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Effects of Mutating Different Steroidogenic Factor-1 Protein Regions on Gene Regulation

Dayami Lopez,[1] Anna C. Nackley,[1] Wendy Shea-Eaton,[1] Jiaping Xue,[2] Bernard P. Schimmer,[2] and Mark P. McLean[1]

[1]Departments of Obstetrics and Gynecology and Biochemistry and Molecular Biology, University of South Florida, Tampa, FL; and [2]The Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

The involvement of cyclic adenosine monophosphate cAMP-dependent protein kinase A (PKA) in the regulation of the steroidogenic acute regulatory protein (StAR) and the high-density lipoprotein receptor (HDL-R) genes by steroidogenic factor-1 (SF-1) and cAMP were examined. Cotransfection studies carried out in Kin 8 cells, a Y1 cell line (mouse adrenal) with a mutation in the type I PKA regulatory subunit, demonstrated that an intact PKA is required for maximal activation and that SF-1 participates in cAMP regulation of these genes. Site-directed mutational analysis was performed to examine which SF-1 regions could be involved in SF-1 transcriptional activation of the StAR and HDL-R genes. SF-1 regions protein analyzed were amino acids Thr 60, Ser 203, Ser 431, Thr 462, and the activation function-2 domain (amino acids 449–462). Plasmids encoding each of the mutated SF-1 proteins were cotransfected with the StAR and HDL-R promoter constructs into human bladder carcinoma (HTB-9) cells in the presence or absence of dibutyryl cAMP. The results of these studies suggest that although SF-1 is required for optimal promoter response to cAMP, transcriptional activation of genes by SF-1 and cAMP are promoter dependent, perhaps resulting from gene-specific interactions of this transcription factor with other regulatory proteins.

Key Words: Steroidogenic acute regulatory protein; high-density lipoprotein receptor; steroidogenic factor-1; cyclic adenosine monophosphate.

Introduction

Steroidogenic factor-1 (SF-1) is a tissue-specific factor involved in sex determination, adrenal development, and development and function of the hypothalamic-pituitary-gonadal axis *(1–3)*. SF-1 also regulates the expression of many important genes along the axis including those involved in steroidogenesis *(4–23)*. Examples of genes regulated by SF-1 are the cholesterol side-chain cleavage cytochrome P450 gene *(4–7),* the bovine 11β-hydroxylase P450 gene *(5,8)*, the aromatase cytochrome P450 gene *(9–11),* the rat 17α-hydroxylase/c17,20 lyase gene *(12),* the mouse steroid 21-hydroxylase P450 gene (*Cyp*21) *(13–15),* the steroidogenic acute regulatory protein (StAR) *(16–21),* and the high-density lipoprotein receptor (HDL-R) *(22–23).*

One of the functions that has been ascribed to the SF-1 protein is the mediation of cyclic adenosine monophosphate (cAMP) induction of numerous genes *(4–7,9–21,23).* The production of cAMP in steroidogenic tissues results from the binding of hormones such as adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG) to their cognate G-protein-coupled receptors and the subsequent activation of adenylate cyclase *(24,25).* The exact mechanism by which SF-1 participates in induction of genes by cAMP is not completely understood. However, a common element to this regulatory process appears to be the requirement for a functional cAMP-dependent protein kinase A (PKA) *(12,13,23,26–28).* It has been previously shown that PKA is able to phosphorylate SF-1 in vitro on serine (Ser) and threonine (Thr) residues (at a ratio of seven serines per threonine residue) *(12)*, even though sequence analysis of the SF-1 protein revealed the presence of a single potential PKA phosphorylation site at Ser 431 *(8).* In addition, a phosphorylated form of SF-1 can be detected in rat granulosa cells after treatment with FSH *(11).* Nevertheless, there is no evidence that this phosphorylated SF-1 form was produced by PKA *(11).* Whether PKA can directly phosphorylate SF-1 in vivo and whether phosphorylation directly affects transactivation of the SF-1 protein remains unclear. The recent finding showing that maximal SF-1 activity requires phosphorylation of the SF-1 protein at Ser 203 by the mitogen-activated protein kinase (MAPK) suggests that direct phosphorylation of SF-1 by PKA could be a possible mechanism through which SF-1 mediates cAMP regulation of genes *(29).*

In the present study, the effects of mutating potential phosphorylation sites within SF-1 (Thr 60, Ser 203, Ser 431,

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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 33606. E-mail: mmclean@com1.med.usf.edu

and Thr 462) and deleting the activation function-2 (AF-2) domain (amino acids 449–462) on SF-1 transcriptional activity of the rat StAR and HDL-R genes, which are known to be regulated by SF-1 and cAMP *(16–23)*, were examined. The consequences of mutation of the various SF-1 protein regions on transcriptional activation of the rat StAR and HDL-R genes by SF-1 and cAMP are promoter dependent, perhaps resulting from gene-specific interactions of SF-1 with other regulatory proteins.

Results

PKA Requirements for cAMP Induction of the StAR Gene

It has been previously shown that genes such as the rat HDL-R and the mouse *Cyp*21 genes require a functional PKA for cAMP induction *(23,26)*. To examine whether PKA may also be involved in transcriptional regulation of the rat StAR gene, cotransfection studies were carried out in Kin 8 cells (a Y1 cell line with a dominant-inhibitory mutation in the type I PKA regulatory subunit that renders PKA resistant to activation by cAMP *[30]*). Basal luciferase activity produced from the StAR and HDL-R promoter constructs in the Kin 8 cells was not significantly different from the basal activity seen in the wild-type Y1 cells (data not shown). However, as shown in Fig. 1, the 8-bromo-cAMP (8-Br-cAMP)-mediated transcriptional stimulation of the StAR promoter observed in Y1 cells ($p < 0.01$) was completely absent in Kin 8. In these studies, the HDL-R promoter was used as a control. The 8-Br-cAMP-mediated transcriptional activation of the HDL-R promoter was not significantly increased relative to the HDL-R promoter basal activity in Kin 8 cells (Fig. 1), confirming previous studies in our laboratory *(23)*.

To determine whether this decrease in 8-Br-cAMP-mediated transcriptional stimulation of the rat StAR gene could influence StAR mRNA levels, Northern blot analysis was carried out. Y1 and Kin 8 cells were incubated for 24 h in the presence or absence of 1 m*M* 8-Br-cAMP, and total RNA was prepared as described in Materials and Methods. As shown in Fig. 2, both Kin 8 and Y1 cells express very low constitutive levels of StAR mRNA. Treatment with 8-Br-cAMP increased StAR mRNA levels 12-fold in Y1 cells (Fig. 2). In Kin 8 cells, however, the increase in endogenous StAR mRNA levels by 8-Br-cAMP was only 1.5-fold; HDL-R mRNA levels (control) were increased only 2-fold (Fig. 2). This correlates with previous immunoblot analysis showing that StAR protein cannot be detected in whole-cell lysates prepared from Kin 8 cells even after ACTH treatment *(31)*. These results clearly suggest that a functional PKA is required for cAMP regulation of the StAR gene.

SF-1 Involvement in cAMP Regulation of the HDL-R and StAR Genes

To determine whether SF-1 may be involved in cAMP regulation of the HDL-R and StAR genes, Y1 and Kin 8 cells were cotransfected with the rat HDL-R or StAR promoter-construct and the SF-1 expression vector ± 8-Br-cAMP. Table 1 gives the results for the HDL-R promoter. The presence of the SF-1 expression plasmid enhanced both basal and 8-Br-cAMP induction of the HDL-R promoter activity in the wild-type Y1 cells about 3.5-fold. However, in the mutant Kin 8 cells, the presence of exogenous SF-1 did not overcome the effects of the PKA deficiency, although the overall numbers for activity were slightly higher when SF-1 was present. Similar results were obtained with the StAR promoter (data not shown). These results suggest that SF-1 is one of the factors that is required for cAMP regulation of the rat HDL-R and StAR genes and confirm the necessity of a functional PKA for this regulatory process.

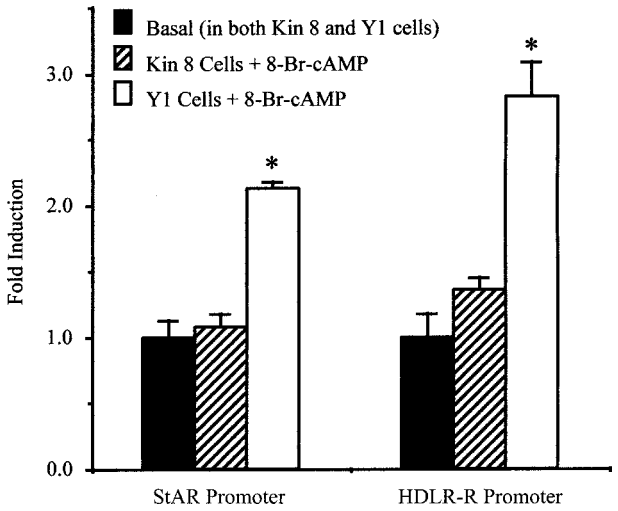

Fig. 1. Effects of 8-Br-cAMP on the expression of luciferase activity under control of the StAR and the HDL-R promoters in Y1 and Kin 8 adrenal cells. Cells were transfected with 2 μg each of either the p-1862 StAR or the p-719 HDL-R construct (and 1 μg Renilla) using Fugene 6 as described in Materials and Methods. 8-Br-cAMP (1 m*M*) was added to some plates 24 h before lysing the cells. Basal refers to the luciferase activity produced from the indicated promoter construct in either Kin 8 or Y1 cells incubated in the absence of 8-Br-cAMP. Data are represented as fold induction in which the value of basal luciferase activity was set at 1.0 and are from three experiments, each performed in triplicate. They are represented as the mean ± SEM. *$p < 0.01$.

Mutational Analysis of the SF-1 Protein

To determine which regions of the SF-1 protein may be involved in transcriptional activation of the StAR and HDL-R genes, site-directed mutational analysis was performed. Figure 3 is a schematic representation of the different SF-1 protein mutations analyzed in this study. These mutations were designed based on previous findings showing that PKA can phosphorylate SF-1 in vitro on Ser and Thr residues *(12)*. The first mutation consisted of introducing a premature stop codon in place of Arg at position 449 of the mouse SF-1 protein, so that most of the carboxy-terminal region including part of the AF-2 domain (amino

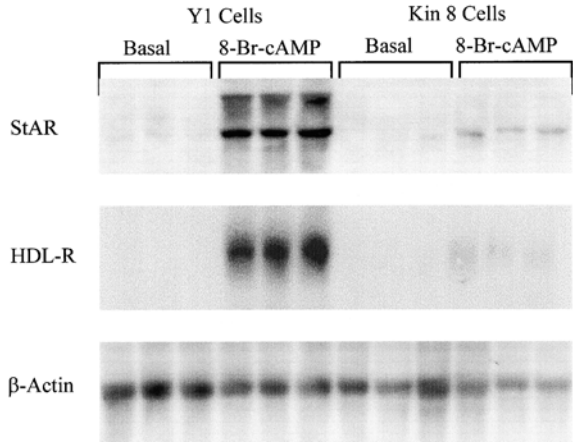

Fig. 2. Effects of 8-Br-cAMP on StAR and HDL-R mRNA levels in Y1 and Kin 8 cells. Y1 and Kin 8 cells were incubated in the presence or absence of 8-Br-cAMP (1 m*M*) for 24 h before lysing. Total RNA isolation and Northern blot analysis were carried out as described in Materials and Methods. 8-Br-cAMP (1 m*M*) was added to some of the flasks 24 h before total RNA preparation. Basal refers to unstimulated (absence of 8-Br-cAMP) mRNA levels seen for the indicated gene in either Kin 8 or Y1 cells. Triplicate samples per each condition were analyzed. Chicken β-actin cDNA probe (internal control) was used to correct for variations in RNA loading.

Table 1
Effects of Lacking an Intact PKA on SF-1 Mediation of the HDL-R Promoter Activity[a]

	Y1 cells	Kin 8 cells
Basal	1.5 ± 0.2	1.5 ± 0.1
Basal + 8-Br-cAMP	4.4 ± 0.9*	2.1 ± 0.3
+SF-1	5.6 ± 1.1*	3.7 ± 0.3
+SF-1 + 8-Br-cAMP	14.5 ± 4.4*	5.5 ± 0.6

[a]Cells were transfected with the p-719 HDL-R construct using Fugene 6 as described in Materials and Methods. 8-Br-cAMP (1 m*M*) was added to 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 twice.

*$p < 0.05$ was obtained by comparing the activity produced from cells treated with and without 8-Br-cAMP within the same group.

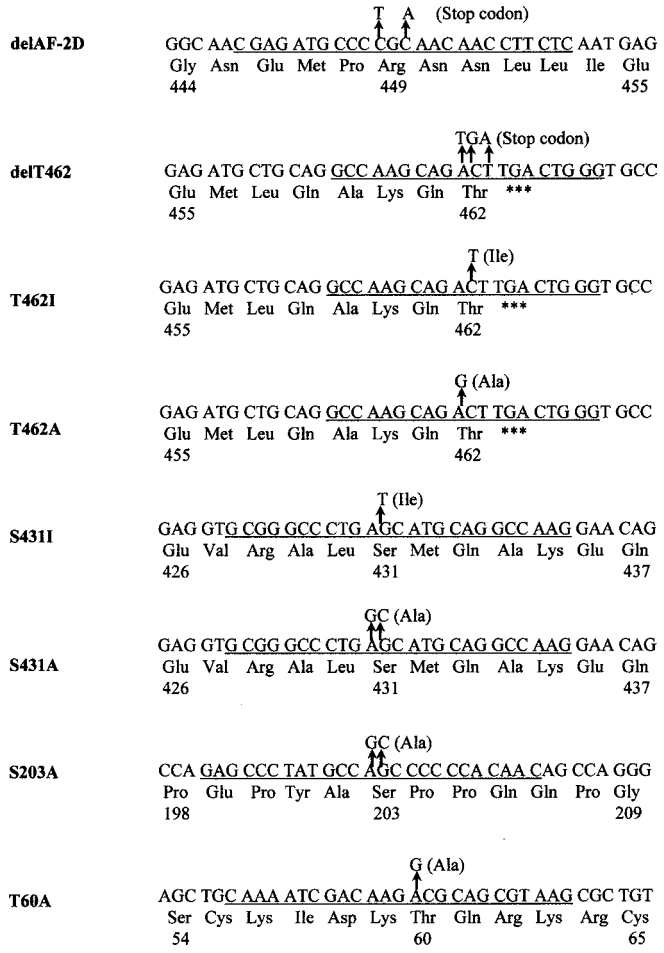

Fig. 3. Schematic representation of the SF-1 protein mutations. The specific nucleotides that have been mutated and their location in the SF-1 protein are shown. Underlined nucleotides correspond to the DNA region used in the designing of mutagenic oligonucleotides.

acids 449–462) is deleted (delAF-2D). Deleting this region has been shown previously to suppress PKA-dependent transactivation of the bovine 17α-hydroxylase/c17,20 lyase gene *(32)* and SF-1 activation of the rat HDL-R gene in the presence or absence of cAMP *(23)*. Since the only amino acid within the AF-2 domain of SF-1 that could be phosphorylated is Thr 462, the next three mutations were prepared to focus on this specific residue. Thr 462 has been proposed as a potential protein kinase C (PKC) phosphorylation site *(8)*. First, a stop codon was introduced instead of Thr 462 at the carboxy-terminal region of the SF-1 protein (delT462). Second, Thr 462 was substituted with an Ile (T462I). Third, Thr 462 was substituted with an Ala (T462A). The next series of mutations replaced Ser 431, the potential PKA phosphorylation site of the SF-1 protein *(8)*, with Ile (S431I) or with Ala (S431A). An additional serine mutation was prepared by replacing Ser 203 with Ala (S203A). Ser 203 is located within the hinge region of the SF-1 protein and has been shown previously to be a MAPK phosphorylation site *(29)*. The final mutation analyzed consisted of substituting Thr 60, which is located within the A-box of SF-1 *(33)*, with Ala (T60A). The A-box (also called the FTZ-F1 region) is a 30 amino acid segment located next to the DNA-binding domain of several nuclear receptors including NGFI-B (or nur77) and SF-1 (reviewed in ref. *33*). This region allows the recognition of additional bases 5' to the AGGTCA hexamer *(33)*. NGFI-B contains an Ser residue at position 354, located within the A-box at a similar position to that of the Thr 60 in the SF-1 protein *(34)*. It has been reported that NGFI-B's Ser 354 is extremely critical for this receptor's abilities to bind its response element fol-

lowing cAMP treatment *(30)*. By analogy, Thr 60 may play a similar role in the SF-1 protein as Ser 354 does in the NGFI-B receptor.

Both wild-type and mutant SF-1 proteins were expressed at comparable levels in HTB-9 cells as determined by Western blot analysis (data not shown). These cells were used because they lack (or have undetectable levels of) HDL-R, StAR, and SF-1. In addition, we have shown previously that in HTB-9 cells, both the StAR *(19)* and the HDL-R *(23)* promoter do not respond to cAMP activation in the absence of SF-1. Thus, by using this system, we were able to study specifically the contributions of SF-1 to the cAMP activation of the StAR and HDL-R genes. The ability of wild-type or mutant SF-1 proteins to bind either the rat StAR or HDL-R promoter was assessed using mobility shift assays. As shown in Fig. 4A, wild-type SF-1 and the mutants S431I, S431A, and S203A bound to a well-characterized SF-1-binding motif *(19)* found in the rat StAR promoter (SFB5). Binding of wild-type SF-1 and the mutants delAF-2D, delT462, T462I, T462A, and T60A to the rat HDL-R SF-1-binding motif (SFBH) *(23)* was also demonstrated (Fig. 4B). Note that in addition to the major complex denoted by an arrow, several minor but specific DNA-protein complexes were observed (Fig. 4). Furthermore, the major complex formed by some of the mutations appears as a doublet (Fig. 4A,B). Binding of the mutants delAF-2D, delT462, T462I, T462A, and T60A to the SFB5 and of the mutants S431I, S431A, and S203A to the SFBH was also observed (data not shown). The combined results of these studies demonstrate that none of the mutations affects the expression or DNA-binding ability of the SF-1 protein. Since mutations of the SF-1 protein did not have a major effect on DNA-binding ability and there was evidence of distinct minor binding complexes as assessed by the mobility shift assays, the possibility exists that these mutations could affect SF-1 interaction with other cofactors resulting in different DNA-protein complexes.

To determine the effects of these mutations on the expression of the luciferase gene under control of the StAR or HDL-R promoter, cotransfection studies were carried out in HTB-9 cells. Dibutyryl cAMP ($[Bu]_2$cAMP) was used because this cAMP analog is more stable than 8-Br-cAMP in HTB-9 cells. Table 2 presents the effects of the SF-1 protein mutants on SF-1 transcriptional activation of the StAR and HDL-R genes. Note the loss of activity in the presence or absence of cAMP with mutations such as the T462A in Table 2 for both the StAR and HDL-R promoters. Table 3 summarizes the effects of the SF-1 mutations in the absence of cAMP. The mutants delAF-2D and T462A, which are located within the SF-1 protein's AF-2 domain, significantly reduced luciferase activity in the rat HDL-R and StAR promoter constructs. Interestingly, the delT462 mutation significantly reduced SF-1-dependent activation of only the HDL-R promoter, whereas the T462I mutation significantly reduced activity only in the StAR promoter. Table 3

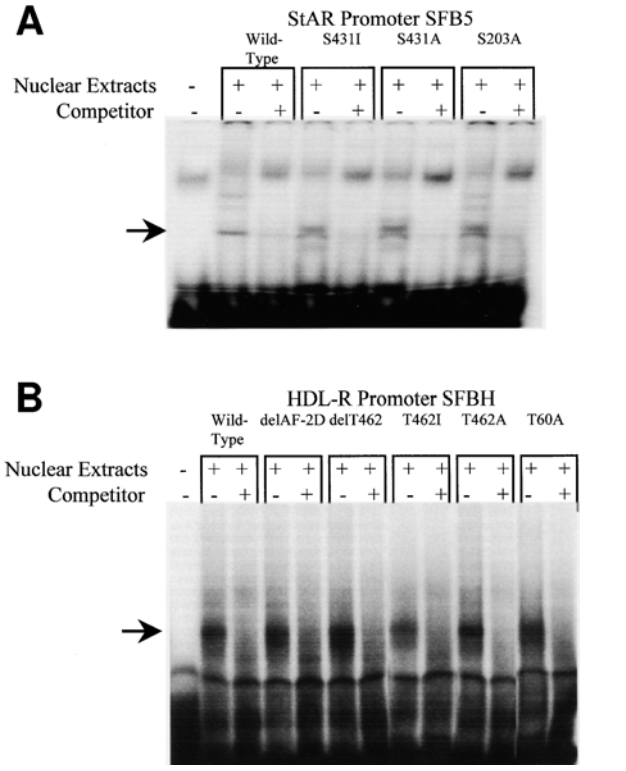

Fig. 4. Binding of wild-type or mutant SF-1 proteins to known SF-1 binding motifs found in the StAR and HDL-R promoter. ^{32}P-labeled double-stranded oligonucleotide probe (100,000 cpm per lane) containing one of the SF-1 motifs found in the StAR promoter (SFB5) (**A**) or the HDL-R SF-1 binding site (SFBH) (**B**) were incubated with wild-type or mutant SF-1 protein prepared as described in Materials and Methods in the presence or absence of 250-fold molar excess of competitor. Representative gel mobility shift assay autoradiographs are presented. Competitor refers to unlabeled SFB5 in (A) and unlabeled SFBH in (B). All SF-1 proteins were expressed equivalently as determined by Western blot analysis (data not shown). Arrows indicate the major DNA-protein complex.

also shows that the S431I mutation, which substituted Ile for Ser 431, decreased SF-1-dependent promoter activity for both genes, although the extent of this reduction was more significant for the HDL-R ($p < 0.01$) than for the StAR promoter ($p < 0.05$). The reduction in HDL-R promoter activity by the mutation delAF-2D confirms previous studies performed in our laboratory *(23)*. Substituting Ser 431 with Ala (S431A), however, had no significant effect on SF-1-dependent promoter activity (Table 3). The effects of this mutation were also examined using a construct containing the SF-1 site at –65 in the *Cyp*21 gene, a gene previously shown to be regulated by SF-1 *(13–15)*. S431A did not significantly affect the SF-1-dependent luciferase activity produced from the p-65 reporter construct (Table 4), similar to the results seen with the StAR and HDL-R genes listed in Tables 2 and 3. The S203A mutation, which substituted Ala for Ser 203, had selective effects on the ability of SF-1 to activate the StAR and HDL-R promoters (Table 3). The S203A mutation significantly re-

Table 2
Effects of Mutating Different Regions of SF-1 Protein on Expression of Luciferase Activity Under Control of StAR and HDL-R Promoters[a]

	StAR	HDL-R
Promoter construct	13.7 ± 1.2	32.7 ± 0.8
+SF-1	132.1 ± 8.7	107.3 ± 13.6
+SF-1 + $(Bu)_2$cAMP	254.2 ± 9.2	284.4 ± 68.1
+delAF-2D	74.8 ± 3.9	57.0 ± 3.6
+delAF-2D + $(Bu)_2$cAMP	111.9 ± 9.7	63.0 ± 8.7
+delT462	104.7 ± 8.4	33.8 ± 6.3
+delT462 + $(Bu)_2$cAMP	319.2 ± 42.5	139.0 ± 18.5
+T462I	71.7 ± 6.3	94.1 ± 14.9
+T462I + $(Bu)_2$cAMP	70.3 ± 6.6	103.8 ± 15.2
+T462A	34.7 ± 6.3	15.3 ± 1.3
+T462A + $(Bu)_2$cAMP	36.2 ± 7.0	15.4 ± 2.2
+S431I	70.1 ± 6.4	38.1 ± 8.8
+S431I + $(Bu)_2$cAMP	101.1 ± 15.6	91.8 ± 18.6
+S431A	128.9 ± 5.9	101.3 ± 13.7
+S431A + $(Bu)_2$cAMP	304.1 ± 29.4	316.3 ± 50.6
+S203A	81.8 ± 4.9	111.4 ± 4.6
+S203A + $(Bu)_2$cAMP	273.7 ± 31.2	170.0 ± 12.6
+T60A	50.7 ± 7.8	38.0 ± 7.5
+T60A + $(Bu)_2$cAMP	55.1 ± 8.2	99.2 ± 15.3

[a]HTB9 cells were transfected with either the p-1862 StAR or the p-719 HDL-R construct using Fugene 6 as described in Materials and Methods. $(Bu)_2$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 three experiments each performed in triplicate.

Table 3
Effects of Mutating Different Regions of SF-1 Protein on Its Ability to Activate StAR and HDL-R Activity[a]

	StAR	HDL-R
+SF-1	100	100
+delAF-2D	57*	53*
+delT462	80	32**
+T462I	54*	88
+T462A	26**	14**
+S431I	53*	36**
+S431A	98	94
+S203A	62*	104
+T60A	38**	35**

[a]These data were derived from Table 2 by dividing the value corresponding to mutated SF-1-dependent promoter activity in the absence of $(Bu)_2$cAMP by the value corresponding to wild-type SF-1-dependent promoter activity in the absence of $(Bu)_2$cAMP. Data are represented as percentage of the wild-type activity. *$p < 0.05$ and **$p < 0.01$ were obtained by comparing to the wild-type SF-1.

Table 4
Effects of S431A Mutation in Putative PKA Phosphorylation Site of SF-1 Protein on Expression of Reporter Plasmid Under Control of SF-1 Site at −65 in *Cyp*21 Gene[a]

	*Cyp*21	*n*
Basal	1.2 ± 0.2	6
+SF-1	73.1 ± 10.2	6
+S431A	90.6 ± 12.5	3

[a]Y1 cells were transfected with the SF-1-dependent plasmid p-65-LUC using calcium chloride as described in Materials and Methods. Data are represented as relative fluorescence (%) ± SEM from *n* determinations each performed in triplicate.

duced SF-1-dependent promoter activity of the StAR gene but not of the HDL-R gene (Table 3). The T60A mutation, which consisted of substituting Ala for Thr 60, reduced SF-1-dependent activity in the rat StAR and HDL-R promoter-constructs (Table 3).

The effects of these mutations on SF-1's ability to mediate cAMP activation of the rat StAR and HDL-R genes were summarized from the data in Table 2 and expressed as fold induction in Table 5. The T462I and T462A mutants diminished SF-1-mediated cAMP activation of both the rat StAR and HDL-R promoters, although the reductions appear to be more dramatic for the HDL-R promoter (2.7 vs 1.1 and 1.0, respectively). The S431I mutation did not significantly affect SF-1's ability to mediate cAMP activation of either promoter. Mutants delT462 and S431A slightly increased SF-1-mediated cAMP activation of the rat StAR and HDL-R promoters. The mutant delAF-2D reduced (2.7 vs 1.1) SF-1-mediated cAMP activation of the HDL-R but had no significant effect on the StAR promoter. By contrast, T60A reduced (1.9 vs 1.1) SF-1-mediated cAMP activation of the StAR promoter but not of the HDL-R promoter. Finally, S203A increased SF-1-mediated cAMP activation of the StAR promoter (1.9 vs 3.3) but decreased SF-1-mediated cAMP activation of the HDL-R promoter (2.7 vs 1.5). These data clearly indicate that although SF-1 is required for optimal promoter response to cAMP, transcriptional activation of genes by SF-1 in the presence or absence of cAMP is promoter dependent.

Discussion

The results obtained from transfection experiments using Y1 and Kin 8 cells clearly demonstrated that like other genes involved in steroid hormone production *(12,23,26, 28)*, a functional PKA is required for transcriptional regulation of the rat StAR gene by SF-1 and cAMP. In addition, the current studies confirmed that SF-1 is required for optimal effects of cAMP on the rat StAR and HDL-R genes. Although these experiments did not show whether these mutations directly affected phosphorylation of the SF-1 protein, they support the supposition that activation of genes by SF-1 in the presence or absence of cAMP is promoter dependent, perhaps resulting from gene-specific interactions of

Table 5
Effects of Mutating Different Regions of SF-1 Protein on Its Ability to Mediate cAMP Activation of StAR and HDL-R Promoter Activity[a]

	StAR	HDL-R
+SF-1	1.9	2.7
+delAF-2D	1.5	1.1*
+delT462	3.1	4.1
+T462I	1.0*	1.1*
+T462A	1.0*	1.0*
+S431I	1.5	2.4
+S431A	2.4	3.1
+S203A	3.3	1.5
+T60A	1.1*	2.6

[a] These data were derived from Table 2 by dividing the value corresponding to wild-type or mutated SF-1-dependent promoter activity in the presence of $(Bu)_2$cAMP by the value corresponding to the activity obtained in the absence of $(Bu)_2$cAMP. Data are represented as fold induction. *$p < 0.05$ was obtained by comparing to the wild-type SF-1.

SF-1 with other regulatory proteins. The presence of different binding complexes formed by the wild-type and mutant proteins shown in the mobility shift assays could suggest that the formation of gene-specific interactions plays a role in the promoter-specific response.

One SF-1 protein region that appears to play an important role in SF-1 transcriptional activation of genes is the AF-2 domain *(23,32)*. The AF-2 transactivation domain, which is found in most ligand-inducible nuclear receptors, is composed of an amphipathic α-helix that appears to be essential for transcriptional activation *(35–37)*. It was demonstrated in the present study that deleting the AF-2 domain of the SF-1 protein (delAF-2D) reduced SF-1-dependent activation of the rat StAR and HDL-R promoters by <50%, although it only affected SF-1-mediated cAMP activation of the HDL-R promoter. The effects of the delAF-2D mutation on the StAR and the HDL-R promoters are less dramatic than those previously found for the bovine *CYP*17 gene *(32)*. In terms of phosphorylation, the AF-2 domain only contains one possible phosphorylation site—Thr 462 *(8)*—which has been proposed as a putative PKC phosphorylation site. In the present study, three mutations of this residue were analyzed: deletion from the C-terminus (delT462), substitution with Ile (T462I), and substitution with Ala (T462A). The results of these mutations suggested that the effect of each mutation and the magnitude of the response is promoter dependent. For example, the T462A mutation decreased SF-1-dependent promoter activity in the presence or absence of cAMP for both promoters (StAR and HDL-R), but these effects were more dramatic for the HDL-R than for the StAR promoter.

It is well known that in addition to the involvement of the AF-2 domain in PKA activation of SF-1, two other functions have been ascribed to this domain *(38–55)*. First, the AF-2 domain is required for interaction with general coactivators such as the steroid receptor coactivator-1 *(38,39)* and the proline-rich nuclear receptor coregulatory protein *(40)* or with gene-specific factors *(6,11,18,41–43)*. Examples of gene-specific factors that have been reported to interact with SF-1 include cAMP response element–binding protein for the rat *Cyp*19 gene *(11)*, estrogen receptor for the fish gonadotropin II-β-subunit gene *(41)*, Sp1 for the bovine *Cyp*11A gene *(6)*, early growth response protein-1 for the mouse and rat LH β-subunit gene *(42–45)*, GATA-4 for the human and mouse Mullerian inhibiting substance gene *(46,47)*, and activator protein-1 (AP-1) for the human ACTH receptor *(48)*. The activation of the human StAR gene requires interaction between SF-1 and CCAAT/enhancer binding protein β (C/EBPβ) or Sp1 *(18,20,21)*, whereas for the mouse gene it requires C/EBPβ or GATA-4, and AP-1 interacting with SF-1 *(18,49,50)*. We have recently determined that the rat StAR *(51)* and HDL-R genes *(52)* require sterol regulatory element binding protein-1a as a coactivator. A recent report by Ivell et al. *(53)* proposed that SF-1 may not be the mediator of LH functions in the bovine StAR gene since the −101 promoter region of this gene, which does not contain any SF-1 binding site, was able to respond to LH stimulation. However, these researchers did not mention a putative SF-1-binding motif located at −47 and a C/EBP binding site also found within this promoter region *(53)* that have been shown to be important for the human StAR promoter *(21)* and that could be the elements responsible for LH-dependent induction.

The second function that has been described for SF-1's AF-2 domain is that it is involved in the activation of SF-1 by oxysterols, the proposed ligands for SF-1 *(54)*. However, it was shown that 25-hydroxy-cholesterol does not act as a ligand for SF-1 in mouse MA-10 cells *(55)*, and there are still some questions about the actual function of the AF-2 domain in this regard. Whether a factor involved in SF-1's ligand production is affected by phosphorylation and whether SF-1 protein phosphorylation affects ligand binding will require further examination.

An important finding of the present study is that phosphorylation at Ser 431 does not appear to be essential for cAMP-dependent activation of the StAR and HDL-R genes as demonstrated by the S431A mutation. The current results and those of a previous report *(23)* showing that replacing Ser 431 with Ile (S431I) dramatically diminished SF-1-dependent activation of the StAR and HDL-R promoters could be explained by the type of residue used in the substitution. Perhaps, because of its size, isoleucine could affect protein folding, which interferes with either coactivator-dependent transactivation or phosphorylation (prevents the exposure of the amino acids that are to be phosphorylated), and, consequently, transcriptional activation is reduced. Interestingly, the S431I mutation did not significantly affect SF-1's ability to mediate cAMP activation of the StAR and

HDL-R promoters, confirming that phosphorylation at this site is not essential for cAMP-dependent activation of these genes. Replacing S431 with alanine could also affect SF-1 protein folding, causing an increase in cAMP enhancement of SF-1-dependent activation of the StAR and HDL-R genes. The substitution of Ala for Ser 431 may facilitate phosphorylation and transactivation, and this may compensate for any decrease in cAMP activation of the StAR and HDL-R promoter activity; however, further studies will be required to confirm these possibilities.

SF-1's Ser 203 has been shown previously to be the major phosphorylated residue in SF-1, and MAPKs were found to be responsible for phosphorylation of SF-1 at this residue *(29)*. In the latter study, it was also shown that replacing Ser 203 with Ala decreased transcriptional activation of the Mullerian-inhibiting substance and the *Cyp*21 genes; however, the decrease was more dramatic for the *Cyp*21 gene than for the Mullerian-inhibiting substance gene *(29)*. In the present study, the S203A mutation significantly decreased SF-1-dependent activation of the StAR promoter but had no effect on the HDL-R, once again confirming the promoter specificity of the SF-1-dependent response. Phosphorylation at Ser 203 has been shown previously to affect cofactor recruitment by SF-1 *(29)*.

The last mutation analyzed herein was the T60A mutation. By analogy with the NGFI-B nuclear receptor, a decrease in SF-1's DNA-binding ability in response to this mutation was expected. However, the mobility shift assay data showed that this mutated SF-1 protein bound to the DNA as well as the wild-type protein. It has been shown that in the case of the NGFI-B nuclear receptor, when Ser 354 (the residue equivalent to SF-1's Thr 60) is phosphorylated, NGFI-B's DNA-binding abilities diminish, and that hormones such as ACTH cause hypophosphorylation of NGFI-B at the Ser 354 residue *(29)*. In the case of the SF-1 protein, it appears that phosphorylation at Thr 60 is not essential for SF-1 binding to the DNA, but it is essential for SF-1 activity. Nevertheless, it is important to point out that the differences between the type of nuclear receptor (NGFI-B vs SF-1) and the type of experiments (phosphorylation studies vs mutational analysis studies) could be responsible for the contradictory results of the two studies. Based on the previous findings that increased in vitro phosphorylation of SF-1 led to a decrease in this transcriptional factor's abilities to bind DNA *(37),* the possibility that a similar effect may occur in the case of SF-1 cannot be ruled out at this point.

In general, these data demonstrate that the consequences of the mutation of the SF-1 protein regions on transcriptional activation by SF-1 are promoter dependent, possibly owing to gene-specific elements interacting with SF-1. This is supported by the mobility shift assay results showing differences in major and minor complex formation among the wild-type and mutated SF-1 proteins and by the transfection studies showing promoter-specific effects. These differences could be an indication of SF-1 interaction with other cofactors. These results parallel those obtained from a recent study using a mutant Y1 cell line with impaired SF-1 protein activity without affecting SF-1's ability to bind DNA *(56)*. In that study, it was found that transcription of SF-1-dependent genes such as cholesterol side-chain cleavage cytochrome P450, 11β-hydroxylase P450, and StAR was reduced in the mutant cell line, but the extent of the reduction was dependent on the type of promoter *(56)*. An important finding of the current study is that although the HTB-9 cells appear to contain endogenous factors that interact with SF-1 in a promoter-dependent manner, these endogenous factors by themselves cannot mediate cAMP activation of the StAR or HDL-R gene. Therefore, SF-1 appears to play a critical role in bringing together the additional cofactors necessary to activate transcription of a specific gene.

Materials and Methods

Materials

All oligonucleotides and primers were synthesized by Integrated DNA Technologies, Inc. (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 (CMV) promoter was obtained from Dr. Keith L. Parker (University of Texas, Southwestern, Dallas, TX). The mouse Y1 adrenal and human HTB-9 bladder carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD). The mouse Kin 8 adrenal cell line was obtained as previously described *(57)*. The QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). The rat HDL-R cDNA was characterized as previously described *(58)*. The StAR cDNA was prepared as previously described *(59)*. [α^{32}P] dCTP (3000 Ci/mmol), the T7 Sequenase DNA Sequencing Kit and poly dI-dC were purchased from Pharmacia Amersham 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 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).

Cell Transfections

The p-719 HDL-R, the p-1862 StAR, and the p-65-LUC reporter constructs were prepared as previously described *(19,23,26)*. Cells (Y1, Kin 8, and HTB-9) were transfected with the specified promoter construct in the presence or absence of SF-1-pCMV by either the Fugene 6 method

according to the manufacturer's conditions (Roche) or the calcium phosphate method as previously described *(13,60)*. Briefly, in the case of the Fugene 6 method, 2 μg of each DNA to be transfected was incubated with Fugene 6 (ratio 2:3) in 100 μL of serum-free DMEM/F12 medium for 15 min. DNA-Fugene 6 complex was then added to the cells (density of 1 to 2×10^5 cells/well, 6-well tissue culture plates). After incubating for 24 h, the medium was replaced, and the cells were allowed to incubate for another 24 h. Either $(Bu)_2$-cAMP or 8-Br-cAMP at a concentration of 1 m*M* was added to some of the plates 24 h before lysing the cells. Cotransfection of a plasmid containing the renilla luciferase gene under control of the SV40 early enhancer/promoter region (1 μg/well) was used as a control to correct for differences in transfection efficiencies. For the calcium phosphate method *(13,60)*, cells (density of 2×10^5 cells/plate, in 60-mm tissue culture dishes) were incubated with the DNA/calcium phosphate precipitates in alpha-minimal essential medium + serum for 24 h. Then the medium + serum was replaced, and cells were incubated for another 48 h before lysis. To correct for variations in transfection efficiencies, separate plates of cells were transfected with a luciferase expression vector under control of the Rous sarcoma virus (RSV) promoter.

Luciferase Assays

For cells transfected by the Fugene 6 transfection method, lysate preparations and luciferase assays were performed by the Dual Luciferase Reporter Assay System using a Turner Designs-20/20 luminometer (Sunnyvale, CA) as previously described *(23)*. For cells transfected by the calcium phosphate method, lysate preparations and reporter gene assays were performed as previously described *(56)* using a Berthold Lumat LB Luminometer.

RNA Isolation and Northern Blot Analysis

Total RNA isolation, electrophoresis, and Northern blot analysis to determine StAR and HDL-R mRNA levels were carried out as previously described *(58,59)*. Probes were labeled by nick translation. A 2.0-kb chicken β-actin cDNA probe was used as an internal control to establish equal RNA loading.

Site-Directed Mutagenesis

Site-directed mutants were obtained using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) as previously described *(23)*. Complementary oligonucleotides used are indicated in Table 6. All mutations were confirmed by sequencing using the T7 Sequenase DNA sequencing kit and [^{35}S]-dATP.

Nuclear Extracts

Nuclear extracts were prepared from HTB-9 cells overexpressing either wild-type or mutated SF-1 protein. HTB-9 cells (density of 10^8 cells/flask) were transiently transfected with 50 μg of the corresponding plasmid using the Fugene 6 transfection method (Roche) as described in the Cell Transfection section and incubated for 48 h at 37°C (5% CO_2). Cells were then washed twice with phosphate-buffered saline and treated with 10 mL of 0.25% trypsin, 1 m*M* EDTA for 10 min. The cell pellet was recovered by centrifugating at 500*g* for 5 min. Nuclear extract preparation, electrophoresis, electrotransfer to nitrocellulose membranes (0.2-μm pore), and Western blot analysis of SF-1 protein were carried out as previously described *(23)*. Nuclear proteins were also used in gel mobility shift assays.

Table 6
Oligonucleotides Used to Mutate Different Regions of SF-1 Protein

Mutation	Oligonucleotide
delAF-2D	5'CGAGATGCCC**T**G**A**AACAACCTTCTC-3'
delT462	5'GCCAAGCAG**TGA**TGACTGGG-3'
T462I	5'-GCCAAGCAGA**T**TTGACTGGG-3'
T462A	5'-GCCAAGCAG**G**CTTGACTGGG-3'
S431I	5'-GCGGGCCCTGA**T**CATGCAGGCCAAG-3'
S431A	5'-GCGGGCCCTG**GC**CATGCAGGCCAAG-3'
S203A	5'-GAGCCCTATGCC**GC**CCCCCCACAAC-3'
T60A	5'-CAAAATCGACAAG**G**CGCAGCGTAAG-3'

[a]Nucleotides in boldface underlined letters correspond to the mutated bases.

Gel Mobility Shift Assay

Complementary oligonucleotides corresponding to the rat StAR SF-1-binding site located at position –846 relative to the transcription start site (SFB5: 5'-TACTCTCGGCCT TGAACGCTTACTGGA-3') or the rat HDL-R SF-1-binding site (SFBH: 5'-GACAGTGCATCAAGGCCGCGAG GGACA-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 oligonucleotides were used as a competitor. Nuclear extracts (20 μg) were 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 bovine serum albumin. After incubation, 100,000 cpm of the radiolabeled probe was added and the mixture incubated for 15 min at 30°C. 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.

Data Analysis

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. To verify equal protein loading, nitrocellulose membranes were stained with 0.1% Ponceau S (in 5% acetic acid) and destained in water. Luciferase data were expressed as the mean ± SEM. Each luciferase assay experiment was performed in triplicate and repeated for the indicated number of times in the figure legends. Data from the individual parameters were compared by analysis of variance followed by Student-Newman-Keuls multiple comparison test when applicable *(61)*. A value of $p < 0.05$ was considered significant for all tests.

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