0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 12
Printed in U.S.A.

Transcriptional Regulation of the Rat Steroidogenic Acute Regulatory Protein Gene by Steroidogenic Factor 1*

TODD W. SANDHOFF, D. BUCK HALES, KAREN H. HALES, AND MARK P. MCLEAN

Departments of Obstetrics and Gynecology (T.W.S., M.P.M.) and Biochemistry and Molecular Biology (T.W.S., M.P.M.), University of South Florida College of Medicine, Tampa, Florida 33606; and Department of Physiology and Biophysics (D.B.H., K.H.H.), University of Illinois at Chicago, Chicago, Illinois 60612

ABSTRACT

Steroidogenic acute regulatory (StAR) protein is synthesized in response to tropic hormones to facilitate cholesterol transport to the inner mitochondrial membrane-bound P450 side-chain cleavage enzyme (P450scc), the first enzymatic step in the steroid hormone biosynthetic pathway. Gonadotropins activate expression of their target genes via the cAMP second messenger system. We have demonstrated that cAMP administration to rat luteal cells stimulates expression of both StAR messenger RNA and protein. Because cholesterol delivery is the first regulated step in steroidogenesis, and because StAR messenger RNA levels are increased in response to tropic hormone and cAMP stimulation, the mechanism by which tropic hormones/cAMP stimulate transcription needs to be elucidated. To this end, approximately 2.7 kb of the rat StAR promoter was isolated and sequenced. Sequence analysis revealed the presence of a TATA-like element as well as multiple regulatory motifs including steroidogenic factor 1 (SF-1) binding sites, an estrogen receptor half-site, and two AP-1 sites within the promoter region. 5′-RACE experiments determined the transcription start site to be located 82 bp upstream of the ATG translation start codon. Electrophoretic mobility shift assays and supershift analysis demonstrated SF-1 binding to three SF-1 binding sites in the rat StAR promoter with high affinity and two SF-1 binding sites with low affinity. Transfection of mouse Y1 adrenal tumor cells and human bladder carcinoma cells (HTB9s) with the rat StAR promoter demonstrated that SF-1 was able to activate transcription of the luciferase reporter gene and that the rat StAR promoter was responsive to cAMP. Nested deletions of the rat StAR promoter (1.9 kb) identified a region between −1413 and −998 that is essential for maximal activation of the rat StAR gene in HTB9 cells; however, deletion of this region does not affect responsiveness to cAMP. 5′-Deletion and site-directed mutagenesis experiments demonstrated that the SF-1 motifs identified within the rat StAR promoter (located at positions −764, −455, and −106) were sufficient to activate transcription as well as confer cAMP responsiveness to the rat StAR gene. Site-directed mutagenesis studies using the smallest promoter fragment demonstrated that the two proximal SF-1 binding sites are crucial for StAR gene transcription, both at a basal level and in response to cAMP stimulation. These studies provide novel insights into the regulation of the rat StAR gene at the transcriptional level by SF-1. (*Endocrinology* **139:** 4820–4831, 1998)

STEROID HORMONES are synthesized in steroidogenic tissues at a basal level and in response to acute tropic hormone stimulation. The biosynthesis of steroid hormones in all steroidogenic tissues begins with the enzymatic conversion of cholesterol to pregnenolone by the cytochrome P450 side-chain cleavage (P450scc) enzyme that is located on the matrix side of the inner mitochondrial membrane (1–5). To maintain steroidogenesis, the cell must provide a continuous supply of cholesterol to the P450scc enzyme complex within the mitochondria for subsequent steroid production.

Using mouse MA-10 Leydig tumor cells, Stocco and colleagues (6–10) have described a series of mitochondrial proteins at 37 kDa, 32 kDa, and 30 kDa that are synthesized in response to luteinizing hormone (LH), human CG (hCG), or with the cAMP analog, dibutyryl cAMP (dbcAMP) (11). Stocco and Sodeman (10) and Epstein and Orme-Johnson (12) have postulated that during the processing of the 37 kDa mitochondrial protein to the 32 and 30 kDa forms, cholesterol may be transferred from the outer to the inner mitochondrial membrane. Clark *et al.* (13) isolated, cloned, and sequenced the 30 kDa LH-induced mitochondrial protein from mouse MA-10 Leydig tumor cells and have referred to this protein as the steroidogenic acute regulatory protein or StAR. StAR has been shown to enhance the mitochondrial conversion of cholesterol into pregnenolone in COS-1 cells when cotransfected with vectors encoding P450scc and adrenodoxin (13–15). The strongest evidence that StAR is required for steroidogenesis was demonstrated by sequencing the StAR genes of individuals with congenital lipoid adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder characterized by impaired synthesis of all adrenal and gonadal steroid hormones. This study demonstrated that the StAR gene in these individuals encoded either a truncated or nonfunctional StAR protein (14), thus supporting the indispensable role that StAR plays in normal adrenal and gonadal steroidogenesis.

Early studies demonstrated an absolute requirement for the synthesis of new proteins for the steroidogenic response to acute hormone stimulation (16), as well as a requirement for phosphorylation of a threonine residue (17); however, the role of transcription in steroidogenesis in response to hormone stimulation required further study. Ferguson and Morita (18) first demonstrated that adrenocorticoid synthesis

Received April 3, 1998.
Address all correspondence and requests for reprints to: Dr. Mark P. McLean, Departments of Obstetrics and Gynecology, 4 Columbia Drive, Room 529 Tampa, Florida 33606.
* This work was supported by NIH Grant R01-HD35163 (to M.P.M.).

in rat adrenal quarters was unaffected by treatment with the RNA synthesis inhibitor actinomycin D. Similar findings were reported by Garren *et al.* (16), which demonstrated that inhibition of *de novo* transcription using actinomycin D had no effect on the acute stimulation of steroid production in the adrenal gland. Studies by Vernikos-Danellis and Hall (19), however, demonstrated that while actinomycin D had essentially no effect on ACTH-stimulated adrenocorticosterone production after 30 min, steroid production was virtually abolished after 24 h. More recent studies by Clark *et al.* (20) have demonstrated that hormone-stimulated StAR protein synthesis and progesterone production are inhibited in MA-10 mouse Leydig tumor cells 1 h after actinomycin D administration. The inhibitory effects of actinomycin D on StAR protein synthesis are diminished by pretreatment of the cells with hCG, but continued synthesis persists only in the absence of actinomycin D and the presence of hCG. These results indicate that while the acute stimulation of steroidogenesis may not require new RNA synthesis, transcription of the StAR gene is essential for maintaining steroidogenesis in adrenal and MA-10 Leydig tumor cells.

To further characterize the mechanism by which gonadotropins exert their effects via intracellular cAMP levels, we have determined the sequence of the promoter region for the rat StAR gene and have demonstrated that StAR gene expression is regulated by the steroidogenic tissue-specific transcription factor, steroidogenic factor 1 (SF-1). Our results further demonstrate that SF-1 is responsible for mediating the enhanced transcriptional activation following cAMP administration. SF-1 was first identified as a transcription factor with limited tissue distribution that recognized a conserved regulatory motif in the proximal promoter regions of genes encoding the cytochrome P450 steroid hydroxylases (21–23). Targeted disruption of the SF-1 gene revealed broader roles for the SF-1 protein, including regulation of the hypothalamic-pituitary-steroidogenic organ axis (24). SF-1 is also thought to be involved throughout the many stages of reproductive development. In fact, SF-1 has recently been shown to be involved with basal StAR gene transcription in the mouse and human genes (25, 26). Only in the human promoter, however, was cAMP able to augment the transcriptional response. These studies provide the first evidence that the rat StAR gene is regulated by SF-1 at the transcriptional level. These studies also characterize five SF-1 binding sites and their role in regulating the rat StAR gene. Our results demonstrate that both high and low affinity SF-1 motifs are used by SF-1 to mediate rat StAR gene transcription, both at a basal level and in response to stimulation with cAMP.

Materials and Methods

Materials

SF-1-specific rabbit polyclonal antisera was purchased from Upstate Biotechnology (Lake Placid, NY), and StAR-specific rabbit polyclonal antisera was generated by D. B. Hales (University of Chicago, Chicago, IL). dbcAMP was purchased from Sigma Chemical Co. (St. Louis, MO). [^{35}S]-deoxy-ATP (1348 Ci/mmol) was purchased from DuPont-New England Nuclear (Wilmington, DE). [α^{32}P]-deoxy-CTP (3000 Ci/mmol) and the Sequenase DNA sequencing kit were obtained from Amersham Corp. (Arlington Heights, IL). BioMax and XAR-5 films were purchased from Eastman Kodak Co. (Rochester, NY). SeaKem and SeaPlaque agarose were purchased from the FMC Corporation (Rockland, ME). All restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN). The Wizard Miniprep DNA purification systems and the Dual-Luciferase Reporter Assay System were purchased from Promega Corp. (Madison, WI). The TA cloning kit was purchased from Invitrogen (San Diego, CA), and the Sephaglas BandPrep kit, the double-stranded nested deletion kit, and poly(dI-dC)·poly(dI-dC) were obtained from Pharmacia Biotech (Piscataway, NJ). Large-scale DNA purification reagents for transfection studies were obtained from Qiagen (Valencia, CA). The Quikchange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). The 5′-RACE system and cell culture reagents were purchased from Gibco BRL (Grand Island, NY), and all PCR reagents were purchased from Perkin Elmer (Branchburg, NJ). Synthetic oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The SuperSignal ULTRA chemiluminescent substrate was obtained from Pierce (Rockford, IL). The mouse Y1 adrenal and human HTB9 cell lines were obtained from ATCC (Rockville, MD). All other chemicals were reagent grade, and were obtained from Fisher Scientific International, Inc. (Norcross, GA) or Sigma Chemical Co.

Luteal cell dispersions

For luteal cell dispersions, 28-day-old female Sprague-Dawley rats were injected with 8 IU PMSG to induce follicular development and ovaries were collected 10 days postovulation for dispersion. Ovaries from 10 animals were collected and dispersed in 5 ml of a solution containing collagenase (0.45 mg/ml) and 5 ml of a solution containing DNAse (0.12 mg/ml) and dispase (6.0 mg/ml). Ovaries were incubated in collagenase-DNAse-dispase solution on a Biostir plate for 30 min stirring gently. Enzyme solution was removed after 30 min and fresh enzyme solution was added for an additional 30 min for a total of three 30-min periods. Cells from each of these aliquots were spun down and resuspended in dispersion media before counting cells by Trypan Blue exclusion. Cells were then plated out in 6-well plates for further experiments.

RNA isolation and electrophoresis

RNA was prepared from ovaries using a modification of the Chomczynski and Sacchi method (27) (TRI-Reagent Method, Molecular Research Center, Inc., Cincinnati, OH). This method consistently yields 5–8 μg RNA/mg tissue. Cultured rat luteal cells were homogenized in 1 ml of TRI-Reagent with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY), and RNA was extracted by the addition of 0.2 ml of chloroform and centrifugation at 12,000 $\times$ *g* for 15 min at 4 C. RNA was precipitated from the aqueous phase with isopropanol, and the RNA pellet was washed in 75% ethanol. The RNA pellet was resuspended in Formazol and quantitated by absorbance at 260 nm in a Beckman Coulter, Inc. DU-70 spectrophotometer (Palo Alto, CA).

For Northern blot analysis, total RNA (20 μg) was denatured at 65 C (15 min) and loaded onto 1% agarose gels containing 3% formaldehyde. Following size fractionation, RNA was blotted onto a nylon membrane (0.45-micron pore size) by capillary transfer and RNA was fixed to the membrane by UV cross-linking (0.3 J/cm^2). Ethidium bromide staining of the gel confirmed that the ribosomal RNAs (18S and 28S subunits) were intact, and determined whether equal amounts of RNA were loaded in each lane.

Northern blot analysis

Northern blot hybridizations were performed using the 867-bp rat StAR cDNA (28). The cDNA inserts were labeled with [α^{32}P] deoxy-CTP using the random-primed DNA labeling method. Northern blots were prehybridized at 62 C for at least 3 h in a 1 M NaCl, 1% SDS solution containing Background Quencher (Molecular Research Center, Inc.). Hybridization was completed in a high-efficiency hybridization solution (Molecular Research Center, Inc.) containing the ^{32}P-labeled probe (1×10^6 dpm/ml; SA = 2×10^8 dpm/μg DNA) at 62 C for at least 16 h. Blots were washed three times at RT (5 min) in 1 $\times$ SSC/1% SDS and three times at RT (10 min) in 0.1 $\times$ SSC/0.1% SDS. RNA:cDNA hybrids were visualized on BioMax film using two intensifying screens and a 12–48 h exposure period.

SDS-PAGE and electrotransfer

Cultured rat luteal cells were homogenized in 0.5 ml ice-cold homogenization buffer, as previously described (29). Ovarian homogenates were assayed for protein concentration by the method of Bradford (30), using BSA as the standard. Ovarian proteins (50 μg protein) were denatured at 100 C in loading buffer (29) for 10 min and subjected to electrophoresis on 7.5–18% gradient SDS-polyacrylamide gels according to the method of Laemmli (31). After electrophoresis, samples were electroblotted onto nitrocellulose (0.2 μm pore) in buffer containing 0.25 M Tris-base (pH 8.3), and 1.92 M glycine for 16 h at 4 C. To verify equal protein loading, nitrocellulose sheets were stained with either 0.1% Ponceau S (in 5% acetic acid) or 0.01% fast green (in 20% methanol and 7% acetic acid) and destained in the same solution without fast green.

Immunoblotting

Ovarian StAR protein contents were estimated by incubating transferred proteins in a 20 mM Tris base-buffered (pH 7.5) sodium chloride (500 mM) solution (TB-NaCl) with 3% milk and 0.05% Tween-20 for 1 h at RT. Buffer was replaced with TB-NaCl containing rabbit polyclonal StAR antiserum diluted 1:1000 in 3% milk and incubated at 4 C for 16 h. Nitrocellulose blots were washed in TB-NaCl containing 0.05% Tween-20 and then incubated in TB-NaCl containing 3% milk and a 1:10,000 dilution of goat antirabbit horseradish peroxidase for 1 h at RT. Blots were rinsed and soaked in Pierce's chemiluminescent super signal substrate for 10 min. Differences in band density on autoradiograms were quantified densitometrically with a Hoefer scanning densitometer (Hoefer Instruments, San Francisco, CA) for statistical analysis.

Long-range PCR

The isolation and sequence determination of the rat StAR promoter was carried out by engineering primers from the rat cDNA sequence for use in screening multiple adaptor-ligated rat genomic libraries by PCR. The StAR-specific primers (GSP1-StAR; GSP2-StAR) were used with adaptor-specific primers to carry out PCRs from five separate rat genomic libraries. The conditions for PCR were denaturation at 94 C for 2 sec followed by a 3 min incubation at 72 C for 7 cycles, followed by 40 cycles of denaturation at 94 C (2 sec) and elongation at 67 C for 3 min. Nested-PCR products were analyzed by 1.2% agarose/EtBr gel electrophoresis. Nested-PCR yielded genomic DNA fragments of 0.4, 2.7, and 4.0 kb. From these fragments, approximately 3.0 kb was sequenced and examined for regulatory motifs using the MacVector program from Oxford Molecular Group.

5′-Rapid amplification of cDNA ends (5′-RACE)

5′-RACE analysis was performed on StAR messenger RNA (mRNA) using the 5′-RACE kit from Gibco BRL. One microgram of ovarian poly A+ RNA was reverse-transcribed using a StAR-specific primer and reverse transcriptase. The StAR-specific primer (GSP3-StAR) was made to position 197 to 216 of the complementary strand of the rat StAR cDNA. GSP3-StAR was used to reverse transcribe mRNA to cDNA. The RNA was then degraded by the addition of RNase H. The single-stranded cDNA was then tailed with terminal deoxynucleotide transferase and dCTP. The tailed cDNA was then used as a template for PCR using nested-PCR primers, GSP1-StAR (CACAGCTTGATGCCTCAGTC-CTTTC) and the abridged anchor primer (GGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG). The parameters for PCR were denaturation at 95 C for 5 min, followed by 35 cycles of denaturation at 95 C for 1 min, annealing at 55 C for 2 min, and extension at 72 C for 3 min. The PCR product was then cloned into the TA vector and sequenced to determine the transcription start site.

Protein expression and purification

The cDNA for the SF-1 DNA-binding domain was obtained from Keith L. Parker (University of Texas, Southwestern Medical School, Dallas, TX) as a fusion protein in the pGEX-1λT vector from Pharmacia Biotech. The recombinant SF-1 fusion protein was expressed in BL21s, a protease-deficient bacterial strain, by inducing expression with the addition of IPTG at a final concentration of 0.1 mM and incubating the bacteria for 4 h at 37 C. After 4 h, the bacteria was spun down, resuspended in PBS, and sonicated, before adding Triton X-100 to a final concentration of 1%. The cells were incubated with the detergent for 30 min before centrifuging the lysate at 12,000 × *g* for 10 min at 4 C. The lysate was then run over a glutathione sepharose column to isolate the GST-SF-1 fusion protein. The column was washed three times with 1 × PBS, and the fusion protein was eluted by the addition of glutathione elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0). The eluted protein was acetone precipitated for 1 h at −20 C and protein concentration was determined by the method of Bradford (30), using BSA as the standard.

Electrophoretic mobility shift assays

Complementary oligonucleotides corresponding to the five putative SF-1 elements identified within the rat StAR promoter were synthesized and annealed. The five synthetic oligonucleotides were:

5′-GGGCAATCATTCCATCCTTGACCCTCTGCA-3′ SFB-1
5′-GGGCCCCCTCCCACCTTGGTCAGCACTGC-3′ SFB-2
5′-GGGCCAGGCTGGCCTTGAACTCAAGAGATC-3′ SFB-3
5′-GGGCTGTGTAGTCCTTGCTGTCCTAGAACT-3′ SFB-4
5′-GGGTACTCTCGGCCTTGAACGCTTACTGGA-3′ SFB-5

that are located at −188, −225, −537, −575, and −846, respectively, relative to the initiation codon. Annealing was performed in annealing buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT) by heating the reaction to 94 C for 3 min, followed by gradually decreasing the temperature until the reaction came to room temperature. The annealed oligonucleotides were phenol/chloroform extracted and labeled with [α^{32}P] deoxy-CTP using the Klenow fragment of DNA polymerase. Annealed oligonucleotides obtained before labeling were used as unlabeled oligonucleotide competitors.

In a typical binding reaction, 1 μg of purified GST-SF-1 was incubated with 2 μg of poly (dI-dC)·poly (dI-dC), and competitor or SF-1 polyclonal antisera as indicated. Incubation reactions were performed in 25 μl (total volume) of binding buffer containing the final concentrations: 12 mM HEPES, pH 7.9, 12% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 4 mM Tris-HCl, pH 8.0. After incubation for 15 min at room temperature, 1 ng of labeled probe was added and the incubation was continued for 15 min at 30 C. Products were resolved by electrophoresis at 30 mA in high ionic strength Tris-glycine electrophoresis buffer (0.25 M Tris base, 1.9 M glycine, and 10 mM EDTA) in a 4% polyacrylamide gel. Gels were dried and autoradiographed. Protein:DNA complexes were visualized on BioMax film using two intensifying screens and a 12–24 h exposure period.

Cell culture

Mouse Y1 adrenal tumor cells and human bladder carcinoma cells (HTB9) were grown in DMEM with 10% FBS and incubated at 37 C with 5% CO_2 until needed for transfection studies. Cells were passed with trypsin-EDTA, and the resulting suspension of cells was centrifuged at 2500 × *g* for 5 min, followed by resuspending cells in fresh media and adding to fresh flasks or 6-well plates.

Transfections and luciferase assays

Cells were plated in six-well plates for use in luciferase assays, and transfections were performed using the calcium phosphate method. Fresh media were added to the cells the day of the transfection. Five micrograms of each plasmid to be transfected was added to the appropriate tube and precipitated with 0.1 volume of NaOAc and three volumes of absolute ethanol. The samples were vortexed and placed at −70 C for 1 h before centrifuging samples at top speed in a microcentrifuge for 15 min at 4 C. The DNA was resuspended in 450 μl of sterile, distilled water before adding 50 μl of 2.5 M $CaCl_2$. The resulting solution was then added drop-wise to 500 μl of a 2 × HEPES-buffered saline solution while bubbling with a 1-ml pipette. The sample was then vortexed for 5 sec and incubated for 20 min at RT before adding to the cells. The DNA was incubated with the cells for 4 h at 37 C with 5% CO_2, followed by washing the cells twice with PBS. After the cells were washed, fresh media were added, and the cells were incubated for 48 h before measurement of luciferase activity. dbcAMP was added to the cells 24 h before the end of the incubation period. At the end of the experiment, cells were washed once with PBS and incubated in 0.5 ml of 1 × passive lysis buffer for 15

min at RT. The resulting lysate was frozen at −80 C until luciferase activity was measured. For this, 20 μl of the lysate was placed in a tube in the Turner Designs 20/20 luminometer (Sunnyvale, CA) and 100 μl of the luciferase enzyme substrate was injected into the tube and the luminescence measured. A control plasmid, which encodes for the Renilla luciferase, a second luciferase protein, was cotransfected into the cells to control for transfection efficiencies. This luciferase enzyme's activity was measured by injecting 100 μl of a second substrate and luminescence measured. The ratio of these readings was used to correct for differences in transfection efficiencies.

Double-stranded nested deletions

Approximately 2 kb of the rat StAR promoter immediately upstream of the StAR translation initiation codon was cloned into the *Mlu*I and *Xho*I sites of the luciferase reporter construct, pGL3-Basic (Promega Corp.), creating pGL3-StAR. Deletions of the 2-kb rat StAR promoter were obtained using the double-stranded nested deletion kit from Pharmacia Biotech according to the manufacturer's protocol. Briefly, 5 μg of pGL3-StAR (50 μl of 0.1 μg/μl) was subjected to restriction digestion with *Kpn*I and *Mlu*I. After digestion was complete, the DNA sample was heated for 10 min at 70 C to inactivate the enzymes. An equal volume of 2 × exonuclease III buffer and 100 U of exonuclease III was added to the linearized plasmid, which was then incubated at 30 C while taking 2 μl aliquots every 4 min for analysis of deletions. The different timed aliquots of digested DNA were then incubated in S1 nuclease buffer with 1.5 U of S1 nuclease to remove all single-stranded DNA regions. S1 nuclease digestion was stopped by adding 1 μl of S1 stop solution and heating to 65 C for 10 min. Deletions were recircularized by ligating linearized DNA samples in 1 × ligation buffer, 5% PEG, and 0.3 U of T4 DNA Ligase. Appropriately sized deletions were used to transform competent JM109 bacteria to select clones with deletions of interest.

Site-directed mutagenesis

Site-directed mutants were obtained using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's protocol. Briefly, two complementary oligonucleotides containing the desired mutations were synthesized and PAGE-purified for the mutation reactions. The mutant oligonucleotides for the SF-1 binding sites were:

5′-GCAATCATTCCATCCTCGACCCTCTGC-3′ SFB-1 (knockout)
5′-CTGCCCCCTCCCAAATTGGTCAGCACTGC-3′ SFB-2 (knockout)
5′-CTCCCACCTTGACTAGCACTGCAGTATGAG-3′ SFB-2 (low to high)

Ten nanograms of the double-stranded DNA template was incubated with 125 ng of the appropriate primer and 1 μl of dNTPs in 50 μl of reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, pH 8.8, 20 mM $MgSO_4$, 1% Triton X-100, and 1 mg/ml nuclease-free 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 sec followed by 35 cycles of denaturation at 95 C for 30 sec, 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 DpnI for 1 h at 37 C to get rid of the parental DNA template. One microliter of the mutant samples was used to transform XL-1 Blues, and the resulting mutations were verified by sequencing.

Data analysis

Data from these individual parameters were compared by ANOVA followed by Student-Newman-Keuls multiple comparison test when applicable (32). All analysis was completed using the Statview program with graphics (Abacus Concepts, Berkeley, CA) on a Macintosh Performa 6400/200 (Macintosh, Apple Computer, Inc., Cupertino, CA). A $P < 0.05$ was considered significant for all tests.

Results

cAMP Regulation of StAR mRNA

LH, or hCG, exerts its effects by binding to its cognate G protein-coupled receptor, thereby activating adenylate cyclase, which results in an increase in intracellular cAMP levels (33–36). To further characterize the mechanism by which hCG causes an increase in steady-state StAR mRNA levels, dispersed luteal cells were untreated or treated with increasing concentrations of dbcAMP for 24 h. Total RNA and protein were isolated and subjected to Northern and Western blot analyses, respectively. The results of these analyses indicated that stimulation of luteal cells with 0.1 and 1.0 mM dbcAMP caused a significant increase in both of the major StAR transcripts as well as StAR protein levels after 24 h of dbcAMP treatment (Fig. 1). Stimulation of cultured luteal cells with levels of dbcAMP below 0.1 mM failed to stimulate a significant change in expression of the StAR gene.

Similar luteal cell studies were performed to determine the time course of cAMP-stimulated StAR gene expression at the mRNA and protein levels *in vitro*. Dispersed luteal cells were treated with or without dbcAMP (1 mM) for 6, 12, and 24 h. Northern and Western blot analyses indicated that by 6 h, StAR mRNA transcripts and StAR protein levels were significantly increased relative to unstimulated luteal cells (Fig. 2). The maximal levels of StAR mRNA and protein expression were seen 24 h following dbcAMP administration.

Rat StAR promoter isolation and characterization

To examine the molecular mechanisms that mediate the actions of gonadotropins to positively regulate StAR gene expression at the transcriptional level, the promoter of the rat StAR gene was isolated, sequenced, and examined for potential regulatory elements. For this, five separate adaptor-ligated rat genomic libraries were screened using StAR-specific and adaptor-specific PCR primers in nested-PCRs. This technique yielded genomic DNA fragments of 0.4, 2.7, and 4.0 kb (Fig. 3). From these fragments, approximately 3 kb of

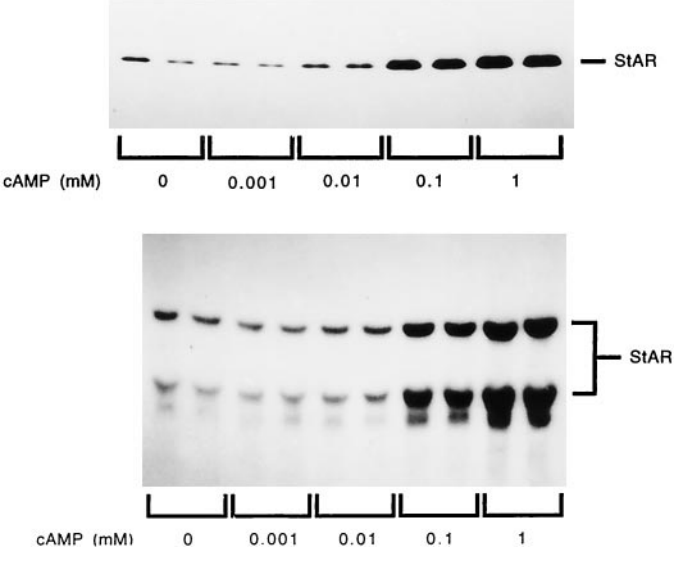

FIG. 1. Dose response of cAMP-stimulated StAR protein and mRNA expression in rat luteal cells. Dispersed luteal cells were untreated (0) or treated with increasing concentrations of dbcAMP (0.001, 0.01, 0.1, and 1 mM) in culture for 24 h. The *top panel* indicates StAR protein levels as detected by Western blot analysis, while the *bottom panel* indicates StAR mRNA transcripts (3.4 and 1.6 kb) as detected by Northern blot analysis following dbcAMP administration.

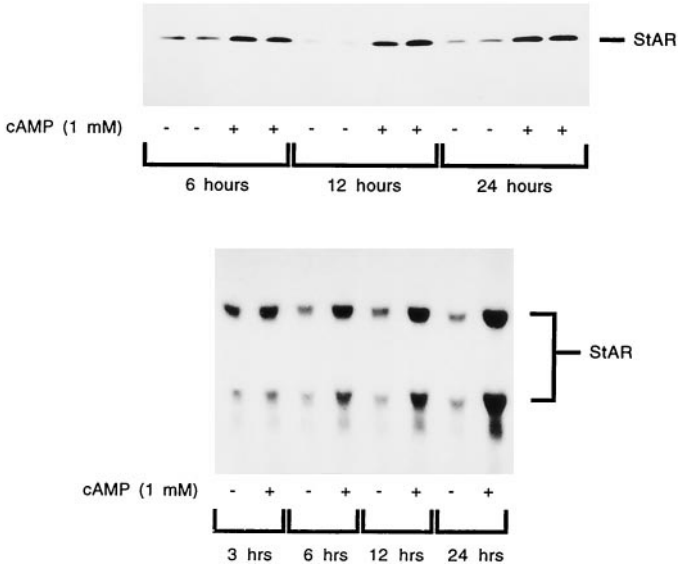

FIG. 2. Time course of stimulation of StAR protein and mRNA expression by cAMP in rat luteal cells. Dispersed luteal cells were treated with or without dbcAMP (1 mM) for 6, 12, and 24 h in culture. The *top panel* indicates StAR protein levels as detected by Western blot analysis, whereas the *bottom panel* indicates StAR mRNA transcripts (3.4 and 1.6 kb) as detected by Northern blot analysis at 6, 12, and 24 h after dbcAMP administration.

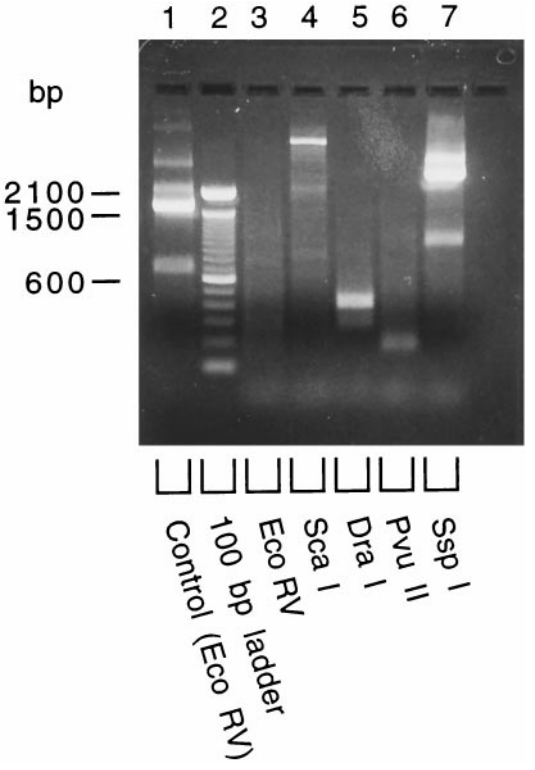

FIG. 3. Isolation of the rat StAR promoter by screening rat genomic libraries using PCR techniques. Adaptor-ligated rat genomic DNA libraries were screened using StAR gene-specific and adaptor-specific primers as outlined in the *Materials and Methods* section. Analysis of nested-PCR products was performed by 1.2% agarose/EtBr gel electrophoresis. Nested-PCR yielded StAR-specific genomic DNA products of 0.4, 2.7, and 4.0 kb (lanes 5, 7, and 4, respectively).

DNA flanking the StAR initiation codon was sequenced using the dideoxy chain termination method (37). Sequence analysis revealed a TATA-like element at bp −113 relative to the StAR initiation codon (Fig. 4).

To determine the start site for transcription of the StAR gene, 5′-RACE analysis was performed. PCRs of the 5′-untranslated region of the rat StAR gene yielded the expected product of approximately 200 bp (data not shown). The PCR product was then cloned into the TA vector and sequenced. Sequence analysis demonstrated that the transcription start site was located 82 bp upstream of the translational start codon (ATG) of the rat StAR gene. This site is 27 bp downstream of the TATA-like element which may aid in transcriptional activation of this gene.

Further promoter sequence analysis using the MacVector sequence analysis program revealed the presence of multiple regulatory elements similar to the consensus sequences for the SF-1 binding site (CCTTG), the estrogen receptor half-site (AGGTCA), and the AP-1 site (GTCGTCA). These regulatory elements included five putative SF-1 binding sites at positions −764 to −754, −493 to −483, −455 to −445, −143 to −132, and −106 to −97, a putative estrogen receptor half-site at position −137 to −132, a putative SP1 site at position −344 to −339, and two putative AP-1 elements at positions −1561 to −1555 and −187 to −181 (Fig. 4). Although the rat StAR gene is regulated by cAMP, sequence analysis of the rat StAR promoter for the presence of cAMP response elements (CRE) did not reveal any regulatory motifs that resembled the classical CRE.

To demonstrate that the steroidogenic tissue-specific transcription factor, SF-1, could recognize and bind to the SF-1 elements identified within the rat StAR promoter, mobility shift assays using oligonucleotides corresponding to these sites were performed with and without the partially purified DNA-binding domain of SF-1. The DNA-binding domain of SF-1 was kindly provided as a GST fusion protein in pGEX-1λT by Keith L. Parker (38). The rat aromatase promoter contains an SF-1 binding site that activates transcription of the aromatase gene. This element (designated RA) has been demonstrated to bind SF-1 (38) and was used as a positive control for binding by the GST-SF-1 fusion protein in lanes 1–3 (Fig. 5). Lanes 1 and 2 contain the RA probe incubated alone and with the GST-SF-1 fusion protein, respectively. The incubation reaction in lane 3 contains SF-1-specific polyclonal antisera, which supershifts the SF-1-containing protein complex. The incubation reactions in lanes 4–6 contain the probe corresponding to the low affinity SF-1 binding site located at position −143 to −132 in the rat StAR promoter. The weak complex formed in lane 4 is competed out by the addition of excess unlabeled oligonucleotide competitor. The incubation reactions for lanes 7–9 contain a probe corresponding to the high affinity SF-1 binding site located at position −106 to −97. The complex formed in lane 7 is competed out with excess unlabeled oligonucleotide (lane 8), and complex formation is diminished in the presence of SF-1-specific antisera (lane 9). Similar experiments were performed using all five SF-1 binding sites identified within the rat StAR promoter. The three high affinity sites are shown in Fig. 6. These sites have been designated SF-1 binding sites 1,

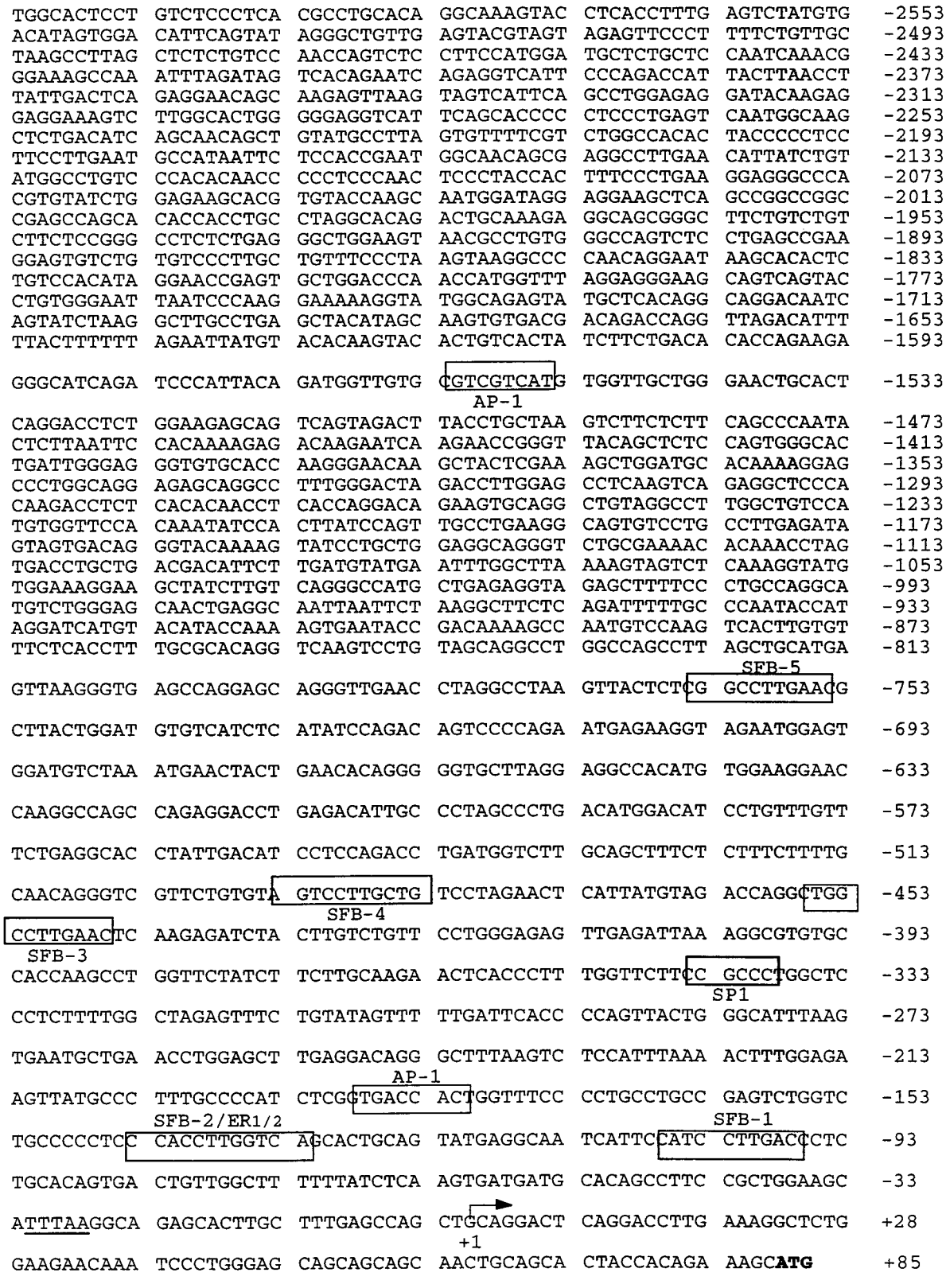

FIG. 4. Sequence analysis of the 5′-flanking region of the rat StAR gene. Approximately 2.5 kb of DNA from the promoter region immediately upstream of the coding region was sequenced and analyzed for regulatory motifs. The transcription start site, determined by 5′-RACE analysis is denoted with an *arrow*. A TATA-like element was identified 31 bp upstream of the StAR transcription start site. Sequence analysis revealed multiple putative SF-1 binding sites at positions −764, −493, −455, −143, and −106, an estrogen receptor half-site at position −137, an SP1 site at position −344, and two AP-1 sites at positions −1561 and −187.

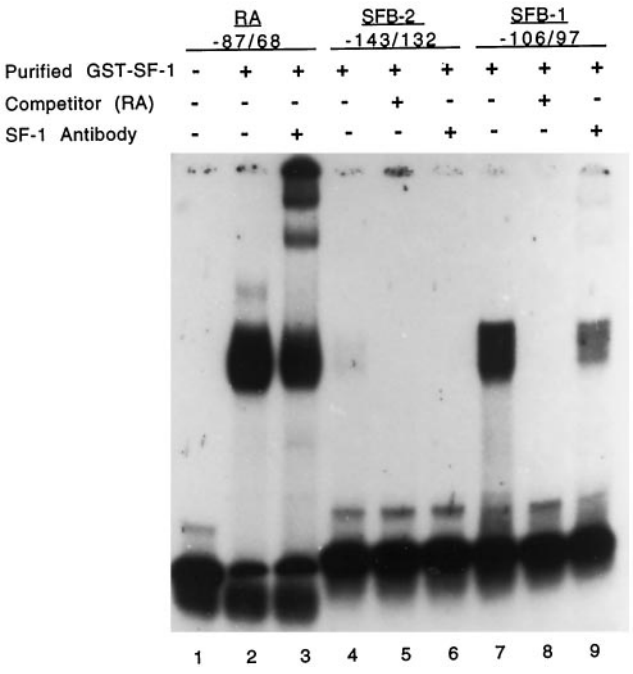

FIG. 5. Electrophoretic mobility shift analysis of SF-1 binding to putative SF-1 binding sites within the rat StAR promoter. Purified GST-SF-1 protein was incubated with labeled double-stranded oligonucleotide probes (see *Materials and Methods*) in the presence or absence of excess unlabeled competitor (RA–rat aromatase oligonucleotide, GGGGTCTCCCAAGGTCATCCTTG) or SF-1-specific antisera as indicated. EMSA results demonstrated SF-1 binding to both the low and high affinity SF-1 site (low −143, SFB-2; high −106, SFB-1). Specific complexes were supershifted (lane 3) and abrogated (lanes 6 and 9) upon addition of SF-1-specific polyclonal antisera.

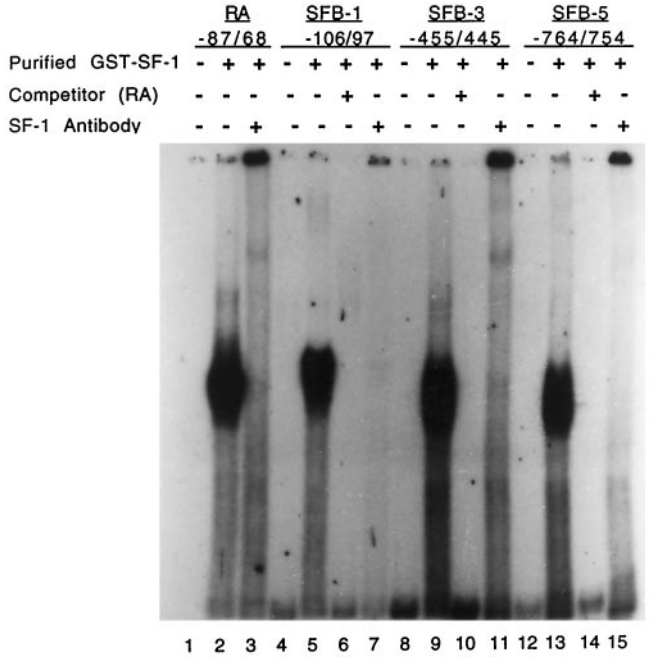

FIG. 6. Electrophoretic mobility shift analysis of SF-1 binding to the high affinity SF-1 binding sites within the rat StAR promoter. Purified GST-SF-1 protein was incubated with labeled oligonucleotide probes (see *Materials and Methods*) in the presence or absence of excess unlabeled RA competitor oligonucleotides or SF-1-specific antisera as indicated. EMSA results demonstrated SF-1 binding to three high affinity SF-1 sites (−106, SFB-1; −455, SFB-3; −764, SFB-5) within the rat StAR promoter. Specific complexes were competed out with the addition of excess unlabeled oligonucleotide (lanes 6, 10, and 14) and complex formation was abrogated upon addition of SF-1-specific polyclonal antisera (lanes 7, 11, and 15).

3, and 5 (SFB-1, SFB-3, and SFB-5). The low affinity SF-1 binding sites (SFB-2 and SFB-4) were bound by the purified GST-SF-1 protein but complexes formed demonstrated relatively low affinity of SF-1 for these two regulatory motifs (Fig. 5 and data not shown).

To further characterize the functional role these putative SF-1 sites play in regulating expression of the StAR gene, approximately 2 kb of the rat StAR promoter was linked to a luciferase reporter gene (pGL3-Basic), creating pGL3-StAR. Transfection of mouse Y1 adrenal tumor cells with pGL3-StAR resulted in a dramatic (90-fold) increase in transcription of the reporter gene compared with the cells transfected with the empty pGL3-Basic vector (Fig. 7A). Stimulation of the cells with dbcAMP resulted in an additional 2-fold stimulation of luciferase activity. To determine whether this activation of transcription was specific for steroidogenic cells, similar transfection studies were carried out using the nonsteroidogenic human bladder carcinoma cell line (HTB9). Luciferase assay results demonstrated that in a nonsteroidogenic cell line, transfection of the StAR promoter-driven luciferase reporter gene had no effect on luciferase activity. However, cotransfection of the SF-1 cDNA in an expression plasmid in the correct orientation (cSF-1) with the 2 kb rat StAR promoter construct could activate transcription of the luciferase reporter gene over 100-fold relative to the promoter construct alone (Fig. 7B). Stimulation of the cells with dbcAMP resulted in a 3-fold further increase in luciferase activity. This activation of transcription was mediated by SF-1 activity because cotransfection of the SF-1 cDNA in the reverse orientation (rSF-1) in parallel cultures did not result in any changes in luciferase activity, both in the unstimulated and cAMP-treated cultures. These data suggest that SF-1 alone was sufficient to drive transcription of the luciferase reporter gene *in vitro*. This study also demonstrated that SF-1 was capable of conferring cAMP-responsiveness to the rat StAR promoter because cAMP administration further enhanced luciferase expression in the cSF-1 transfected cells and had no effect on the rSF-1 transfected, cAMP-treated cultures.

To determine which SF-1 sites were important for the induction of StAR gene expression, nested deletions of the rat StAR promoter (2 kb) were generated and linked to the luciferase reporter gene. Transfection of mouse Y1 adrenal tumor cells with the various deletions of the rat StAR promoter demonstrated that two of the proximal high affinity SF-1 binding motifs, designated SFB-1 and SFB-5, are critical for maximal activation of transcription of the rat StAR gene (Fig. 8A). Deletion of SF-1 binding site 5 reduced basal transcriptional activation of the reporter gene to 60% of control levels. Cotransfection of the various deletions with the SF-1 cDNA into the nonsteroidogenic cell line HTB9, however, demonstrated that maximal levels of basal StAR expression were only obtained when the region between −1413 and −998 was present within the promoter (Fig. 8B). Interest-

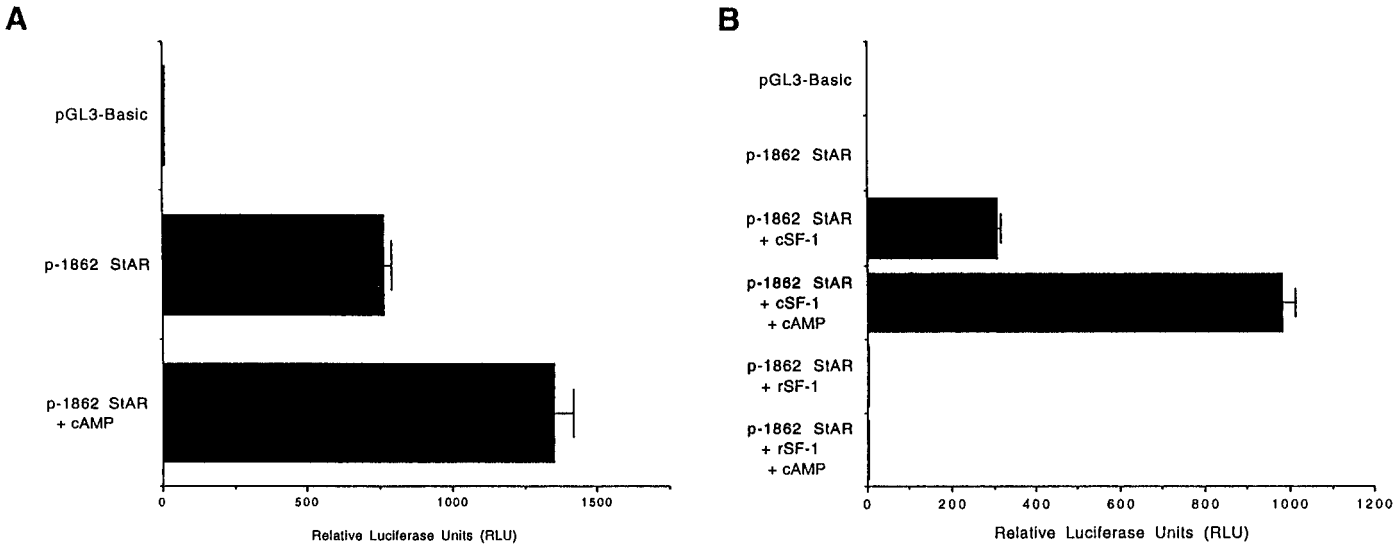

FIG. 7. StAR promoter-driven luciferase activity in Y1 adrenal (A) and human bladder carcinoma (B) cells expressing SF-1 ± cAMP. The full-length rat StAR promoter (2 kb) linked to a luciferase reporter gene (p-1862 StAR) was transfected into Y1 adrenal cells (A) or HTB9 cells (B) treated with or without dbcAMP (1 mM) for 24 h. HTB9 cells (nonsteroidogenic cells) were cotransfected with the SF-1 cDNA in either the correct (c) or reverse (r) orientation. Luciferase activities in cell lysates prepared 48 h after transfection were determined and normalized to the Renilla luciferase activity, which contains the SV40 early enhancer/promoter region driving luciferase expression. Luciferase activity was measured in triplicate and the results presented.

ingly, cAMP responsiveness was not adversely affected by deletion of this region.

To determine whether the luciferase activity obtained with the smallest promoter fragment (p-342 StAR) was due to the SF-1 sites within the p-342 StAR deletion, site-directed mutagenesis was used to knockout the high affinity site, the low affinity site, both SF-1 sites, or to transform the low affinity SF-1 site into a high affinity SF-1 binding site. The first mutation obtained was a T→C mutation in the core SF-1 binding motif (CCTTG→CCTCG) of the high affinity SF-1 binding site, creating p-342 StAR M1. The results of the luciferase activity in the HTB9 cells with and without cotransfection of the SF-1 expression plasmid are shown in Fig. 9. Luciferase activity in cells transfected with the wild-type 450 bp rat StAR promoter (p-342 StAR) increased 13-fold with the cotransfection of the SF-1 expression plasmid. Luciferase activity was further stimulated 16-fold upon addition of dbcAMP. A single base change in the core region of the proposed SF-1 binding site reduced SF-1 transcriptional activation to 26% and 22% of wild-type luciferase levels in unstimulated and cAMP-treated cultures, respectively.

To determine whether the low affinity SF-1 binding site was responsible for activating transcription of the reporter gene above background levels in the high affinity site mutant, site-directed mutagenesis was used to obtain a low affinity site mutant (p-342 StAR M2), a double SF-1 site mutant (p-342 StAR M1/2), and a mutated low to high affinity SF-1 binding site (p-342 StAR M3). The results using the low affinity site mutation (M2) demonstrated a reduction in basal and cAMP-stimulated luciferase levels to 66% and 54% of wild-type levels, respectively, which is consistent with the electrophoretic mobility shift assays, further supporting the low affinity of SF-1 binding and activation of transcription through this site (SFB-2). To determine if mutating both SF-1 sites, high and low affinity, could completely abolish SF-1 activation of gene expression, a mutant with both sites knocked out was obtained and used in this system. The results of this experiment support this hypothesis. Luciferase levels in the unstimulated cultures were reduced to less than the unstimulated, non-SF-1 transfected control levels. The dbcAMP-stimulated luciferase levels were increased compared with the unstimulated cells but still were reduced to less than 10% of appropriate control levels. The last mutant used in these studies had the low affinity SF-1 binding site (SFB-2) changed to a high affinity SF-1 binding site (CCACCTTGGTCA→ CCACCTTGACTA; M3). The results using mutant M3 demonstrated that the presence of two high affinity SF-1 sites in close proximity can additively affect transcription. The cells transfected with M3 alone had background levels of luciferase activity. When the SF-1 expression plasmid was cotransfected into these cells, luciferase activity increased 12-fold. Further stimulation of the cells with dbcAMP caused luciferase activity to increase an additional 10-fold. Both basal and cAMP-stimulated cultures cotransfected with the M3 mutation expressed luciferase levels that were 2-fold higher than the wild-type promoter-driven luciferase gene (Fig. 9).

Discussion

These studies have demonstrated that the rat StAR promoter is responsive to stimulation by cAMP and that this activation by cAMP involves SF-1. This is consistent with the mechanism of gonadotropin stimulation, which acts through activation of adenylate cyclase. Our studies have shown that StAR mRNA and protein levels are increased in response to cAMP stimulation in a time- and dose-dependent manner.

In the past 3 yr, the mouse, rat, and human StAR genes have been isolated and sequenced. All three genes are comprised of seven exons and six introns that span 6.5, 7, and 8

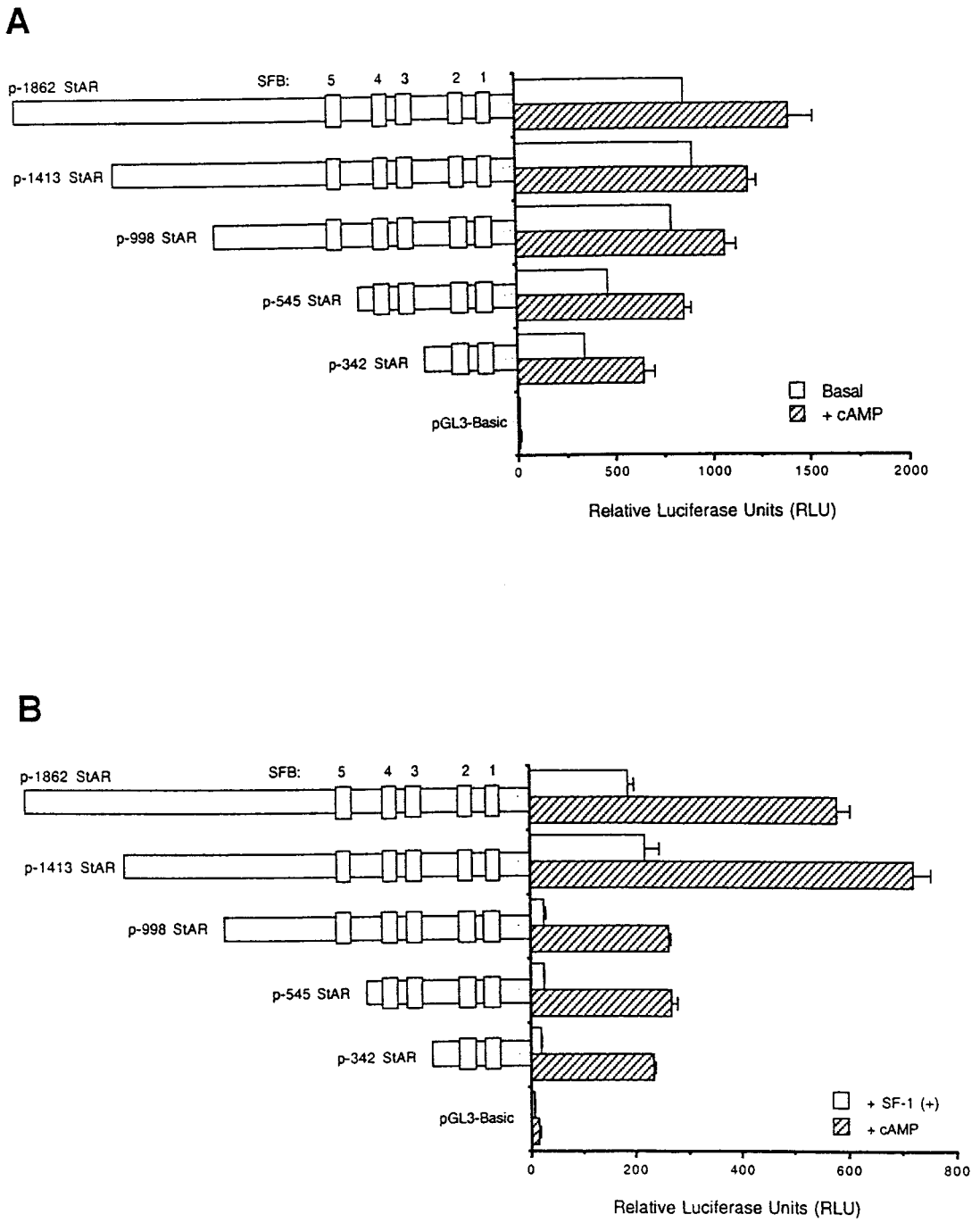

FIG. 8. Deleted StAR promoter-driven luciferase activity in Y1 adrenal cells (A) and HTB9 bladder cells (B) expressing SF-1 ± cAMP. Various deletions of the rat StAR promoter (p-1862 StAR, p-1413 StAR, p-998 StAR, p-545 StAR, and p-342 StAR) were transfected into Y1 adrenal cells (A) or HTB9 bladder cells (B) treated with or without dbcAMP (1 mM) for 24 h. HTB9 cells (nonsteroidogenic cells) were cotransfected with the SF-1 cDNA in the correct (c) orientation. Luciferase activities in cell lysates prepared 48 h after transfection were determined and normalized to the Renilla luciferase activity, which contains the SV40 early enhancer/promoter region driving luciferase expression. Luciferase activity was measured in triplicate and the results presented.

kb for the mouse, rat, and human genes, respectively (39–41). The transcription start site for the rat StAR gene, as determined by 5′-RACE analysis, is similarly located within the promoter region as that determined for the mouse and human transcription start sites (25, 41). The rat, mouse, and human promoter regions, unlike many genes expressed in a highly regulated fashion, lack the canonical TATA box. While StAR transcription is positively regulated in response to cAMP stimulation, the rat, mouse, and human StAR promoters all lack a classical CRE. Thus, the mechanism whereby StAR transcription is activated by gonadotropins via cAMP has only recently been determined to be due in part

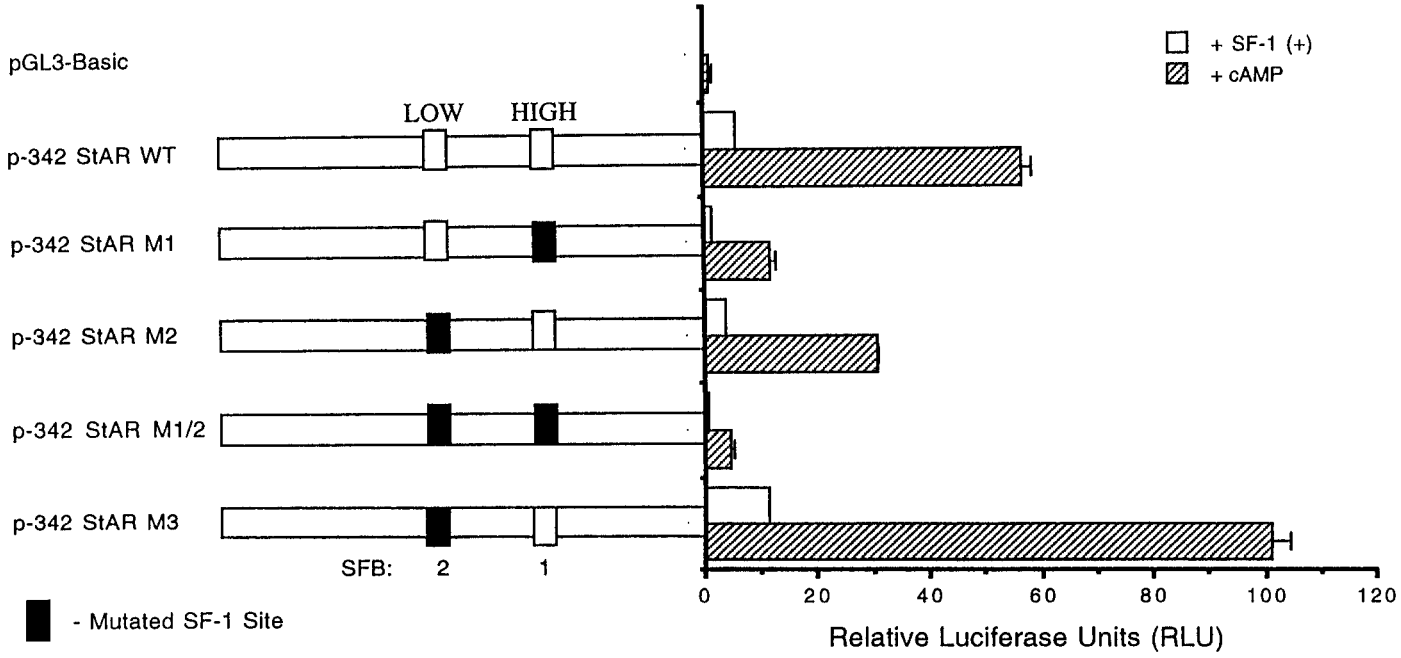

FIG. 9. Luciferase activity of various p-342 StAR mutants in HTB9 cells expressing SF-1 ± cAMP. The minimal rat StAR promoter (p-342 StAR) and the various rat StAR promoter mutants (p-342 StAR-M1, M2, M1/2, and M3) were linked to the luciferase reporter gene and cotransfected with the SF-1 cDNA into HTB9 cells treated plus or minus dbcAMP (1 mM) for 24 h. Luciferase activities in cell lysates prepared 48 h after transfection were determined and normalized to the Renilla luciferase activity, which contains the SV40 early enhancer/promoter region driving luciferase expression. Luciferase activity was measured in triplicate and the results presented.

to SF-1 activation. The presence of AP-1 sites, which traditionally recruit cJun/cFos heterodimers to positively regulate transcription is currently being characterized in our laboratory.

SF-1 was first shown to be an essential regulator of the cytochrome P450 steroid hydroxylase gene (21–23) and was subsequently linked to the expression of the gene encoding Müllerian-inhibiting substance (42, 43). SF-1 is an orphan member of the nuclear receptor superfamily and is thought to bind to the consensus sequence PyCAAGGPyCPu (38). This study and studies using the human StAR promoter (26) have demonstrated that SF-1 binds to regulatory elements containing a CCTTG motif, which is the complement of the SF-1 consensus sequence shown above. SF-1 is thought to activate transcription of its target genes upon binding these sequence motifs within the promoter regions. Analysis of the StAR promoter region in the rat, mouse, and human has revealed the presence of multiple SF-1 binding sites. Two sequence motifs that match the known requirements for binding of SF-1 were found in the mouse promoter region at positions −135 to −128 (CCACCTTGG) and at −46 to −42 relative to the transcriptional start site (25). While approximately 3 kb of the 5′-flanking region of the mouse StAR gene was sequenced and analyzed for DNA regulatory motifs, only two SF-1 binding sites were found, both of which were located within the first 200 bp of the promoter. The primary site (−135) was able to bind SF-1 by mobility shift analysis, and mutation of this site was able to decrease basal levels of transcription, but neither site influenced cAMP stimulation of transcription. The human StAR promoter contained three SF-1 binding sites at positions −926 to −918 (TGACCTTGA), −105 to −95, and −42 to −35 relative to the transcription start site (26). Mutation of the distal or proximal *cis*-elements substantially reduced SF-1-supported StAR promoter activity. In the rat StAR promoter, however, five potential SF-1 sites have been identified within the first kb of the transcription start site. Three sites exhibit high affinity for SF-1 binding and two sites exhibit relatively low affinity; however, all sites seem to be required for maximal activation of rat StAR gene transcription.

These studies have also demonstrated that SF-1 is involved in cAMP responsiveness of the rat StAR promoter using cotransfection studies and luciferase assays. Studies in a nonsteroidogenic cell line have demonstrated that SF-1 alone was capable of stimulating transcription of the reporter gene. The rat StAR promoter was unable to activate transcription of the reporter gene in the nonsteroidogenic human bladder carcinoma cell line HTB9. Cotransfection of the SF-1 cDNA in the correct orientation (cSF-1) activated transcription of the reporter gene over 100-fold. Cotransfection of the SF-1 cDNA in the reverse orientation (rSF-1) had no effect on luciferase activity, which remained at background levels. Cotransfection of the correct and reverse SF-1 cDNAs also demonstrated the fact that SF-1 is involved with cAMP-responsiveness of the rat StAR promoter. dbcAMP administration to cells transfected with the cSF-1 cDNA resulted in increased luciferase activity compared with unstimulated cSF-1 transfected controls. However, luciferase activity in cells transfected with the rSF-1 cDNA and stimulated with cAMP remained at background levels indicating that SF-1 was required to mount a transcriptional response.

There is the possibility that ubiquitous transcription factors necessary for assembling the transcriptional machinery are involved with this transcriptional activation. Recent stud-

ies have demonstrated that SP1 and SF-1 can interact *in vivo* and direct regulation of a CYP11A promoter-linked luciferase reporter gene in a cooperative manner (44). Consistent with this hypothesis, an SP1 site was identified within the rat StAR promoter at position −344 to −339. Further studies are required to characterize the role SP1 may play in regulating StAR gene expression.

Promoter deletion studies and site-directed mutagenesis were used to determine which SF-1 binding sites within the StAR promoter region were important for transcriptional activation. Analysis of the rat StAR promoter region (2 kb) identified three high affinity SF-1 binding sites as determined by electrophoretic mobility shift analysis. The promoter was then subjected to deletion analysis to yield promoter fragments with one, two, or all three high affinity SF-1 binding sites to determine which sites were critical for StAR gene transcription. Deletion experiments in Y1 adrenal tumor cells demonstrated that as each high affinity SF-1 site was deleted, luciferase levels were decreased. Minor differences in the rat StAR promoter deletion data between the HTB9 cells and the Y1 adrenal tumor cells stem from the fact that the HTB9 cells are a nonsteroidogenic cell line and lack steroidogenic tissue-specific factors necessary for transcriptional activation of the reporter gene. On the other hand, the Y1 cells are a steroidogenic cell line that synthesize large amounts of steroids and possess the necessary proteins required for regulation of genes encoding proteins involved in steroidogenesis.

To determine whether the luciferase activity generated by the smallest deletion (p-342 StAR) was due to the presence of SF-1 sites, the p-342 StAR promoter fragment was then subjected to site-directed mutagenesis to further characterize SF-1 interaction with and regulation of the minimal rat StAR promoter. The wild-type p-342 StAR promoter construct activated luciferase gene expression one-tenth as much as the full-length StAR promoter. This data suggests that multiple SF-1 sites within the StAR promoter may act cooperatively to regulate transcription of the StAR gene. The p-342 StAR M1 mutant with the high affinity SF-1 binding site altered by a single base change in the core region of the proposed SF-1 binding site, reduced SF-1 transcriptional activation to approximately one quarter of wild-type luciferase levels in unstimulated and dbcAMP-treated cultures, respectively. This study reflects the importance of the high affinity SF-1 binding site in the regulation of the StAR gene, however, the mutation of the single high affinity site does not completely inhibit reporter gene expression. This may be due to the presence of a low affinity SF-1 binding site, which has been demonstrated to bind SF-1.

When the low affinity SF-1 binding site alone was mutated, luciferase levels fell significantly, but not as dramatically as the high affinity SF-1 binding site mutant. The fact that mutating the low affinity SF-1 binding site resulted in decreased luciferase activity suggests that this low affinity SF-1 site is important for maximal regulation of the StAR gene at the transcriptional level. To determine if these two SF-1 sites were completely responsible for the increase in reporter gene expression, both sites were knocked out by site-directed mutagenesis. The luciferase levels in the unstimulated cultures were reduced to control levels. The cAMP-stimulated luciferase levels were not increased significantly compared with the unstimulated cells and were reduced to less than 10% of dbcAMP-stimulated p-342 StAR wild-type control levels.

To determine whether the SF-1 transcriptional response might be enhanced by changing the low affinity SF-1 binding site into a high affinity SF-1 site, we altered the low affinity site to more closely resemble the SF-1 consensus sequence for binding. The presence of two high affinity SF-1 sites in close proximity additively affected transcription. The fact that both basal and cAMP-stimulated cultures cotransfected with the M3 mutation expressed luciferase levels that were twice as high as the wild-type promoter-driven luciferase gene, sheds some insight into the binding characteristics of SF-1. All five SF-1 elements within the rat StAR promoter share the core CCTTG nucleotides; however, the three high affinity sites and the mutated low affinity site all contain the additional adenosine residue following the guanosine residue (CCTTGA).

The results of this investigation indicate that cAMP administration to rat luteal cells enhances expression of rat StAR mRNA and protein levels in a time and dose-dependent manner. Furthermore this study indicates that SF-1 binds to five regulatory motifs within the rat StAR promoter and activates StAR gene transcription at a basal level and in response to cAMP administration. These studies are the first to demonstrate that the rat StAR promoter is regulated by SF-1 and that SF-1 alone confers cAMP-responsiveness to the rat StAR promoter.

Acknowledgments

The DNA-binding domain of SF-1 was kindly provided as a GST fusion protein in pGEX-1λT by Keith L. Parker at the University of Texas, Southwestern Medical School, Dallas, TX (38). Dr. Parker also provided the full-length SF-1 cDNA in the pCMV expression plasmid in both the correct (cSF-1) and the reverse (rSF-1) orientation (22). We acknowledge Dr. Xia Liu for her technical assistance.

References

1. **Iida S, Papadopoulos V, Hall PF** 1989 The influence of exogenous free cholesterol on steroid synthesis in cultured adrenal cells. Endocrinology 124:2619–2624
2. **Jefcoate CR, DiBartolomeos MJ, Williams CA, McNamara BC** 1987 ACTH regulation of cholesterol movement in isolated adrenal cells. J Steroid Biochem 27:721–729
3. **Lambeth JD, Xu XX, Glover M** 1987 Cholesterol sulfate inhibits adrenal mitochondrial cholesterol side chain cleavage at a site distinct from cytochrome P-450scc. Evidence for an intramitochondrial cholesterol translocator. J Biol Chem 262:9181–9188
4. **Privalle CT, Crivello JF, Jefcoate CR** 1983 Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. Proc Natl Acad Sci USA 80:702–706
5. **Privalle CT, McNamara BC, Dhariwal MS, Jefcoate CR** 1983 ACTH control of cholesterol side-chain cleavage at adrenal mitochondrial cytochrome P-450scc. Regulation of intramitochondrial cholesterol transfer. Mol Cell Endocrinol 53:87–101
6. **Stocco DM** 1992 Further evidence that the mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are involved in the acute regulation of steroidogenesis. J Steroid Biochem Mol Biol 43:319–333
7. **Stocco DM, Ascoli M** 1993 The use of genetic manipulation of MA-10 Leydig tumor cells to demonstrate the role of mitochondrial proteins in the acute regulation of steroidogenesis. Endocrinology 132:959–967
8. **Stocco DM, Chen W** 1991 Presence of identical mitochondrial proteins in unstimulated constitutive steroid-producing R2C rat Leydig tumor and stimulated nonconstitutive steroid-producing MA-10 mouse Leydig tumor cells. Endocrinology 128:1918–1926
9. **Stocco DM, Clark BJ** 1993 The requirement of phosphorylation on a threonine residue in the acute regulation of steroidogenesis in MA-10 mouse Leydig tumor cells. J Steroid Biochem Mol Biol 46:337–347
10. **Stocco DM, Sodeman TC** 1991 The 30-kDa mitochondrial proteins induced by

hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. J Biol Chem 266:19731–19738
11. **Stocco DM, Kilgore MW** 1988 Induction of mitochondrial proteins in MA-10 Leydig tumor cells with human chorionic gonadotropin. Biochem J 249:95–103
12. **Epstein LF, Orme-Johnson NR** 1991 Regulation of steroid hormone biosynthesis. Identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells. J Biol Chem 266:19739–19745
13. **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. J Biol Chem 269:28314–28322
14. **Lin D, Sugawara T, Strauss III JF, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL** 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267:1828–1831
15. **Sugawara T, Holt JA, Driscoll D, Strauss III JF, Lin D, Miller WL, Patterson D, Clancy KP, Hart IM, Clark BJ, Stocco DM** 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. Proc Natl Acad Sci USA 92:4778–4782
16. **Garren LD, Ney RL, Davis WW** 1965 Studies on the role of protein synthesis in the regulation of corticosterone production by ACTH *in vivo*. Proc Natl Acad Sci USA 53:1443–1450
17. **Green EG, Orme-Johnson NR** 1991 Inhibition of steroidogenesis in rat adrenal cortex cells by a threonine analogue. J Steroid Biochem Mol Biol 40:421–429
18. **Ferguson JJ, Morita Y** 1964 RNA synthesis and adrenocorticotropin responsiveness. Biochim Biophys Acta 87:348–350
19. **Vernikos-Danellis J, Hall M** 1965 Inhibition of adrenocortical responsiveness to ACTH by actinomycin D *in vivo*. Nature 207:766
20. **Clark BJ, Combs R, Hales KH, Hales DB, Stocco DM** 1997 Inhibition of transcription affects the synthesis of the steroidogenic acute regulatory protein (StAR) and steroidogenesis in MA-10 mouse Leydig tumor cells. Endocrinology 138:4893–4901
21. **Morohash K, Honda S, Inomata Y, Handa H, Omura T** 1992 A common trans-acting factor, Ad4-binding protein, binds to the promoters of steroidogenic P450s. J Biol Chem 267:17913–17919
22. **Ikeda Y, Lala DS, Luo Kim E, Moisan M-P, Parker KL** 1993 Characterization of the mouse FTZ-F1 gene, which encodes an essential regulator of steroid hydroxylase gene expression. Mol Endocrinol 7:852–860
23. **Honda S-I, Morohash K-I, Nomura M, Takeya H, Kitajima M, Omura T** 1993 AD4BP regulating steroidogenic P450 gene is a member of steroid hormone receptor superfamily. J Biol Chem 268:7494–7502
24. **Parker KL, Schimmer BP** 1997 Steroidogenic factor 1:A key determinant of endocrine development and function. Endocr Rev 18:361–377
25. **Caron KM, Ikeda Y, Soo S-C, Stocco DM, Parker KL, Clark BJ** 1997 Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. Mol Endocrinol 11:138–147
26. **Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F, Strauss III JF** 1997 Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor 1. Steroids 62:5–9
27. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
28. **Sandhoff TW, McLean MP** 1996 Hormonal regulation of steroidogenic acute regulatory (StAR) protein messenger ribonucleic acid expression in the rat ovary. Endocrine 4:259–267
29. **McLean MP, Nelson S, Billheimer JT, Gibori G** 1992 Differential capacity for cholesterol transport and processing in large and small rat luteal cells. Endocrinology 131:2203–2212
30. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
31. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
32. **Zar JH** 1974 In: Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ, pp 101–127
33. **Rommerts FFG, Cooke BA, van der Molen HJ** 1974 The role of cyclic AMP in the regulation of steroid biosynthesis in testis tissue. J Steroid Biochem 5:279–285
34. **Hseuh AJW, Adashi EY, Jones PBC, Welsh TH** 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev 5:76–127
35. **Marsh JM** 1975 The role of cyclic AMP in gonadal function. Adv Cyclic Nucl Res 6:137–199
36. **Sala GB, Hayashi K, Catt KJ, Dufau ML** 1979 Adrenocorticotropin action in isolated adrenal cells. The intermediate role of cyclic AMP in stimulation of corticosterone synthesis. J Biol Chem 254:3861–3875
37. **Sanger F, Nicklen S, Coulson AR** 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
38. **Lynch JP, Lala DS, Peluso JJ, Luo W, Parker KL, White BA** 1993 Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. Mol Endocrinol 7:776–786
39. **Clark BJ, Soo S-C, Caron KM, Ikeda Y, Parker KL, Stocco DM** 1995 Hormonal and developmental regulation of the steroidogenic acute regulatory protein. Mol Endocrinol 9:1346–1355
40. **Kim Y-C, Ariyoshi N, Artemenko I, Elliot ME, Bhattacharyya KK, Jefcoate CR** 1997 Control of cholesterol access to cytochrome P450scc in rat adrenal cells mediated by regulation of the steroidogenic acute regulatory protein. Steroids 62:10–20
41. **Sugawara T, Lin D, Holt JA, Martin KO, Javitt NB, Miller WL, Strauss III JF** 1995 Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. Biochemistry 34:12506–12512
42. **Shen W-H, Moore CCD, Ikeda Y, Parker KL, Ingraham HA** 1994 Nuclear receptor steroidogenic factor 1 regulates MIS gene expression: a link to the sex determination cascade. Cell 77:651–661
43. **Ikeda Y, Luo X, Abbud R, Nilson JH, Parker KL** 1995 The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. Mol Endocrinol 9:478–486
44. **Liu Z, Simpson ER** 1997 Steroidogenic factor 1 (SF-1) and SP1 are required for regulation of bovine CYP11A gene expression in bovine luteal cells and adrenal Y1 cells. Mol Endocrinol 11:127–137