Synergistic Activation of the Human Type II 3β-Hydroxysteroid Dehydrogenase/Δ⁵-Δ⁴ Isomerase Promoter by the Transcription Factor Steroidogenic Factor-1/Adrenal 4-binding Protein and Phorbol Ester*

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Steroidogenic factor-1/adrenal 4-binding protein (SF-1/Ad4BP) is an orphan nuclear receptor/transcription factor known to regulate the P450 steroid hydroxylases; however, mechanisms that regulate the activity of SF-1/Ad4BP are not well defined. In addition, little is known about the mechanisms that regulate the human steroidogenic enzyme, type II 3β-hydroxysteroid dehydrogenase (3β-HSD II), the major gonadal and adrenal isofrom. Regulation of the 3β-HSD II promoter was examined using human adrenal cortical (H295R; steroidogenic) and cervical (HeLa; non-steroidogenic) carcinoma cells. H295R cells were transfected with a series of 5’ deletions of 1251 base pairs (bp) of the 3β-HSD II 5’-flanking region fused to a chloramphenicol acetyltransferase (CAT) reporter gene followed by treatment with or without phorbol ester (phorbol 12-myristate 13-acetate; PMA). CAT assay data indicated that the region from −101 to −52 bp of the promoter was required for PMA-induced expression. A putative SF-1/Ad4BP regulatory element, TCAAGGTTA, was identified by sequence homology at −64 to −56 bp of the promoter. Cotransfection of HeLa cells with the −101 3β-HSD-CAT construct and an expression vector for SF-1/Ad4BP increased CAT activity 49-fold. Subsequent treatment with PMA induced an unexpected synergistic increase in transcriptional activity 540-fold over basal. Mutation of the putative response element (TCAAGGTTA to TCAATTAAA) abolished SF-1-induced CAT activity and the synergistic response to PMA. Gel mobility shift assays confirmed that SF-1/Ad4BP interacts with the putative element and transcripts for SF-1/Ad4BP were detected in H295R cells by Northern analysis. These data are the first to demonstrate 1) regulation of a non-cytochrome P450 steroidogenic enzyme promoter by SF-1/Ad4BP, 2) a powerful synergistic effect of PMA on SF-1/Ad4BP-induced transcription, and 3) the importance of the SF-1/Ad4BP regulatory element in the regulation of the 3β-HSD II promoter.

The steroidogenic enzyme 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴-ene-isomerase (3β-HSD) is essential for the biosynthesis of all classes of steroid hormones and catalyzes the dehydrogenation and isomerization of Δ⁵-3β-hydroxysteroids including pregnenolone, 17α-hydroxyprogrenolone, dehydroepiandrosterone, and 5-androstene-3β,17β-diol to the Δ⁴-3-ketosteroids progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone, respectively. Subsequent tissue-specific metabolism of Δ⁴-3-ketosteroids by various cytochrome P450 enzymes results in the production of glucocorticoids, mineralocorticoids, estrogens, and androgens. This crucial enzyme is present in classical steroidogenic tissues such as the adrenal cortex, ovary, testis, and placenta, and was more recently localized to peripheral tissues such as prostate, mammary gland, and skin (1). In the human, 3β-HSD exists as two isoforms (type I and II) derived from the tissue-specific expression of two highly related but distinct genes (2, 3). Human type I 3β-HSD is predominantly expressed in placenta and skin and is the major form found in breast tissue (2). In contrast, type II 3β-HSD expression is almost exclusively localized to the adrenal, ovary, and testis (2).

The essentiality of this enzyme in adrenal and gonadal steroidogenesis is underscored by the severe physiological consequences that arise in cases of 3β-HSD deficiency. Congenital adrenal hyperplasia, which can be fatal if not detected and treated early, occurs in response to deficiencies in any one of the steroidogenic enzymes involved in the biosynthesis of cortisol, including 3β-HSD (4). However, because 3β-HSD is also involved in gonadal steroidogenesis, insufficient levels of 3β-HSD may impair sexual differentiation, resulting in pseudohemaphroditism with incomplete masculinization of the external genitalia in males and mild virilization in females (5, 6). Furthermore, ovarian synthesis of progesterone is required for the establishment and maintenance of early pregnancy (7) and a reduction in the duration or concentration of systemic progesterone, luteal phase insufficiency, is associated with impaired fertility and repeated first trimester abortion (8, 9).

Numerous studies have demonstrated the role of the tran-
scription factor steroidogenic factor-1 (SF-1; also called adrenal 4-binding protein; Ad4BP) in the CaMP-mediated transactivation of cytochrome P450 steroid hydroxylase genes (10–15) in adrenal and gonadal tissues. This transcription factor is a member of the steroid hormone receptor superfamily (11, 16) and is classified as an orphan nuclear receptor because an endogenous ligand has not been identified. SF-1/Ad4BP and a closely related isoform, embryonal long terminal repeat-binding protein (ELP), are transcribed from the same gene (17, 18) and are homologs of fushi tarazu factor 1 (Ftz-F1), an orphan nuclear receptor that controls the fushi tarazu homeobox gene in Drosophila (19). In addition to its regulatory actions on steroid hydroxylase gene expression, targeted disruption of the Ftz-F1 gene in mice proved this transcription factor to be essential for sexual differentiation and development of the adrenal gland and gonads (20) and critical for normal development of the ventromedial hypothalamus and pituitary gonadotrophs (21). Interestingly, this nuclear receptor mediates transcriptional control over a number of genes that are involved in various aspects of reproductive function including the α-subunit of pituitary glycoprotein hormones (22), Müllerian inhibiting substance (23), and oxytocin (24) genes. Additionally, pituitaries of Ftz-F1-disrupted mice lack transcripts for gonadotropin-releasing hormone receptor, as well as β-subunits of luteinizing hormone and follicle-stimulating hormone (25), all of which are requisite for reproductive competence.

Although it is well established that 3β-HSD is vital for the synthesis of essential adrenal glucocorticoid and mineralocorticoid hormones, as well as gonadal production of progesterone, as well as gonadal production of progesterone, 3β-HSD isozyme and treatment of the cells with either angiotensin II or phorbol ester results in enhanced expression of 3β-HSD mRNA and synthesis of aldosterone, presumably through activation of protein kinase C (PKC; Refs. 27 and 28). The objective of the present study was to identify the specific regions of the type II 3β-HSD gene that confer basal and responsive promoter activity and treatment of the cells with either angiotensin II or phorbol ester results in enhanced expression of 3β-HSD isozyme and treatment of the cells with either angiotensin II or phorbol ester results in enhanced expression of 3β-HSD mRNA and synthesis of aldosterone, presumably through activation of protein kinase C (PKC; Refs. 27 and 28).

MATERIALS AND METHODS

Cell Culture—H295R (human adrenocortical tumor; Ref. 27) cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1, 15 mM HEPES; Life Technologies, Inc.) supplemented with 2% fetal calf serum (HyClone Laboratories, Logan, UT) and insulin (6.25 mg/ml), transferrin (6.25 mg/ml), selenium (6.25 mg/ml), and linoleic acid (5.35 mg/ml; 1% ITS + , Collaborative Research, Bedford, MA). HeLa (human cervical carcinoma) cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 with 10% fetal calf serum. All media contained 50 μg/ml gentamicin (Sigma). Phorbol ester (phorbol 12-myristate 13-acetate (PMA), Sigma) treatment and control media supplemented to cells during transient transfection, or cells destined for nuclear extract preparation or RNA isolation contained 200 μM PMA (added as 10,000-fold stock in dimethyl sulfoxide; MeSO) or an equivalent amount of MeSO as carrier, respectively.

Plasmids and Reporter Plasmid Construction—Plasmid (BlueScript KS II+ vector; Stratagene, San Diego, CA) containing 1251 bp of 5’-flanking and 820 bp of downstream sequence (~1251 to 820 bp, relative to the transcriptional start site) of the h3βHSD-II gene (3) subcloned into the HindIII site was a kind gift of Dr. Van Lau-The, Laval University, Quebec, Canada. Initially, sequence from ~1251 to +45 bp was amplified by the polymerase chain reaction (PCR) using Taq polymerase (Promega, Madison, WI), a 5’ oligonucleotide primer identical to the published T7 promoter sequence in the BlueScript vector and a 3’ oligonucleotide primer that spanned from +27 to +45 bp of untranslated exon I and was designed to contain a penultimate HindIII site. Following agarose gel purification, the ~1251 to +45 bp fragment was subcloned into the PCR II vector (InVitrogen, San Diego, CA), subjected to HindIII digestion, and ultimately fused to the chloramphenicol acetyltransferase (CAT) gene in the pCAT-Basic vector (~101 CAT, Promega).

Progressive 5’ deletion mutants of the ~1251 to +45 bp fragment of the h3βHSD-II gene were generated using PCR. Five 3’ oligonucleotide primers were used in separate reactions with the previously described 3’ oligonucleotide primer to generate five constructs with sequence progressively deleted from the 5’ end of the promoter. The five primer sequences were 1051 to 1028, 1051 to 1025, 1051 to 1021, 1051 to 678 (~701 CAT), 301 to ~276 (~301 CAT), ~101 to ~78 (~101 CAT), and ~52 to ~32 (~52 CAT) bp of the promoter. All oligonucleotide tides contained a penultimate HindIII restriction endonuclease site to facilitate subcloning. Following PCR, expected sizes of the promoter fragments were confirmed by agarose gel electrophoresis, extracted from the gel, and subcloned into the PCR II vector. Clones containing the five promoter fragments were digested with HindIII and the resulting fragments subjected to agarose gel purification prior to ligation into the pCAT-basic vector. All constructs were confirmed by sequencing through the ligation sites using the dyeoxy chain-termination method (29) and Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.).

The ~101M CAT mutant promoter construct (~101M CAT) was generated by synthesizing the top and complementary DNA strands (~101 to +1 bp) in three sections that, when annealed, yielded 3 double-stranded oligonucleotides containing 6-bp overhangs to facilitate ligation. The putative SF-1 response element was mutated by substituting two thymidine residues for two guanine residues (underlined) in the sequence so that TCAAGTATA became TCAATTATA. Both 5′ and 3′ ends of the construct were designed to contain HindIII restriction sites to facilitate subcloning into the pCAT-basic vector. The entire ~101 mutant promoter CAT construct was sequenced in both directions to confirm mutation of the putative SF-1/Ad4BP response element.

The pRC/RSV expression vector (InVitrogen) containing the full-length SF-1/Ad4BP cDNA (RSV-SF-1/Ad4BP) was described previously (30). The pSV2-luciferase (pSV2-luc) plasmid was used to normalize for differences in transfection efficiency among samples. All reporter constructs and expression vectors were prepared for transfection using the Qiagen Plasmid Mega kit (Qiagen Inc., Chatsworth, CA).

Transient Transfections—H295R and HeLa cells were transiently transfected using a modification of the calcium phosphate co-precipitation method (31). Briefly, adherent H295R and HeLa cells were cultured in 65% confluent in 100-mm tissue culture dishes (Corning Scientific Products, Corning, NY) in 10 ml of the appropriate medium. Calcium phosphate-DNA co-precipitates were formed by dropwise addition of equal volumes (0.5 ml) of solution A (0.24 mM CaCl2 containing 10 μg of promoter-CAT construct, 10 μg of control pGEM-3Z plasmid (Promega), plus 2 μg of pSV2-luc plasmid DNA for H295R cells and 10 μg of promoter-CAT construct, 10 μg of control pGEM-3Z plasmid, 2 μg of pSV2-luc plasmid DNA for HeLa cells) to Solution B (2% Hepes-buffered saline; 50 mM Hepes, 1.4 mM NaHPO4, 0.28 mM NaCl (pH 7.1)). Calcium phosphate-DNA precipitates were incubated at 23 °C for at least 20 min and added to single 100-mm dishes of cells containing 9 ml of fresh medium. H295R and HeLa cells were incubated with precipitate for 4 h at 37 °C (5% CO2 and 95% air), shocked for 3 min on 30 s, respectively, with 15% (v/v) glycerol in Dulbecco’s phosphate-buffered saline (D-PBS; 0.137 mM NaCl, 0.137 mM NaCl, 0.5 mM MgCl2, 6.45 mM NaHPO4, 1.5 mM KH2PO4), washed with D-PBS, and incubated at 37 °C for 24 or 36 h, respectively. During the final 24 h of incubation, cells were cultured in the presence or absence of PMA or carrier as described previously. Cells were harvested using trypsin/EDTA (Life Technologies, Inc.), pelleted, resuspended in 0.25 mM Tris–HCl (pH 7.4), and stored at ~70 °C until assayed for CAT activity. Transfections were performed in triplicate with mock (no plasmid) serving as negative controls. Experiments were repeated identically at least twice and at least three times with modifications.

CAT and Luciferase Assays—Frozen cell pellets were thawed on ice and lysed by sonication. Soluble extract was separated from cell debris by centrifugation, divided into aliquots for CAT and luciferase assays, and frozen at ~70 °C prior to use. Prior to CAT assay, pellets were heated to 60 °C for 5 min to denature any endogenous acetylase/ deacetylase enzymes. Fluorescent CAT assays were performed as described (32) with some modification using the FLASH CAT assay kit (Stratagene). Acetyl coenzyme A (CoA) was synthesized by reaction of CoA (Pharmacia Biotech Inc.) with acetic anhydride (Sigma) as described elsewhere (33) and stored at ~70 °C until use. H295R cell
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extracts (45 μl) and HeLa cell extracts (1 μl of a 1:10 dilution) were incubated in 0.25 x Tris-HCl (pH 7.4) in a total reaction volume of 125 μl with acetyl-CoA (8.2 mM) and fluorescent borondipyrromethene di-fluoride (BODIPY) chloromethanaphenyl (CAM) substrate (1:12.5 dilution) at 27 °C for 8 and 1 h, respectively. Reactions were terminated by addition of cold ethyl acetate (850 μl) and incubated for 30 min. An aliquot (800 μl) of extracted substrate and acetylated products was removed (organic phase), dried under vacuum, and resuspended in ethyl acetate (20 μl) prior to separation on thin-layer chromatography plates (LK6, Whatman, Clifton, NJ) with chloroform:methanol (9:1) for 30 min. Substrate and products were visualized under long-wave UV light in the dark and photographed (Type 55 positive/negative film, Polaroid, Cambridge, MA). Substrate and combined product bands were scraped from the plates, extracted and diluted 1:10 in methanol prior to measurement with a fluorometer. Percent conversion of BODIPY CAM substrate to 1-, 3-, and 1,3-acetylated BODIPY CAM products was computed after correction for background activity.

Differences in transfection efficiency between samples were monitored using a portion of the cell extracts for luciferase assays (34) with minor modification. Briefly, 10 μl of cell extract was added to 350 μl of reaction buffer (25 mM glycylglycine, 5 mM ATP, and 15 mM MgSO4 (pH 7.6)). Luciferase (1 μl; Boehringer Mannheim) was injected into the reaction and relative light output was measured using a Monolight luminometer (Analytical Fluorescence Labs, San Diego, CA). Transfection efficiency did not vary significantly between triplicate culture dishes within any single treatment group or between treatment groups.

Preparation of Nuclear Extracts—Several 225-cm2 flasks of H295R cells were maintained as described previously. At 55–65% confluency, cells were cultured in the presence or absence of 200 nM PMA for 24 h at which time cells were harvested using trypsin/EDTA, pelleted, washed with D-PBS, pelleted and resuspended in D-PBS (1.0 ml), and maintained on ice. Crude nuclear extracts were prepared according to the method of Dignam and co-workers (35) as modified by Andrews and Faller (36). Briefly, after pelleting and removal of D-PBS, cells were allowed to swell for 10 min on ice in a hypotonic buffer (10 mM HEPES-KOH (pH 7.9) at 4 °C, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) followed by vortexing and centrifugation. The supernatant was discarded and the pellet resuspended in a high salt buffer (20 mM HEPES-KOH (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) for 20 min to extract nuclear proteins. The suspension was centrifuged, and the supernatant containing nuclear proteins was aliquoted and stored in liquid nitrogen. Protein concentrations were determined using the BCA method (Pierce) as modified for the presence of sulphydryl reagents (37).

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays (EMSA) were performed as described (38) with some modification. Single-stranded oligonucleotides containing the putative SF-1/Ad4BP element (underlined) and 10–11 bases of 5' and opposite strand 5’-TCAGCCCTTATTACCTT-3’ (SacI-GAGCCTGTA-3’ and opposite strand 5’-TCAGCCCTTATTACCTT-3’ (SacI-GAGCCTGTA-3’ and opposite strand 5’-TCAGCCCTTATTACCTT-3’) were synthesized using an automated DNA synthesizer (Oligo 1000, Beckman Instruments Inc., Palo Alto, CA). Double-stranded SF-1/Ad4BP probe was prepared by annealing 50 ng of each oligonucleotide strand for 2 min at 65 °C, followed by slow cooling to room temperature. The probe was end-labeled using [γ-32P]dATP-labeled cDNA probe. SF-1/Ad4BP probe was generated using random primers, exon S2; Refs. 43 and 44) generously provided by Dr. Kelly Mayo, Northwestern University, Evanston, IL. After hybridization, the blot was washed once with 2 x SSC, 1% (w/v) SDS at 45 °C for 15 min; twice with 2 x SSC, 0.1% (w/v) SDS at 45 °C for 15 min; and once with 0.1 x SSC, 0.1% (w/v) SDS for 15 min at 60 °C. Autoradiography was performed at −70 °C with intensifying screens using Kodak X-Omat AR or BioMax MR film. Following autoradiography, blots were stripped of probe by washing for 1 h at 80 °C. The blot was de-glyoxalated in 0.1 M sodium phosphate (pH 8.0) for 1 h at 65 °C, followed by prehybridization in 100 ml of hybridization buffer (50% v/v deionized formamide, 5 % SSC, 0.025 M sodium phosphate buffer (pH 7.0), 5% w/v SDS, 0.1% w/v BSA, 0.1% w/v Ficoll 400, 0.1% w/v polyvinylpyrrolidone, and 0.05% w/v fish sperm DNA) for 1 h at 42 °C prior to addition of 50 μCi of [α-32P]dATP-labeled DNA probe. SF-1/Ad4BP probe was generated using random primers, exon S2; Refs. 43 and 44) generously provided by Dr. Kelly Mayo, Northwestern University, Evanston, IL. After hybridization, the blot was washed as described above, except that the duration of the final wash was 10 min.

Digital Imaging—Autoradiographs and Polaroid negatives were scanned using an Agfa Arcus II flatbed scanner and Adobe Photoshop software. Digitized images were saved as TIFF files, and all cropping and text enhancements were carried out using Aldus PageMaker or Freehand programs.

RESULTS

To determine the region(s) of the promoter that confer phorbol ester-mediated transcriptional regulation of the type II 3β-HSD gene, a series of 5’ deletions of the promoter fused to a CAT reporter gene were used to transiently transfect human adrenocortical carcinoma (H295R) cells followed by treatment for 24 h in the absence (basal) or presence of PMA. As shown in Fig. 1, deletion of the promoter sequence from −101 to −52 bp results in a nearly 4-fold and 500-fold reduction in basal and PMA-stimulated CAT activity, respectively, suggesting that this region is important for both basal and PMA-induced regulation of type II 3β-HSD expression. Additionally, the region from −1251 to −301 bp may contain a negative regulatory element that inhibits PMA-induced transcription since an approximately 5-fold increase in CAT activity was observed following...
lowing the deletion of −1251 to −301 bp of the promoter.

A subsequent search of the −1251 to −252 bp promoter se-
quence revealed a putative SF-1/Ad4BP regulatory element,
TCAAGGTAA, at −64 to −56 bp that differed from the reported
SF-1 (PyCAAGGPyCPu; Ref. 13) and Ad4BP (C/TCAAGGT/
CC/T; Ref. 10) elements by a single nucleotide (underlined). To
determine if the putative SF-1/Ad4BP element was functional,
non-steroidogenic HeLa cells, which do not express SF-1/
Ad4BP, were transiently cotransfected with the
−1251 CAT, −252 CAT, and −1251M CAT constructs in the presence and
absence of an expression vector for SF-1/Ad4BP (RSV-SF-1/
Ad4BP). In addition, transfected cells were cultured for 24 h in
the presence or absence of PMA (200 nM) to investigate the
ability of phorbol ester to modulate SF-1/Ad4BP-mediated ac-
tivity of the 3β-HSD promoter (Fig. 2).

Cotransfection of HeLa cells with −101 CAT and RSV-SF-1/
Ad4BP increased CAT activity 49-fold over basal, whereas only
small increases were observed (6- and 2-fold, respectively) in
those cells transfected with −52 CAT, which lacked the putative
SF-1/Ad4BP element, or −101M CAT constructs in the presence and
absence of an expression vector for SF-1/Ad4BP (RSV-SF-1/
Ad4BP). In addition, transfected cells were cultured for 24 h in
the presence or absence of PMA (200 nM) to investigate the
ability of phorbol ester to modulate SF-1/Ad4BP-mediated ac-
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Cotransfection of HeLa cells with −101 CAT and RSV-SF-1/
Ad4BP increased CAT activity 49-fold over basal, whereas only
small increases were observed (6- and 2-fold, respectively) in
those cells transfected with −52 CAT, which lacked the putative
SF-1/Ad4BP element, or −101M CAT, in which two nucle-
etides in the putative response element had been mutated.
PMA treatment increased CAT activity 20-fold over basal in those
cells transfected with −101 CAT alone. PMA had a sim-
ilar effect on cells transfected with −52 CAT (17-fold increase);
however, CAT activity in response to PMA was considerably
less in those cells transfected with −101M CAT (5-fold).
Interestingly, an unexpectedly powerful synergistic effect of SF-1/
Ad4BP and PMA on CAT activity was observed in cells cotrans-
F.1. Deletion mutagenesis of human type II 3β-HSD promoter CAT constructs and promoter activities in transiently trans-
fected H295R cells. H295R cells were transfected with a series of 5′ deletion mutants of the type II 3β-HSD promoter fused to a CAT reporter
gene using the calcium phosphate coprecipitation method followed by treatment for 24 h with PMA (200 nM; solid bars) or Me2SO carrier (open
bars) as described under “Materials and Methods.” Data represent the mean ± S.E. of triplicate cultures from a single representative experiment of two experiments.

FIG. 2. Synergistic effect of PMA on SF-1/Ad4BP-mediated ac-
tivation of the type II 3β-HSD promoter in transiently trans-
fected HeLa cells. HeLa cells were cotransfected with −101, −52, or
−101M CAT reporter constructs and an expression vector for SF-1/
Ad4BP (RSV-SF-1/Ad4BP) followed by treatment for 24 h with PMA
(200 nM) or Me2SO carrier as described under “Materials and Methods.”
Data represent the mean ± S.E. of triplicate cultures from a single
representative experiment of two experiments. Numbers above bars
indicate -fold increases above basal for each construct.

the response in a physiologically relevant human steroidogenic
cell line. To address this issue, adrenocortical carcinoma
(H295R) cells were transfected with the −101, −52, and
−101M CAT constructs followed by treatment with or without
phorbol ester. As shown in Fig. 3, PMA treatment of H295R
cells transfected with the −101 CAT construct containing the
putative SF-1 regulatory element increased promoter activity
18-fold over basal. In contrast, deletion (−52 CAT) or mutation

Although phorbol ester activation of the human type II 3β-
HSD promoter in non-steroidogenic HeLa cells appeared to be
primarily mediated by SF-1, it was also necessary to evaluate
(−101M CAT) of the putative SF-1 regulatory element dramatically reduced PMA responsiveness to 3-fold and 2-fold over that for basal, respectively. Collectively, these data and those from the preceding HeLa cell experiments clearly demonstrate the importance of the SF-1 regulatory element in mediating phorbol ester regulation of the human type II 3β-HSD promoter in both steroidogenic and non-steroidogenic cells.

Interaction of SF-1 with the putative regulatory element was examined by EMSA using nuclear extracts prepared from H295R cells cultured in the presence or absence of PMA and a 32P-labeled double-stranded oligonucleotide containing the putative SF-1/Ad4BP site and 10–11 bases of 5′ and 3′ flanking type II 3β-HSD promoter sequence. As shown in Fig. 4, multiple protein-DNA complexes were formed when nuclear extracts from untreated and PMA-treated H295R cells were incubated with the oligonucleotide probe. However, when extracts were incubated in the presence of increasing concentrations of unlabeled oligonucleotide, the appearance of one complex was markedly diminished as compared with the others. Preincubation of the extracts with SF-1/Ad4BP antiserum abolished the formation of that particular complex, confirming the participation of SF-1/Ad4BP. Additionally, the intensity of the band representing the SF-1/Ad4BP specific protein-DNA complex appeared to be slightly darker for control versus PMA treatment. These data may be interpreted to suggest reduced DNA binding in those extracts derived from cells treated with PMA as compared with control. However, given that the cells were harvested at only one time point and that the kinetics of PMA-induced expression of SF-1/Ad4BP mRNA are currently unknown, the results of this comparison appear to be of limited value.

To further test the specificity of DNA binding, nuclear extracts from untreated H295R cells were incubated in the presence of a nonspecific control consisting of antiserum against a secreted epididymal protein in rats or increasing concentrations of unlabeled heterologous oligonucleotides (containing GAS or PIE response elements) or unlabeled oligonucleotide (−101M) containing a mutated form of the putative SF-1/Ad4BP response element flanked by type II 3β-HSD promoter sequence (Fig. 5). Incubation of nuclear extracts with nonspecific antibody or increasing concentrations of unlabeled −101M, GAS, or PIE oligonucleotides failed to diminish the appearance of the complex that was abolished by SF-1/Ad4BP antiserum, suggesting that formation of this protein-DNA complex is specific to SF-1/Ad4BP.

Finally, Northern analysis was used to determine if SF-1/Ad4BP was expressed in H295R, HeLa, and mouse F9 teratocarcinoma cells. As shown in Fig. 6, SF-1/Ad4BP transcripts were detected in H295R cells and expression appeared to increase slightly upon treatment with PMA. In addition, a slightly smaller transcript was detected in F9 cells after longer autoradiography times. As anticipated, no transcripts for SF-1/Ad4BP were detected in HeLa cells. Collectively, results of the EMSA and Northern analysis demonstrate that SF-1/Ad4BP is expressed in adrenal cortical carcinoma cells and interacts with the putative SF-1/Ad4BP regulatory element present in the promoter of the human type II 3β-HSD gene.

DISCUSSION

In the present study we sought to determine regions of the human type II 3β-HSD promoter important for regulation us-
The putative SF-1/Ad4BP element found in the type II 3β-HSD gene, which is predominantly expressed in the placenta, most likely differs from that of the type II isoform. HSD gene, which is predominantly expressed in the placenta.

**Arrowhead**

**Materials and Methods.**

EMSA were performed using nuclear extracts from control H295R cells (20 μg) and labeled oligonucleotide containing the SF-1/Ad4BP regulatory element present in the type II 3β-HSD promoter in the presence or absence of SF-1/Ad4BP antiserum (1 μl), nonspecific antiserum (1 μl), or increasing molar concentrations (50 or 500-fold) of unlabeled 101M, GAS, or PIE oligonucleotide as described under "Materials and Methods." Arrowheads denote loss of gel-shifted complex in extracts incubated with SF-1/Ad4BP antiserum.

Expression of 3β-HSD and synthesis of aldosterone in H295R cells are regulated through the action of angiotensin II via the type I angiotensin II receptor coupled to polyphosphoinositide-C and subsequent increases in intracellular calcium (28). Because this effect can be mimicked by phorbol ester (27), it is presumed to be mediated via protein kinase C. Our results are consistent with PKC-mediated regulation of 3β-HSD expression in H295R cells. PMA treatment of H295R cells transfected with a series of 9 deletion mutants of the 3β-HSD promoter increased reporter gene activity, for all constructs except 52 bp, greater than that observed for untreated transfected cells. The novel aspect of this finding is that the PMA-induced increase in transcriptional activity appeared to be mediated by SF-1/Ad4BP. Deletion of promoter sequence from -101 to -52 bp, later found to contain a functional SF-1/Ad4BP element, abolished PMA-stimulated CAT activity. Additionally, mutation of the putative SF-1/Ad4BP regulatory element also inhibited PMA-induced promoter activation. To date, no similar effect of phorbol ester has been reported for any of the genes known to be regulated by SF-1/Ad4BP.

**Arrowheads**

**Materials and Methods.**

Cotransfection of HeLa cells with the -101 CAT construct and RSV-SF-1/Ad4BP expression vector yielded a significant 49-fold increase in transcriptional activity due to SF-1/Ad4BP alone, as well as a distinct synergistic effect of PMA on SF-1/Ad4BP-mediated transcription. The 49-fold increase in CAT activity in the absence of any other treatment is greater than that reported for isolated SF-1/Ad4BP elements from other genes similarly cotransfected into non-steriodogenic cell lines (13, 15, 23, 24, 49). SF-1/Ad4BP alone was unable to activate MIS gene expression in HeLa cells, whereas coexpression of a
mutant form of SF-1/Ad4BP lacking the putative ligand binding domain slightly increased transcriptional activity (23), suggesting that ligand or a cofactor is necessary for activation of the MIS promoter by SF-1/Ad4BP. In the case of some steroid hydroxylase genes, the addition of protein kinase A activators such as cAMP and forskolin or coexpression of the catalytic subunit of protein kinase A is necessary for appreciable activation by SF-1/Ad4BP in nonsteroidogenic cells. In our case, high levels of transcriptional activity due to SF-1/Ad4BP alone may result from promoter-specific sequence that confers a greater sensitivity of the type II 3β-HSD gene to stimulation by SF-1/Ad4BP. A number of reports have suggested that nucleotides 5′ of the response elements for SF-1/Ad4BP and another orphan nuclear receptor, nerve growth factor-inducible factor B (NGFI-B), may influence binding affinity (10, 15, 23, 50). Additionally, a brief survey of reported functional SF-1/Ad4BP response elements in comparison to that for type II 3β-HSD indicates that the putative 3β-HSD SF-1/Ad4BP element is a unique variant of the AGGTCA consensus motif in that the penultimate cytidine at the 3′ end of the sequence has been replaced with an adenine resulting in AGGTAA, which has not been reported for other genes. This difference could be in part responsible for the enhanced SF-1/Ad4BP activation of 3β-HSD gene expression in HeLa cells.

The mechanism underlying the stimulatory effect of PMA on 3β-HSD promoter activity in HeLa cells in the absence of SF-1/Ad4BP is less clear and may be independent of a functional SF-1/Ad4BP response element because removal of the element, −52 CAT, failed to reduce CAT activity in response to PMA. It is possible that a yet unidentified regulatory element is present in the sequence from −52 to +45 bp of the promoter and perhaps PMA induces the synthesis of a HeLa cell-specific regulatory protein(s) that is responsive to PMA in the absence of SF-1/Ad4BP. Loss of PMA-induced CAT activity after mutation, but not removal, of the putative SF-1/Ad4BP response element (−101M CAT) is seemingly more complex. However, it is possible that the PMA-responsive region of the promoter may partially overlap that for SF-1/Ad4BP binding and that the loss of several nucleotides from the 5′-end of the PMA responsive sequence may be well tolerated whereas mutation of several of those nucleotides severely compromises PMA responsiveness. Interestingly, HeLa cells express an orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor (COPUT-TF; Ref. 51), that has been reported to bind to recognition elements that partially and totally encompass the SF-1/Ad4BP elements in the 17α-hydroxylase (15) and oxytocin (24) promoters, respectively.

The highly synergistic effect of PMA on SF-1/Ad4BP activation of the 3β-HSD promoter in HeLa cells has not been reported previously, and the mechanism responsible for this cell specific response is presently unknown. Because PMA is a potent activator of PKC and SF-1/Ad4BP has 10 consensus PKC phosphorylation sites by sequence homology (16) and is a phosphoprotein, it is tempting to speculate that the profound response to PMA is due, at least in part, to direct phosphorylation of the transcription factor. Alternatively, it is possible that treatment with PMA induces the production and(or) phosphorylation of a HeLa cell factor, that significantly augments SF-1/Ad4BP activation of the type II 3β-HSD promoter. Synergistic effects of phorbol ester and chola toxin on estradiol-stimulated transcription of a synthetic estrogen-responsive reporter gene were also found to be cell-specific and appeared to result from the stabilization or facilitation of the receptor with components of the transcriptional apparatus, possibly as a result of phosphorylation of the receptor or other necessary proteins (52). It is also possible that PMA provokes synthesis of an undiscovered ligand for SF-1/Ad4BP in nonsteroidogenic HeLa cells. Recent data indicate that SF-1/Ad4BP expression and action are not limited to steroidogenic tissues and the regulation of steroidogenic enzymes (22–25). Thus, it is possible that the undiscovered ligand for SF-1/Ad4BP is not a steroid, as has been hypothesized (15, 17, 53); instead, it may be a molecule that is present in steroidogenic as well as nonsteroidogenic tissues, with specificity of activation relying solely on the tissue-specific expression of SF-1/Ad4BP.

There is evidence to indicate that other members of the steroid/thyroid hormone receptor superfamily, normally activated by ligand binding, may elicit their actions at steroid-responsive regulatory elements through ligand-independent processes that involve cross-talk between membrane-bound receptor signaling pathways and the specific nuclear steroid receptor. Dopamine can activate progesterone, estrogen (ER), vitamin D, and thyroid hormone receptor-β, but not glucocorticoid, receptor-mediated activation of target response elements in transfected cells in the absence of steroid ligand and in the case of progesterone receptor, activation required the presence of a specific serine phosphorylation site on the receptor (54). More recently, epidermal growth factor (EGF) was reported to activate estrogen-independent transcription of a consensus estrogen response element cotransfected with an expression vector for the mouse ER into human endometrial adenocarcinoma cells (55). In the presence of estrogen, EGF had a synergistic effect on transcription. Although not determined, ER phosphorylation and(or) activation of other regulatory proteins were hypothesized as plausible mechanisms mediating the effects of EGF.

In HeLa cells, co-transfection of SF-1/Ad4BP followed by treatment with PMA results in promoter activity levels that greatly exceed the additive effect of each treatment alone and fulfills the criteria for synergism in transcriptional activation as discussed by Herschlag and Johnson (56). Therefore, the synergism implies that the two activating mechanisms function in the same pathway. While these high levels of activation might occur without physical interaction between the two activating agents, it is also highly likely that the activation of protein kinase C isoforms by phorbol ester treatment may alter the phosphorylation state of SF-1 with a corresponding change in activity.

Our investigation of basal and phorbol ester-mediated regulation of type II 3β-HSD gene expression in human adrenocortical carcinoma cells has resulted in several novel findings. Both basal and PMA-induced transcription of the gene in H295R cells required the presence of promoter sequence containing an SF-1/Ad4BP recognition element. This is the first demonstration of SF-1/Ad4BP-mediated regulation of a non-cytochrome P450 steroidogenic enzyme promoter. Cotransfection of the reporter construct containing the putative SF-1/Ad4BP element or a mutated version of the element and an expression vector for SF-1/Ad4BP into HeLa cells confirmed the essentiality and functionality of this response element in a non-steroidogenic cell line. We also discovered a previously unreported synergistic effect of PMA on the regulation of transcription by SF-1/Ad4BP. Additionally, results of EMSA with SF-1/Ad4BP antiserum indicated that H295R cell nuclear extracts contained SF-1/Ad4BP, which specifically interacted with the putative 3β-HSD SF-1/Ad4BP response element. Northern analysis also confirmed the presence of SF-1/Ad4BP transcripts in H295R but not HeLa cells. Collectively, these data provide considerable evidence to support a role for SF-1/Ad4BP in the regulation of the type II 3β-HSD gene in adrenal cortical cells.

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2 K. Morohashi, unpublished data.
However, further research is needed to more clearly define the mechanisms underlying phorbol ester-mediated regulation of this gene in both H295R and HeLa cells.

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