Multiple Steroidogenic Factor 1 Binding Elements in the Human Steroidogenic Acute Regulatory Protein Gene 5′-Flanking Region Are Required for Maximal Promoter Activity and Cyclic AMP Responsiveness†

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ABSTRACT: A proximal element from the human StAR gene promoter, containing the sequence $^{-105}$TATCCTTGAC^{-95}, was shown to confer responsiveness to 8-Br-cAMP in the presence of steroidogenic factor 1 (SF-1) when placed behind a minimal thymidine kinase promoter or an SV40 promoter and transfected into BeWo cells which normally lack StAR and SF-1. This element was also transactivated by SF-1 in a yeast one-hybrid system. The −105 to −95 sequence was protected by SF-1 in footprint analysis and a double-stranded oligonucleotide containing the element bound SF-1 specifically in electrophoretic mobility shift assays. Another SF-1-binding sequence 35 bp upstream of the transcription start site ($^{-42}$CAGCCTTC^{-35}) was identified in the DNase 1 footprint analysis and, when mutated, markedly reduced SF-1-dependent and 8-Br-cAMP-stimulated StAR promoter activity in BeWo cells. The two proximal SF-1 response elements were shown to be critical for StAR promoter function in human granulosa-lutein cells, which express SF-1 and respond to cAMP with increased transcription of the StAR gene. Mutation of either element substantially reduced basal and forskolin-stimulated promoter activity, although mutation of the −105 to −95 element had more pronounced effects. Mutation of a third, more distal, SF-1-binding site at −926 to −918 also reduced basal but not forskolin-stimulated promoter activity in the granulosa-lutein cells. These findings demonstrate that multiple SF-1 response elements are required for maximal StAR promoter activity and regulation by cAMP.

Steroidogenic acute regulatory protein (StAR) plays a critical role in the initial step in steroid hormone synthesis, the conversion of cholesterol to pregnenolone (Lin et al., 1995; Stocco & Clark, 1996). This reaction takes place in the mitochondria, catalyzed by cytochrome P450scc. StAR, by mechanisms that remain to be determined, appears to increase the delivery of substrate cholesterol to the inner mitochondrial membrane where P450scc is located (Arakane et al., 1996; Stocco & Clark, 1996). Mutations in the StAR gene cause lipoid congenital adrenal hyperplasia, a disease characterized by a striking impairment of the formation of all adrenal and gonadal steroid hormones and massive accumulation of cholesterol in the steroidogenic cells (Lin et al., 1995).

The StAR gene is expressed in a restricted pattern in human tissues. StAR mRNA is detected in adrenal cortex, testis, ovary, and to a lesser extent in kidney, but not in placenta, liver, or brain (Sugawara et al., 1995a). The human (Sugawara et al., 1995b) and mouse (Clark et al., 1995) StAR gene promoters each contain sequences resembling binding sites for the orphan nuclear transcription factor steroidogenic factor 1 (SF-1), also called AdBP4 (Parker & Schimmer, 1995; Morohashi et al., 1995). SF-1 plays a key role in regulating the expression of steroidogenic enzymes and other proteins involved in the control of reproductive tissues. The stimulation of transcription of some genes encoding steroidogenic enzymes by cAMP is also dependent upon SF-1. We recently reported that the StAR gene promoter is SF-1 dependent and that SF-1 renders the StAR promoter cAMP responsive in the context of cells that do not express endogenous SF-1 (e.g., BeWo choriocarcinoma cells, HeLa cells, and SK-OV-3 cells) (Sugawara et al., 1996). Here we demonstrate that the proximal promoter of the human StAR gene contains three SF-1-controlled elements that all must be intact for maximal promoter activity and cAMP responsiveness.

MATERIALS AND METHODS

Plasmids. The human StAR promoter constructs were fused to a luciferase reporter gene in the pGL2 vector (Promega) as previously described (Sugawara et al., 1996). Mutations were produced using the Transformer system (Clontech). Copies of the wild-type and mutated −105 to −95 elements were inserted behind a Herpes simplex virus minimal thymidine kinase promoter coupled to a luciferase reporter in the pTKLuc vector or the pGL-promoter vector (Promega) in which inserts are placed behind the SV40 promoter. Control plasmids, pCH110 or RSV-β-gal, were used to assess transfection efficiency (Sugawara et al., 1996; Kiriakidou et al., 1996). Plasmids were prepared for transfection studies using the Qiagen Maxiprep system.

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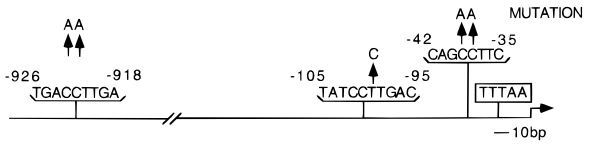

FIGURE 1: The human StAR gene promoter and its SF-1 response elements.

Cell Culture and Transfection. BeWo choriocarcinoma cells (b30 clone), a gift from Dr. A. Schwartz (Washington University, St. Louis), were cultured in Delbecco's minimal essential medium containing 25 mM glucose and 25 mM HEPES, supplemented with 10% fetal bovine serum and 50 μg/mL gentamycin. Cultures of proliferating human granulosa-lutein cells were prepared and cultured as described by McAllister et al. (1994). BeWo cells were transfected using Lipofectamine as described by Sugawara et al. (1996). Proliferating granulosa-lutein cells were transfected using a calcium phosphate coprecipitation method as described by Kiriakidou et al. (1996). Some BeWo cell cultures were treated with 1 mM 8-B-cAMP (Sugawara et al., 1996). Twenty micromolar forskolin was used to stimulate some of the granulosa-lutein cell cultures as described by Kiriakidou et al (1996).

Luciferase and β-Galactosidase Assays. Cells were collected into lysis buffer, and luciferase and β-galactosidase assays were performed using reagent systems purchased from Promega as previously described (Sugawara et al., 1996). Relative luciferase units were calculated as the ratio of luciferase light units/β-galactosidase activity.

DNase 1 Footprint Analysis. DNase 1 footprinting was carried out using a 243 bp segment of the human StAR gene promoter, obtained by digesting a promoter fragment from -235 to $+39$ in the pGL2 vector with *Hae*II and *Bgl*II (Sugawara et al., 1995b). The fragment was labeled on the antisense strand with reagents purchased from Promega using the supplier's suggested protocol. Footprinting was conducted using a murine SF-1-glutathione-S-transferase (GST) fusion protein, kindly provided by Dr. Keith Parker (Duke University), with the Core Footprinting System (Promega). GST was used as a control.

Electrophoretic Mobility Shift Assays (EMSAs). The wild-type -105 to -95 site double-stranded oligonucleotide probes were constructed from the following oligonucleotides: 5′-AATCGCTCTATCCTTGACCCCTTCCTTTG-3′ and 5′-GCAAAGGAAGGGGTCAAGGATAGAGCGAT-3′. The mutant site probe was constructed from the following oligonucleotides: 5′-AATCGCTCTATCCT**C**GACCCCT-TCCTTTG-3′ and 5′-GCAAAGGAAGGGGTC**G**AGGATA-GAGCGAT-3′ as described by Sugawara et al (1996). The -42 to -35 site oligonucleotide probe was constructed from the following oligonucleotides: 5′-ATGATGCACAGCCT-TCAGC-3′ and 5′-CGCTGAAGGCTGTGCATCA-3′. The mutant probe was constructed from the following oligonucleotides: 5′-ATGATGCACAG**AA**TTCAGC-3′ and 5′-CGCTGAA**TT**CTGTGCATCA-3′. The double-stranded synthetic oligonucleotides were labeled with T4 polynucleotide kinase and [γ^{32}P]ATP.

EMSAs were conducted with SF-1-GST fusion protein as described by Sugawara et al. (1996) with the important exception that the binding reactions and electrophoresis were carried out in Tris-acetate-EDTA (TAE) buffer, produced by mixing 67 mL of 1 M Tris-HCl (pH 7.5), 11 mL of 3 M Na0Ac (pH 6.0), 40 mL of 0.25 M EDTA, and 882 mL of water to yield a 10$\times$ buffer solution or a binding buffer consisting of 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 6.25 mM MgCl2, 10% glycerol, and 0.5 mM dithiothreitol. These buffers have lower ionic strength than the Tris-glycine-EDTA (TGE) buffer employed for binding reactions and electrophoresis in our previous study (Sugawara et al., 1996). Briefly, GST-SF-1 protein, 2×10^5 cpm of ^{32}P-labeled double-stranded oligonucleotide probe (2 ng), 2$-$4 μg of poly (d*I*d*C*/d*I*d*C*) with or without unlabeled competitor probe were

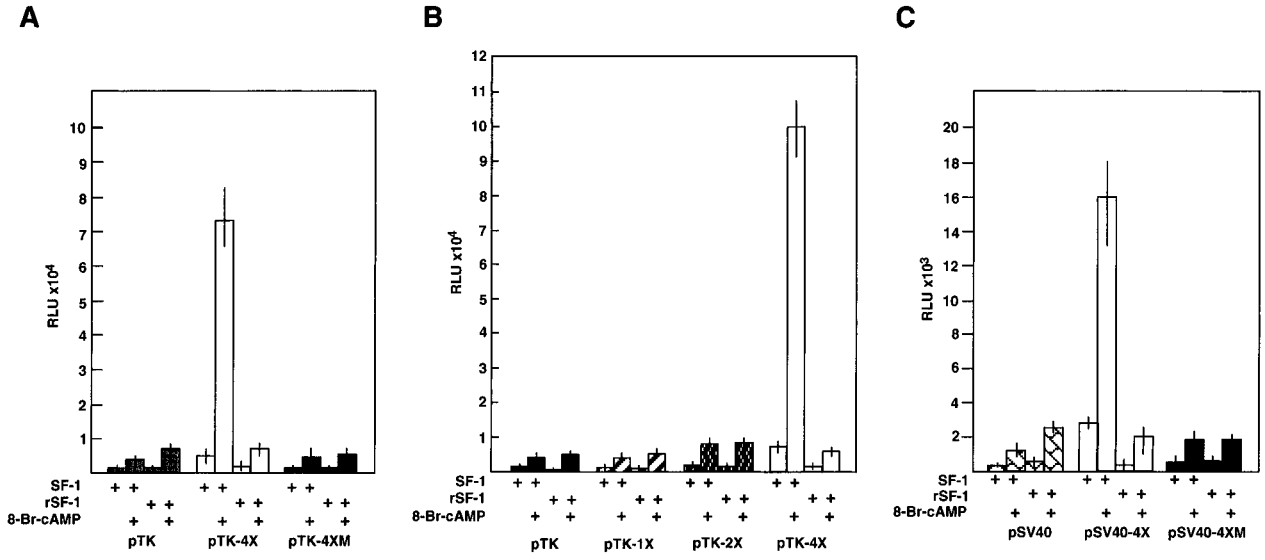

FIGURE 2: The -105 to -95 SF-1 response element confers 8 Br-cAMP-responsiveness on a minimal promoter in the presence of SF-1 in BeWo choriocarcinoma cells. (A) Four copies of the -105 to -95 element (pTK-4X) or four copies of the mutated element (pTK-4XM) were placed behind a minimal thymidine kinase promoter and transfected into BeWo choriocarcinoma cells with SF-1 expression plasmids. Some cultures were treated with 1 mM 8-Br-cAMP as indicated. Values are means $\pm$ SE from three separate experiments with triplicate cultures in each treatment group. rSF-1 denotes the plasmid with the SF-1 cDNA in the reverse orientation. RLU denotes relative luciferase activity. (B) Effect of copy number on function of the chimeric reporter. One, two, or four copies of the -105 to -95 element were placed behind a minimal thymidine kinase promoter and activity in the presence or absence of 1 mM 8-Br-cAMP with the indicated SF-1 expression plasmids was tested. Values are means $\pm$ SD from an experiment with triplicate cultures in each treatment. (C) Function of four copies of the -105 to -95 element (pSV40-4X) or the mutated element (pSV40-4XM) behind an SV40 promoter. The promoter constructs were tested in BeWo cells as described in panel A. Results are means $\pm$ SD from an experiment with triplicate cultures in each treatment group.

incubated in a total volume of 20−30 μL. Reaction mixtures were incubated at room temperature for 30 min and then subjected to PAGE at 150 V for 1.5 h and autoradiography.

Yeast One-Hybrid System. Yeast strain YM4271 (Clontech) with genotype MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3 112trp1-903, tyr1-501 was used for target−reporter construct integration. Two vectors, pHiSi, which carries the His3 reporter gene, and pLacZi, which carries the LacZi reporter gene, were used to generate new yeast strains containing four copies of the target element. Four tandem copies of the sequence containing the −105 to −95 element SF-1-binding site (5′-AATCGCTCTATCCTTGACCCCTTCCTTTG-3′) were produced by polymerase chain reaction (PCR) using a 5′-primer flanked by an *Eco*RI site and a 3′-primer flanked by *Xba*I and *Sal*I sites. The amplified fragments were digested with *Eco*RI and *Xba*I or *Eco*RI and *Sal*I and cloned into pHISi and pLacZi, respectively, upstream of the His3 and the LacZi promoters. The target−reporter constructs were then transformed into the YM4271 strain using the lithium acetate method. Briefly, competent cells were prepared according to standard methods (Ito et al., 1983; Schiestl & Gietz, 1989; Hill et al., 1991). Dual reporter His^+ and Ura^+ yeast strains were obtained by sequential transformation of the reporter−target constructs linearized at the His3 and Ura3 loci to facilitate homologous recombination. The new His^+ and $Ura3^+$ yeast strain was used to screen a target independent activation domain fused with a murine SF-1 cDNA insert in the correct and opposite orientations. Competent YM4271 His^+ and Ura^+ cells were transformed with the SF-1 constructs, and the transformation mixture was plated on to selection media lacking His and Ura for His^+ and Ura^+ strain selection and Leu for activation domain selection. 3-Aminotriazole was added to the medium at a concentration of 35 mM to prevent growth of colonies due to leaky histidine expression. Colonies that grew on the medium described above were considered to demonstrate interaction between the target construct and specific DNA binding domains that allowed histidine production in yeast cells after transcriptional activation of the His3 reporter.

To insure stringent screening, β-galactosidase assays were performed. Colonies were lifted on to Whatman no. 5 filter papers, cells were permeabilized by a freezing−thawing cycle in liquid nitrogen and then placed on a filter presoaked in X-Gal solution and incubated at room temperature for up to 8 h. Colonies that turned blue produce β-galactosidase after transcriptional activation of the LacZi reporter that follows the interaction between the target sequence and specific DNA-binding domain.

RESULTS AND DISCUSSION

Element −105 to −95 Confers 8-Br-cAMP responsiveness in the Presence of SF-1 When Placed behind a Minimal Promoter. We previously reported that a proximal motif resembling an SF-1-binding site at −105 to −95 was essential for StAR promoter activity in BeWo choriocarcinoma cells cotransfected with a plasmid expressing SF-1 (Figure 1) (Sugawara et al., 1996). Placing four copies of this element (underlined) and its surrounding sequences (5′-AATCGCTCTATCCTTGACCCCTTCCTTTG-3′), which deviates from the high-affinity SF-1 consensus binding sequence of PyCAAGGPyPyPur described by Honda et al. (1993) by one nucleotide, behind a minimal thymidine kinase promoter, conferred 8-Br-cAMP responsiveness on the

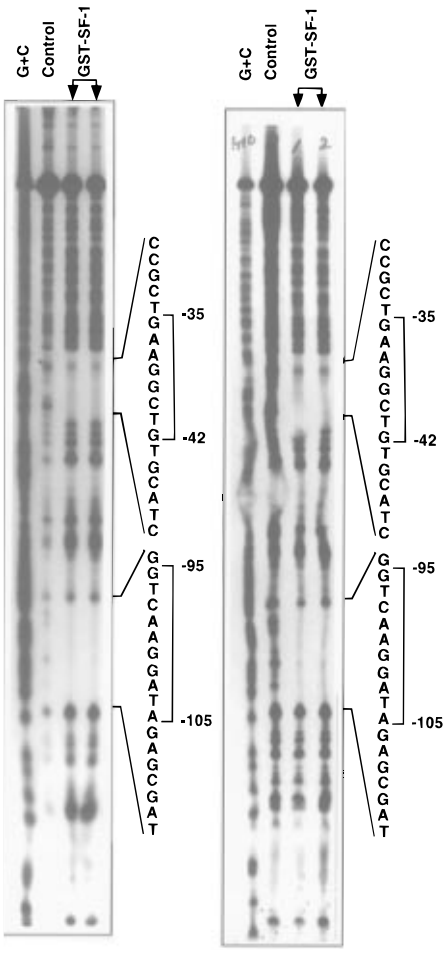

FIGURE 3: DNA footprint analysis of the human StAR promoter with recombinant murine SF-1. DNA footprint analyses were carried out on two separate occasions as described in Materials and Methods with a recombinant murine SF-1-GST fusion protein (1 μg, left lane, and 4 μg/reaction, right lane). G+C denotes sequence lane. Two protected sequences were identified, one encompassing the −105 to −95 element, the other an SF-1-like binding sequence at −42 to −35.

chimeric promoter in the presence of SF-1 in BeWo cells (Figure 2A). The cAMP analog increased reporter activity 7−10-fold with SF-1, but four copies of the element did not respond to cotransfection with a plasmid containing the SF-1 cDNA in the reverse orientation in the absence or presence of 8-Br-cAMP. More than two copies of the element were required to enhance reporter gene expression, as no increment in luciferase activity was seen with promoter constructs containing a single copy or two copies of the element (Figure 2B). When the element was mutated to 5′-TATCC**C**TGA-3′, no response to SF-1 and 8-Br-cAMP was observed (Figure 2A).

The activity of the −105 to −95 element was not dependent upon the thymidine kinase promoter. Four copies of the wild-type element also increased reporter gene expression in BeWo cells when placed behind an SV40 promoter in the presence of SF-1 and 8-Br-cAMP (Figure 2C). Four copies of the mutated element behind the SV40 promoter did not support increased reporter gene expression in the presence of SF-1 and 8-Br-cAMP.

Element −105 to −95 Binds SF-1. We originally reported that the −105 to −95 motif had only modest affinity for SF-1 compared to a more distal consensus SF-1-binding sequence at −926 to −918 (Sugawara et al., 1996). DNase 1 footprint analysis using recombinant murine SF-1-GST

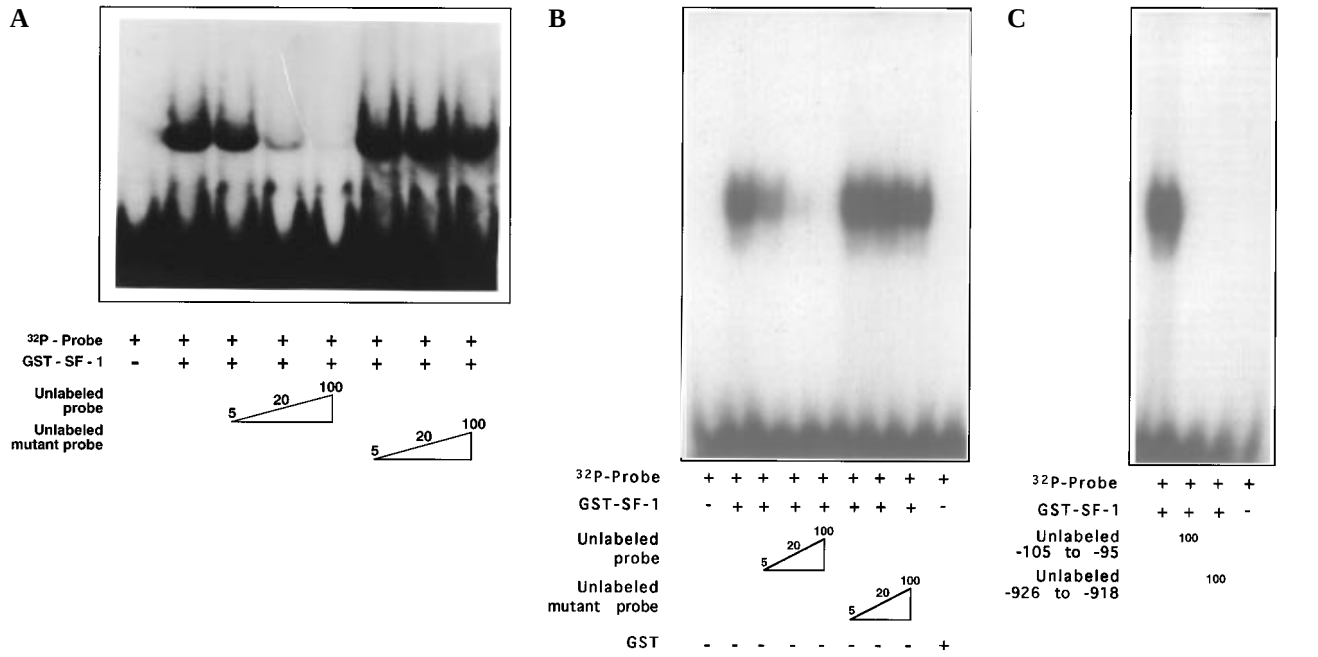

FIGURE 4: The −105 to −95 and −42 to −35 motifs bind SF-1. (A) Binding of SF-1-GST to the −105 to −95 probe. Complex formation was suppressed by unlabeled probe, but not by mutant probe. (B) The −42 to −35 probe is bound by SF-1-GST, but not GST alone, in a specific fashion. Unlabeled −42 to −35, but not mutated −42 to −35 probe, inhibits formation of labeled −42 to −35 probe-SF-1-GST complex. (C) The specific binding of SF-1-GST to the −42 to −35 probe can be competed by unlabeled −105 to −95 and −926 to −918 probes. Figures denoting unlabeled competitor levels indicate molar excesses.

fusion protein demonstrated that the recombinant protein protected a sequence encompassing the −105 to −95 element (5′-ATCGCTCTATCCTTGACC-3′) (Figure 3). In addition, a sequence 35 bp upstream of the transcription start site, 5′-GATGCACAGCCTTCAGCGG-3′, was protected. The latter protected sequence contained the underlined motif, which like the −105 to −95 element, deviates by only one nucleotide from the consensus high-affinity SF-1-binding site sequence (Honda et al., 1993). This protected site was subsequently tested for SF-1-binding and functional activity (vide infra). When GST was used as a control in the DNase 1 footprinting studies no promoter sequences were protected.

To document the specificity of the SF-1 interactions with the StAR promoter sequences protected in the DNase 1 footprint analysis, we carried out EMSAs using oligonucleotide probes corresponding to these sequences. When EMSAs were carried out in buffers with a lower ionic strength than the TGE buffer used in our previous experiments, we found that the −105 to −95 element specifically bound SF-1-GST fusion protein (Figure 4A). The complex of SF-1-GST and labeled double-stranded oligonucleotide containing the −105 to −95 site was inhibited in a concentration dependent manner by unlabeled wild-type probe, but not by unlabeled mutated probe. The −42 to −35 sequence protected in the DNase 1 footprint analysis also bound SF-1-GST fusion protein in a specific fashion (Figure 4B). The binding of SF-1-GST was inhibited by unlabeled probe, but not by the mutated probe containing the sequence 5′-CAG**AA**TTCA-3′. SF-1-GST binding to the −42 to −35 probe was also inhibited by competition with the unlabeled −105 to −95 and −926 to −918 elements (Figure 4C). Additional experiments performed with murine Y1 adrenal tumor cell nuclear extracts which contain endogenous SF-1 demonstrated specific complexes with the −105 to −95 and −42 to −35 probes that were ablated by an antibody to SF-1 (data not shown). These observations document that the two elements at −105 to −95 and −42 to −35 are SF-1-binding sites.

SF-1 Transactivates at the −105 to −95 Element in a Yeast One-Hybrid System. To confirm that SF-1 is capable of transactivating at the −105 to −95 element, we inserted four copies of the sequence behind the His3 and LacZi promoters and introduced these constructs into yeast cells. The cells were subsequently transformed with a vector harboring a murine SF-1-Gal 4 activation domain fusion protein or with the vector containing the SF-1 cDNA in the opposite orientation. Yeast cells transformed with the SF-1-Gal 4 activation domain fusion protein and the two plasmids containing four copies of the wild-type −105 to −95 element grew on selection media (Figure 5). Yeast did not grow when transformed with the control plasmid containing the SF-1 cDNA in the reverse orientation. We also inserted four copies of the mutated element behind the His3 promoter and transformed yeast cells containing the SF-1-Gal 4 activation domain fusion protein. Occasional small colonies grew, slowly, in the selection medium as compared to the numerous large colonies which grew when the SF-1-Gal 4 activation domain fusion protein was in the presence of four copies of the wild-type element. The SF-1-Gal 4 activation domain fusion protein could not support growth of yeast cells harboring vectors containing the His3 and LacZi vectors alone (data not shown).

Importance of an Element near the Transcription Start Site for SF-1-Supported cAMP-Stimulated StAR Promoter Activity. To determine if the −42 to −35 site is important for SF-1 regulation of StAR gene promoter activity, we mutated the sequence in the 1.3 kb human StAR promoter and tested the activity of the wild-type and mutant promoters in BeWo cells cotransfected with SF-1, in the absence or presence of 8-Br-cAMP. Mutation of the element (see Figure 1) substantially reduced basal and 8-Br-cAMP-stimulated StAR promoter activity by more than 60% and approximately

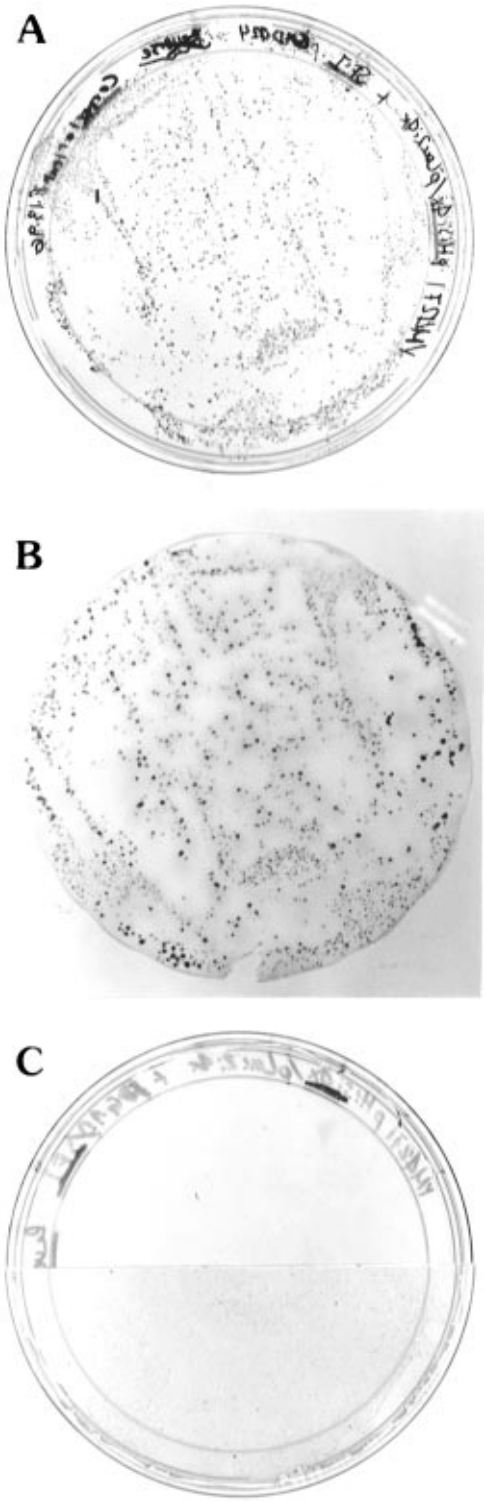

FIGURE 5: SF-1 transactivates the −105 to −95 SF-1 response element in a yeast one-hybrid system. (A) Yeast colonies growing on selection medium when transformed with 4 copies of the −105 to −95 element coupled to the His3 and LacZ genes (4XpHIsi and 4XpLacZi) and a SF-1-Gal 4 activation domain fusion protein. (B) X-Gal staining of filter lift of the plate shown in panel A demonstrating that growing colonies express the LacZ gene. (C) Failure of yeast colonies to grow on selection medium when transformed with 4XpHIsi and 4XpLacZi and a Gal 4 activation domain coupled to a mouse SF-1 cDNA insert in the opposite orientation (upper half of the plate) or when yeast are transformed with the SF-1-Gal 4 activation domain fusion protein and a plasmid containing four copies of the mutant −105 to −95 element and the His3 gene (4XMpHISi) (lower half of the plate).

90%, respectively (Table 1). This degree of inhibition of StAR promoter activity in BeWo cells cotransfected with an expression plasmid for SF-1 is similar to that observed in our previous studies in which the −926 to −918 and −105 to −95 elements were mutated individually (Sugawara et al., 1996). Thus, in the context of host cells that do not express endogenous SF-1, all three of the SF-1-binding sites appear to govern basal and cAMP-stimulated StAR promoter activity when presented with exogenous SF-1. Disruption of any one of these cis elements has a significant impact on promoter function.

Table 1: SF-1-Binding Site-Like Element, −42 to −35, Is Important for SF-1 Supported cAMP-Stimulated StAR Promoter Activity in BeWo Cells[a]

plasmid	relative luciferase activity × 10^{-3} expt 1	expt 2
pGL1.3 kb StAR + SF-1	8.5 ± 4.8	5.4 ± 0.49
pGL1.3 kb StAR + SF-1 + 8-Br-cAMP	193 ± 47	34.9 ± 6.7
pGL-42 to −35 M + SF-1	0.9 ± 0.2	2.2 ± 0.57
pGL-42 to −35 M + SF-1 + 8-Br-cAMP	13.8 ± 1.4	4.5 ± 0.28
pGL1.3 kb StAR + rSF-1	0.3 ± 0.04	0.4 ± 0.10
pGL1.3 kb StAR + rSF-1 + 8-Br-cAMP	4.9 ± 0.2	2.4 ± 0.21
pGL-42 to −35 M + rSF-1	0.2 ± 0.1	0.2 ± 0.01
pGL-42 to −35 M + rSF-1 + 8-Br-cAMP	1.8 ± 0.3	0.8 ± 0.12

[a] BeWo choriocarcinoma cells were transfected with the indicated plasmid constructs, and luciferase reporter gene expression was determined as described in Materials and Methods. pGL 1.3 kb StAR is the wild-type promoter. The 1.3 kb promoter constructs with mutations in the −42 to −35 site are indicated as pGL-42 to −35 M. SF-1 denotes the SF-1 expression plasmid with the cDNA insert in the correct orientation. rSF-1 denotes the expression plasmid with the SF-1 cDNA in the reverse orientation. Some cultures were treated with 1 mM 8-Br-cAMP. Values presented are means ± SD from two separate experiments with triplicate dishes in each treatment group.

cis Elements −42 to −35 and −105 to −95 Are Critical for StAR Promoter Activity in Human Granulosa-Lutein Cells. To test the relative importance of the distal (−926 to −918) and proximal (−105 to −95 and −42 to −35) SF-1-binding elements in a host cell which expresses StAR and SF-1, we transfected cultures of proliferating granulosa-lutein cells with 1.3 kb human StAR promoter−luciferase fusion constructs, with or without mutations in the SF-1-binding elements, and examined basal and forskolin-stimulated promoter activity.

Forskolin treatment produced a greater than 6-fold increase in reporter gene expression driven by the wild-type promoter (Figure 6). This is similar to the increase in steady state levels of StAR mRNA in granulosa-lutein cells exposed to 8-Br-cAMP (Kiriakidou et al., 1996). Mutation of the distal element at −926 to −918 reduced basal promoter activity by approximately 50%, but had no significant influence on forskolin-stimulated promoter activity. Mutation of the −105 to −95 cis element had a more profound effect, reducing basal and forskolin-stimulated promoter activity by greater than 90 and 80%, respectively. Mutation of the −42 to −35 element reduced basal and forskolin-stimulated promoter activity, respectively, by more than 50 and 80% (Figure 6).

To test the relationship between the input of promoter construct DNA to transcriptional activity with promoter constructs containing three intact SF-1-binding sites or only one SF-1-binding site, we transfected granulosa-lutein cells with a constant amount of plasmid DNA using varying amounts of the wild-type promoter construct or a construct in which the −926 to −918 and −105 to −95 SF-1-binding sites were mutated, leaving only the −42 to −35 element intact. A promoterless plasmid was added to bring the total

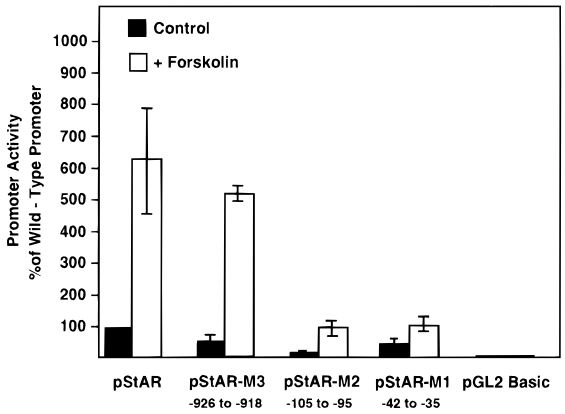

FIGURE 6: Relative importance of the three SF-1-binding elements in the human StAR promoter in human granulosa-lutein cells. The wild-type 1.3 kb human StAR promoter fused to a luciferase reporter or mutant constructs containing mutations in the −926 to −918 (pStAR-M3), −105 to −95 (pStAR-M2) and −42 to −35 (pStAR-M1) elements or the promoterless plasmid control (pGL2Basic) were transfected into propagatable human granulosa-lutein cells. Some cultures were treated with 20 μM forskolin. StAR promoter activity is expressed relative to that of the wild-type 1.3 kb fragment in the basal state. Values presented are means ± SE from three separate experiments with triplicate cultures in each group.

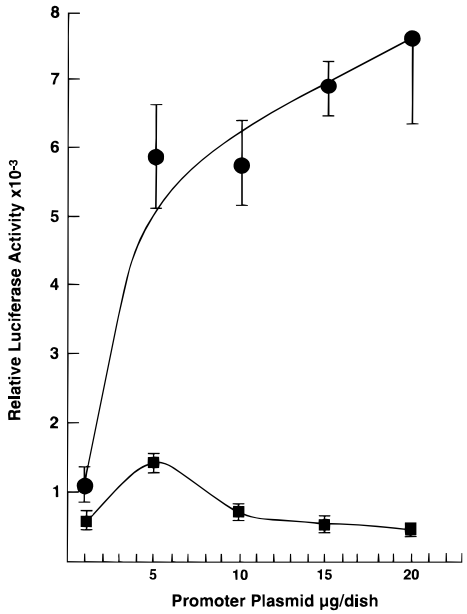

FIGURE 7: Effect of increasing promoter construct input on reporter activity. Granulosa-lutein cells were transfected with the indicated amounts of wild-type promoter construct (closed circles) or a promoter construct that contained mutations in two of the three SF-1-binding sites (−926 to −918 and −105 to −95) (closed squares). A total of 20 μg of plasmid DNA was transfected, and the balance was made up by the promoterless vector along with the β-galactosidase plasmid (5 μg/dish) for normalization of transfection efficiency. All cultures were treated with forskolin. Relative luciferase activity was determined and values presented are means ± SE from six replicate dishes from two separate experiments. The relative luciferase activity observed in granulosa-lutein cells transfected with 20 μg/dish of the promoterless plasmid was 291 ± 35.

amount of plasmid DNA transfected to 20 μg/dish, and all cultures were treated with forskolin. Luciferase activity increased with increasing amounts of wild-type promoter construct transfected, indicating that the endogenous SF-1 was not limiting (Figure 7). In contrast, the reporter activity of the mutated construct, which as expected was substantially lower than the wild-type promoter, was biphasic, increasing at lower plasmid concentrations and then declining as larger amounts of promoter construct DNA were added, falling to a level close to that measured when only promoterless plasmid vector was transfected. The biphasic pattern cannot be attributed to a toxic effect of high concentrations of the mutated promoter construct because the β-galactosidase activity of the cotransfected control plasmid was constant over all mutated promoter concentrations tested. These findings suggest that the presence of three intact SF-1-binding sites sustains transcriptional activity despite increasing promoter DNA content and a constant endogenous level of SF-1. This sustained transcriptional response is not seen when only one SF-1-binding site is intact, perhaps because the transcription apparatus is less stable when only a single SF-1 site is functional.

The mouse StAR gene promoter shares similar structure to the promoter of the human gene, including the presence of SF-1-binding motifs (Clark et al., 1995). Caron et al. (1997) recently demonstrated that the mouse StAR promoter is regulated by SF-1, findings that are consonant with our studies on the human gene. Interestingly, Caron et al. (1997) found that only one cis element that binds SF-1 in the mouse promoter is important and that this SF-1-binding site does not mediate the cAMP responsiveness of the promoter in murine host cells (Y1 adrenal tumor cells and MA-10 Leydig cells), observations that conflict with our results from experiments on the human StAR promoter studied in human granulosa-lutein cells. The differences between our findings and those of Caron et al. (1997) may be due to the different host cells employed in the transfection studies. Adrenal cortical cells and testicular Leydig cells differ from granulosa-lutein cells in that the latter cells undergo terminal differentiation (luteinization) in response to tropic hormones that activate adenylyl cyclase. Alternatively, the murine and human StAR gene promoters may be functionally different.

Our observations establish that the human StAR gene promoter contains at least three distinct SF-1 responsive elements (Figure 1). Each of these elements must be intact for maximal basal promoter activity, since mutation of one substantially reduces promoter function even though the others remain intact. A similar situation has been described in the P450c21 promoter, which also has three SF-1-binding sites (Rice et al., 1990). These cis elements confer cAMP responsiveness to the P450c21 promoter, as has been described for SF-1-binding sites in other genes (Michael et al., 1995). Likewise, our findings implicate the two more proximal SF-1-binding sites in the human StAR gene promoter as being critical for cAMP responsiveness in human granulosa-lutein cells. However, it is possible that these elements are less important in regulating cAMP responsiveness in Leydig cells and adrenal cortical cells.

There appears to be a hierarchy of importance of the SF-1 elements in the human StAR gene promoter in the context of human granulosa-lutein cells, since mutations in the −105 to −95 produced a greater effect than mutations in the −926 to −918 and −42 to −35 elements. Interestingly, the distal element is a consensus SF-1-binding sequence while the two proximal elements diverge by one nucleotide from this consensus, high-affinity, binding sequence. Thus, the range of cis element core sequences upon which SF-1 can act is broader than originally believed. In the human StAR promoter, the distal site appears to be important for basal StAR promoter activity, whereas the more proximal sites affect basal as well as cAMP-stimulated promoter function.

Although the present observations demonstrate a critical role for SF-1 in regulating human StAR gene expression, particularly in response to cAMP, other factors may be required for the induction of StAR gene transcription by the cyclic nucleotide in granulosa-lutein cells. Cyclic AMP analogues increase StAR gene transcription and steady state StAR mRNA levels in human granulosa-lutein and thecal cells, but these responses occur only after several hours and are blocked by cycloheximide, an inhibitor of protein synthesis (Kiriakidou et al., 1996). The slow response time and the requirement for on-going protein synthesis strongly suggest that new proteins need to be synthesized in order for cAMP to activate transcription of the StAR gene. Since SF-1 is expressed in human granulosa-lutein and thecal cells in culture in the absence of stimulation (Sugawara et al., 1996), it is unlikely that cAMP acts by increasing expression of SF-1. Thus, other proteins that serve as coactivators may be needed to permit cAMP-mediated stimulation of StAR transcription. The nature of these putative proteins and the means by which they interact with SF-1 are, at present, obscure.

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