motif was able to eliminate the DNA-SF-1 protein complex almost completely. Likewise, the addition of increasing amounts of SF-1 antibody gradually reduced the DNA-SF-1 protein complex while the supershifted complex increased (Fig. 5B, lanes 7 and 8).

To examine whether this SCP2 promoter region was actually involved in SF-1 binding, DNase I footprinting analysis of this promoter region was performed as described

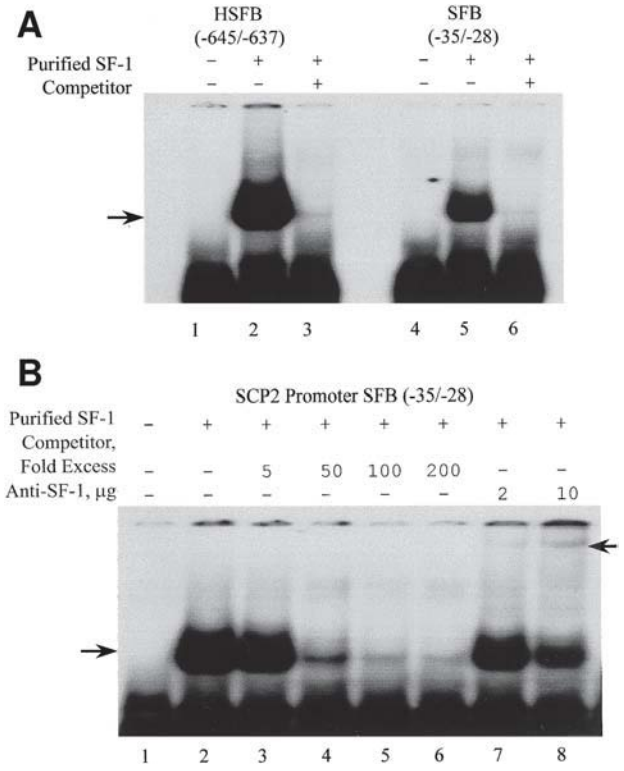

Fig. 5. Binding of SF-1 to the SF-1-binding site (SFB) in the SCP2 promoter. A ^{32}P-labeled double-stranded oligonucleotide probe (200,000 cpm/lane) containing SFB was incubated with 2.5 µg of purified GST-SF-1 fusion protein in either the absence or presence of competitor as described under Materials and Methods. The DNA protein complexes were resolved on a 4% nondenaturing acrylamide gel at 4°C in 1X TBE. The gel was then vacuum-dried and autoradiographed. (**A**) A representative gel mobility shift assay autoradiograph is presented. HSFB, which refers to a known SF-1-binding site from the rat HDL-R promoter (–645/–637), was used as a positive control. Competitor (250-fold excess) refers to unlabeled HSFB oligonucleotide in lane 3 and unlabeled SFB oligonucleotide in lane 6. This experiment was repeated three times. (**B**) Competition studies of the SCP2 SF-1-binding site. For competition studies, binding reactions were carried out in the presence of increasing levels of either unlabeled SFB (5–200X) or SF-1 antibody (2–9 µg of IgG). A representative mobility shift assay autoradiograph is presented. This experiment was repeated twice.

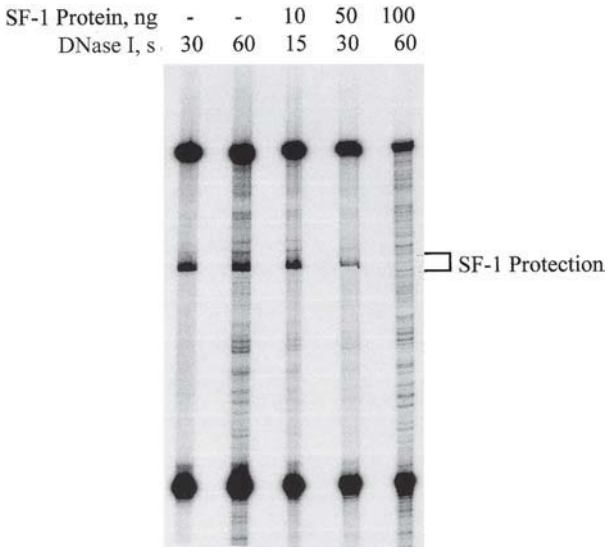

Fig. 6. DNase I footprint analysis of the SCP2 SF-1-binding site. A 400-bp DNA fragment of the SCP2 promoter was ^{32}P-end-labeled on the top strand. Binding reactions were performed in either the presence or absence of recombinant SF-1 protein. DNase I footprinting analysis was performed on two separate samples using the Sure Track Footprinting Kit according to the manufacturer's specifications. This experiment was repeated two times with identical results. A single gel is shown and the protected region is indicated.

under Materials and Methods. As shown in Fig. 6, incubation of labeled SCP2 promoter with purified recombinant SF-1 protein revealed the presence of a protected region within this promoter that was not observed in the absence of SF-1.

To confirm further SF-1 specificity to bind and activate the SCP2 SF-1-binding motif, radiolabeled, mutated oligonucleotide probes (see Materials and Methods) were used in mobility shift assays. Figure 7A shows the sequences for the wild-type and mutated oligonucleotides used in these experiments. Mut1 and Mut2 either increased or had no effect on SF-1 binding to the DNA, whereas Mut3 and Mut4 completely eliminated binding. Mut4 was then tested in cotransfection studies. As shown in Fig. 8, cotransfecting this mutation into Y1 cells reduced both basal and cAMP-induced luciferase activity compared to the wild-type promoter.

To examine whether protein kinase A (PKA) was involved in transcriptional regulation of the rat SCP2 gene by SF-1 and cAMP, cotransfection studies were carried out in Kin 8 cells. This Y1 cell line is resistant to cAMP activation as a result of a dominant mutation in the PKA regulatory subunit *(31,32).* As shown in Fig. 9, both basal and 8-Br-cAMP-mediated transcriptional stimulation of the SCP2 promoter were diminished in Kin 8 cells compared with the wild-type Y1 cells. Basal luciferase levels were reduced by 50% ($p < 0.01$), whereas 8-Br-cAMP-induced activity was reduced by 75% ($p < 0.01$) (Fig. 9).

Discussion

The human SCP2/SCPx gene spans about 80 kb of chromosome lp32 *(33,34)*, whereas the mouse gene spans about 100 kb of chromosome 4C5→D1 *(35).* It has been established that the human gene contains two promoters: a 5'-promoter, which directs transcription of the SCPx mRNA, and a promoter 45 kb downstream in intron 11, which directs transcription of the SCP2 mRNA *(36).* In the present study, we reported partial sequences of the rat SCP2 promoter and demonstrated that at least one of the putative regulatory elements identified in this promoter fragment, the SF-1-binding motif, could be involved in the regulation of the SCP2 gene by tropic hormones. These data correlate

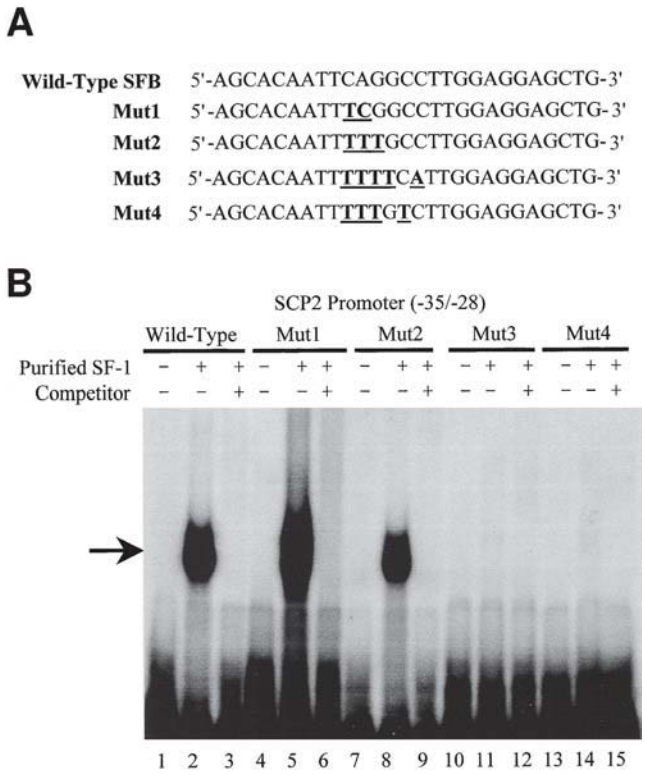

Fig. 7. Mutational analysis of the SCP2 SF-1-binding site (SFB). (**A**) Sequences for both wild-type and mutated oligonucleotide probes are shown. (**B**) ^{32}P-labeled double-stranded oligonucleotide probes (50,000 cpm/lane) containing either the wild-type or mutated SFB were incubated with 2.5 µg of purified GST-SF-1 fusion protein in either the absence or presence of unlabeled competitor (250-fold molar excess). A representative mobility shift assay autoradiograph is presented. Competitor refers to SFB in lane 3, Mut1 in lane 6, Mut2 in lane 9, Mut3 in lane 12, and Mut4 in lane 15. This experiment was repeated four times.

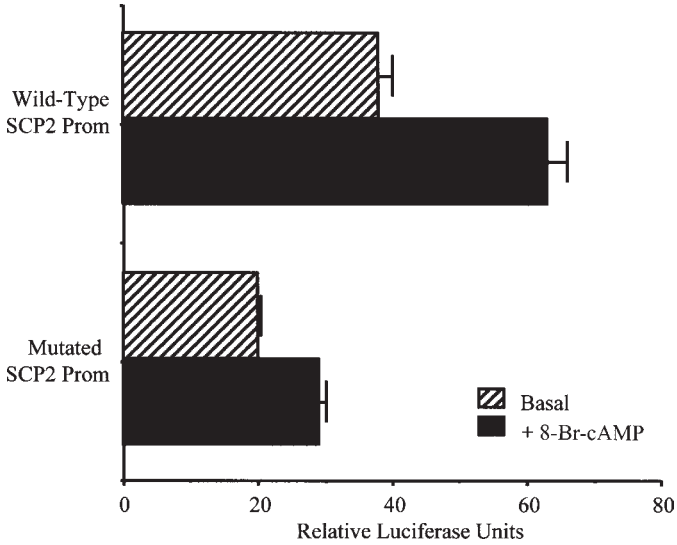

Fig. 8. Effects of mutating the SF-1-binding site found in the SCP2 promoter on the expression of luciferase activity in Y1 cells. Cells were transfected with wild-type or mutated SCP2 promoter construct as described under Materials and Methods. 8-Br-cAMP (1 m*M*) was added to some plates 24 h before lysing the cells. Data are represented as relative luciferase activity ± SEM and are from a typical experiment performed in triplicate. This experiment was repeated two times.

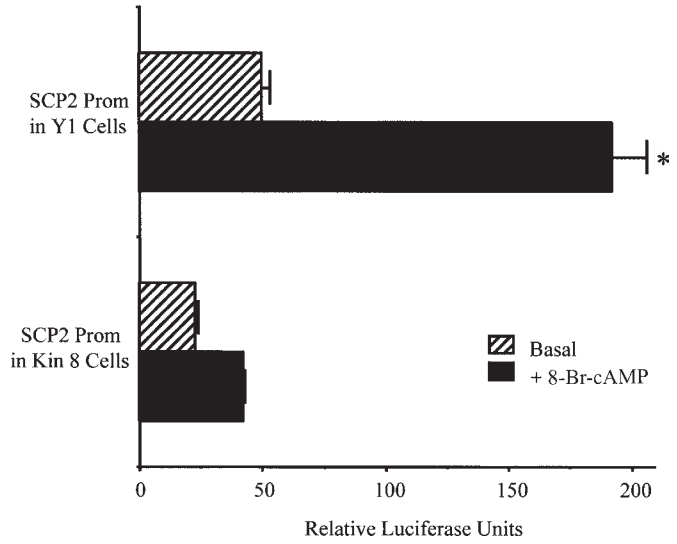

Fig. 9. Effects of cAMP on the expression of luciferase activity under control of the SCP2 promoter in Y1 and Kin 8 adrenal cells. Cells were transfected with the SCP2 promoter construct as described under Materials and Methods. 8-Br-cAMP (1 m*M*) was added to some plates 24 h before lysing the cells. Data are represented as relative luciferase activity ± SEM and are from a typical experiment performed in triplicate. This experiment was repeated twice. *$p < 0.01$.

with the proposed role of SCP2 on steroid hormone synthesis *(1–5)*. In addition, it has been shown that elevated SCP2 levels are associated with increased activity of steroidogenic enzymes such as cholesterol side-chain cleavage cytochrome P450 (P450scc) *(37)*. The finding that cAMP increased SCP2 mRNA levels in luteal cells is consistent with previous reports showing that tropic hormones induce both SCP2 protein and mRNA levels *(6–9)*. Apparently, the increase in ovarian steroidogenic capacity depends on increased sterol transport, a process mediated by SCP2. The opposite effect has been demonstrated in earlier studies showing that prostaglandin F2α, an antisteroidogenic agent, reduces SCP2 levels *(38)*.

We also found that the transcript corresponding to the SCP2-related protein, SCPx, was induced by cAMP. We have recently isolated the promoter of the SCPx gene and found that this promoter contains two SF-1-binding motifs through which SF-1 binds and activates SCPx gene transcription (unpublished observations).

An important consideration is whether SF-1 by itself mediates cAMP regulation of this gene. It has been shown for genes such as StAR *(39–41)*, bovine CYP11A *(42)*, rat CYP19 *(43)*, the fish gonadotropin II β-subunit *(44)*, rat *(45)* and mouse *(46,47)* LHβ subunit, and the ACTH receptor *(48)* that SF-1 requires interaction with other factors to fully activate transcription of these genes. SF-1 coactivators that have been reported include CAAT/enhancer bind-

ing protein *(39,40)*, GATA-4 *(40),* Sp1 *(41,42,45)*, cAMP response element binding protein *(43),* estrogen receptor *(44),* early growth response protein *(45–47)*, and AP-1 *(48).* In addition to the SF-1 site, this SCP2 promoter fragment contains a putative IRE and a PPRE motif, which are characteristic of peroxisomal proteins, and four AP-1 motifs. Characterization of each of these putative binding sites and determination of the importance of these sites in the cAMP regulation of this gene are still required.

Another region that appears to be critical for SF-1 binding to the SCP2 promoter includes the two base pairs immediately upstream of the SF-1-binding motif, as demonstrated by the mutation analysis studies using Mut1 and Mut2. Mut1 consisted of changing the cytidine and adenosine bases upstream of the SF-1-binding site with thymidine and cytidine bases, respectively. This mutation enhanced recombinant SF-1 protein binding to the SCP2 SF-1-binding motif. However, when those same bases were changed to thymidines, as shown in Mut2, no changes in SF-1's DNA binding ability were observed, suggesting that although these bases may not bind SF-1 directly, they may influence SF-1 binding to the SCP2 SF-1-binding motif. Only Mut3 and Mut4, which involve changes within the SF-1-binding motif, completely eliminated SF-1 binding to the SCP2 promoter. Because the cytidine and adenosine bases analyzed in Mut1 and Mut2 are within a putative AP-1-binding motif, it would be of great interest to examine whether SF-1 directly interacts with AP-1 to regulate the SCP2 gene.

The finding that a functional PKA is required for basal and SF-1-mediated cAMP regulation of the SCP2 gene appears to be common to other genes regulated by SF-1. These include the HDL-R *(26), Cyp*21 *(49),* P450scc *(50, 51),* and steroid 11β-hydroxylase (11β-OHase) *(51,52)* genes. All of these genes showed diminished gene expression in Kin 8 cells *(49–52).* However, there appear to be some differences in the absolute requirement for PKA activity depending on the gene involved *(51).* In the case of the P450scc gene, both basal and 8-Br-cAMP-induced mRNA levels were markedly reduced but still detectable in the absence of a functional PKA, whereas in the case of the 11β-OHase gene, both basal and 8-Br-cAMP-induced mRNA levels completely disappeared as a result of the PKA mutation *(51).* Nevertheless, the lack of a functional PKA only affected cAMP-induced transcription in the case of the HDL-R gene *(26).* Whether these differences in the absolute requirement for PKA activity are related to a gene-specific coactivator that interacts with SF-1 in each case needs to be determined.

In summary, the results of the present study demonstrate, for the first time, that the SCP2 gene, like other genes involved in steroid hormone production, is regulated by SF-1 and cAMP. The results confirm that hormone regulation of steroidogenesis occurs at several levels including cholesterol transport.

Materials and Methods

Materials

All oligonucleotides and primers were synthesized by Integrated DNA Technologies (Coralville, IA). The pGL3-basic luciferase vector, renilla luciferase vector, and the Dual Luciferase Reporter Assay System were obtained from Promega (Madison, WI). The murine SF-1 cDNA under the control of the cytomegalovirus promoter was obtained from Dr. Keith L. Parker (University of Texas, Southwestern, Dallas, TX). The mouse adrenocortical Y1 and human bladder carcinoma HTB9 cell lines were obtained from American Type Culture Collection (Rockville, MD). The mouse Kin 8 adrenal cell line was obtained from Dr. Bernard P. Schimmer (University of Toronto, Toronto, Canada). The QuickChange Site-directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). The SCP2 cDNA was prepared as previously described *(28).* [α^{32}P]dCTP (3000 Ci/mmol), poly dI-dC, and the T7 Sequenase DNA Sequencing Kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [^{35}S]dATP (1000–1500 Ci/mmol) was obtained from Dupont/New England Nuclear (Wilmington, DE). The Fugene 6 transfection reagent and the Nick Translation Kit were obtained from Roche (Indianapolis, IN). Dulbecco's modified Eagle's medium:nutrient mixture F-12 (DMEM/F12) was obtained from Gibco-BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Summit Biotechnology (Ft. Collins, CO). BioMax-MR films were obtained from Fisher (Norcross, GA). All other chemicals were purchased from Fisher or Sigma (St. Louis, MO).

Animals

Twenty-eight-day-old Sprague-Dawley rats were purchased from Harlan (Madison, WI). All protocols to treat animals were approved by the University of South Florida Animal Care Committee. Throughout the experiment, animals had free access to food and water and were housed under a 12-h dark, 12-h light cycle. Follicular development and ovulation were induced in rats by injection with 8 IU of pregnant mare's serum gonadotropin (PMSG). By this method, rats ovulate approx 72 h following treatment with PMSG. Rats were euthanized by clipping the diaphragm while under ether anesthesia.

Luteal Cell Dispersion

For luteal cell dispersions, ovaries were collected 10 d postovulation. Ovaries from 10 animals were collected and dispersed in 10 mL of a solution containing 0.23 mg/mL of collagenase, 0.03 mg/mL of DNase, and 1.5 mg/mL of dispase and then incubated on a Biostir plate for 30 min with gentle stirring. After incubation, the collagenase-DNase-dispase solution was changed, and the ovaries were incubated for another 30 min. This process was repeated three

times. Cells were then centrifuged and resuspended in dispersion medium for trypan blue exclusion cell viability analysis.

Cell Culture

Luteal cells were plated in 6-well plates (approx 10^6 cells/well) and incubated with DMEM/F12 medium + 10% FBS for 37 h at 37°C (5% CO_2). The medium was changed 2 h before adding $(Bu)_2$cAMP. After incubating the cells with the specified dose of $(Bu)_2$cAMP for the indicated time, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Cells were then used for RNA isolation.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method employing TRI Reagent. This method consistently yields 5–8 µg of RNA/mg of tissue. Cells were scraped with a rubber policeman into 2 mL of TRI Reagent and transferred to an RNase-free centrifuge tube. Cells were then homogenized with a Polytron homogenizer (Brinkmann, Westbury, NY) and centrifuged at 11,000*g* for 15 min at 4°C. RNA was precipitated from the aqueous phase with isopropanol. The RNA pellet was washed in 75% ethanol and then resuspended in Formazol. Electrophoresis and Northern blot analysis to determine SCP2 mRNA levels were carried out as previously described *(37).* The rat SCP2 cDNA was labeled with [α^{32}P]dCTP using the nick translation methods. Blots were then stripped and reprobed with the internal control β-actin.

Cloning of SCP2 Promoter

Cloning of the SCP2 promoter was performed using the Advantage *Tth* Polymerase Mix and the PromoterFinder DNA Walking Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Two nested primers, GSP1 (5'-AGCTGGCAGCTTCGGGAAAACCCAT-3') and GSP2 (5'-GCTG-AAATCTGGTGCGTTCTGAAGG-3'), were designed to the rat SCP2 cDNA sequence *(28).* These primers were then used with adapter-specific primers in screening five separate adapter-ligated rat genomic libraries by PCR. The conditions for PCR were 7 cycles of denaturation at 94°C for 2 s and elongation at 72°C for 3 min, followed by 40 cycles of denaturation at 94°C for 2 s and elongation at 67°C for 3 min. Nested PCR products were analyzed by 1.2% agarose/ethidium bromide gel electrophoresis. All DNA fragments were cloned into the pCR 2.1 TA cloning vector and sequenced in both directions using the T7 Sequenase DNA Sequencing Kit and [^{35}S]-dATP. The SCP2 promoter obtained using this method was then subcloned into pGL3-basic luciferase vector using *Xho*I and *Kpn*I sites.

Site-Directed Mutagenesis

Site-directed mutants were obtained using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Briefly, 10 ng of plasmid was incubated with 125 ng of the appropriate complementary oligonucleotides (given subsequently) and 1 µL of dNTPs in 50 µL of reaction buffer (100 m*M* KCl; 100 m*M* $[NH_4]_2SO_4$; 200 m*M* Tris-HCl, pH 8.8; 20 m*M* $MgSO_4$; 1% Triton X-100; and 1 µg/mL of nuclease-free bovine serum albumin [BSA]). One microliter of *Pfu* DNA polymerase (2.5 U/µL) was added to the reaction, and each reaction was heated to 95°C for 30 s followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 12 min. After the cycling reaction, samples were subjected to digestion with *Dpn*I for 1 h at 37°C to remove the parental DNA template. One microliter of the mutant samples was used to transform XL-1 Blue bacterial cells. The mutations were confirmed by sequencing using the T7 Sequenase DNA Sequencing Kit and [^{35}S]-dATP. Oligonucleotides used for site-directed mutagenesis to mutate the SCP2 SF-1-binding motif were 5'-AGCACA-ATT**TTT**G**T**CTTGGAGGAGCTG-3' and its complement. The nucleotides in boldface underlined letters correspond to the mutated bases.

Cell Transfections

Cells were transfected with the SCP2 promoter-luciferase gene construct in either the presence or absence of SF-1-pCMV using the Fugene 6 transfection method according to the manufacturer's instructions. Cells were first plated either in 6-well tissue culture plates at a density of 3×10^6 cells/well or in 12-well tissue culture plates at a density of 5×10^4 cells/well and incubated for 24 h at 37°C (5% CO_2). Fresh DMEM/F12 medium + 10% FBS was added before transfections. Two micrograms (or 0.5 µg for 12-well plates) of each plasmid to be transfected was incubated with Fugene 6 (ratio 2:3) in 100 µL of medium for 15 min. DNA-Fugene 6 complex was then added to the cells. After incubating for 4 h, the medium was replaced, and the cells were allowed to incubate for 48 h at 37°C (5% CO_2). Either $(Bu)_2$cAMP or 8-Br-cAMP (1 m*M*) was added to some plates 24 h prior to the end of the incubation period. Cells were washed twice with PBS and treated with a passive lysis buffer for 20 min. Lysates were transferred to a microcentrifuge tube and stored at −80°C until determination of luciferase activity. Cotransfection of a plasmid containing the renilla luciferase gene under control of the SV40 early enhancer/promoter region was used as a control to correct for differences in transfection efficiencies.

Luciferase Assays

Luciferase assays were performed using the Dual Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Briefly, 50 µL of luciferase

substrate was added to 10 μL of lysate, and luciferase activity was measured using a Turner Designs-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Fusion Protein Production

GST-SF-1 fusion protein was overexpressed in *Escherichia coli* by induction of midlogarithmic phase cultures with 1 m*M* isopropyl-β-D-thiogalactopyranoside. After incubating for 6 h at 27°C, cells were sedimented by centrifuging at 7700*g* for 10 min at 4°C. The cell pellet was resuspended in PBS and sonicated using a Sonic Dimembrator 60 (Fisher) with a 1/4-in. tip at full strength in 10-s bursts until cells were lysed. Triton X-100 was then added to a final concentration of 1%, and the sample was incubated for 30 min at 4°C. The suspension was centrifuged at 12,000*g* for 10 min at 4°C. Affinity purification of the fusion protein was performed using the GST-fusion Purification Kit (Pharmacia Biotech) per the manufacturer's recommendations. Briefly, cleared lysate was passed through the glutathione Sepharose 4B column. After washing the column with PBS, the fusion protein was eluted with 10 m*M* reduced glutathione in 50 m*M* Tris-HCl, pH 8.0. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Purified GST-fusion protein was used in mobility shift assays.

Gel Mobility Shift Assay

Complementary oligonucleotides corresponding to the SCP2 promoter region from −46 to −20 (SCP2 SF-1-binding motif: 5'-AGCACAATTCAGGCCTTGGAGGAGCTG-3') with GGG overhangs at the 5' ends were synthesized and annealed in 10 m*M* Tris-HCl, pH 7.5; 1 m*M* EDTA; 25 m*M* NaCl; 10 m*M* $MgCl_2$, and 1 m*M* dithiothreitol (DTT). The oligonucleotide probe was then labeled using the Klenow fragment of DNA polymerase and [α^{32}P] dCTP (3000 Ci/mmol). Unlabeled SF-1-binding motif oligonucleotide was used as a competitor in some experiments. Mut1 (5'-AGCACAATT**TC**GGCCTTGGAGGAGCTG-3'), Mut2 (5'-AGCACAATT**TTT**GCCTTGGAGGAGCTG-3'), Mut3 (5'-AGCACAATT**TTTT**C**A**TTGGAGGAGCTG-3'), and Mut4 (5'-AGCACAATT**TTT**G**T**CTTGGAGGA-GCTG-3') are identical to the SCP2 SF-1-binding motif except for the boldfaced and underlined bases. In some experiments, Mut1-4 were also ^{32}P-labeled and used as probe. Purified GST-SF-1 fusion protein (2.5 μg) was incubated in either the presence or absence of competitor for 30 min at room temperature in binding buffer (12 m*M* HEPES, pH 7.9; 12% glycerol; 60 m*M* KCl; 1 m*M* EDTA; 1 m*M* DTT; and 4 m*M* Tris-HCl, pH 8.0), 2 μg of poly dI-dC, and 0.4 μg of BSA. After incubation, 200,000 cpm of the radiolabeled probe was added and the mixture incubated for 15 min at 30°C. Where indicated, SF-1 antibody was also added to the reaction for supershift analysis. The DNA-protein complexes were resolved on a 4% nondenaturing acrylamide gel at 4°C in 1X TBE (0.05 *M* Tris, 0.05 *M* boric acid, and 0.001 *M* EDTA). Gels were then vacuum-dried and exposed to BioMax-MR films at −80°C for 12–24 h.

DNase I Footprint Analysis

The 400-bp rat SCP2 promoter was excised from the pGL3-basic vector using *Xho*I and gel purified using the Sephaglas BandPrep kit (Amersham/Pharmacia Biotech) and then treated with calf intestinal phosphatase (CIP) (Promega). CIP was inactivated by phenol-chloroform extraction. The DNA was precipitated and vacuum dried. Three picomoles of probe was labeled using T4 polynucleotide kinase (Promega) and [γ^{32}P] ATP. *Eco*RI was used to generate a DNA probe labeled on only one end. The radiolabeled probe was phenol-chloroform extracted and ethanol precipitated. DNA footprinting analysis was performed using the SureTrack Footprinting Kit (Amersham/Pharmacia Biotech) according to the manufacturer's specifications. Briefly, 10–20 fmol of labeled probe (30,000–60,000 cpm) was incubated in either the presence or absence of 100–500 ng of recombinant SF-1 protein and incubated at room temperature for 20 min. BSA was used as a negative control in these experiments. $CaCl_2$ and $MgCl_2$ were added to the binding reaction to a final concentration of 1 and 0.5 m*M*, respectively, and incubated for 1 min. Next, 0.6–5 U of DNase I was added and the mixture incubated for another minute. The DNA was purified by phenol-chloroform extraction and ethanol precipitation. Samples were resuspended in formamide loading buffer and resolved on an 8% sequencing gel. Gels were then vacuum-dried and either exposed to BioMax-MR films at −80°C for 12–24 h or to a phosphor screen for 6–12 h.

Data Analyses

Northern blot results were analyzed using a Hoefer Scanning Densitometer (Hoefer, San Francisco, CA). Equal RNA loading was verified by ethidium bromide staining of the agarose gel. Luciferase data were expressed as the mean ± SEM. Each luciferase assay experiment was performed in triplicate and repeated for the number of times indicated in the legends to Figs. 3, 4, 8, and 9. Data from the individual parameters were compared by analysis of variance followed by Student-Newman-Keuls multiple comparison test when applicable *(53).*

Acknowledgments

We thank Marie J. Trinidad for technical assistance and Drs. Keith L. Parker and Bernard P. Schimmer for gifts of materials. This work was supported by a grant from the National Institutes of Health (R29 HD 31644) to M. P. McLean. D. Lopez was supported by AHA Florida Affiliate Post-doctoral Fellowship no. 9703004.

References

1. Vahouny, G. V., Chanderbhan, R. F., Kharroubi, A., Noland, B. J., Pastuszyn, A., and Scallen, T. J. (1987). *Adv. Lipid Res.* **22,** 83–113.
2. Chanderbhan, R., Tanaka, T., Strauss, J. F., Irwin, D., Noland, B. J., Scallen, T. J., and Vahouny, G. V. (1983). *Biochem. Biophys. Res. Commun*. **117,** 702–709.

3. Tanaka, T., Billheimer, J. T., and Strauss, J. F. III. (1984). *Endocrinology* **114**, 533–540.
4. Chanderbhan, R. F., Kharroubi, A. J., Noland, B. J., Scallen, T. J., and Vahouny, G. V. (1986). *Endocr. Res.* **12**, 351–370.
5. Yamamoto, R., Kallen, C. B., Babalola, G. O., Rennert, H., Billheimer, J. T., and Strauss, J. F. III. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 463–467.
6. Trzeciak, W. H., Simpson, E. R., Scallen, T. J., Vahouny, G. V., and Waterman, M. R. (1987). *J. Biol. Chem.* **262**, 3713–3717.
7. Van Noort, M., Rommerts, F. F. G., van Amerongen, A., and Wirtz, K. W. A. (1988). *Mol. Cell Endocrinol.* **56**, 133–140.
8. McLean, M. P., Puryear, T. K., Khan, I., Azhar, S., Billheimer, J. T., Orly, J., and Gibori, G. (1989). *Endocrinology* **125**, 1337–1344.
9. Rennert, H., Amsterdam, A., Billheimer, J. T., and Strauss, J. F. III. (1991). *Biochemistry* **30**, 11,280–11,285.
10. Menon, K. M. J. and Gunaga, K. P. (1974). *Fertil. Steril*. **25**, 732–750.
11. Waterman, M. R. and Keeney, D. S. (1996). *Vitam. Horm.* **52**, 129–148.
12. Enmark, E. and Gustafsson, J. A. (1996). *Mol. Endocrinol.* **10**, 1293–1307.
13. Monte, D., DeWitte, F., and Hum, D. W. (1998). *J. Biol. Chem.* **273**, 4585–4591.
14. Guo, I. C., Tsai, H. M., and Chung, B. C. (1994). *J. Biol. Chem.* **269**, 6372–6379.
15. Morohashi, K. I., Zanger, U. M., Honda, S. I., Hara, M., Waterman, M. R., and Omura, T. (1993). *Mol. Endocrinol.* **7**, 1196–1204.
16. Michael, M. D., Kilgore, M. W., Morohashi, K., and Simpson, E. R. (1995). *J. Biol. Chem.* **270**, 13,561–13,566.
17. Lynch, J. P., Lala, D. S., Peluso, J. J., Luo, W., Parker, K. L., and White, B. A. (1993). *Mol. Endocrinol.* **7**, 776–785.
18. Carlone, D. L. and Richards, J. S. (1997). *Mol. Endocrinol.* **11**, 292–304.
19. Zhang, P. and Mellon, S. H. (1996). *Mol. Endocrinol.* **10**, 147–158.
20. Mason, J. I., Keeney, D. S., Bird, I. M., Rainey, W. E., Morohashi, K. I., Leers-Sucheta, S., and Melner, M. H. (1997). *Steroids* **62**, 164–168.
21. Sugawara, T., Holt, J. A., Kiriakidou, M., and Strauss, J. F. III. (1996). *Biochemistry* **35**, 9052–9059.
22. Sugawara, T., Kiriakidou, M., McAllister, J. M., Holt, J. A., Arakane, F., and Strauss, J. F. III. (1997). *Steroids* **62**, 5–9.
23. Clark, B. J., Soo, S. C., Caron, K. M., Ikeda, Y., Parker, K. L., and Stocco, D. M. (1995). *Mol. Endocrinol.* **9**, 1346–1355.
24. Sandhoff, T. W., Hales, D. B., Hales, K. H., and McLean, M. P. (1998). *Endocrinology* **139**, 4820–4831.
25. Cao, G., Garcia, C. K., Wyne, K. L., Schultz, R. A., Parker, K. L., and Hobbs, H. H. (1997). *J. Biol. Chem.* **272**, 33,068–33,076.
26. Lopez, D., Sandhoff, T. W., and McLean, M. P. (1999). *Endocrinology* **140**, 3034–3044.
27. McLean, M. P., Parmer, T. G., Biener, Y., Billheimer, J. T., Azhar, S., and Gibori, G. (1994). *Endocrine* **2**, 823–831.
28. Billheimer, J. T., Strehl, L. L., Davis, G. L., Strauss, J. F. III, and Davis, L. G. (1990). *DNA Cell Biol.* **9**, 150–165.
29. Keri, R. A. and Nilson, J. H. (1996). *J. Biol. Chem.* **271**, 10,782–10,785.
30. Halvorson, L. M., Kaiser, U. B., and Chin, W. W. (1996). *J. Biol. Chem.* **271**, 6645–6650.
31. Doherty, P. J., Tsao, J., Schimmer, B. P., Mumby, M., and Beavo, J. A. (1982). *J. Biol. Chem.* **257**, 5877–5883.
32. Rae, P. A., Gutmann, N. S., Tsao, J., and Schimmer, B. P. (1979). *Proc. Natl. Acad. Sci. USA* **76**, 1896–1900.
33. Ohba, T., Rennert, H., Pfeifer, S. M., He, Z., Yamamoto, R., Holt, J. A., Billheimer, J. T., and Strauss, J. F. III. (1994). *Genomics* **24**, 370–374.
34. Vesa, J., Hellsten, E., Barnoski, B. L., Emanuel, B. S., Billheimer, J. T., Mead, S., Cowell, J. K., Strauss, J. F. III, and Peltone, L. (1994). *Hum. Mol. Genet.* **3**, 341–346.
35. Raabe, M., Seedorf, U., Hameister, H., Ellinghaus, P., and Assmann, G. (1996). *Cytogenet. Cell Genet.* **73**, 279–281.
36. Ohba, T., Holt, J. A., Billheimer, J. T., and Strauss, J. F. III. (1995). *Biochemistry* **34**, 10,660–10,668.
37. Chanderbhan, R., Noland, B. J., Scallen, T. J., and Vahouny, G. V. (1982). *J. Biol. Chem.* **257**, 8928–8934.
38. McLean, M. P., Billheimer, J. T., Warden, K. J., and Irby, R. B. (1995). *Endocrinology* **137**, 3370–3378.
39. Reinhart, A. J., Williams, S. C., Clark, B. J., and Stocco, D. M. (1999). *Mol. Endocrinol.* **13**, 729–741.
40. Silverman, E., Eimer, S., and Orly, J. (1999). *J. Biol. Chem.* **274**, 17,987–17,996.
41. Sugawara, T., Saito, M., and Fujimoto, S. (2000). *Endocrinology* **141**, 2895–2903.
42. Liu, Z. and Simpson, E. R. (1997). *Mol. Endocrinol.* **11**, 127–137.
43. Carlone, D. L. and Richards, J. S. (1997). *Mol. Endocrinol.* **11**, 292–304.
44. Le Drean, Y., Liu, D., Wong, A. O. L., Xiong, F., and Hew, C. L. (1996). *Mol. Endocrinol.* **10**, 217–229.
45. Kaiser, U. B., Halvorson, L. M., and Chen, M. T. (2000). *Mol. Endocrinol.* **14**, 1235–1245.
46. Lee, S. L., Sadovsky, Y., Swirnoff, A. H., Polish, J. A., Goda, P., Gavrilina, G., and Milbrandt, J. (1996). *Science* **273**, 1219–1222.
47. Halvorson, L. M., Ito, M., Jameson, J. L., and Chin, W. W. (1998). *J. Biol. Chem.* **273**, 14,712–14,720.
48. Sarkar, D., Kambe, F., Hayashi, Y., Ohmori, S., Funahashi, H., and Seo, H. (2000). *Endocr. J.* **47**, 63–75.
49. Parissenti, A. M., Parker, K. L., and Schimmer, B. P. (1993). *Mol. Endocrinol.* **7**, 283–290.
50. Handler, J. D., Schimmer, B. P., Flynn, T. R., Szyf, M., Seidman, J. G., and Parker, K. L. (1988). *J. Biol. Chem.* **283**, 13,068–13,073.
51. Wong, M., Rice, D. A., Parker, K. L., and Schimmer, B. P. (1989). *J. Biol. Chem.* **264**, 12,867–12,871.
52. Mouw, A. R., Rice, D. A., Meade, J. C., Chua, S. C., White, P. C., Schimmer, B. P., and Parker, K. L. (1989). *J. Biol. Chem.* **264**, 1305–1309.
53. Zar, J. H. (1974). In: Biostatistical Analysis: Multiple Comparisons. McElroy, W. D. and Swanson, C. P. (eds.). Prentice-Hall: Englewood Cliffs, NJ, pp. 151–162.