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Human P450scc Gene Transcription Is Induced by Cyclic AMP and Repressed by 12-*O*-Tetradecanoylphorbol-13-Acetate and A23187 through Independent *cis* Elements

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Long-term regulation of mammalian steroid hormone synthesis occurs principally by transcriptional regulation of the gene for the rate-limiting cholesterol side-chain cleavage enzyme P450scc. Adrenal steroidogenesis is regulated primarily by two hormones: adrenocorticotropin, which works via cyclic AMP (cAMP) and protein kinase A, and angiotensin II, which works via Ca^{2+} and protein kinase C. Forskolin and 8-bromo-cAMP stimulated, while prolonged treatment with a phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate [TPA]) and a calcium ionophore (A23187) additively suppressed accumulation of endogenous P450scc mRNA in transformed murine adrenal Y1 cells. In Y1 cells transfected with 2,327 base pairs of the human P450scc promoter fused to the bacterial gene for chloramphenicol acetyltransferase (CAT), forskolin increased CAT activity 900% while combined TPA plus A23187 reduced CAT activity to 15% of the control level. Forskolin induced the P450scc promoter as rapidly as a promoter containing two cAMP-responsive elements fused to a simian virus 40 promoter, a system known to respond directly to cAMP. Basal expression was increased by sequences between −89 and −152 and was increased further by sequences between −605 and −2327. This upstream region also conferred inducibility by cAMP. TPA plus A23187 transiently increased CAT activity before repressing it, reflecting the complex actions of angiotensin II in vivo. Repression by prolonged treatment with TPA plus A23187 was mediated by multiple elements between −89 and −343. Induction of CAT activity by forskolin was not diminished by treatment with TPA plus A23187, nor were the regions of the promoter responsible for regulation by the two pathways coisolated. Thus, the human gene for P450scc is repressed by TPA plus A23187 by mechanisms and sequences independent of those that mediate induction by cAMP.

Steroid hormones, which work by controlling transcription of specific genes, are critical regulators of physiological processes (5). The first, rate-limiting, and hormonally regulated step in the generation of steroid hormones is conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme P450scc. This mitochondrial cytochrome P450 enzyme receives electrons from NADPH via two protein intermediates, a flavoprotein, adrenodoxin reductase, and an iron-sulfur protein, adrenodoxin. By using these electrons, P450scc catalyzes three sequential reactions—22 hydroxylation, 20 hydroxylation, and $C_{20,22}$ bond cleavage—apparently at a single active site (reviewed in reference 39). As side-chain cleavage is the rate-limiting step in steroidogenesis (51), it is important to understand both the hormonal regulation and tissue-specific expression of this gene.

The developmental patterns of expression and hormonal regulation of P450scc are specific to each steroidogenic tissue. Increased steroidogenesis and accumulation of P450scc mRNA are stimulated by adrenocorticotropin in the human adrenal zonae fasciculata and reticularis, by luteinizing hormone and follicle-stimulating hormone in human ovarian granulosa cells, and by luteinizing hormone and human chorionic gonadotropin (hCG) in human testicular Leydig cells and placental cytotrophoblasts (11, 14, 22, 45, 52, 53). In all of these cases, the stimulatory hormone binds a cell surface receptor that activates a G protein (G_s) to increase intracellular cyclic AMP (cAMP). This, in turn, increases transcription of the gene for P450scc (28, 38). By contrast, angiotensin II (AII) acutely stimulates mineralocorticoid production in the adrenal zona glomerulosa by alterations of intracellular Ca^{2+} and activation of protein kinase C (PKC) (reviewed in reference 4). However, prolonged stimulation with AII or agents that activate the PKC pathway represses the quantities and activities of steroidogenic enzymes in cultured adrenal cells (12, 18, 36, 37). Thus, regulation of adrenal steroidogenesis is complex and is controlled by at least two different intracellular second-messenger systems.

The regions of the P450scc promoter responsible for cAMP induction and basal expression remain poorly characterized. In transient transfections of mouse adrenal Y1 tumor cells, 5.4 kilobases of 5′-flanking DNA from the human gene for P450scc mediated cAMP induction of expression of the bacterial gene for chloramphenicol acetyltransferase (CAT) to 750% of the control value, but the sequences responsible were not localized (28). The region between −2500 and −573 of the human promoter conferred strong basal expression in adrenocortical Y1 cells but not in human placental cytotrophoblast JEG3 cells (10), suggesting that it contains an adrenal-specific element. Data for shorter fragments of the bovine P450scc promoter fused to different reporter genes were internally inconsistent, and the CAT constructions responded weakly in Y1 cells treated with cAMP or forskolin (about twofold) (1).

The mechanism by which cAMP regulates P450scc mRNA is controversial. In primary cultures of bovine adrenal cells,

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induction of bovine P450scc gene transcription by cAMP appeared to be slow and require protein synthesis (31). Those investigators suggested that cAMP induced expression of a protein that then activated transcription of P450scc. We, and others, have shown that cAMP induction of P450scc can be rapid and independent of protein synthesis (22, 38). Although it was possible that these differences were species specific, tissue specific, or both, the group reporting a requirement for protein synthesis was unable to confirm this in either Y1 cells or primary cultures of bovine adrenal cells transiently transfected with bovine P450scc-CAT constructs (1). Thus, the mechanism for cAMP-mediated induction is unclear and the *cis*-acting sequences responsible for either basal expression or cAMP induction have not been localized. Furthermore, transcriptional regulation of the gene for P450scc by the Ca^{2+}-PKC second-messenger pathway has not been studied.

In this study, we characterized the regions of the human P450scc promoter necessary for basal expression and transcriptional regulation mediated by both cAMP and Ca^{2+}-PKC. Basal expression was significantly increased by two separate regions of 5′-flanking DNA. The upstream region (−2327 to −605) is also responsible for cAMP induction and contains the proposed adrenal-specific element (10). The kinetics of cAMP induction are rapid, suggesting a direct effect on transcription. The promoter region between −343 and −89 contains multiple elements that function as novel, hormonally dependent transcriptional repressors that respond strongly to a combination of A23187, a Ca^{2+} ionophore, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester. As the regions responsible for induction by cAMP and repression by the Ca^{2+}-PKC pathway do not colocalize, the two second-messenger pathways must alter P450scc transcription by independent mechanisms.

MATERIALS AND METHODS

Tissue culture. Y1 cells, a generous gift from B. Schimmer, were maintained in 50% DME H16–50% Ham F12 with 15% heat-inactivated horse serum, 2.5% fetal bovine serum, and 50 μg of gentamicin per ml at 37°C in 5% CO_2. For hormonal treatment, the cells were switched to medium supplemented with only 0.5% fetal bovine serum and 50 μg of gentamicin per ml. Unless indicated otherwise, treatments lasted for 12 h with 20 μM forskolin, 500 μM A23187, 30 nM TPA, or 1 mM 8-bromo-cAMP. Forskolin, A23187, TPA, and 8-bromo-cAMP were all purchased from Sigma Chemical Co.

RNA isolation. Cells were harvested from the tissue culture plates with phosphate-buffered saline (PBS) free of Ca^{2+} and Mg^{2+} (PBS-CMF), pelleted by a brief spin in a clinical centrifuge, and lysed with 1.0 ml of 5 M guanidinium isothiocyanate–50 mM Tris (pH 7.8)–0.5% sarcosyl–0.1 g of $CsCl_2$ per ml–10% β-mercaptoethanol. Total cellular RNA was isolated by pelleting through a 500-μl cushion of 5.7 M $CsCl_2$–100 mM EDTA. The RNA pellet was suspended in 300 μl of 10 mM Tris (pH 7.5)–0.1 mM EDTA–1% sodium dodecyl sulfate, extracted once with a 1:1 solution of buffered phenol-chloroform (pH 7.0), and then extracted with 200 μl of ether saturated with water before precipitation with 1/10 of a volume of 3 M sodium acetate and 2.2 volumes of ice-cold ethanol.

RNA transfer blots. The appropriate amount of RNA was pelleted and then suspended in 16 μl of 1 M glyoxal–50% dimethyl sulfoxide–10 mM NaH_2PO_4. RNA was denatured in this solution at 50°C for 60 min, cooled to room temperature, and loaded with 4 μl of 50% glycerol–0.01 M NaH_2PO_4–0.4% bromophenol blue into a 1.0% agarose–10 mM NaH_2PO_4 (pH 7.0) gel. The samples were subjected to electrophoresis at 40 to 50 V until the bromophenol blue migrated 8.0 cm and then transferred for 24 h to Hybond-N (Amersham Corp.) membranes. The transferred RNA was cross-linked to the membrane by using UV irradiation (1,200 μJ applied with a Stratagene UV StrataLinker). Membranes were prehybridized overnight at 42°C in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin)–50 mM NaH_2PO_4 (pH 8.0)–0.5% sodium dodecyl sulfate–250 μg each of Torula yeast RNA and herring sperm DNA per ml. Hybridization was for 24 h in the same buffer except at pH 7.0 and containing only 100 μg each of Torula RNA and of herring sperm DNA per ml with 10^6 cpm of probe (generated by random priming from a cDNA template) per ml. Templates were gel purified fragments of full-length human P450scc cDNA (11), a 1.2-kilobase fragment of rat P450scc cDNA (21), and a 700-base-pair (bp) *Hin*dIII fragment of human lamin A cDNA (20). Nonspecific hybridization was removed by washing in either 0.1× SSC–1% sodium dodecyl sulfate or 0.5× SSC, as indicated, for 15 min at room temperature, followed by two 30-min washes at 55°C. Autoradiography was done with one intensifying screen at −70°C.

Plasmids. The construction of our *cat* expression vector, pACAT, is described elsewhere (S. T. Brentano, J. Picardo-Leonard, S. H. Mellon, C. C. D. Moore, and W. L. Miller, Mol. Endocrinol., in press) and was modified further by deletion of sequences from the *Nde*I site to the polyadenylation signal and removal of the polylinker sequence between the *Xba*I and *Sph*I sites to yield pAnΔCATS/X (Fig. 1A).

A plasmid containing about 2,500 bp of the 5′-flanking and untranslated DNA for the human gene for P450scc was generously provided by Bon-chu Chung. This plasmid was used to generate a series of 5′ deletion constructs (Fig. 1B) by using unique or rare restriction sites for internal deletions. Each deletion was created such that a *Kpn*I site was always located on the 5′ end. The 3′ ends of all of the constructs were defined by ligating the *Pvu*II site at +49 to a filled-in *Bam*HI site, thus regenerating the *Bam*HI site. Each deletion clone was digested with *Kpn*I and *Bam*HI, and the fragment was purified by gel electrophoresis and then ligated into the *Kpn*I and *Bam*HI sites of the pAnΔCATS/X vector. The resulting P450scc–CAT plasmids are designated pAn−XCAT, where X designates the 5′ end of the construct, in base pairs, relative to the transcriptional initiation site of human P450scc (Fig. 1B). pAnRSVCAT was generated by cloning the 587-bp *Hin*dIII fragment of the Rous sarcoma virus (RSV) promoter-enhancer from RSVβ-gal (17) into the *Hin*dIII site of pAnΔCAT. Similarly, the *Bam*HI-to-*Bgl*II fragment of TKCAT that contains the herpes simplex virus thymidine kinase promoter out to −109 bp relative to the transcriptional start site was cloned into the pAnΔCAT *Bam*HI site to generate pAnTK−109CAT. All constructs were confirmed by restriction mapping and by sequencing across all cloning junctions.

Transfections. Plasmids were isolated by using the Triton X-100 cleared-lysate protocol, purified by one cycle of equilibrium density centrifugation through $CsCl_2$, treated with RNase A and proteinase K, and extracted several times with phenol-chloroform (50:50, pH 7.0). Cells were transfected with a slightly modified calcium phosphate coprecipitation procedure of Chen and Okayama (8) by using HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) instead of BES (*N*,*N*-bis(2-hydroxyethyl)-2-amino-ethane-

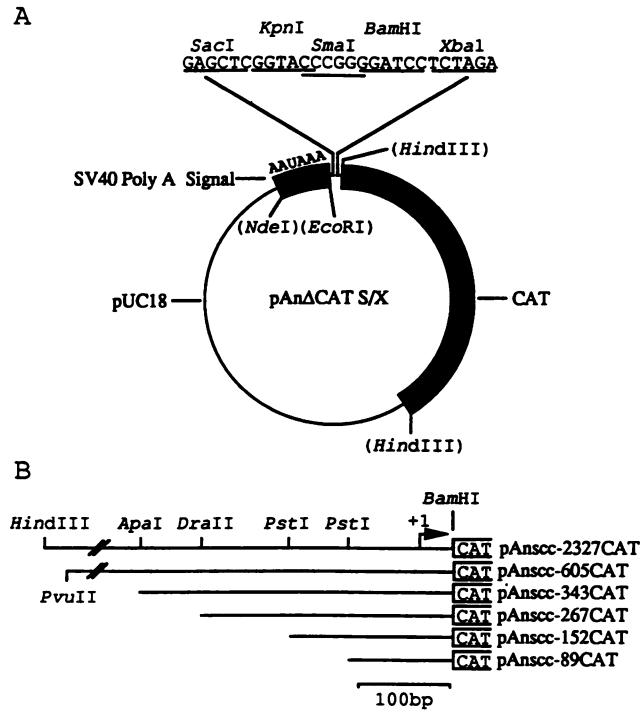

FIG. 1. Schematic representation of pAnsccXCAT 5′ deletion plasmids. (A) The parent vector, pAnΔCATS/X, contains the CAT sequence, the simian virus 40 early polyadenylation signal cloned in the sense orientation relative to the CAT-coding sequence, and the *Sac*I-to-*Bam*HI sites of the pUC18 polylinker. Restriction enzyme sites within brackets were destroyed in the cloning process. (B) The series of human P450scc deletion plasmids used in this study, with the largest P450scc fragment ending at the *Hin*dIII site at −2327 bp upstream from the transcriptional initiation site (+1) indicated by the arrow. The plasmids were generated in Bluescript vectors by using the sites indicated such that *Bam*HI and *Kpn*I sites defined the 3′ and 5′ ends, respectively, and were used to clone into pAnΔCATS/X.

sulfonic acid) buffer. Precipitates contained 15 μg of DNA which, unless otherwise indicated, consisted of 10 μg of the experimental CAT plasmid with 5 μg of transfection control plasmid RSVβGal (17). DNA precipitates were left on the cells for 12 h at 37°C in 2.5% CO_2, and then the transfection medium was replaced with medium containing the appropriate hormone(s).

CAT assays. The cells were harvested with 10 ml of PBS-CMF, pelleted, rinsed with 1.0 ml of PBS, pelleted again, and suspended in 100 μl of 250 mM Tris (pH 7.5)–0.1% Triton X-100. Cells were lysed by being incubated on ice for 5 min and vortexed vigorously several times; cellular debris was then removed by pelleting in a microcentrifuge at 4°C for 10 min. From this cleared extract, 50 μl was used for the β-galactosidase assay (17) and 50 μl was used for the two-phase CAT assay (16, 44). A standard curve was always performed with purified CAT enzyme (Sigma) to ensure that experimental values fell within the linear range of the assay. Background was determined by a mock-transfected extract and subtracted from the experimental CAT values, which were then normalized for transfection efficiency with the β-galactosidase data. Each experimental treatment was performed in triplicate with independent DNA precipitates and repeated at least three times with at least three different plasmid preparations. Unless indicated otherwise, each value presented is the mean of three or more experiments ± the standard error of the mean (SEM).

RNase protection. RNA was harvested as described above. Templates for transcribing the RNA probes were generated by cloning the appropriate promoter fragment into Bluescript vectors in the antisense orientation with respect to the T7 promoter and then linearized at an appropriate restriction enzyme site (see Fig. 8). Probes were synthesized in a 25-μl volume containing 1 μg of the linearized template; CTP, ATP, and GTP each at 400 μM; 50 μCi of UTP (800 Ci/mmol, Amersham); 25 U of placental RNase inhibitor (RNasin; Promega Biotec); 10 U of T7 polymerase; and 5 μl of 5× T7 buffer supplied with the enzyme. The transcription reaction was done at 37°C for 30 min, followed by one phenol-chloroform extraction, ethanol precipitation, and purification of the full-length transcripts by gel electrophoresis on denaturing 5% polyacrylamide–7 M urea gels. RNA samples were precipitated with ethanol and suspended in 30 μl of 80% formamide–400 mM NaCl–40 mM PIPES [piperazine-*N*,N′-bis(2-ethanesulfonic acid)] (pH 6.7)–1 mM EDTA containing 5 × 10^5 cpm of control probe (pAnRSV-CAT) and 5 × 10^5 cpm of the scc-CAT probe. Hybridization was done overnight at either 55 or 58°C, after which 300 μl of ice-cold 10 mM Tris (pH 7.5)–1 mM EDTA–300 mM NaCl–0.35 to 0.7 U of DNase-free RNase (Boehringer Mannheim Biochemicals)–0.5 μg of RNase T1 was added, followed by incubation at either 30 or 37°C for 60 min. The samples were then treated with 20 μg of proteinase K–1 μg of tRNA–sodium dodecyl sulfate to 1% for 30 min at 65°C, extracted with phenol-chloroform, and precipitated with ethanol twice. The pellets were suspended in 3 μl of 90% formamide–0.5 mM EDTA–0.04% xylene cyanol–0.04% bromophenol blue, subjected to electrophoresis on a denaturing 7% polyacrylamide–8 M urea gel, and autoradiographed as described above.

RESULTS

Expression and regulation of endogenous murine P450scc mRNA in Y1 cells. Y1 cells are a stably transformed cell line created from a mouse adrenocortical tumor (57). These cells possess many features of adrenal cortex cells, including the presence of high-affinity receptors for both adrenocorticotropin and AII, and they respond to these hormones with increases in steroidogenesis (6, 46). Although Y1 cells lack most adrenal steroidogenic enzymes, they express P450scc activity (49). To determine the suitability of Y1 cells for the study of transcriptional regulation of the human gene for P450scc, we treated them with several known regulators of adrenal steroidogenesis and measured the response of the endogenous murine P450scc mRNA. Northern (RNA) blots of Y1 cell mRNA probed with a rat probe for P450scc showed that both forskolin, a cAMP agonist (Fig. 2), and 8-bromo-cAMP (data not shown) strongly increased the abundance of P450scc mRNA. By contrast, prolonged treatment with both the Ca^{2+} ionophore A23187 and the phorbol ester TPA diminished mouse P450scc mRNA abundance in Y1 cells; furthermore, the effects of these drugs were additive (Fig. 2A). This decrease was not due to general inhibition of transcription or selective cell death: reprobing of the Northern blots showed no effect on nuclear lamin (Fig. 2B), and staining of Y1 cells treated with TPA plus A23187 with the vital dye trypan blue showed no effect on cell viability (data not shown). In Y1 cells treated with combinations of forskolin, TPA, and A23187, the PKC agonists reduced the abundance of P450scc mRNA but did not abolish induction by forskolin (Fig. 2A).

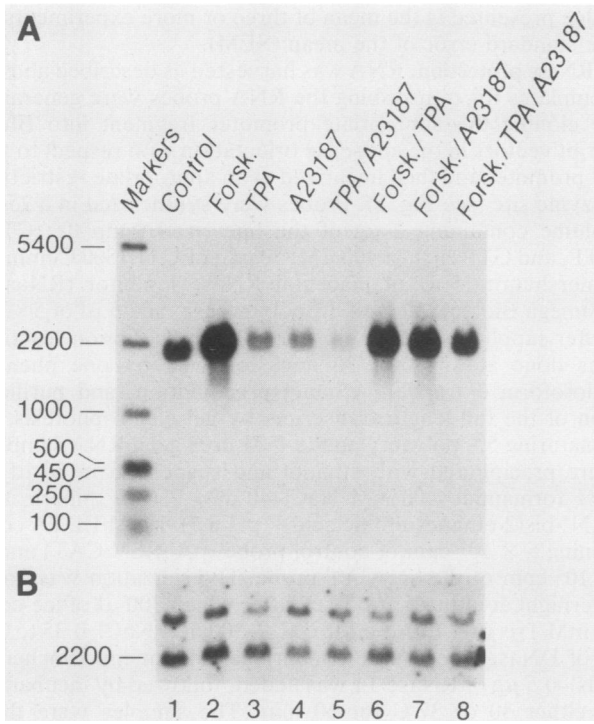

FIG. 2. Regulation of murine P450scc mRNA in Y1 cells. Y1 cells were grown to a density of about 10^7/10-cm-diameter dish and then exposed to the indicated drugs for 12 h (forskolin [Forsk.], 2.0 $\times$ 10^{-5} M; TPA, 3.0 $\times$ 10^{-8} M; A23187, 5.0 $\times$ 10^{-7} M). Total cellular mRNA was prepared, and 20-μg samples were subjected to Northern analysis. (A) The blot was probed with a 1.2-kilobase fragment of rat P450scc cDNA and washed in 0.1× SSC at 55°C. (B) The blot was boiled and then reprobed with a 750-bp *Hin*dIII fragment of human nuclear lamin cDNA and washed in 0.5× SSC at 55°C. Molecular size markers (sizes on the left in base pairs) are end-labeled, *Hin*dIII-cut bacteriophage PM2.

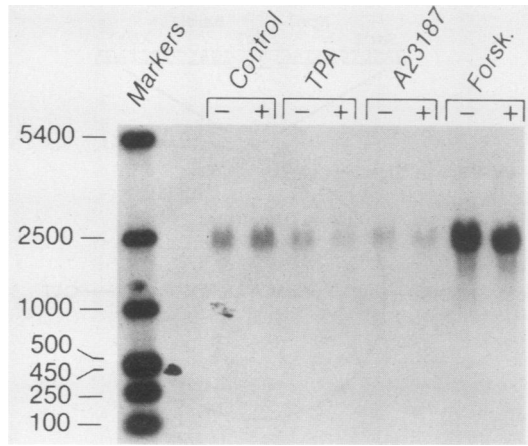

FIG. 3. Effect of the $Ca_3(PO_4)_2$ transfection protocol on P450scc regulation. Northern analysis of 20 μg of endogenous murine P450scc mRNA from Y1 cells which were transfected (+) or mock transfected without $Ca_3(PO_4)_2$ (−) and then treated with forskolin (Forsk.), TPA, or A23187 as described in the legend to Fig. 2. The blot was probed with a 1.2-kilobase fragment of rat P450scc cDNA and washed in 0.1× SSC at 55°C. Molecular size markers (sizes on the left in base pairs) are end-labeled, *Hin*dIII-cut bacteriophage PM2.

Expression and regulation of a transfected human P450scc-CAT fusion construct. To study the human P450scc promoter, we constructed a series of plasmids containing progressively shorter segments of the 5′-flanking DNA from the human gene for P450scc fused to the bacterial reporter gene for CAT (Fig. 1). The parental vector, pAnΔCAT, contains a simian virus 40 polyadenylation signal upstream from the human P450scc sequences to reduce spurious transcription generated within the vector (Brentano et al., submitted). CAT activity from this promoterless vector was seldom detected above the background; therefore, the data for basal *cat* expression are normalized to the shortest plasmid, pAnscc−89CAT.

As extracellular Ca^{2+} is an important regulator of steroidogenesis (4), we determined whether our transient transfection protocol, which used $Ca(PO_4)_2$-DNA coprecipitation, could artifactually alter transcription of P450scc. Y1 cells were transfected or mock transfected without Ca^{2+} and then treated with A23187, TPA, and forskolin. Northern blots of endogenous murine P450scc mRNA showed that the transfection protocol had no significant quantitative effect on regulation of murine P450scc by the various drug treatments (Fig. 3).

Y1 cells were then transiently transfected with pAnscc−2327CAT or control plasmid pAnTK−109CAT and treated with forskolin, TPA, and A23187. The changes in CAT activity (Fig. 4) were qualitatively equivalent to the changes seen in murine P450scc mRNA abundance (Fig. 2A). Thus, many, if not all, of the regulatory elements responsible for transcriptional regulation lay within the 2,377-bp fragment (−2327 to +49) used.

Testing for interactions between the two pathways required knowledge of the maximal responses for each; therefore, we performed dose-response and time course experiments (Fig. 5). Forskolin induced P450scc promoter-dependent CAT activity maximally at 3 $\times$ 10^{-6} M and half-maximally at about 10^{-6} M (Fig. 5A). High concentrations of TPA can desensitize the PKC pathway (25), probably accounting for the rise in CAT activity with TPA concentrations greater than 10^{-7} M (Fig. 5B). To test this,

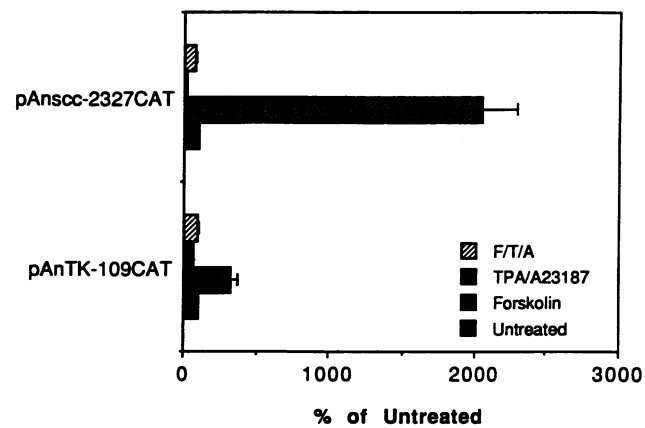

FIG. 4. Regulated expression of the transiently transfected human P450scc promoter. Y1 cells were transiently transfected with either pAnscc−2327CAT or control plasmid pAnTKCAT and treated with forskolin, TPA plus A23187, or a combination of all three (F/T/A) as described in the legend to Fig. 2. The data are from one experiment done in triplicate and presented as the mean percent change from the untreated condition ± the SEM.

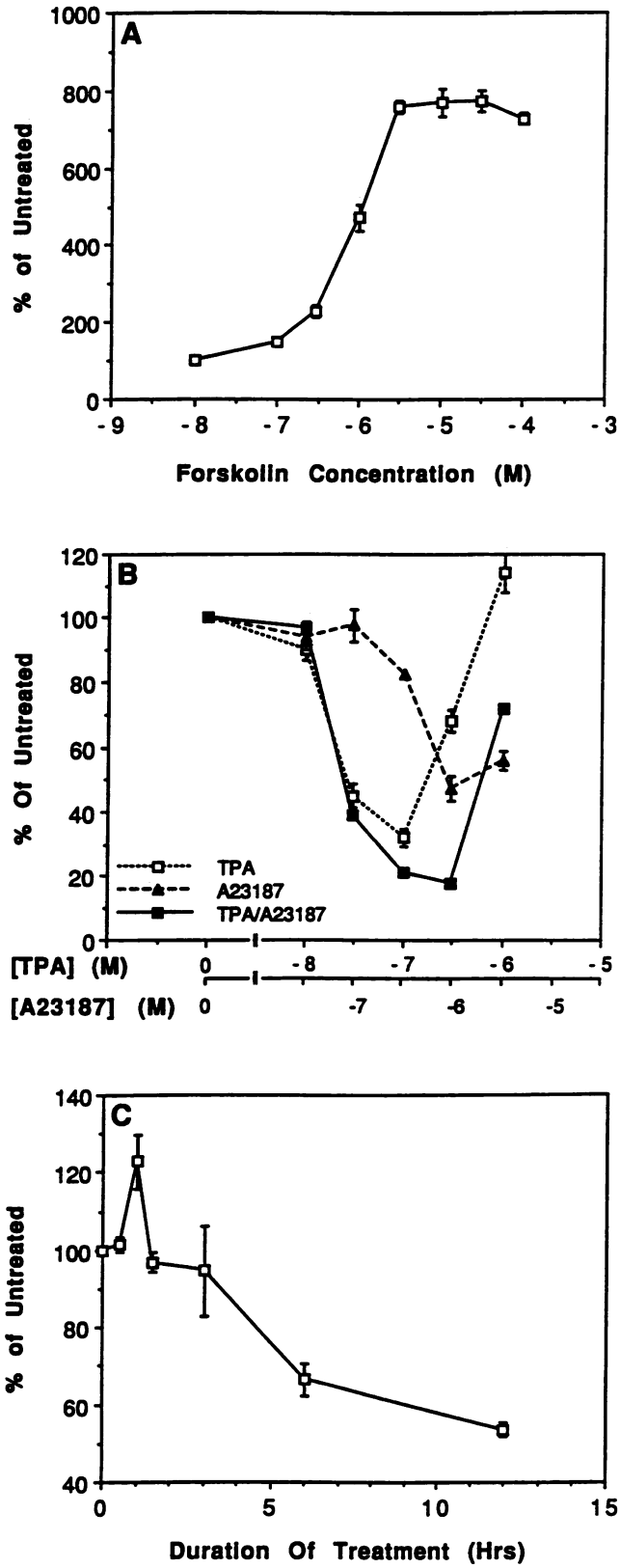

FIG. 5. Dose-response and time course experiments. Y1 cells were transiently transfected in triplicate with pAnscc−2327CAT and treated as described below, and then CAT extracts were analyzed. (A) Dose response for forskolin induction of pAnscc−2327CAT. (B) Dose responses for treatment with TPA, A23187, or a combination of TPA plus A23187. This experiment was done with 2.0×10^{-5} M forskolin to prevent repression below the background level. (C) Induction kinetics for treatment with TPA plus A23187 (TPA, 3.0×10^{-8} M; A23187, 5.0×10^{-7} M). The values show mean percent change ± the SEM compared with the appropriate untreated extract for one experiment done in triplicate.

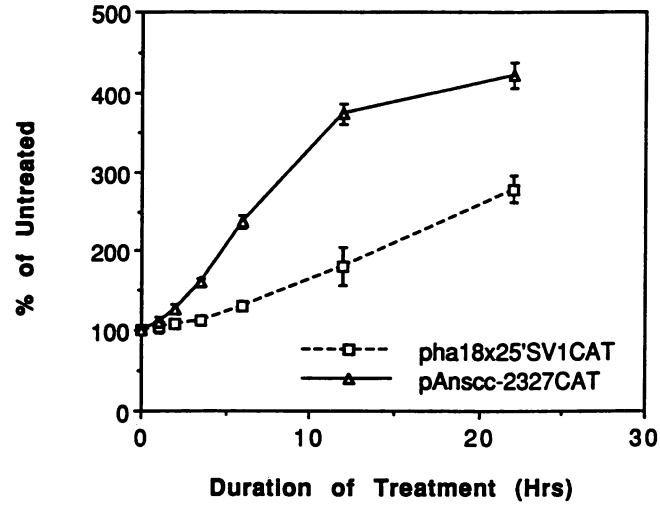

FIG. 6. Comparison of the kinetics of pAnscc−2327CAT induction by cAMP with those of the hCGα subunit CRE. Y1 cells were transiently transfected with either pAnscc−2327CAT or phα 18×25'SV1CAT, which contains two tandem copies of the α subunit CRE. Forskolin (2.0×10^{-5} M) treatment for the longest duration (22 h) began 12 h posttransfection. The values are mean percent change ± the SEM compared with the appropriate untreated extract for one experiment.

we transiently transfected Y1 cells with pAnscc−2327CAT for 12 h and then pretreated some cells with 300 nM TPA for 12 h before treating them acutely with 20 μM forskolin with or without 30 nM TPA. Forskolin induced CAT activity to 344 ± 22% of the untreated-control value, but addition of 30 nM TPA reduced this forskolin-induced CAT activity nearly to control values (148 ± 10%). However, pretreatment of cells with 300 nM TPA for 12 h apparently desensitized the PKC pathway so that acute treatment with forskolin plus 30 nM TPA raised CAT activity to 298 ± 16%, indicating that the acute inhibitory effect of 30 nM TPA had been abolished.

The kinetics of the response to TPA plus A23187 were biphasic (Fig. 5C): CAT activity was induced mildly at 60 min, fell back to the basal value by 90 min, and decreased below the basal level thereafter.

Mechanism for cAMP induction of the P450scc promoter. The rapid kinetics of forskolin induction of pAnscc−2327CAT (Fig. 6) suggests that induction of the human P450scc promoter in Y1 cells by cAMP is direct. We compared expression of our pAnscc−2327CAT with a promoter that responds rapidly and directly to cAMP. This construct contains two tandem copies of the cAMP-responsive element (CRE) from the gene for the α subunit of hCG (hCGα) fused to a simian virus 40 promoter driving *cat* expression (19). This CRE contains the consensus sequence TGACGTCA, which responds rapidly and directly to changes in intracellular cAMP (7, 29, 30, 40). Temporal regulation of the human P450scc promoter by cAMP was even more rapid than that of the hCGα CRE (Fig. 6). Whether this induction is direct or requires protein synthesis

is unknown, as Y1 cells treated with 40 μM cycloheximide increased the abundance of murine P450scc mRNA to the same level as that seen with forskolin alone (data not shown).

Regulation by forskolin in the presence of TPA and A23187. Northern analysis (Fig. 2A) showed that TPA or A23187 treatment of cultures also treated with forskolin reduced the abundance of endogenous Y1 P450scc mRNA compared with forskolin treatment alone. The effect was strongest when TPA and A23187 were combined. To determine whether some of this repression was mediated by compromising the cAMP pathway, we measured forskolin induction of pAnscc−2327CAT with or without TPA and A23187. Addition of forskolin to cultures treated with TPA plus A23187 increased CAT activity 841 ± 56% (mean ± SEM) over cultures treated with only TPA plus A23187 (data not shown). However, induction by forskolin treatment of cultures not exposed to TPA or A23187 was 983 ± 95% (data not shown); these values are not significantly different, suggesting that repression by TPA plus A23187 was not due to interaction between the two second-messenger pathways.

Promoter regions important for basal expression of the human gene for P450scc. The series of deletion constructions (Fig. 1) was transiently transfected into Y1 cells and assayed for CAT activity (Fig. 7A). The basal expression of all of the deletion plasmids was detectable above the background. Adding sequences to −152 bp increased transcription 600% above the level for the shortest construct, pAnscc−89CAT. Adding more 5′ sequence to −605 bp reduced basal activity slightly, but addition of sequences between −605 and −2,327 bp increased basal activity further, to about 1,000% of that of pAnscc−89CAT. Thus, two regions of the promoter appear to contribute significant basal activity: the first is between bases −89 and −152, and the second is between bases −605 and −2327.

Promoter regions necessary for induction by cAMP and repression by TPA plus A23187. To identify regions involved in hormonally regulated transcription, Y1 cells transfected with the series of deletion plasmids were treated with forskolin (Fig. 7B) or TPA plus A23187 (Fig. 7C). Forskolin induced CAT activity in all of the constructions, but only the activity of pAnscc−2327CAT was greater than that of the negative control, pAnTK−109CAT. These results isolate the principal *cis* element(s) for cAMP induction within the −605- to −2,327-bp fragment. In transfected Y1 cells treated with TPA plus A23187, significant repression below that seen for control plasmid pAnTK−109CAT began with pAn scc−152CAT and decreased further with constructs pAnscc−267CAT and pAnscc−343CAT (Fig. 7C). This indicates that repression by TPA plus A23187 is mediated by multiple elements between −89 and −343.

RNase protection experiments (Fig. 8) demonstrated that the deletion constructs initiated transcription from the correct site for both basal expression and drug-regulated expression. The clustered family of bands surrounding the correct initiation site in the RNase protection experiments was also seen with human P450scc mRNA extracted from JEG-3 cells (data not shown). The RNase protection data in Fig. 8B confirm that forskolin did not increase transcription from deletion plasmids smaller than pAnscc−2327CAT (Fig. 7B), as they were not induced more than the internal control, pAnRSVCAT. The apparent forskolin induction of the shorter deletion plasmids, as well as the two control plasmids, pAnTK−109CAT and pAnRSVCAT, may have been due to a general increase in transcription of genes transcribed by RNA polymerase II or an effect of forskolin on

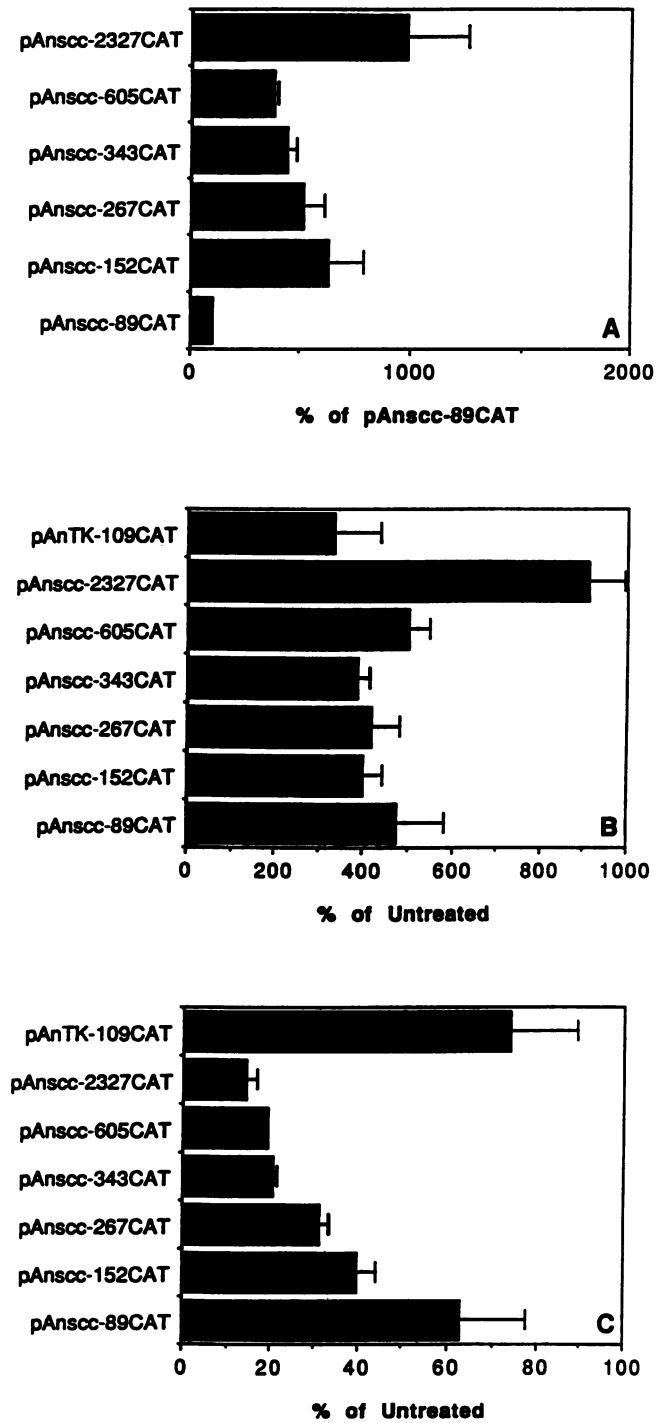

FIG. 7. Basal and regulated expression levels for human P450scc XCAT deletion plasmids. Y1 cells were transfected with the indicated plasmids in triplicate, and CAT assays were performed on cell extracts after 12 h of incubation in medium with or without drugs. (A) Basal level of expression of transiently transfected deletion plasmids in Y1 cells. The values are percent differences from the shortest construct, pAnscc−89 CAT, and represent the mean ± the SEM of at least three separate transfections. (B) Forskolin induction of the various deletion plasmids expressed as mean percent change ± the SEM compared with the same construct not treated with forskolin from at least three separate transfections. (C) Repression by TPA plus A23187 of the deletion plasmids expressed as mean percent changes ± the SEM from the same construct not treated with TPA plus A23187 from at least three separate transfections.

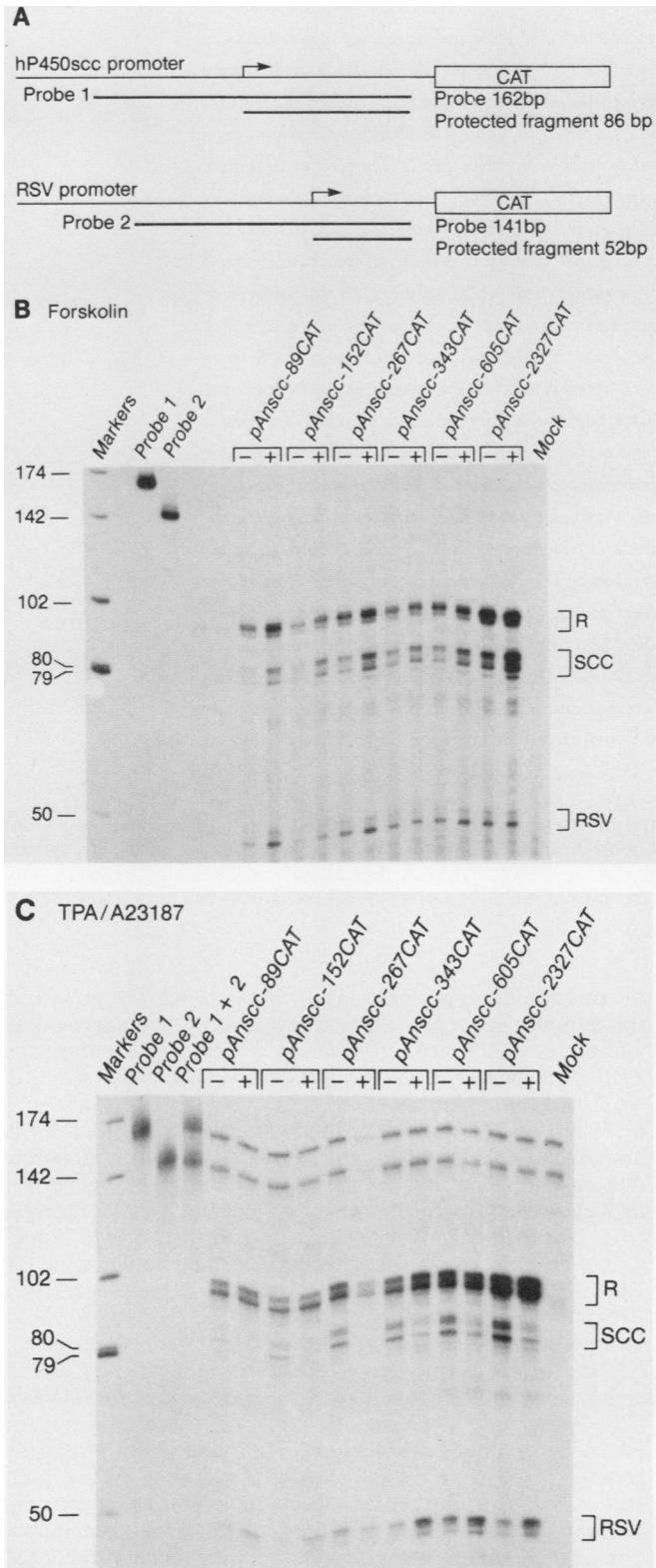

FIG. 8. RNase protection analysis of deletion plasmids. (A) Schematic of the RNase protection probes used for the analysis in panels B and C. Probe 1, for human P450scc, protects the 86 bases between the P450scc mRNA cap site and the *Bam*HI site, where the *cat* sequences were cloned (Fig. 2). Probe 2, for RSV mRNA, protects a 52-bp fragment. (B) RNase protection analysis of 20 μg of mRNA from Y1 cells transiently transfected with the series of deletion plasmids plus the pAnRSVCAT control plasmid and treated with (+) or without (−) forskolin (2.0×10^{-5} M). (C) Similar to panel B, except that cells were treated with (+) or without (−) TPA plus A23187 (TPA, 3.0×10^{-8} M; A23187, 5.0×10^{-7} M). Probe 1, Probe for the human scc-CAT transcripts; Probe 2, probe for the RSVCAT internal control transcripts; R, readthrough transcripts and/or probe annealed to the transfected plasmid; SCC, correctly initiated transcripts from the scc promoter; RSV, protected probe from the RSVCAT internal control vector; Mock, 20 μg of RNA from mock-transfected cells. Molecular size markers (sizes on the left in base pairs) are end-labeled *Hae*III-cut Bluescript SK+.

the transfection protocol in Y1 cells. Similarly, RNase protection experiments (Fig. 8C) confirmed that transcription was initiated from the correct site and repressed by treatment with TPA plus A23187 (Fig. 7C). The specificity of this repression of the human P450scc promoter by TPA plus A23187 was demonstrated by the increased repression seen with constructs longer than pAnscc−89CAT, by the relatively smaller repression seen with the pAnTK−109CAT control, and by induction of the pAnRSVCAT internal control plasmid used in the RNase protection experiments. As the transfection control plasmid RSVβ-gal contains the same RSV promoter fragment which was induced by treatment with TPA plus A23187 (Fig. 8C), the slight repression of pAnTK−109CAT and pAnscc−89CAT was probably an artifact of normalization of the data.

DISCUSSION

Y1 cells as a model system for analyzing transcriptional regulation of the human gene for P450scc. Primary cultures of human adrenocortical cells are not readily used for studying transcription of the human gene for P450scc, and a stably transformed human adrenocortical cell line does not exist. Y1 murine adrenocortical cells retain endogenous P450scc activity, and although their responses to adrenocorticotropin and AII can be variable and unstable, they respond reproducibly to intracellular agonists of steroidogenesis in a manner similar to that of murine, bovine, and human adrenocortical cells; thus, they have been used to analyze transcriptional regulation of several steroidogenic enzymes (1, 10, 24, 42, 47). Since the endogenous murine gene for P450scc responds to treatment with forskolin, TPA, and A23187 (Fig. 2), Y1 cells should be useful for analysis of transcriptional regulation of the transfected human gene for P450scc.

Promoter regions necessary for basal transcription. All of our human P450scc deletion constructions gave detectable CAT activity in transiently transfected Y1 cells. The shortest construct, pAnscc−89CAT, contains a TATA box and a possible CAAT motif (CATT at −63), as well as a sequence between −86 and −71 that closely resembles the basal transcription element recently described for the human gene for liver P450c (56). Constructions containing bases between −152 and −605 of the human P450scc 5′-flanking DNA were all transcribed at between 400 and 600% of the level of the pAnscc−89CAT construct. This indicates that an element for basal expression lies between −89 and −152. A likely candidate is the sequence between bases −117 and −108, GGGGAGGAGC, which matches at 9 of 10 bases with the SP-1 consensus G/TGGGCGGG/AG/AC/T (15).

Basal expression increased further with the region between −605 and −2327, which is also necessary for cAMP induction. The colocalization of these two functions is seen in the murine genes for P450c21, P450c11, and P450scc (24,

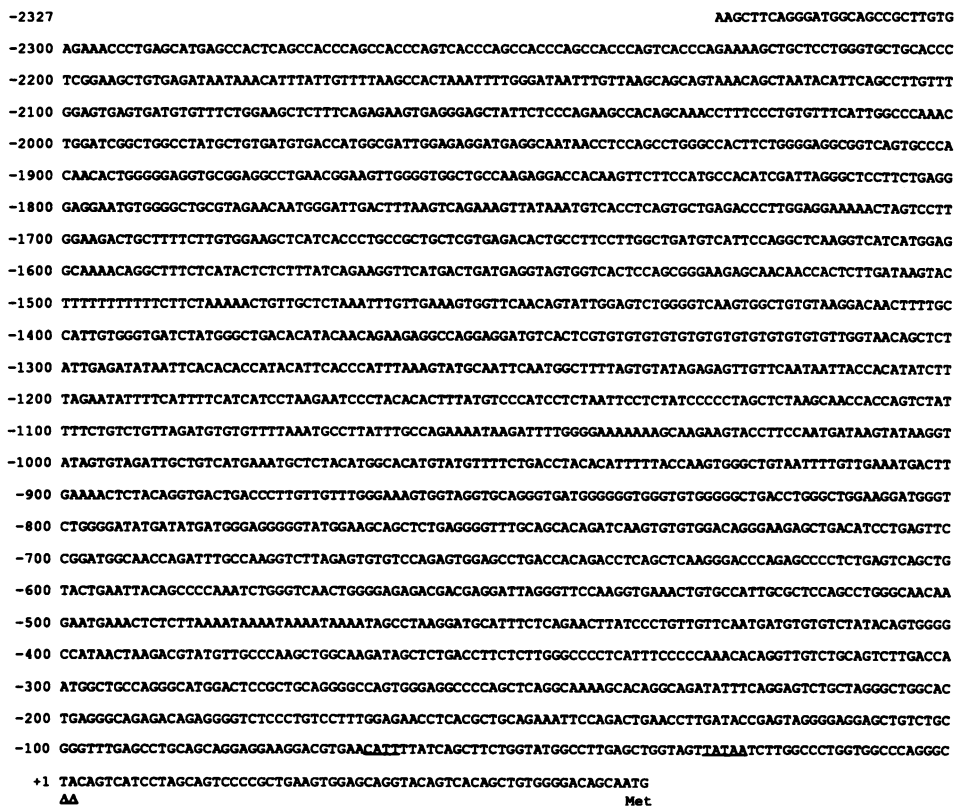

FIG. 9. Sequence for the human gene for P450scc from −2,327 bp relative to the transcriptional start site to the translation initiation codon, ATG. Both strands were sequenced by dideoxy-chain termination of double-stranded templates. The sequence out to −605 corresponds exactly to that of Morohashi et al. (42), except that our sequence contains two fewer TAAAA repeats between bases −467 and −486. Arrowheads mark the sites of transcriptional initiation as determined by Inoue et al. (28) and Chung et al. (10). The CAAT and TATA motifs are underlined.

43, 54). In those experiments, basal expression correlated with the activity of type I cAMP-dependent protein kinase A (PKA) in Y1 cells with a variety of PKA mutations. In addition, point mutations in the putative CRE in the murine P450c11 promoter eliminated basal activity (43). These results suggested that the basal activity of the PKA pathway helps dictate the basal level of expression of these genes (24). The murine gene for P450scc was only partially sensitive to the diminished activity of PKA in the various Y1 PKA mutants (54). Similarly, basal expression of the human P450scc promoter was not severely compromised in our shorter P450scc–CAT constructs that lack the cAMP-responsive region from −2327 to −605.

Promoter regions involved in cAMP regulation. Several hormones that regulate steroidogenesis use cAMP as a second messenger. cAMP can regulate gene transcription through *cis*-acting CREs (consensus binding site, TGACGTCA) which bind a family of related CRE-binding proteins (23, 26), as well as a number of other proteins (discussed in reference 13). Alternatively, cAMP can induce transcription through *cis*-acting TPA response elements (TREs; consensus binding site, CCCCAGGC or TGAC/GTCA) (25, 27; for a review, see reference 32). The cAMP-responsive region of the human P450scc promoter (bases −605 to −2327) contains one sequence, TGATGTCA, between bases −1626 and −1633 (Fig. 9) that matches at 7 of 8 bases with the consensus CRE. The identical core sequence, TGATGTCA, binds CRE-binding proteins with about 5% of the affinity of the consensus sequence when in the context of the phosphoenolpyruvate carboxykinase CRE (7); however, the flanking sequences, which are also critical for binding and activity (26), are different. In addition, one consensus TRE sequence, TGAGTCA, lies between bases −605 and −611 (Fig. 9). Whether these CRE- or TRE-like sequences mediate cAMP induction of the human P450scc promoter is unknown. Other genes for steroidogenic enzymes, including the murine gene for P450c21 and the bovine gene for P450c17, are also regulated by cAMP but lack consensus CRE or TRE sequences (24, 35). In contrast, cAMP induction of both the murine gene for P450c11 and human gene for P450c17 appears to involve CRE or CRE-like sequences (47; Brentano et al., submitted).

An alternate, indirect pathway has been proposed to explain the apparently slow kinetics of induction by cAMP and cycloheximide sensitivity of the bovine gene for P450scc (31). However, experiments with the human gene for P450scc in human granulosa cells (22) and with the murine gene for P450scc in mouse Leydig MA10 cells (38) demonstrated no sensitivity of P450scc to inhibitors of protein synthesis. Our comparison of the kinetics of forskolin induction of the human P450scc promoter with those of the CRE in the hCGα promoter indicate that the human P450scc promoter is stimulated very rapidly, with a $t_{1/2}$ of about 6 h. Similarly, the bovine P450scc promoter transiently transfected into Y1 cells was induced rapidly and was not impaired by inhibitors of protein synthesis (1). The rapid induction we observed is consistent with direct activation of human P450scc transcription by cAMP in Y1 cells.

Promoter elements involved in Ca^{2+}-PKC regulation of P450scc gene transcription. A variety of experiments indicate that the response of adrenal cells to AII is biphasic. Short-term stimulation (0.5 to 2.0 h) of bovine adrenal cells with AII or a combination of TPA plus A23187 rapidly increased aldosterone synthesis and secretion (33); however, the effects on P450scc synthesis are unknown. In contrast, long-term stimulation (24 to 48 h) of primary cultured human fetal adrenal cells with TPA lowered the abundance of P450scc mRNA and protein and blocked the ability of cAMP to increase the abundance of P450scc (36, 37). The kinetics for the response of the human P450scc promoter to treatment with TPA plus A23187 is consistent with a such a biphasic response. Treatment of the transiently transfected pAn scc−2327CAT construction with TPA plus A23187 for 1 h resulted in mild induction, but longer incubations (2 to 12 h) strongly repressed transcription. Such repression of adrenal steroidogenesis by prolonged stimulation of the PKC pathway may modify both the amounts and types of steroids produced by adrenals (37).

Although cAMP and PKA may play a role in basal transcription of the human gene for P450scc, it is unlikely that repression by TPA plus A23187 indicates loss of analogous basal induction by the Ca^{2+}-PKC pathway. The pretreatment experiment showed that desensitization of PKC abolishes TPA repression. Furthermore, desensitization of PKC did not reduce CAT activity below the basal level (data not shown). Thus, TPA repression requires an intact PKC pathway and a desensitized PKC pathway does not compromise basal expression.

Repression of P450scc promoter activity by TPA plus A23187 does not involve inhibition of the cAMP-PKA pathway. Such an interaction was an attractive hypothesis, in view of the apparent role of cAMP-PKA in determining basal expression of P450scc, the known ability of AII to inhibit adenylate cyclase via a G protein (55), and the convergence of the PKA and PKC pathways on both CRE and TRE *cis*-acting elements (25, 27). However, induction by cAMP and repression by TPA plus A23187 map to separate *cis*-acting regions of the human P450scc promoter; thus, interaction between these pathways is not responsible for repression by TPA plus A23187.

Repression by TPA plus A23187 is mediated by multiple DNA-protein interactions as repression increases progressively with longer promoter fragments between −89 and −343 bp. Whether this is because TPA and A23187 work through separate *cis* elements or converge through a common *cis* element in multiple regions of the promoter is unknown. No repressive *cis*-acting elements responsive to TPA plus A23187 have been described; however, several *cis*-acting elements, including the serum response element and the binding sites for Jun/AP-1, AP-2, AP-3, and NFκB, mediate transcriptional activation by TPA (3, 9, 27, 34, 50). TPA combined with A23187 also activates the murine granulocyte-macrophage colony-stimulating factor promoter through conserved lymphokine element 2 and motif GC (41). The human P450scc promoter region between −89 and −343 contains several regions of limited homology to some of these activation sequences, but their role, if any, in repression of the human P450scc promoter by TPA plus A23187 is unknown.

Most models for transcriptional repression involve either interference with an activating protein(s) or direct interaction with the RNA polymerase II transcription complex (41). We have eliminated the possibility that repression of the human P450scc promoter by TPA plus A23187 is through interference with the CRE(s): the two effects map to distinctly different regions of the promoter, and removal of the CRE does not eliminate repression by TPA plus A23187. Another possibility is that TPA plus A23187 represses the activity of an unidentified basal transcription factor(s). Part of the region that contains the repression elements responsive to TPA plus A23187 also contains a basal activation element(s). Furthermore, the repression by TPA plus A23187 to 15 to 20% of the control level in pAnscc−343CAT is just enough to account for the 500% increase in basal activity conferred by sequences between −89 and −152. Thus, the repression may be a reversal of the activation associated with this region of the promoter, analogous to glucocorticoid repression of the bovine prolactin and human glycoprotein α-subunit promoters (2, 48). If this is true, a simple binding site competition model, as proposed for glucocorticoid repression (2, 48), cannot explain all of the repression of P450scc because the basal activation and the effects of TPA plus A23187 map to the same as well as different regions of the promoter. This could be explained by protein-protein interactions between the two types of elements.

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LITERATURE CITED

1. **Ahlgren, R., E. R. Simpson, M. R. Waterman, and J. Lund.** 1990. Characterization of the promoter/regulatory region of the bovine CYP11A (P450scc) gene. J. Biol. Chem. **265**:3313–3319.
2. **Akerblom, I. E., E. P. Slater, M. Beato, J. D. Baxter, and P. L. Mellon.** 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science **241**: 350–353.
3. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell **49**:729–739.
4. **Barrett, P. Q., W. B. Bollag, C. M. Isales, R. T. McCarthy, and H. Rasmussen.** 1989. Role of calcium in angiotensin II-mediated aldosterone secretion. Endocrinol. Rev. **10**:496–518.
5. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56**:335–344.
6. **Begeot, M., D. Langlois, I. Vilgrain, and J. M. Saez.** 1987. Angiotensin II (A-II) steroidogenic refractoriness in Y-1 cells in the presence of A-II receptors negatively coupled to adenylate cyclase. Endocrinol. Res. **13**:301–316.
7. **Bokar, J. A., W. J. Roesler, G. R. Vandenbark, D. M. Kaetzel, R. W. Hanson, and J. H. Nilson.** 1988. Characterization of the cAMP responsive elements from the genes for the α-subunit of glycoprotein hormones and phosphoenolpyruvate carboxykinase (GTP). J. Biol. Chem. **263**:19740–19747.
8. **Chen, C. A., and H. Okayama.** 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques **6**:632–638.
9. **Chiu, R., M. Imagawa, R. J. Imbra, J. R. Bockoven, and M.**

Karin. 1987. Multiple *cis*- and *trans*-acting elements mediate transcriptional response to phorbol esters. Nature (London) **329**:648–651.

10. **Chung, B., M.-C. Hu, C.-C. Lai, and C.-H. Lin.** 1989. The 5′-region of the P450XIA1 (P450scc) gene contains a basal promoter and an adrenal-specific activating domain. Biochem. Biophys. Res. Commun. **160**:276–281.
11. **Chung, B., K. J. Matteson, R. Voutilainen, T. K. Mohandas, and W. L. Miller.** 1986. Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. Proc. Natl. Acad. Sci. USA **83**:8962–8966.
12. **Cozza, E. N., M. D. C. Vila, M. Acevedo-Duncan, R. V. Farese, and C. E. Gomez-Sanchez.** 1990. Treatment of primary cultures of calf adrenal glomerulosa cells with adrenocorticotropin (ACTH) and phorbol esters: a comparative study of the effects on aldosterone production and ACTH signaling system. Endocrinology **126**:2169–2176.
13. **Dean, D. C., J. J. McQuillan, and S. Weintraub.** 1990. Serum stimulation of fibronectin gene expression appears to result from rapid serum-induced binding of nuclear proteins to a cAMP response element. J. Biol. Chem. **265**:3522–3527.
14. **Di Blasio, A. M., R. Voutilainen, R. B. Jaffe, and W. L. Miller.** 1987. Hormonal regulation of messenger ribonucleic acids for P450scc (cholesterol side-chain cleavage enzyme) and P450c17 (17 α-hydroxylase/17,20-lyase) in cultured human fetal adrenal cells. J. Clin. Endocrinol. Metab. **65**:170–175.
15. **Dynan, W. S., S. Sazer, R. Tjian, and R. T. Schimke.** 1986. Transcription factor Sp1 recognizes a DNA sequence in the mouse dihydrofolate reductase promoter. Nature (London) **319**: 246–248.
16. **Eastman, A.** 1987. An improvement to the novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques **5**:730–732.
17. **Edlund, T., M. D. Walker, P. J. Barr, and W. J. Rutter.** 1985. Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5′ flanking elements. Science **230**:912–916.
18. **Enyedi, P., B. Szabo, and A. Spat.** 1985. Reduced responsiveness of glomerulosa cells after prolonged stimulation with angiotensin II. Am. J. Physiol. **248**:E209–E224.
19. **Fenstermaker, R. A., A. Milsted, J. B. Virgin, W. L. Miller, and J. H. Nilson.** 1989. The transcriptional response of the human chorionic gonadotropin β-subunit gene to cAMP is mediated by cis-acting sequences different from that found in the α-subunit gene. Mol. Endocrinol. **3**:1070–1076.
20. **Fisher, D. Z., N. Chaudhary, and G. Blobel.** 1986. cDNA sequencing of nuclear lamins A and C reveals primary and secondary homology to intermediate filament proteins. Proc. Natl. Acad. Sci. USA **83**:6450–6454.
21. **Goldring, N. B., J. M. Durica, J. Lifka, L. Hedin, S. L. Ratoosh, W. L. Miller, J. Orly, and J. S. Richards.** 1987. Cholesterol side-chain cleavage P450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. Endocrinology **120**:1942–1950.
22. **Golos, T. G., W. L. Miller, and J. F. Strauss.** 1987. Human chorionic gonadotropin and 8-bromo cyclic adenosine monophosphate promote an acute increase in cytochrome P450scc and adrenodoxin messenger RNAs in cultured human granulosa cells by a cycloheximide-insensitive mechanism. J. Clin. Invest. **80**:896–899.
23. **Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. I. Biggs, W. W. Vale, and M. R. Montminy.** 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature (London) **337**:749–752.
24. **Handler, J. D., B. P. Schimmer, T. R. Flynn, M. Szyf, J. G. Seidman, and K. L. Parker.** 1988. An enhancer element and a functional cyclic AMP-dependent protein kinase are required for expression of adrenocortical 21-hydroxylase. J. Biol. Chem. **263**:13068–13073.
25. **Hoeffler, J. P., P. J. Deutsch, J. Lin, and J. F. Habener.** 1989. Distinctive adenosine 3′,5′-monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. Mol. Endocrinol. **3**:868–880.
26. **Hoeffler, J. P., T. E. Meyer, Y. Yun, J. L. Jameson, and J. F. Habener.** 1988. Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. Science **242**:1430–1433.
27. **Imagawa, M., R. Chiu, and M. Karin.** 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51**:251–260.
28. **Inoue, H., Y. Higashi, K. I. Morohashi, and Y. Fujii-Kuriyama.** 1988. The 5′-flanking region of the human P-450(SCC) gene shows responsiveness to cAMP-dependent regulation in a transient gene-expression system of Y-1 adrenal tumor cells. Eur. J. Biochem. **171**:435–440.
29. **Jameson, J. L., and C. M. Lindell.** 1988. Isolation and characterization of the human chorionic gonadotropin β subunit (CGβ) gene cluster: regulation of a transcriptionally active CGβ gene by cyclic AMP. Mol. Cell. Biol. **8**:5100–5107.
30. **Jameson, J. L., A. C. Powers, G. D. Gallagher, and J. F. Habener.** 1989. Enhancer and promoter element interactions dictate cyclic adenosine monophosphate mediated and cell-specific expression of the glycoprotein hormone α-gene. Mol. Endocrinol. **3**:763–772.
31. **John, M. E., M. C. John, V. Boggaram, E. R. Simpson, and M. R. Waterman.** 1986. Transcriptional regulation of steroid hydroxylase genes by corticotropin. Proc. Natl. Acad. Sci. USA **83**:4715–4719.
32. **Karin, M.** 1989. Complexities of gene regulation by cAMP. Trends Genet. **5**:65–67.
33. **Kojima, I., K. Kojima, D. Kruetter, and H. Rasmussen.** 1984. The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. J. Biol. Chem. **259**:14448–14457.
34. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741–752.
35. **Lund, J., R. Ahlgren, D. Wu, M. Kagimoto, E. R. Simpson, and M. R. Waterman.** 1990. Transcriptional regulation of the bovine CYP17 (P-45017α) gene. J. Biol. Chem. **265**:3304–3312.
36. **Mason, J. I., B. R. Carr, and W. E. Rainey.** 1986. The action of phorbol ester on steroidogenesis in cultured human fetal adrenal cells. Endocrinol. Res. **12**:447–467.
37. **McAllister, J. M., and P. J. Hornsby.** 1988. Dual regulation of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, dehydroepiandrosterone sulfotransferase by adenosine 3′,5′-monophosphate and activators of protein kinase C in cultured human adrenocortical cells. Endocrinology **122**:2012–2018.
38. **Mellon, S. H., and C. Vaisse.** 1989. cAMP regulates P450scc gene expression by a cycloheximide-insensitive mechanism in cultured mouse Leydig MA-10 cells. Proc. Natl. Acad. Sci. USA **86**:7775–7779.
39. **Miller, W. L.** 1988. Molecular biology of steroid hormone synthesis. Endocrinol. Rev. **9**:295–317.
40. **Milsted, A., R. P. Cox, and J. H. Nilson.** 1987. Cyclic AMP regulates transcription of the genes encoding human chorionic gonadotropin with different kinetics. DNA **6**:213–219.
41. **Miyatake, S., M. Seiki, M. Yoshida, and K.-I. Arai.** 1988. T-cell activation signals and human T-cell leukemia virus type I-encoded p40× protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. Mol. Cell. Biol. **8**:5581–5587.
42. **Morohashi, K.-I., K. Sogawa, T. Omura, and Y. Fuji-Kuriyama.** 1987. Gene structure of human cytochrome P-450(SCC), cholesterol desmolase. J. Biochem. **101**:879–887.
43. **Mouw, A. R., D. A. Rice, J. C. Meade, S. C. Chua, P. C. White, B. P. Schimmer, and K. L. Parker.** 1989. Structural and functional analysis of the promoter region of the gene encoding mouse steroid 11β-hydroxylase. J. Biol. Chem. **264**:1305–1309.
44. **Neumann, J. R., C. A. Morency, and K. O. Russion.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques **5**:444–447.
45. **Picado-Leonard, J., R. Voutilainen, L.-C. Kao, B.-C. Chung, J. F. I. Strauss, and W. L. Miller.** 1988. Human adrenodoxin:

cloning of three cDNAs and cycloheximide enhancement in JEG-3 cells. J. Biol. Chem. **263**:3240–3244.

46. **Pierson, R. W. J.** 1967. Metabolism of steroid hormones in adrenal cortex tumor cultures. Endocrinology **81**:693–707.
47. **Rice, D. A., L. D. Aitken, G. R. Vandenbark, A. R. Mouw, A. Franklin, B. P. Schimmer, and K. L. Parker.** 1989. A cAMP-responsive element regulates expression of the mouse steroid 11β-hydroxylase gene. J. Biol. Chem. **264**:14011–14015.
48. **Sakai, D. D., S. Helms, J. Carlstedt-Duke, J.-A. Gustafsson, F. M. Rottman, and K. R. Yamamoto.** 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2**:1144–1154.
49. **Schimmer, B. P.** 1979. Adrenocortical Y1 cells. Methods Enzymol. **53**:570–574.
50. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
51. **Stone, D., and O. Hechter.** 1955. Studies on ACTH action in perfused bovine adrenals: aspects of progesterone as an intermediary in corticosteroidogenesis. Arch. Biochem. Biophys. **54**:121–126.
52. **Voutilainen, R., and W. L. Miller.** 1987. Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450scc, in human steroidogenic tissues. Proc. Natl. Acad. Sci. USA **84**: 1590–1594.
53. **Voutilainen, R., J. Tapanainen, B. Chung, K. J. Matteson, and W. L. Miller.** 1986. Hormonal regulation of P450scc (20,22-desmolase) and P450c17 (17α-hydroxylase/17,20-lyase) in cultured human granulosa cells. J. Clin. Endocrinol. Metab. **63**: 202–207.
54. **Wong, M., D. A. Rice, K. L. Parker, and B. P. Schimmer.** 1989. The roles of cAMP and cAMP-dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11β-hydroxylase genes in mouse adrenocortical tumor cells. J. Biol. Chem. **264**:12867–12871.
55. **Woodcock, E. A., and C. I. Johnston.** 1984. Inhibition of adenylate cyclase in rat adrenal glomerulosa cells by angiotensin II. Endocrinology **115**:337–341.
56. **Yanagida, A., K. Sogawa, K.-I. Yasumoto, and Y. Fujii-Kuriyama.** 1990. A novel *cis*-acting DNA element required for a high level of inducible expression of the rat P-450c gene. Mol. Cell. Biol. **10**:1470–1475.
57. **Yasumura, Y., V. Buonassisi, and G. Sato.** 1966. Clonal analysis of differentiated function in animal cell cultures. I. Possible correlated maintenance of differentiated function and the diploid karyotype. Cancer Res. **26**:529–535.