Steroidogenic Factor 1-Dependent Promoter Activity of the Human Steroidogenic Acute Regulatory Protein (StAR) Gene†

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ABSTRACT: Steroidogenic acute regulatory protein (StAR) is required for efficient adrenal cortical and gonadal but not trophoblast steroid hormone synthesis. StAR gene expression in gonadal cells is stimulated by tropic hormones acting through the intermediacy of cAMP. DNA sequence analysis of the human StAR gene promoter revealed two motifs resembling binding sites for steroidogenic factor 1 (SF-1), a member of the orphan nuclear receptor transcription factor family that controls expression of steroidogenic hydroxylases. The 5′-most sequence (distal site) is a consensus SF-1 binding site. The proximal site is a consensus estrogen receptor binding half-site. The StAR gene promoter is not active in BeWo choriocarcinoma cells, COS-1 cells, HeLa cells, or SK-OV-3 ovarian adenocarcinoma cells, all of which do not express significant levels of SF-1 mRNA. Introduction of SF-1 into these cells stimulated StAR promoter activity, particularly in response to cAMP. Two orphan nuclear transcription factors that bind to sequences similar to SF-1 sites, NGFI-B/Nur77 and RNR-1, did not support cAMP-stimulated StAR promoter activity in BeWo cells. Mutation of the distal putative SF-1 binding site reduced basal and cAMP-stimulated promoter activity in BeWo cells by 82% and 71%, respectively. Mutation of the proximal putative SF-1 binding site reduced basal and cAMP-stimulated promoter activity by 89% and 96%, respectively. Mutations in both sites reduced basal promoter activity to 7% of wild type promoter activity and cAMP-stimulated promoter activity to less than 5% of the wild type. Deletion analyses of promoter activity were consistent with the mutation studies. Electrophoretic mobility shift assays (EMSAs) demonstrated that the distal site binds to SF-1 expressed in COS-1 cells and to an SF-1−GST fusion protein with high affinity, but that the mutated distal sequence does not. An anti-SF-1 antibody ablated the characteristic SF-1−DNA complex with the distal sequence. The proximal site formed a number of protein−DNA complexes with COS-1 cell extracts, but appeared to have at best only very modest affinity for SF-1. Collectively, our findings demonstrate that SF-1 plays a key role in controlling the basal and cAMP-stimulated expression of the StAR gene. SF-1 can function at two distinct sites in the human StAR gene promoter, apparently by two different types of interaction, to control transcription.

The first reaction in the biosynthesis of steroid hormones is catalyzed by the cholesterol side-chain cleavage enzyme (P450scc). This reaction is acutely stimulated by tropic hormones acting through the intermediacy of cAMP. This acute steroidogenic response involves the translocation of cholesterol from the outer to inner mitochondrial membranes, a process that is effected by steroidogenic acute regulatory protein (StAR). Congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which synthesis of adrenal and gonadal steroids is severely impaired, is caused by mutations in the StAR gene (Lin et al., 1995; Tee et al., 1995).

Expression of StAR is directly correlated with steroidogenic activity in adrenal and gonadal cells (Epstein & Orme-Johnson, 1991; Stocco & Sodeman, 1991). The abundance of StAR mRNA in gonadal cells is regulated by cAMP (Sugawara et al., 1995a; Clark et al., 1995). This regulation is evidently at the transcriptional level since cAMP-induced increases in StAR mRNA in human granulosa are associated with increased StAR mRNA synthesis, as determined by nuclear run-on assays; the increase in StAR mRNA is blocked by actinomycin D and is not associated with a change in StAR mRNA half-life (Kiriakidou, McAllister, and Strauss, unpublished observations).

The StAR gene is not expressed in the human placenta (Gradi et al., 1995; Sugawara et al., 1995a), an organ that produces substantial amounts of progesterone. Moreover, the StAR gene promoter is not active in trophoblast-derived cells (Sugawara et al., 1995b). We previously suggested that the 1.3 kb of proximal StAR promoter contained the cis elements necessary for tissue-specific expression since a reporter gene fused to this promoter DNA was expressed in adrenal cells (Y1 cells) but not in trophoblast-derived cells (BeWo choriocarcinoma cells) (Sugawara et al., 1995b).

Our initial review of the human StAR promoter DNA sequence (Sugawara et al., 1995b) revealed two sequences that could represent binding sites for the orphan nuclear receptor transcription factor, steroidogenic factor 1 (SF-1), also called Ad4BP, which is known to have a major role in the expression of steroidogenic P450 enzymes (Parker & Schimmer, 1995). The promoter of the murine StAR gene (Clark et al., 1995) shares a number of structural features

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with the human StAR promoter, including the presence of putative SF-1 binding sites, reinforcing the notion that SF-1 could play a role in StAR gene expression. Clark et al. (1995) also reported that, developmentally, SF-1 mRNA appears before StAR transcripts in mouse adrenal cortex and gonads, consistent with the idea that SF-1 regulates StAR gene expression.

The present studies were designed to explore the role of SF-1 in human StAR gene expression. Here we demonstrate that SF-1 allows the StAR gene promoter to function in several different primate cells, including human trophoblast cells, and also confers cAMP-responsiveness on the promoter.

MATERIALS AND METHODS

Plasmid Constructs. A 1.3 kb *Hin*dIII fragment of the StAR gene (nt −1300 to +39) was cloned into the plasmid vector pGL_2 (Promega), which contains the firefly luciferase as a reporter gene. Truncated promoter sequences (nested deletions) were produced by PCR. Mutations in the promoter sequences were introduced using the Transformer Site-Directed Mutagenesis kit (Clontech). The DNA sequences of all promoter constructs were verified by DNA sequence analysis using an Applied Biosystems Inc. automated DNA sequencer. The mouse SF-1 cDNA inserted into pCMV-5 in the correct and reverse orientations were kindly provided by Dr. Keith L. Parker (Duke University). cDNAs for mouse NGFI-B/Nur77 and rat RNR-1 (regenerating liver nuclear receptor 1) in pCMV-5 and a control reporter gene construct consisting of one Nur77 response element (TCGAGAAAG-GTCAC) coupled to the β-globin promoter in pGL_2Basic were generously provided by Dr. Rebecca Taub (University of Pennsylvania). An expression plasmid for the catalytic subunit of protein kinase A, MTCEVα-neo (Clegg et al., 1992), and the empty vector were geneously provided by Dr. Stanley McKnight (University of Washington). Other plasmids used in these experiments included the pGL_2Basic vector, which contains no promoter sequences; pGL_2Control, which places the luciferase gene under the control of the SV40 promoter and enhancer; and pCH110, a plasmid in which the *lacZ* gene is under control of the early SV40 promoter (Pharmacia).

Cell Culture and Transfections. BeWo choriocarcinoma cells, COS-1 cells, HeLa cells, and SK-OV-3 human ovarian adenocarcinoma cells were cultured in 35 mm plastic dishes in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50 μg of gentamycin/mL. The BeWo cells (clone b30) were a gift from Dr. Arnold Schwartz, Washington University, St. Louis, MO. COS-1, HeLa cells, and SK-OV-3 cells were obtained from the American Type Culture Collection (Rockville, MD). Plasmids used for transfection were purified using the Maxiprep reagent system (Qiagen). Cell cultures at 40%−60% confluence were washed twice with serum-free medium before adding 1 mL of serum-free medium containing 1 μg of pGL_2 plasmid constructs, in some experiments 0.5 or 1 μg of SF-1 expression plasmid, and 1 μg of pCH110 plasmid with 10 μL of Lipofectamine (GIBCO/BRL). After 5 h incubation, the medium was replaced with 1 mL of medium with 20% serum. Medium was changed after 18 h with 2 mL of medium supplemented with 10% serum. Cells were harvested after 48 h of culture. Some cultures were treated with 8-Br-cAMP (1 mM) during the final 24 h of culture.

Luciferase and β-Galactosidase Assays. Cells were harvested 48 h after transfection, and extracts were made in Promega lysis buffer. One aliquot (40 μL out of 400 μL total extract volume) was used for luciferase assays with Promega reagents, and another 150 μL was taken for β-galactosidase assay with Promega reagents. The "blank" luciferase value measured in untransfected cell extracts was subtracted from luciferase readings of transfected cell extracts. The luciferase assay results were normalized to β-galactosidase activity to compensate for variations in transfection efficiency. Each treatment group contained at least triplicate cultures, and each experiment was repeated two or three times.

Northern Blot and RT-PCR Analysis of SF-1 Expression. To determine the relative expression of endogenous SF-1 in the host cells used in the promoter studies, total RNA was isolated from these cells as well as from cultured human luteinized granulosa and human thecal cells by the method of Chomczynski and Sacchi (1987). The RNA was subjected to Northern blotting using a human SF-1 cDNA generated by RT-PCR using primers (forward, 5′-TGTCCGGCTAC-CACTACGGAC-3′; reverse, 5′-CGTCAGGCACTTCTG-GAAGCG-3′) that yield a 170 bp amplification product derived from SF-1 mRNA. The primers were designed from the human SF-1 partial genomic sequence deposited in Genbank (accession no. U32592). The amplification product used for probing the Northern blots was sequenced to verify identity with SF-1 cDNA. The membranes were later re-probed with a labeled 18s rRNA oligonucleotide probe in order to assess the loading of lanes.

Preparation of Protein Extracts and SF-1−GST Fusion Protein. COS-1 cells were transfected with pCMV-5 vectors containing the murine SF-1 cDNA in the correct and reverse orientations using Lipofectamine as described above. 36 h after transfection, the cells were scraped into a buffer consisting of 40 mM Tris, 10 mM EDTA, and 150 mM NaCl, pH 7.9. The cell pellet was washed with phosphate-buffered saline, suspended in 500 μL of 10 mM HEPES, pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT, and 1 mM PMSF, and subjected to freeze−thawing as described by Bakke and Lund (1995). The protein concentration of the extracts was determined by the Bio-Rad dye binding assay.

A murine SF-1−GST fusion protein construct, provided by Dr. Keith Parker, was expressed in bacteria as described by Lala et al. (1992) and used for EMSAs as reported by Lynch and colleagues (1993).

Electrophoretic Mobility Shift Assays (EMSAs). EMSAs were carried out with 5 μg of COS-1 cell protein extract or 1−4 μL of SF-1−GST fusion protein preparation (0.1 μg/μL) in a total vol of 22 μL with 2×10^5 cpm of ^{32}P-labeled double-stranded synthetic oligonucleotide probe (2 ng), 2−4 μg of poly (dI·dC/dI·dC) with or without unlabeled competitor probe according to Sambrook et al. (1989) and Lund et al. (1990). In some experiments 2 μL of antibody to mouse SF-1 (Ikeda et al., 1993) or non-immune serum were added to the mixture 30 min prior to addition of labeled probe. The reaction mixtures were incubated at room temperature for 30 min and then subjected to PAGE at 150 V for 1.5 h and autoradiography.

The sequences of the oligonucleotides used to assemble the double-stranded probe to assess the distal putative SF-1

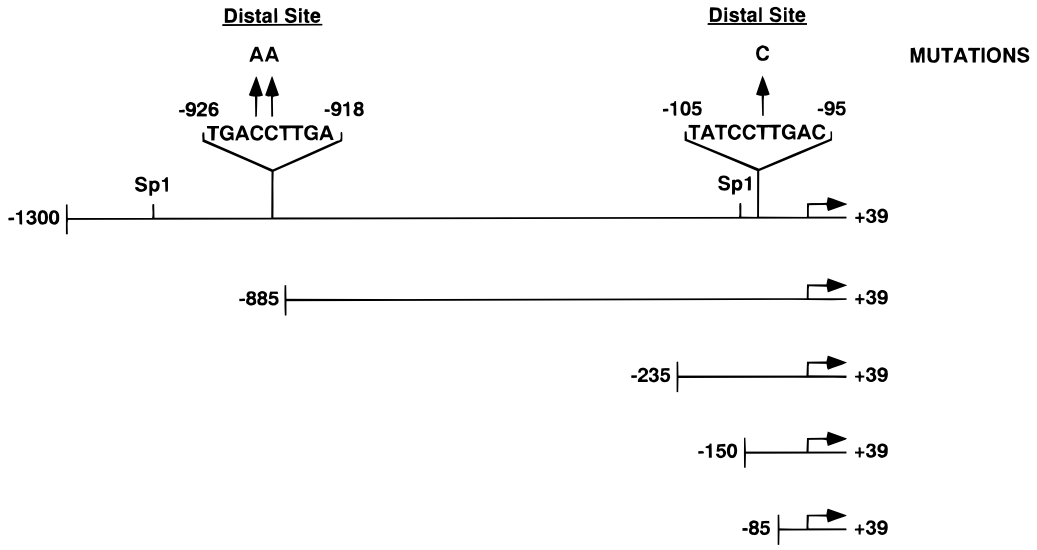

FIGURE 1: Schematic representation of the human StAR gene promoter and constructs employed in these studies.

site were 5′-ACTGGCCAGCTGTTTGACCTTGAACAAGTCA-3′ and 5′-CTGACTTGTTCAAGGTCAAACAGCTGGCCAG-3′. To generate a probe representative of the mutated distal site the following oligonucleotides were employed: 5′-ACTGGCCAGCTGTTTGAAATTGAACAAGTCA-3′ and 5′-CTGACTTGTTCAATTTCAAACAGCTGGCCAG-3′. The putative proximal SF-1 binding site was analyzed using a double-stranded oligonucleotide probe produced from the following sequences: 5′-AATCGCTCTATCCTTGACCCCTTCCTTTG-3′ and 5′-GCAAAGGAAGGGGTCAAGGATAGAGCGAT-3′. The probe reflective of the mutant proximal putative SF-1 site was constructed with the following oligonucleotides: 5′-AATCGCTCTATCCTCGACCCCTTCCTTTG-3′ and 5′-GCAAAGGAAGGGGTCGAGGATAGAGCGAT-3′. The double-stranded synthetic oligonucleotides were labeled using T4 polynucleotide kinase and [γ^{32}P]ATP.

RESULTS

SF-1 Activates the Human StAR Promoter in Various Primate Cells and Confers cAMP-Responsiveness. We previously demonstrated that the human StAR promoter is active in Y1 adrenal cortical tumor cells but not in BeWo choriocarcinoma cells (Sugawara et al., 1995b). The StAR promoter, presented schematically in Figure 1, contains several sequences resembling SF-1 binding sites. The 5′-most putative SF-1 binding site (distal site) is a consensus SF-1 binding sequence. The 3′-most putative SF-1 binding site (proximal site) is an estrogen receptor half-site. We postulated that the relative absence of SF-1 expression in BeWo cells, as well as certain other non-steroidogenic cell types, could explain, in part, the lack of StAR promoter activity in these cells.

We could not detect SF-1 transcripts in BeWo cells, COS-1 cells, HeLa cells, and SK-OV-3 ovarian adenocarcinoma cells by Northern blot analysis of total RNA, but found the 3.5 kb SF-1 transcript in cultures of human theca and granulosa cells (Figure 2). From densitometric analysis, it appears that human granulosa and theca cells have at least 10-fold more endogenous SF-1 mRNA than the BeWo, COS-1, HeLa, and SK-OV-3 cells.

The introduction of murine SF-1 into each of these cell lines enhanced the ability of the 1.3 kb human StAR promoter (nt −1300 to +39) to drive luciferase expression (Figure 3). The extent of stimulation of promoter activity by SF-1 varied among the different cell types, being greatest for HeLa cells and SK-OV-3 cells and least for COS-1 cells. The plasmid containing the SF-1 cDNA in the reverse orientation did not significantly increase StAR promoter function. Expression of SF-1 also conferred cAMP-responsiveness on the StAR promoter; 8-Br-cAMP increased promoter activity 10-fold over basal levels in BeWo cells and more than 2-fold in HeLa and SK-OV-3 cells. COS-1 cells are not responsive to exogenous cAMP analog in our hands (unpublished observations). To examine the influence of cAMP-regulated signal transduction pathways on SF-1-supported StAR promoter activity in these cells, we co-transfected an expression plasmid for the catalytic subunit of protein kinase A or the empty plasmid vector into the COS-1 cells. The addition of the protein kinase A catalytic subunit increased SF-1-supported StAR promoter activity more than 2-fold.

Neither NGFI-B/Nur77 nor RNR-1 (Wilson et al., 1993; Scearce et al., 1993), orphan nuclear receptors which can bind to SF-1-like response elements, could support StAR promoter activity in BeWo cells (Table 1). However, these two transcription factors stimulated expression of luciferase under the direction of a construct containing a single Nur77 response element (1XNBRE) coupled to the β-globin promoter (Table 1). This control demonstrates that the NGFI-B/Nur77 and RNR-1 expression plasmids are functional in BeWo cells.

Mutations in Putative SF-1 Binding Sites Reduce Basal and cAMP-Stimulated StAR Promoter Activity Supported by SF-1. We produced several fusion gene constructs in which 5′-sequences of the 1.3 kb promoter fragment were deleted. The Δ −885/+39 construct removes a consensus Sp1 binding site at −1160 to −1152 and a consensus SF-1 binding sequence at −926 to −918 (5′-TGACCTTGA-3′). Two other constructs, Δ −235/+39 and Δ −150/+39, pruned down the promoter but retained an SF-1-like binding site at −105 to −95 (5′-TATCCTTGACCCC-3′). The former construct also retained a consensus Sp1 binding sequence,

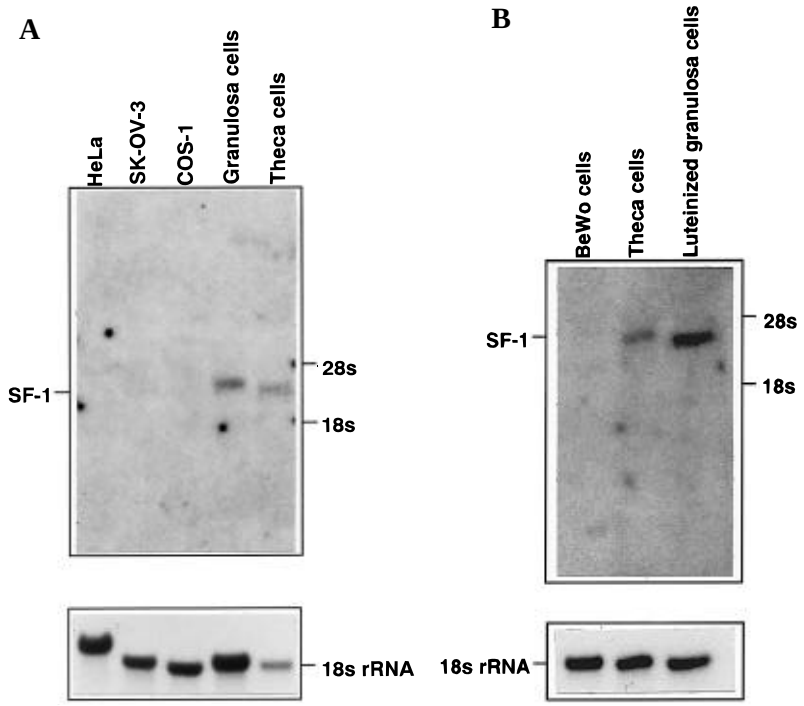

FIGURE 2: SF-1 mRNA is not detectable in BeWo choriocarcinoma cells, COS-1 cells, HeLa cells and SK-OV-3 cells. (A) Northern blot of total RNA (5 μg/lane) from BeWo cells, human theca cells, and human granulosa cells. The blot was probed with an SF-1 partial cDNA, stripped, and reprobed with an 18s rRNA probe. (B) Northern blot of total RNA from HeLa, SK-OV-3, COS-1, granulosa, and theca cells (5 μg/lane for all samples except theca, which is 1 μg). Probed as described above.

which was deleted in the latter construct. Deletion of promoter sequences containing the distal SF-1 consensus binding site reduced basal and 8-Br-cAMP-stimulated promoter activity in the presence of SF-1 by 83% and 64%, respectively, in BeWo cells (Table 2). Removal of additional sequences up to the consensus Sp1 binding sequence did not have any additional impact on basal or cAMP-stimulated promoter activity, whereas removal of the Sp1 binding sequence diminished basal and cAMP-stimulatable promoter activity by 91% and 87%, respectively. Deletion of both the distal and proximal putative SF-1 binding sites ablated basal and cAMP-stimulated promoter activity.

Mutation of the distal putative SF-1 binding site (5′-TGACCTTGA-3′ to 5′-TGA**AA**TTGA-3′) reduced basal and cAMP-stimulated promoter activity in BeWo cells by 82% and 71%, respectively. These findings are consistent with the observed reduction in basal and cAMP-stimulated promoter activity seen when the region of the promoter containing the distal site was removed in the deletion analysis. Mutation of the proximal putative SF-1-like binding site (5′-TATCCTTGACCCC-3′ to 5′-TATCCT**C**G-ACCCC-3′), which could disrupt each of the potential SF-1 binding sites in this sequence (TATCCTT and TGACCCC), resulted in a 89% reduction in basal promoter activity and a 96% reduction in cAMP-stimulated promoter activity (Table 3). Mutation of both sites essentially abolished basal and cAMP-stimulated promoter activity. Similar results were obtained in a transfection study using COS-1 cells as hosts (data not shown).

EMSA of SF-1 Binding to the Putative SF-1 Binding Sites in the Human StAR Gene Promoter. EMSAs demonstrated that the synthetic double-stranded oligonucleotide containing the distal site, which is a consensus SF-1 binding sequence, forms a major protein−DNA complex with extracts from COS-1 cells transfected with the SF-1 expression plasmid (Figure 4A). This complex was not detected when labeled probe was incubated with extracts from cells transfected with a plasmid harboring the SF-1 cDNA in the reverse orientation. Whereas the wild-type distal site competed for binding of the labeled probe by the extracts of the COS-1 cells transfected with SF-1, the mutated distal site did not, nor did the proximal wild type and mutated site (Figure 4A,B). These findings suggest that the proximal site has a lower affinity for SF-1 than the distal site. Confirmation that the specific complex formed between the distal site and the SF-1-containing COS-1 cell extracts indeed represented the binding of SF-1 to the labeled double-stranded oligonucleotide was obtained with the aid of a specific antiserum raised against SF-1 (Ikeda et al., 1993) that has been documented to abolish SF-1−DNA complexes (Lynch et al., 1993; Bakke & Lund, 1995). When incubated with SF-1-containing cell extracts prior to addition of labeled distal probe, the antibody to SF-1, but not non-immune serum, ablated the characteristic protein−DNA complex, possibly causing a super shift of the band (Figure 4B).

The labeled proximal putative SF-1 binding site produced a number of protein−DNA complexes with COS-1 cell extracts. A faint band (complex 2) with a mobility similar to that of the distal site−SF-1 complex was observed after prolonged exposure in two out of five separate experiments (Figure 5A). The formation of the faint band was not the result of deficiencies in the COS-1 cell extracts, since the same extracts generated the strong specific distal site probe−protein complex in simultaneously run EMSAs. The faint proximal site probe−protein complex was not seen when the labeled proximal probe was incubated with extracts of COS-1 cells transfected with the SF-1 expression plasmid containing the cDNA in the reverse orientation. Major DNA−protein complexes were suppressed by the addition of excess unlabeled distal or proximal probes (Figure 5B).

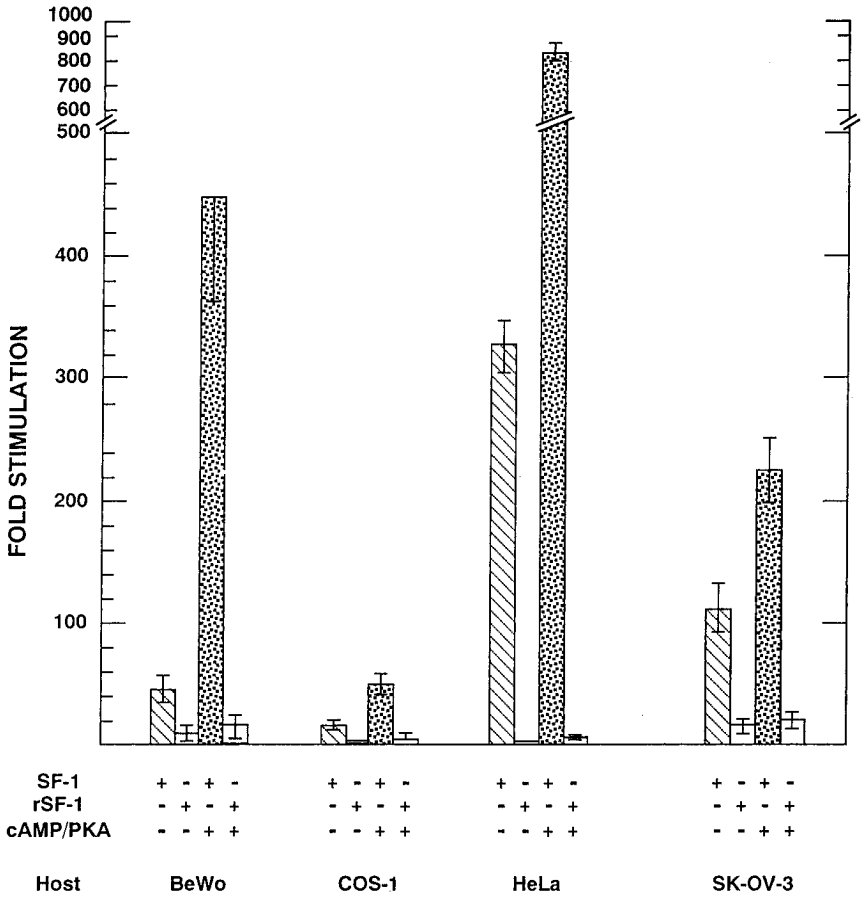

FIGURE 3: SF-1 stimulates StAR promoter activity in BeWo, COS-1, HeLa, and SK-OV-3 cells. Cells were transfected with the indicated plasmids and pCH110 as described in Materials and Methods. The reporter activity is expressed relative to the pGL_2Basic plasmid transfected in combination with the SF-1 expression plasmid. Luciferase activities observed with pGL_2Basic were not significantly affected by any of the other treatments. In studies with COS-1 cells, a protein kinase A catalytic subunit expression plasmid or the empty vector was transfected along with the reporter construct and the SF-1 vectors in lieu of treatment with 8-Br-cAMP. The results presented are means ± SE for $N = 3$ or 4 experiments.

Table 1: Inability of NGFI-B/Nur77 and RNR-1 To Support StAR Promoter Activity in BeWo Choriocarcinoma Cells[a]

plasmid	treatment	RUL × 10^{-3}
pGLBasic + SF-1		0.86 ± 0.36
pGLBasic + SF-1	8-Br-cAMP	1.83 ± 1.03
pGLControl + SF-1		14.26 ± 1.10
pGLControl + SF-1	8-Br-cAMP	17.70 ± 2.84
pGL1.3kbStAR + SF-1		5.18 ± 2.74
pGL1.3kbStAR + SF-1	8-Br-cAMP	48.20 ± 10.5
pGL1.3kbStAR + NGFI-B/Nur77		0.8 ± 0.2
pGL1.3kb + NGFI-B/Nur77	8-Br-cAMP	1.8 ± 0.2
pGL1.3kbStAR + RNR-1		0.4 ± 0.1
pGL1.3kbStAR + RNR-1	8-Br-cAMP	1.5 ± 0.4
1XNBRE + pCMV-5		0.6 ± 0.1
1XNBRE + pCMV-5	8-Br-cAMP	1.6 ± 0.4
1XNBRE + NGFI-B/Nur77		54.3 ± 6.5
1XNBRE + NGFI-B/Nur77	8-Br-cAMP	220.8 ± 41.3
1XNBRE + RNR-1		115.1 ± 5.8
1XNBRE + RNR-1	8-Br-cAMP	274.3 ± 24.1

[a] BeWo cells were transfected with the indicated plasmids and pCH110 as described in Materials and Methods. Some cultures were treated with 1 mM 8-Br-cAMP. Values presented are means ± SE relative luciferase units (RLU) × 10^{-3} from two separate experiments in which each treatment group contained three replicate cultures for studies on StAR promoter activity and from a single experiment for analysis of the 1XNBRE construct.

Table 2: Deletion Analysis of the StAR Promoter[a]

plasmid	promoter activity (% of pGL_2Control)	
	−	+
pGLBasic	0.14 ± 0.07	0.22 ± 0.08
pGLControl	100	85.3 ± 18
pGL1.3kbStAR	32.4 ± 5.76	343 ± 165
pGLΔ-885/+39	5.5 ± 3.05	124 ± 82
pGLΔ-235/+39	9.8 ± 6.94	114 ± 68
pGLΔ-150/+39	2.9 ± 0.82	45.0 ± 10
pGLΔ−85/+39	1.6 ± 0.45	4.1 ± 0.8

[a] BeWo cells were transfected with the indicated plasmids and the SF-1 expression plasmid with pCH110 as described in Materials and Methods. Cultures were incubated with vehicle (−) or with 1 mM 8-Br-cAMP (+). Values presented are mean promoter activities ± SE relative to the pGL_2Control promoter construct from three separate experiments in which each treatment group consisted of three replicate cultures.

Although the unlabeled mutated distal and proximal probes diminished several of the prominent labeled DNA−protein complexes, they did not affect the intensity of complex 2. Anti-SF-1 abolished complex 2, whereas non-immune serum did not (Figure 5B). These findings collectively suggest that the weak band occassionally formed with the proximal site probe represents a low-affinity SF-1−DNA complex, which is not unexpected given the deviation of the proximal site sequence from the consensus SF-1 binding site motif.

An SF-1−GST fusion protein, expressed in bacteria, formed specific complexes with the labeled distal site probe (Figure 6A,B). On prolonged exposure, a modest complex was formed with the labeled proximal site probe and the SF-1−GST fusion protein (Figure 6A). The binding of labeled distal site probe to the SF-1−GST fusion protein was

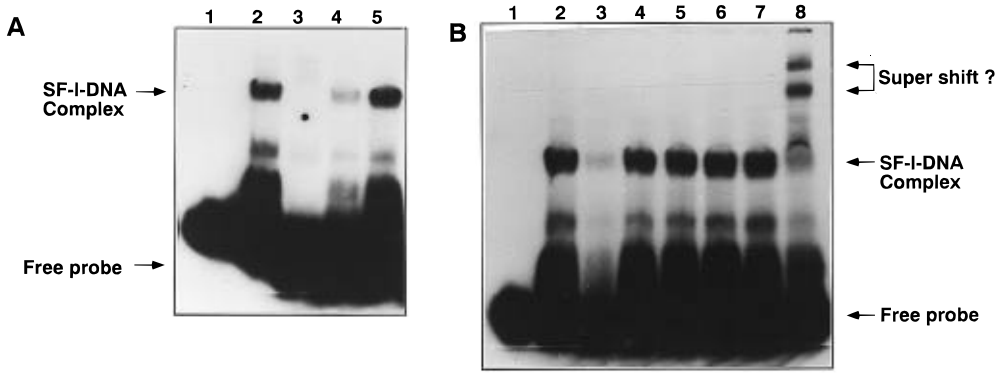

FIGURE 4: Distal putative SF-1 site forms a complex with a protein in COS-1 cells transfected with SF-1 expression plasmid. EMSA using a ^{32}P-labeled double-stranded oligonucleotide probe representing the distal putative SF-1 site were carried out as described in Materials and Methods. (A) Lane 1, labeled probe; lane 2, extract from COS-1 cells transfected with a plasmid containing the mouse SF-1 cDNA in the correct orientation and labeled probe; lane 3, extract from COS-1 cells transfected with a plasmid in which the SF-1 cDNA was inserted in the reverse orientation and labeled probe; lane 4, extract from COS-1 cells transfected with SF-1 expression plasmid, labeled probe, and a 50-fold excess of unlabeled distal probe; lane 5, extract from COS-1 cells transfected with SF-1 expression plasmid, labeled probe and 50-fold excess of unlabeled mutated distal probe. (B) Lane 1, labeled probe; lane 2, extract of COS-1 cells transfected with SF-1 expression plasmid and labeled probe; Lane 3: extract, labeled probe and 20-fold excess of unlabeled distal probe; lane 4, extract, labeled probe, and 20-fold excess of unlabeled proximal probe; lane 5, extract, labeled probe, and 20-fold excess of unlabeled mutated distal probe; lane 6, extract, labeled probe, and 20-fold excess of unlabeled mutated proximal probe; lane 7, extract, labeled probe and non-immune serum; lane 8, extract, labeled probe, and anti-SF-1 serum.

Table 3: Mutations in Putative SF-1 Binding Sites Ablate StAR Promoter Activity in BeWo Cells

plasmid	relative promoter activity	
	−	+
pGL1.3kbStAR	100	100
pGL1.3kbStAR (CC→AA)	18.5 ± 0.8	29.0 ± 6.7
pGL1.3 kbStAR (T→C)	11.5 ± 1.8	4.4 ± 1.0
pGL1.3kbStAR (T→C, CC→AA)	6.5 ± 1.3	3.4 ± 1.3
pGLBasic	0.05	0.01

[a] BeWo cells were transfected with the indicated plasmids with pCH110 as described in the Materials and Methods. Cultures were treated with vehicle (−) or 1 mM 8-Br-cAMP (+). Values presented are means ± SE of promoter activities relative to the wild type 1.3 kb StAR promoter fragment from three separate experiments in which each treatment group contained three replicate cultures.

inhibited in a dose dependent fashion by unlabeled distal site probe, but not by the mutated distal site double-stranded oligonucleotide (Figure 6B).

DISCUSSION

The objective of the present study was to evaluate the role of SF-1 in the regulation of the human StAR gene promoter. Our strategy was to introduce a StAR promoter−reporter gene construct and SF-1 into cells that do not normally express StAR or SF-1. We utilized BeWo choriocarcinoma cells for the majority of these experiments because we have previously shown that they do not express StAR mRNA and have negligible SF-1 message.

Co-transfection of an SF-1 expression plasmid with the 1.3 kb StAR promoter construct allowed the human StAR promoter to function in a variety of cell hosts that do not normally express StAR or SF-1 mRNA, including BeWo cells, COS-1 cells, HeLa cells, and SK-OV-3 cells. The expression of SF-1 also conferred the ability of the promoter fragment to respond to cAMP-activated signal transduction pathways. Two other transcription factors that bind to DNA sequences that are similar to those to which SF-1 binds to could not support basal or cAMP-stimulated StAR promoter activity, suggesting that the action of SF-1 on the StAR promoter is specific.

Analysis of a series of 5′-deletion constructs documented that the DNA sequences containing the two putative SF-1 binding sites are essential for SF-1 supported basal and cAMP-stimulated activity in BeWo cells. Furthermore, mutation of these sites individually substantially reduced promoter activity. Mutation of both sites essentially abolished basal and cAMP-stimulated reporter gene expression in BeWo cells. This situation is reminiscent to observations made on the mouse P450c21 gene promoter, which has three elements that bind SF-1. Deletion of any of the three SF-1 binding sites causes a dramatic reduction in P450c21 promoter activity in Y1 cells and in mice (Rice et al., 1990; Crawford et al., 1995).

Our findings that putative SF-1 sites mediate cAMP-responsiveness is in agreement with studies on the promoters of several steroid hydroxylases (Bakke & Lund, 1995; Michael et al., 1995; Morohashi et al., 1995). The cAMP enhancement of SF-1 effects varied from 2- to 10-fold, depending on the host cell. This is in the range of responses observed by other investigators who examined cAMP amplification of SF-1 effects on steroidogenic enzyme gene promoters. cAMP could lead to phosphorylation of SF-1 and hence alter its functional activity. Theoretically, cAMP could also induce the synthesis of an SF-1 co-activator or an SF-1 ligand (Morohashi et al., 1993; Parker & Schimmer, 1995). The mechanism underlying the cAMP response in the host cells that we used and the factors contributing to the differential responses among the host cells remain to be determined.

EMSAs demonstrated that the distal site, which contains a consensus SF-1 binding sequence, forms a specific complex with SF-1 expressed in COS-1 cells and an SF-1−GST fusion protein expressed in bacteria. The mutated distal sequence that markedly reduces SF-1-supported promoter activity in BeWo cells does not form these complexes. These observations are consistent with the notion that SF-1 is the trans acting factor that binds to the distal cis element in the StAR promoter.

The proximal site, which has a sequence that differs from the consensus SF-1 binding sequence, formed several

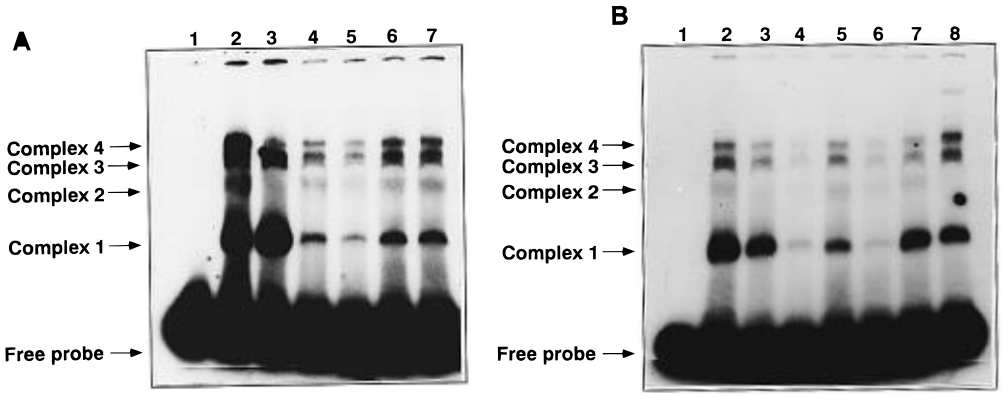

FIGURE 5: Proximal putative SF-1 binding site forms multiple complexes with proteins present in extracts of COS-1 cells transfected with SF-1 expression plasmid. ^{32}P-labeled double-stranded oligonucleotide probe representing the putative proximal SF-1 binding site was incubated with extracts of COS-1 cells transfected with SF-1 expression plasmids containing the cDNA insert in the correct (A and B) or reverse orientations (A). (A) Lane 1, labeled probe; lane 2, extract from COS-1 cells transfected with SF-1 expression plasmid with cDNA in the correct orientation and labeled probe; lane 3, extract of COS-1 cells transfected with expression plasmid with SF-1 cDNA in the reverse orientation and labeled probe; lane 4, extract of COS-1 cells transfected with SF-1 expression plasmid, labeled proximal site probe and a 10-fold excess of unlabeled proximal site; lane 5, extract of COS-1 cells transfected with SF-1 expression plasmid, labeled proximal site probe, and a 20-fold excess of unlabeled proximal site; lane 6, extract of COS-1 cells transfected with SF-1 expression plasmid, labeled proximal site probe, and a 10-fold excess of unlabeled mutated proximal site; lane 7, extract of COS-1 cells transfected with SF-1 expression plasmid, labeled proximal site probe, and a 20-fold excess of unlabeled mutated proximal site. (B) Lane 1, labeled probe; lane 2, extract from COS-1 cells transfected with SF-1 expression plasmid and labeled probe; lane 3, extract, labeled probe, and 20-fold excess of unlabeled distal probe; lane 4, extract, labeled probe, and 20-fold excess of unlabeled proximal probe; lane 5, extract, labeled probe, and 20-fold excess unlabeled mutated distal probe; lane 6, extract, labeled probe, and 20-fold excess unlabeled proximal probe; lane 7, extract, labeled probe, and non-immune serum; lane 8, extract, labeled probe, and anti-SF-1 serum.

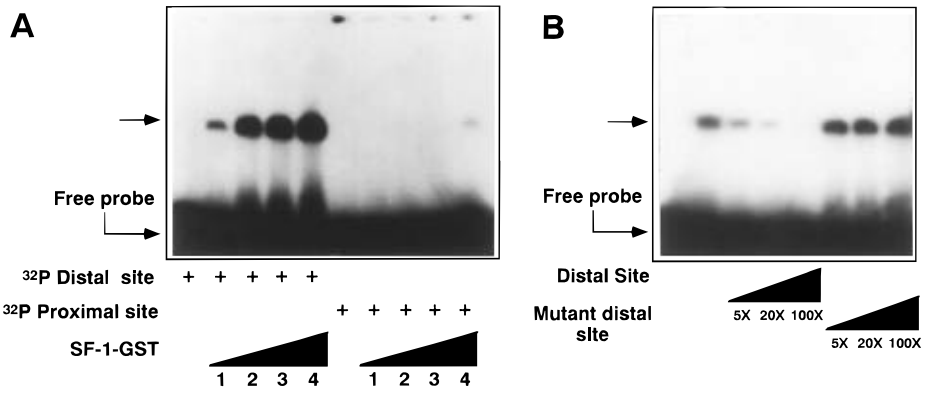

FIGURE 6: Distal site, but not the proximal site, binds to an SF-1−GST fusion protein. (A) Increasing amounts of SF-1−GST fusion protein (1−4 μL of fusion protein preparation) were incubated with labeled distal or proximal site probe. A long exposure is presented to demonstrate the only modest affinity of the proximal site for the SF-1−GST fusion protein. (B) Association of the distal site with the SF-1−GST fusion protein is specific. Unlabeled distal site probe in a 5−100-fold molar excess, but not the mutated distal site probe, inhibited formation of the labeled distal site SF-1−GST fusion protein complex in a dose-dependent manner.

complexes with proteins extracted from COS-1 cells transfected with the SF-1 expression plasmid. One of the complexes that was occassionally observed, and did not represent a major band, had a mobility identical to that formed by the distal site. Formation of this complex was prevented by an anti-SF-1 antibody. However, the labeled proximal site probe did not readily form complexes with the SF-1−GST fusion protein. These findings suggest that the proximal site has at best a very low affinity for SF-1. Mutation of the proximal site markedly reduces SF-1-supported StAR promoter activity, demonstrating that SF-1 in some way influences this cis element, even if it does not have a high affinity for the sequence. Thus, SF-1 may control StAR gene transcription at the distal and proximal sites through different mechanisms: binding as a monomer to the distal site and indirectly at the proximal site. Further studies are required to determine the nature of SF-1 regulation of the proximal site and the nature of SF-1 action on this cis element.

The actions of SF-1 on the StAR promoter are not unique to the human gene. As noted previously, the murine StAR gene contains a consensus SF-1 binding site sequence (Clark et al., 1995). The murine StAR promoter has recently been shown to be responsive to SF-1 (K. Caron and K. L. Parker, personal communication).

Our experiments have focused on the role of SF-1 in the control of StAR promoter function. It is possible and indeed very likely that other transcription factors play important roles in controlling this gene besides SF-1 and SF-1-dependent factors. The StAR gene promoter contains several consensus Sp1 binding site sequences. These cis elements could have some importance, since Sp1 has been demonstrated to be important in cAMP-stimulated transcription of steroidogenic enzymes (Momoi et al., 1992).

SF-1 has been implicated in the regulation of gonadotropin, ovarian oxytocin, Mullerian inhibitor substance, and steroid hydroxylase gene expression (Wehrenberg et al., 1994; Parker & Shimmer, 1995). Our findings extend the role of

SF-1 in gene expression to a non-enzymatic protein involved in the rate-limiting step in steroid hormone synthesis.

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