

# Transcription of Cholesterol Side-Chain Cleavage Cytochrome P450 in the Placenta: Activating Protein-2 Assumes the Role of Steroidogenic Factor-1 by Binding to an Overlapping Promoter Element

MICHA BEN-ZIMRA, MORIAH KOLER, AND JOSEPH ORLY

*Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel 91904*

Progesterone is essential to the sustenance of pregnancy in humans and other mammals. From the second trimester on, the human placenta is the sole origin of *de novo* synthesized steroid hormones. In mice, placentation at midgestation is accompanied by a temporal rise of steroid hormone synthesis commencing in the giant cells of the mouse trophoblast. In doing so, the giant trophoblasts, as any other steroidogenic cell, express high levels of the key steroidogenic enzyme, cholesterol side-chain cleavage cytochrome P450 (P450<sub>scc</sub>). Because steroidogenic factor 1 (SF-1), the transcription factor required for expression of P450<sub>scc</sub> in the adrenals and the gonads, is not expressed in the placenta, we hypothesized that placenta-specific nuclear factor(s) (PNF) assumes the role of SF-1 by binding to the same promoter region that harbors the SF-1 recognition site in the P450<sub>scc</sub> gene. To address this possibility, we used SCC1, a well conserved proximal region in the P450<sub>scc</sub> genes (–60/–32 in the rat gene) to purify PNF from human term placenta. Sequencing of the purified PNF revealed that it is the  $\alpha$  isoform of the

human activating protein-2 (AP-2 $\alpha$ ). Specific antibodies tested in EMSA confirmed that AP-2 $\alpha$  is the predominant isoform that binds SCC1 in the human placenta, whereas AP-2 $\gamma$  is the only mouse placental protein that binds this oligonucleotide. Functional studies showed that coexpression of the rat P450<sub>scc</sub> promoter (–378/+8 CAT) and AP-2 isoforms ( $\alpha$  or  $\gamma$ ) in human embryonic kidney 293 cells results in a marked activation of chloramphenicol acetyltransferase (CAT) transcription that is dependent on an intact AP-2 motif, GCCTTGAGC. This motif conforms with consensus sequences previously determined for binding of the AP-2  $\alpha$  and  $\gamma$  isoforms. Mutations of the AP-2 element ablated binding of AP-2 to SCC1, as well as severely diminished the promoter activity in primary mouse giant trophoblasts and human choriocarcinoma JAR cells. Collectively, these studies suggest that expression of placental P450<sub>scc</sub> is governed by AP-2 factors that bind to a *cis*-element that largely overlaps the sequence required for recognition of SF-1 in other steroidogenic tissues. (*Molecular Endocrinology* 16: 1864–1880, 2002)

**P**ROGESTERONE AND ESTROGEN are essential to establish and sustain pregnancy in mammals (1–6). The origin of these steroid hormones during pregnancy can be the ovarian corpus luteum, the placenta, and the fetal adrenal (6, 7). In these steroidogenic tissues, steroid hormone synthesis initiates when cholesterol is converted in the mitochondria to the first steroid, pregnenolone. This reaction is cata-

lyzed by a specialized enzyme complex that includes the cholesterol side-chain cleavage cytochrome P450 (P450<sub>scc</sub>) and other accessory proteins (8, 9). Further processing of pregnenolone to active steroids requires endoplasmic reticulum-bound enzymes such as 3 $\beta$ -hydroxysteroid dehydrogenase/isomerase (3 $\beta$ -HSD) (10) and cytochrome P450 aromatase (11, 12).

In humans, the corpus luteum is active during the first trimester, and the placenta assumes the steroidogenic role of the ovary at 8 wk of pregnancy. The principal steroid-producing cells in the human placenta are the syncytiotrophoblasts that, at term, can secrete up to 300 mg of progesterone per d. The use of antiprogesterin mifepristone (RU486) for therapeutic abortion during the second trimester (13), together with the dramatic decrease of placental steroidogenesis observed in women who experienced pregnancy failure during wk 8–12 of gestation (14), suggest that placental progesterone production is essential for maintenance of pregnancy. In this regard, several mechanisms by which placental progesterone affects

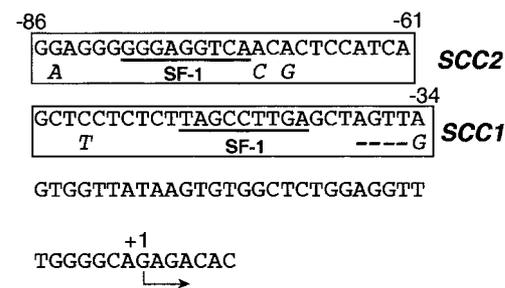
Abbreviations: AP-2, Activating protein-2; 8-Br-cAMP, 8-bromo-cAMP; CAT, chloramphenicol acetyltransferase; COUP-TF, chick ovalbumin upstream promoter transcription factor; DNase, deoxyribonuclease; DR0, direct repeat with 0 bp spacing element; DTT, dithiothreitol; E9.5, embryonic d 9.5; GCNF, germ cell nuclear factor; GFP, green fluorescent protein; hCG, human chorionic gonadotropin; HEK, human embryonic kidney; 3 $\beta$ -HSD, 3- $\beta$ -hydroxysteroid dehydrogenase; p.c., postcoitus; PEI, polyethyleneimine; PMSF, phenylmethyl sulfonyl fluoride; PNF, placental nuclear factor; P450<sub>scc</sub>, cholesterol side-chain cleavage cytochrome P450; RAR, retinoic acid receptor; RXR, retinoid X receptor; SF-1, steroidogenic factor-1; Sp1, selective promoter 1; StAR, steroidogenic acute regulatory; WT, wild-type.

various myometrial and endometrial activities to prevent premature parturition have been proposed (15–17). The latter roles of progesterone in sustaining pregnancy are also relevant in rodents. However, the role of placental progesterone production in rodents is less obvious because luteal progesterone secretion is indispensable throughout term (18). Nevertheless, by the time of placentation at midpregnancy, a marked rise in the expression of the steroidogenic genes, including P450scc (19–21), steroidogenic acute regulatory factor (StAR), 3 $\beta$ -HSD (22), and P45017 $\alpha$  (20, 21), is observed in the giant trophoblast cells. This transient expression of genes required for placental progesterone production has been associated with various speculations related to protection of the conceptus from maternal immunorejection (19, 23), or effects of locally produced steroids on sex determination of the fetus (24). It is noteworthy that after implantation, expression of P450scc and 3 $\beta$ -HSD has been demonstrated also in decidual cells of the uterine wall (21, 22, 25), suggesting that a local uterine production of steroid hormones is also important during early phases of pregnancy.

It is well accepted that expression of P450scc and other steroidogenic genes in the gonads and the adrenal is provoked by trophic hormones via the intermediary role of cAMP (26–28). It is less clear what hormones or other signaling cues up-regulate the steroidogenic genes in the placenta. At the transcriptional level, steroidogenic factor 1 (SF-1) (29), also known as Ad4BP (30), is the pivotal tissue-specific factor that mediates hormone-induced expression of P450scc and other steroid hydroxylases in the adrenal and gonads (11, 30–34). Furthermore, targeted ablation of the mouse SF-1 gene also impairs the embryonic development of those organs (35–37). Nevertheless, P450scc expression and normal development of SF-1-deficient placenta allow live birth of mutant pups (35, 37). These observations agree with the apparent absence of SF-1 in human trophoblasts (6), rat chorionicarcinoma Rcho-1 cells, and midpregnancy rat placenta (38). Collectively, these findings suggest that the expression of placental P450scc does not require SF-1. The present study aimed to find what *trans*-acting protein(s) could possibly control P450scc transcription in the absence of SF-1 in the mouse and human placenta.

Among several *cis*-acting DNA sequences that have been implicated in cAMP-mediated transcription of P450scc (32, 39–41), two proximal regions, named SCC1 and SCC2 (Ref. 33 and Table 1) harbor SF-1 binding motifs required for functional activity of the promoter in rat ovarian and adrenal cells (31, 33). In particular, the sequence of SCC1 is highly conserved (Table 2) among the rat, mouse, porcine, ovine, bovine, and human genes (31, 39, 42–46). Therefore, we postulated that the placenta should express a protein that can “substitute” for SF-1 action by associating with a *cis*-element in the SCC1 region and mimic SF-1 activation of the P450scc promoter in trophoblast

**Table 1.** Sequence of the Proximal Rat P450scc Promoter (33)



Boxed nucleotides are SCC1 and SCC2 probes used for this study; underlined nucleotides are putative SF-1 binding sites; italicized subscripts denote mismatched nucleotides of the mouse P450scc.

cells. Our studies revealed that these criteria are met by a placental nuclear factor, initially called PNF, which was later purified and identified as the activating protein-2 (AP-2).

## RESULTS

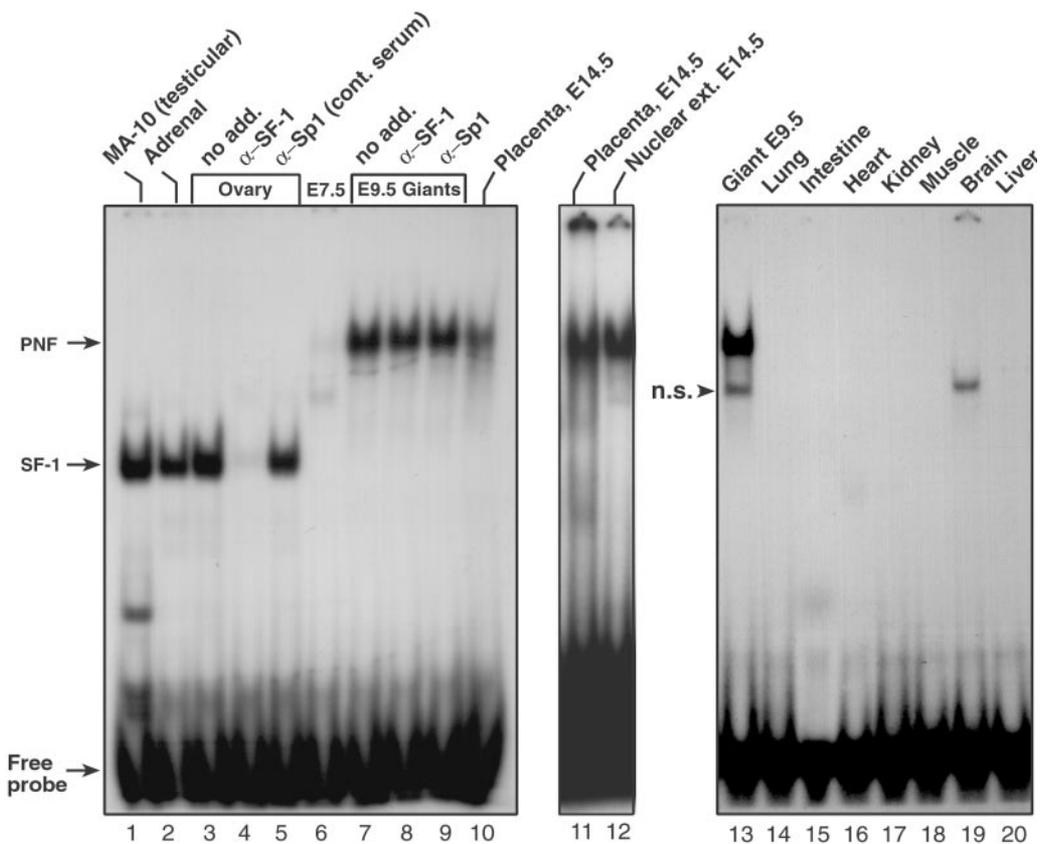
### DNA Binding Activity

Because the SCC1 region of the rat P450scc promoter was shown to be important for regulation of P450scc transcription (31, 33), we attempted to identify placental proteins that might bind to a <sup>32</sup>P-SCC1 probe (Table 1). Figure 1 shows that protein extracts prepared from either mouse embryonic d 9.5 (E9.5) giant trophoblast cells (lane 7), or E14.5 whole placenta (lane 10), formed a slow migrating complex with SCC1 that is clearly distinct from the typical SF-1 binding patterns observed with cell extracts from either testicular Leydig MA-10 mouse cell line, mouse adrenal, or mouse ovary (lanes 1, 2, and 3, respectively). The SCC1-bound trophoblast protein(s) was not affected by SF-1 antiserum (lane 8), whereas this antiserum (47) readily ablated the ovarian SF-1 band shift (lane 4). These results suggested that the placental protein(s) that formed a complex with SCC1 did not include SF-1. An identical DNA-protein band shift was also observed with placenta nuclear extract (lane 12). Therefore, for reasons of convenience we named the putative placental protein that binds SCC1 “placental nuclear factor” (PNF). Control nonspecific antiserum to selective promoter 1 (Sp1) did not affect PNF or SF-1 binding to SCC1 (Fig. 1, lane 9 or 5, respectively). This result indicated that Sp1 is not involved in the PNF-SCC1 complex. The amount of PNF expressed in E7.5 preplacentation sites was very low (lane 6). Screening of various adult mouse tissue extracts for the presence of PNF-like binding activity did not reveal the typical PNF band shift in the lung, intestine, heart,

**Table 2.** Sequence Alignment (Sense Strand) of the SCC1 Region in Various P450scc Genes (Ref. 46)

Rat	-60	5' -	GCTcCTC	CTCT	TAGCCTT	GAGCTAG	TTAGT	-3'	-32
Mouse	-56	5' -	GCTTCTC	CTCT	TAGCCTT	GAGCT	G---GT	-3'	-32
Porcine	-57	5' -	GCTTCTC	GC	TAAcCCTT	GAGCT	G---GT	-3'	-33
Ovine	-56	5' -	GCTTCTC	CATT	TAGCCTT	GAGCT	G---GT	-3'	-32
Bovine	-57	5' -	GCTTCTC	ACTT	TAGCCTT	GAGCT	G---GT	-3'	-33
Human	-56	5' -	GCTTCTG	GTA	TgGCCTT	GAGCT	G---GT	-3'	-32

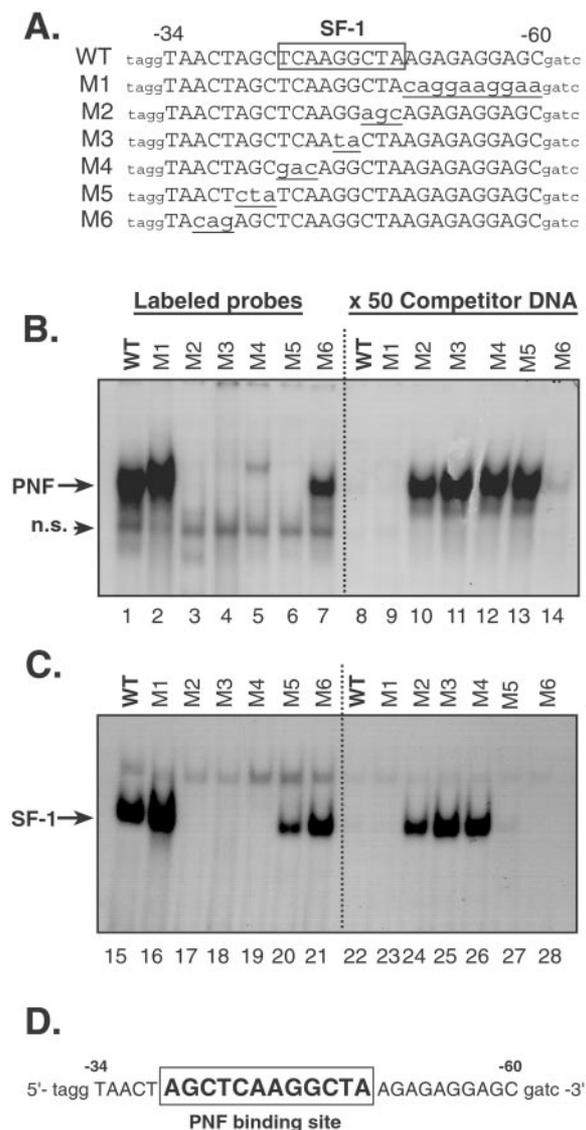
This alignment is based on known rat (42), mouse (31), porcine, (43), ovine (44), bovine (39), and human (45) P450scc gene sequences. Highly identical sequences are boxed. Lowercase letters denote mismatched bases. Gaps (-) were introduced to maximize sequence identity.

**Fig. 1.** A Non-SF-1 Protein from Trophoblast Giant Cells Forms a Complex with a Proximal SF-1 Binding Element in the P450scc Promoter (SCC1)

Extracts were prepared from the following cells and tissues: giant trophoblast cells from pregnant mice on d 9.5 post coitum (E9.5); E7.5 decidua; whole placenta of E14.5; MA-10 mouse testicular Leydig cell line; adrenals, ovaries and the indicated organs (lanes 14–21) prepared from d 9.5 pregnant mice. In addition, nuclear extracts were prepared from E14.5 placenta. EMSAs were performed after incubation of cell extracts (15–30  $\mu$ g), or nuclear extracts (3  $\mu$ g), with radiolabeled SCC1 probe corresponding to the -34/-60 sequence of the rat P450scc (see *Materials and Methods*). Where indicated, extracts were preincubated with antiserum to either SF-1 or Sp1 (nonspecific control) before addition of the probe. Arrows denote the following band shifts: SF-1; PNF; n.s., nonspecific complex.

kidney, muscle, brain, and liver (lanes 14–20). These results suggested that, in comparison to the placenta, the maternal tissues do not contain detectable levels of PNF activity.

To identify the sequence required for PNF binding in SCC1, mutated probes of SCC1 (antisense, Fig. 2A) were tested by EMSA using giant cell extract. PNF did not complex with any mutants of the SF-1 binding



**Fig. 2.** Characterization of PNF Binding Element

A, WT SCC1 probe (antisense strand) was mutated (*underlined lowercase* characters) as indicated in M1–M6 mutants. B, EMSA was conducted using giant trophoblast extracts incubated with either  $^{32}$ P-labeled mutant probes (lanes 1–7), or labeled WT probe competed with 50-fold molar excess of the indicated SCC1 mutants (lanes 8–14). C, Ovarian extract expressing SF-1 was used for EMSA studies as described in panel B. *Arrows* denote the band shifts of PNF, SF-1, and a minor nonspecific protein-DNA complex (n.s.). D, PNF binding site.

element (Fig. 2B, lanes 3–5). Moreover, PNF binding required the presence of an additional 5'-nucleotide triplet (AGC) upstream of the SF-1 site (M5, lane 6). These results were corroborated by competition assays using wild-type (WT)  $^{32}$ P-SCC1 as a probe and a 50-fold molar excess of the unlabeled DNA mutants (lanes 8–14). Additional EMSA studies using ovarian extracts (Fig. 2C) confirmed that the DNA motif required for SF-1 binding is 5'-TCAAGGCTA, whereas

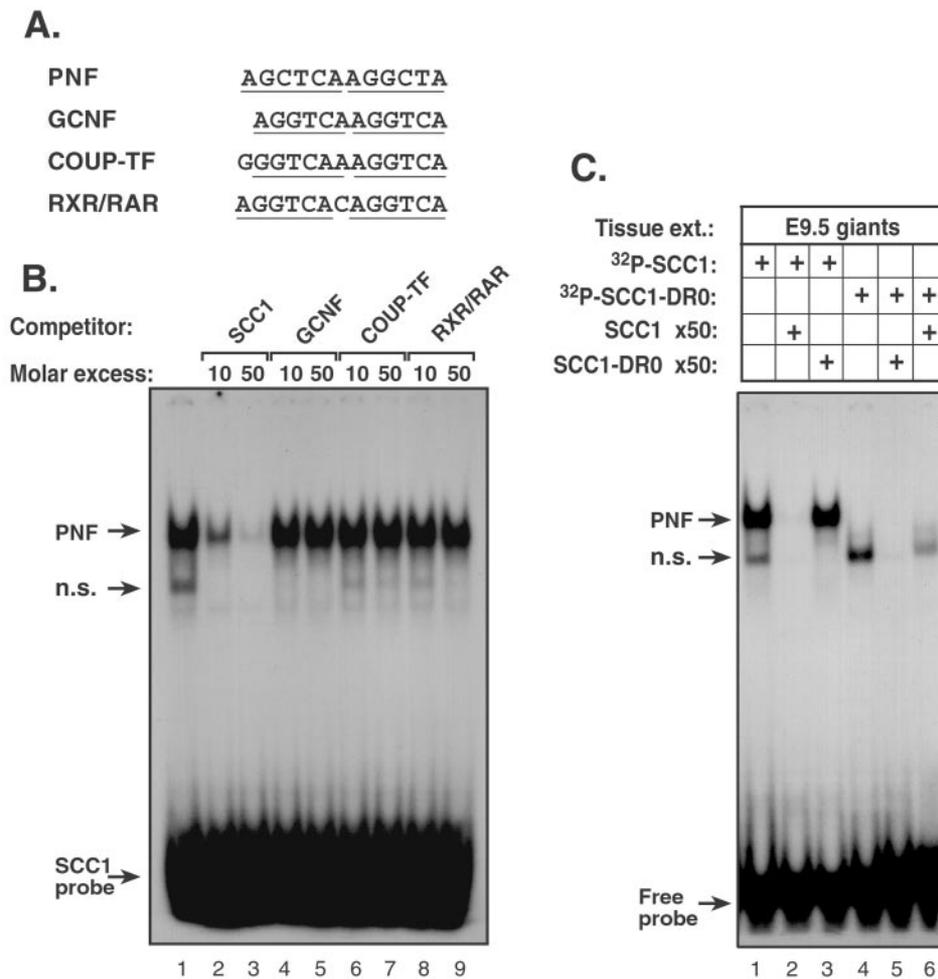
PNF binding required an extended element, 5'-AGCTCAAGGCTA (Fig. 2D). At first, this sequence seemed to be a near-consensus variation of a direct repeat with 0 bp spacing element (DR0), AGGTCAAGGTCA. Therefore, we tested whether direct repeat elements recognizing various known nuclear receptors, like chicken ovalbumin upstream promoter transcription factor (COUP-TF) (48), germ cell nuclear factor (GCNF) (49), and retinoid X receptor/retinoic acid receptor (RXR/RAR) (50) can also bind PNF. Figure 3B provides a compelling evidence that COUP-TF, GCNF, and RXR/RAR binding elements cannot compete for PNF binding to SCC1 (lanes 4–9). A similar inability to compete for PNF binding activity was also observed in the presence of molar excess of the following motif sequences (not shown) that bear relevant resemblance to the PNF binding element: nerve growth factor inducible-B response element (NGRE)-Nur77 (51), cAMP response element (52), estrogen response element (53), Sp1 (54), and CCAAT enhancer binding protein- $\beta$  (CEBP/ $\beta$ ) (54). Finally, Fig. 3C shows that SCC1 probe in which the putative PNF recognition site (AGCTCAAGGCTA) was replaced with a consensus DR0 motif, AGGTCAAGGTCA (SCC1-DR0), was incapable of PNF binding (lanes 3–6). This result suggested that the minor differences between the sequence of consensus DR0 and the sequence of PNF binding element are, nevertheless, critical for recognition of the placental protein.

#### PNF Does Not Bind SCC2

The promoter region SCC2 is located upstream of SCC1 (Table 1) and nests a second site (GGGAGGTCA), previously proposed to be a putative SF-1 binding motif important for functional activity of the P450scc promoter in Y-1 adrenal cells (31). To test whether SCC2 can associate with PNF, we compared the binding characteristics of SCC2 and SCC1 using E9.5 giant trophoblast cell extract (Fig. 4). Clearly, SCC2 did not complex with PNF (lane 4), nor could it compete for SCC1 binding to PNF (lanes 3). Control experiments with extract of corpora lutea from the same 9.5 postcoitus (p.c.) females showed that SF-1 did not bind the SCC2 probe either (lane 8). However, a large molar excess of SCC2 moderately displaced SCC1 binding to SF-1 (lane 7). The latter result conformed with earlier studies showing a weak SCC2 binding to rat ovarian SF-1 (33). Collectively, these results suggest that SCC2 does not contain sequences of interest related to binding of PNF or other potential factors in the placenta.

#### Functional Studies

To test whether transcription of the P450scc promoter in placental cells requires PNF binding, we conducted promoter-reporter analysis in primary cultures of mouse giant trophoblast cells. A typical culture of giant cells (Fig. 5A) includes a minor fraction of unavid-



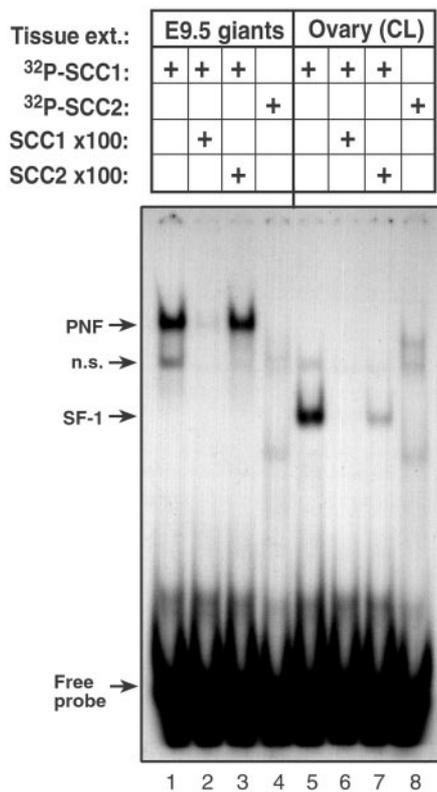
**Fig. 3.** PNF Does Not Bind Direct Repeat Elements Recognized by Known Nuclear Receptors

A, DR0 and DR1 probes recognized by the indicated nuclear receptors were prepared as described in *Materials and Methods*. Underline indicates half-site core motifs. B, EMSA was conducted using giant trophoblast extracts (E9.5) incubated with labeled SCC1 probe in the absence or presence of 10-fold and 50-fold molar excess of the indicated unlabeled oligonucleotides. Arrows denote PNF band shift and a nonspecific (n.s.) complex. C, The PNF core element in SCC1 was replaced with consensus DR0 motif (AGGTCAAGGTCA), and the resulting probe (SCC1-DR0) was examined by EMSA with E9.5 giant cell extract. Positive control EMSA included authentic SCC1 probe. Competing nonlabeled probes (50-fold) were included as indicated. Arrows denote the band shifts of PNF and a minor nonspecific protein-DNA complex (n.s.).

able nongiant placenta cells that underlay the mesometrial giant cell layers. Therefore, to examine in which of the placental cell types the P450scc promoter can be activated, we constructed chimeric genes based on the  $-378/+8$  region of the rat P450scc gene, placed upstream of a green fluorescent protein (GFP) coding sequence (*Materials and Methods*). Additional GFP reporter plasmids were constructed to include site-specific mutations in the SCC1 core element, aiming to impair the potential binding of PNF (M3 and M5, Figs. 2A and 5B). After transfection, cells were incubated with or without 8-bromo-cAMP (8-Br-cAMP) in serum-free medium and inspected daily by confocal microscopy. Figure 5C shows visual promoter activities in live cells depicted by a pseudocolor-coded presentation of GFP fluorescence levels. The highest expression of GFP was observed in cells

containing the WT promoter and treated with cAMP (panel D'). Site-specific mutations M3 and M5 markedly ablated the GFP expression in cells treated with cAMP (panels F' and H'), or incubated without the cyclic nucleotide (panels E' and G'). The low expression of M3 and M5 mutants in the absence of cAMP suggest that PNF is responsible for both the basal and the inducible promoter activity in the placental cells. In all cases, a predominant expression of GFP was observed in the giant cells, whereas GFP staining of other placental cell types was not detectable (panels A'–D').

The visual GFP studies were corroborated by quantitative analyses of chloramphenicol acetyltransferase (CAT) expression. To this end, the  $-378/+8$  region of the rat P450scc gene (WT) and site-specific mutants of this region (M3 and M5; Fig. 6, upper right panel) were ligated into a pCAT-Basic plasmid and transiently ex-



**Fig. 4.** PNF Does Not Bind to an Upstream Putative SF-1 Binding Site, SCC2

Cell extracts were prepared from either E9.5 giant trophoblast cells or ovarian corpora lutea (CL) obtained from the same animals. EMSA was performed as described in Fig. 1 using either radiolabeled SCC1 or SCC2 oligonucleotides. Where indicated, the binding assay was performed in the presence of excess unlabeled oligonucleotides. Arrows denote the band shifts of PNF, SF-1, and a minor nonspecific protein-DNA complex (n.s.).

pressed in giant trophoblast cells. Because 8-Br-cAMP-treated cells expressed much higher levels of the GFP reporter gene, we examined the CAT constructs in cAMP-induced giant cells. Figure 6A shows that mutations unable to bind PNF (M3 and M5), lost 70–75% of their ability to activate CAT expression in cAMP-treated cells. A similar loss of CAT expression by the M3 and M5 promoter mutants was also observed in control cultures without cAMP, although the overall CAT activities were considerably lower (not shown).

To demonstrate the difference between PNF action in the giant cells and the role of SF-1 in the ovary, we compared the activity of the same promoter constructs in hormone-induced granulosa cells. FSH generated a nearly 10-fold increase of the  $-378/+8$  CAT activity (WT, Fig. 6B). The M3 construct, in which the SF-1 binding motif is mutated, showed a 75% reduction of the promoter activity. By contrast, the promoter plasmid that harbors the M5 mutation, which does not affect the SF-1 binding motif, generated a normal 10-fold response to FSH.

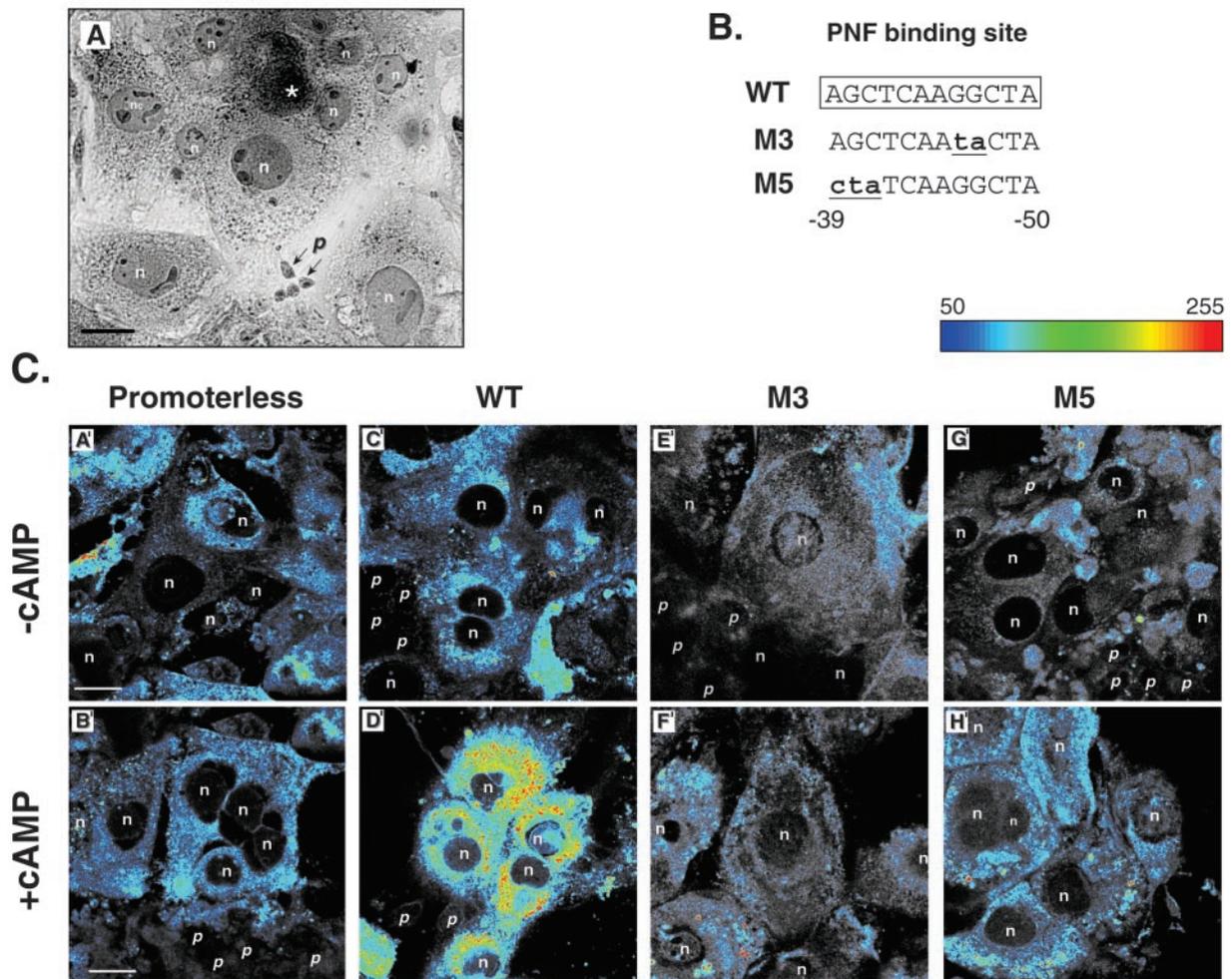
## Human PNF

In view of the fact that the SCC1 region is highly conserved in the P450scc gene of many species, including man (Ref. 46 and Table 2), we questioned whether human placenta contains a PNF-like binding activity. The band shift patterns shown in Fig. 7 indicated that, indeed, human term placenta contains PNF activity and, as expected, a 100-fold molar excess of either SCC1-M3 mutant (lane 3), or a GCNF probe (lane 4), did not compete for SCC1 binding to the human protein. Importantly, excess of SCC1-M3 and GCNF (DR0) oligonucleotides efficiently displaced a nonspecific complex that was observed in the mouse tissues (Figs. 1–4) and became predominant in extracts of the human term placenta (n.s., Fig. 7). Therefore, we used SCC1-DRO to displace binding of this nonspecific protein to the SCC1 affinity column during purification of PNF from human term placenta (*Materials and Methods*).

Similar binding characteristics of human PNF were observed when EMSA was performed using extracts of human choriocarcinoma JAR cells (Fig. 8A). Radiolabeled SCC1 formed a typical PNF band shift, which was absent when either SCC1-M3 or SCC1-M5 mutants were used as probes. In accordance, transient transfection assays in JAR cells revealed that SCC1-M3 and SCC1-M5 mutants of the  $-378/+8$  CAT gene were significantly less active (50%) when compared with the basal activity of the WT promoter (Fig. 8B). Altogether, these results suggested that PNF may be relevant for regulation of the P450scc gene in the human placenta.

## UV Cross-Linking

In an attempt to approximate the size of the SCC1-PNF complex, we conducted UV cross-linking experiments by modifying a previously described procedure (55). As detailed in *Materials and Methods*, a 50-mer <sup>32</sup>P-SCC1 probe was incubated with either an extract of E14.5 mouse placenta or an extract of human term placenta. The typical DNA-PNF band shift was UV irradiated within the EMSA gel, and slices containing the SCC1-PNF complex were then electroeluted and resolved by SDS-PAGE analysis without a preceding deoxyribonuclease (DNase) digest. Figure 9 shows that protein-DNA complexes of similar size were cross-linked in the presence of either mouse or human placenta extracts. The size of the predominant complex was 90 kDa, which suggested that a protein of approximately 50–60 kDa was covalently cross-linked to the DNA probe. To test whether the protein cross-linked to SCC1 is the PNF observed in EMSA, we used a 25-fold excess of unlabeled WT SCC1, or SCC1-M3 mutant, added before cross-linking. The presence of WT SCC1, but not SCC1-M3, markedly reduced the labeling of the predominant band of 90 kDa, suggesting that PNF is indeed the protein covalently bound to SCC1. It should be noted that SCC1 labeled



**Fig. 5.** Expression of GFP Reporter Driven by P450scc Promoter in Giant Trophoblast Cells

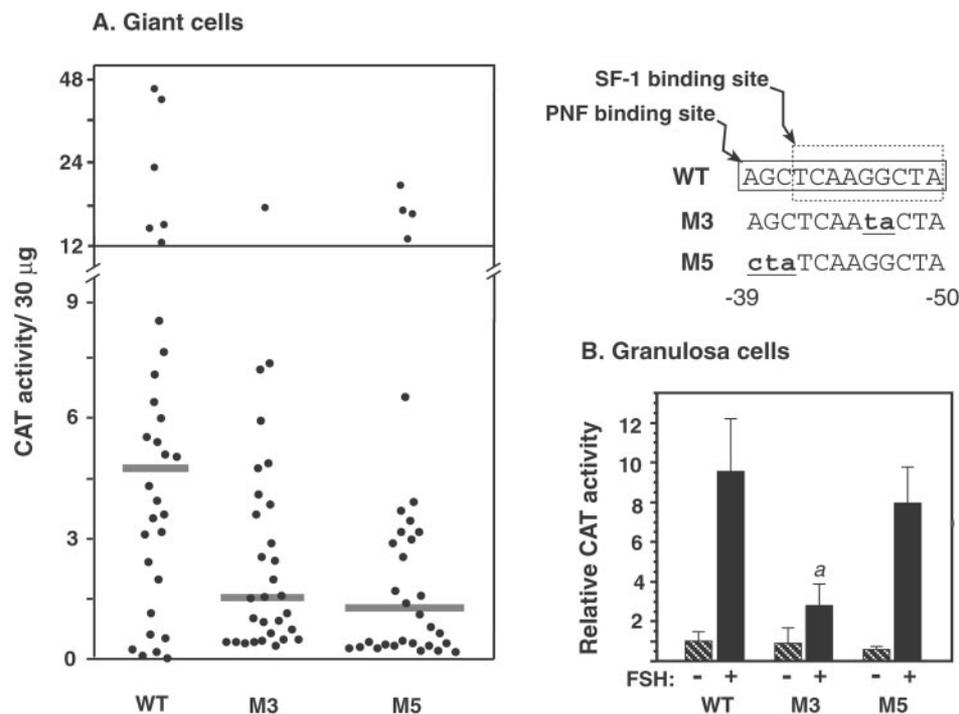
A, Primary cultures of giant trophoblast cells (E9.5) were prepared by surgical removal of the trophoblast layers from individual implantation sites, followed by collagenase-dispase dissociation and seeding of the cells on glass coverslips (*Materials and Methods*). Shown is a phase-contrast viewfield of a typical culture at the time of transfection (24 h), depicting multiple giant cells, some of which were not fully spread (\*). Note minor contamination with small non-giant placenta cells (p). n, Giant cell nucleus; nc, nucleolus. Bar, 50  $\mu$ m. B, A proximal region of the rat P450scc promoter (–378/+8) was mutated within the sequence of the PNF binding site, as shown (M3 and M5). The WT promoter (WT) and its mutants were then ligated to pEGFP promoterless plasmid (*Materials and Methods*). C, Transient transfections were performed by use of the following constructs: a promoterless GFP plasmid (Promoterless); P450scc p–378/+8 GFP construct (WT); two mutants of the –378/+8 region designated M3-GFP and M5-GFP, as shown in panel B. Cells were incubated with or without 0.5 mM 8-Br-cAMP (cAMP) in serum-free medium and the presented confocal microscope analysis of the GFP fluorescence in live cells was performed after 6 d in culture (*Materials and Methods*). Fluorescence intensity is presented by pseudocolor scale of 50–255 gray levels. Cell contour was visualized by enhanced brightness of each micrograph to simulate cellular counterstaining. n, Giant cell nuclei; p, small, non-giant placenta cells. Bar, 50  $\mu$ m.

additional complexes of higher molecular mass, implying that PNF-SCC1 complex includes more than one polypeptide component.

#### PNF Is AP-2 Transcription Factor

As human term placenta contains ample PNF activity, we used this tissue as a rich source of PNF for purification by affinity chromatography procedures (*Materials and Methods*). Sequencing of the purified human PNF protein by mass spectrometry identified it as

human activating protein-2 $\alpha$  (AP-2 $\alpha$ ). The molecular mass of human AP-2 $\alpha$ , 48 kDa (56), agrees well with the expected size of human PNF as determined by UV cross-linking experiments (Fig. 9). To test whether the SCC1-PNF complex observed in EMSA contains AP-2 $\alpha$ , we incubated extracts from human term placenta with monoclonal antiserum to AP-2 $\alpha$ . Figure 10 shows that this antiserum caused a supershift of the entire SCC1-PNF complex (lanes 1 and 2). By contrast, antiserum to AP-2 $\gamma$  barely affected the PNF-DNA band (lane 3), suggesting that the predominant AP-2 isoform



**Fig. 6.** Selective Effects of Site-Directed Mutations on Activation of the P450scc Promoter in Giant Trophoblast and Ovarian Granulosa Cells

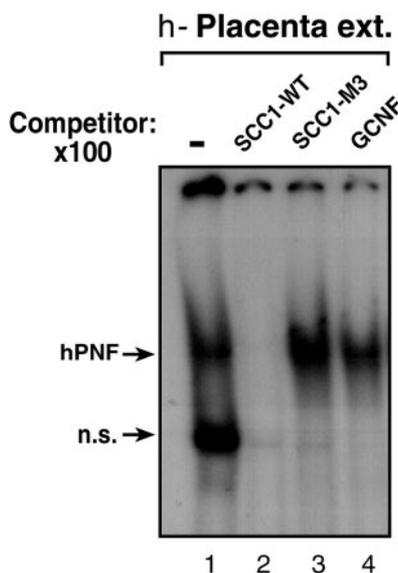
The proximal region of the rat *P450scc* promoter (−378/+8) was mutated within the sequence of the PNF binding site as shown in the upper right panel (M3, M5). The WT promoter (WT) and its variants were ligated into pCAT-Basic plasmid (*Materials and Methods*). A, Primary cultures of giant trophoblast cells from E9.5 were transiently transfected with the CAT constructs, and cell extracts were prepared after a 24-h incubation of the cells with 0.5 mM 8-Br-cAMP. Promoter activity in individual culture wells was determined by use of 5–30  $\mu$ g protein and a 16-h CAT assay. The results are presented (solid circles) as percent of  $^{14}$ C-chloramphenicol converted to its acetylated products. The median values (horizontal bars) are 4.79 (>3.075, 97.8%), 1.52 (<2.85, 95%), and 1.02 (<2.97, 95%) for WT, M3, and M5, respectively. This distribution-free statistical method was selected to accommodate the large variation of the CAT activities observed in the primary giant cell cultures. B, Primary granulosa cell cultures were transiently transfected with the CAT constructs described in panel A and treated for 6 h with FSH (100 ng/ml) as described in *Materials and Methods*. Control culture wells did not receive hormone treatment. CAT activity was determined by use of 5–10  $\mu$ g protein and a 4-h CAT assay. Results are presented (mean  $\pm$  SE) relative to the basal activity of the WT construct (=1). Three independent transfections were performed for each construct. a,  $P < 0.05$  when compared with the FSH-induced activities of WT or M5.

in human placenta is AP-2 $\alpha$ . Furthermore, identical binding characteristics (lanes 4–6) were observed when extract of human placenta was incubated with a  $^{32}$ P-SCC1 sequence of the human *P450scc* gene (−49/−32). Control nonrelevant antiserum to c-Jun did not affect the protein-DNA complex at all (lane 7). Interestingly, incubation of AP-2 antisera with extract of JAR cells showed that AP-2 $\gamma$  is the predominant isoform in these immortalized cells (lanes 8–10). Finally, EMSA studies testing extracts of the mouse E9.5 giant trophoblast cells revealed that murine PNF is solely AP-2 $\gamma$  (lanes 11–13).

Examination of the sequence we previously perceived as “PNF binding element” (sense 5′-TAGCCTTGAGCT) revealed a nested motif, GCCTTGAGC, that complies with a consensus element previously determined for specific binding of AP-2 $\alpha$  and AP-2 $\gamma$ ,  $G_{\text{C}}/C\text{CCNN}^A/C_{\text{G}}^G/A\text{G}^G/C_{\text{T}}$  (57). Therefore, we tested a SCC1 oligonucleotide in which we mutated three bases of the formerly considered PNF binding site, so

that the putative AP-2 motif remained intact (SCC1-AP-2) (Fig. 11). Indeed, Radiolabeled SCC1-AP-2 oligonucleotide readily associated with murine AP-2 $\gamma$  (Fig. 11, lane 4), and a 20-fold molar excess of nonlabeled SCC1-AP-2 DNA competed for binding of the AP-2 $\gamma$  to WT  $^{32}$ P-SCC1 (Fig. 11, lanes 1 and 3). These experiments confirmed that AP-2 $\gamma$  binds to the GCCTTGAGC motif. However, the competition experiments suggest that WT SCC1 oligonucleotide binds AP-2 $\gamma$  with somewhat better affinity than SCC1-AP-2 DNA (Fig. 11, compare lane 2 with lane 3).

To test whether the *P450scc* promoter can respond to the presence of AP-2 proteins, we cotransfected human embryonic kidney 293 (HEK293) cells with −378/+8 CAT constructs and expression vectors for either AP-2 $\alpha$  or AP-2 $\gamma$ . Both AP-2 isoforms activated the WT promoter 25- to 40-fold over its basal activity (Fig. 12). The promoter activity was abolished when the AP-2 binding element was modified by the M3 or M5 mutations. These functional studies strongly sup-



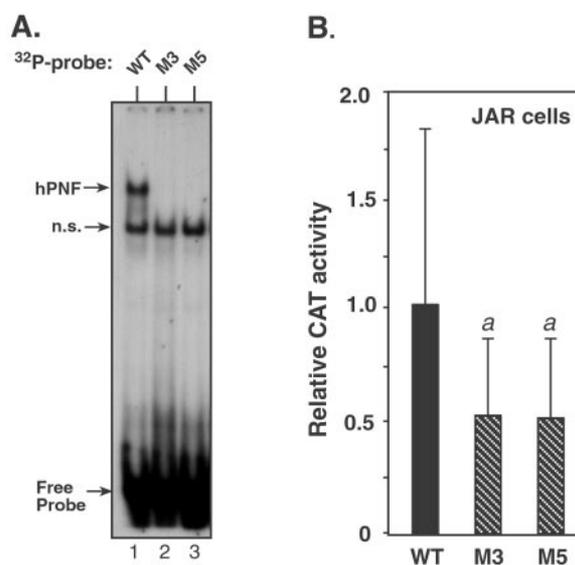
**Fig. 7.** PNF-Like Binding Activity in Human Placenta

Extracts were prepared from human term placenta and EMSA was performed using radiolabeled SCC1. Where indicated, the binding assay was performed in the presence of excess unlabeled oligonucleotides. Arrows denote the band shifts of human PNF (hPNF) and a nonspecific protein-DNA complex (n.s.).

ported the notion that AP-2 proteins can activate transcription of P450scc.

## DISCUSSION

Placentalation in mice has been shown to be accompanied by a rise of *de novo* steroid hormone production in giant trophoblast cells of midpregnancy (24). Later reports by us and others have shown that the genes involved in progesterone synthesis, including P450scc, adrenodoxin, adrenodoxin-reductase, StAR, and 3 $\beta$ -HSD, are expressed in the rat and mouse giant trophoblast cells of d 8.5–10.5 p.c. (20–22). Because SF-1, required for expression of P450scc in the adrenals and the gonads, is not expressed in the placenta (35, 37), an alternative regulatory protein(s) must substitute for the role of SF-1 in this organ. We hypothesized that such a placental factor(s) may bind to the same promoter region that harbors the SF-1 recognition site. To address this possibility, we used a well conserved promoter sequence of the P450scc gene, previously termed SCC1 (33), to probe a potential protein(s) that can bind this oligonucleotide. Our initial EMSA studies showed that, indeed, giant trophoblast and other placental cell types do express a putative placenta-specific nuclear factor, PNF. At first, it seemed that PNF binds a dodecamer sequence (antisense strand 5'-AGCTCAAGGCTA) positioned at -50/-39 within SCC1. Triple-base mutations along this sequence functionally disabled binding of PNF

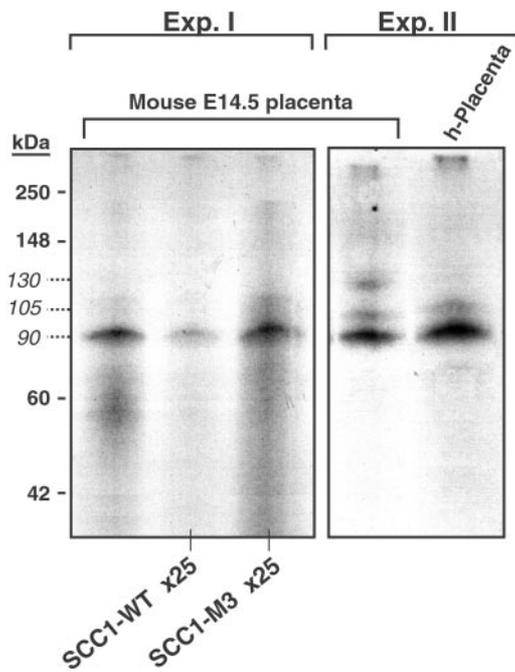


**Fig. 8.** PNF Binding Motif Is Required for Activation of P450scc Promoter in Human Placental Cell Line

A, Cell extracts were prepared from JAR cells and EMSA was performed using either of the indicated  $^{32}$ P-probes. Arrows denote a DNA-PNF-like complex (hPNF) and a nonspecific band shift (n.s.). B, JAR cell cultures were transiently transfected (*Materials and Methods*) with the WT, M3, and M5 CAT constructs described in Fig. 6A. After a 24-h incubation, the promoter activity was determined by use of 5–30  $\mu$ g protein and a 16-h CAT assay. Results are presented (mean  $\pm$  SE) relative to the activity of the WT construct. Six independent transfections were performed for each construct. a,  $P < 0.05$  when compared with the activity of WT.

and severely diminished the promoter activity in primary giant cell culture and in a human choriocarcinoma JAR cell line. Using GFP and CAT reporter assays, our functional analyses suggested that the promoter element required for PNF binding is essential to activate both basal and cAMP-responsive transcription.

Despite the fact that the PNF-binding element seemed closely related to a DR0 motif (AGGTCAAGGTCA), oligonucleotide containing various direct repeat motifs were unable to compete for PNF binding. This result implied that PNF might not be a member of the nuclear receptor family after all. To identify the placental protein, we purified PNF from human term placenta by use of affinity chromatography approaches. Sequencing of the purified protein revealed that human PNF is the  $\alpha$ -isoform of the human activating protein-2 (AP-2 $\alpha$ ). Specific antibodies tested in EMSA confirmed that AP-2 $\alpha$  is the predominant isoform in human term placenta. This protein readily binds to the SCC1 region in both the human and rat P450scc genes. Similar EMSA also showed that AP-2 $\gamma$  is the only isoform that is expressed in the trophoblast cells of the mouse placenta. This observation agrees with earlier studies demonstrating that of the three AP-2 genes known to date, AP-2 $\gamma$  is the most abundant one in the mouse placenta (58).

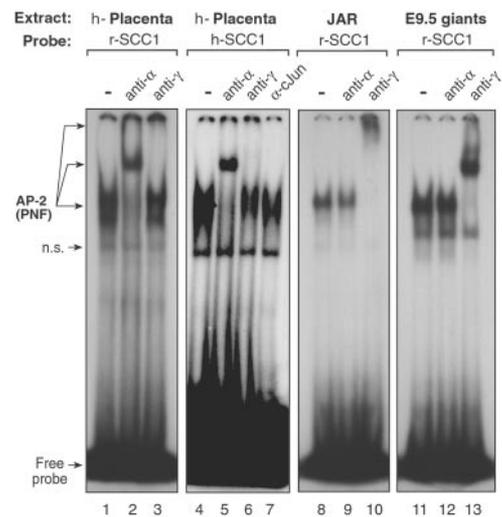


**Fig. 9.** UV Cross-Linking of SCC1-PNF Complex

Two independent UV cross-linking experiments (*left and right panels*) are presented using a 50-mer SCC1 labeled probe (*Materials and Methods*) and extracts from either E14.5 mouse placenta or human term placenta. EMSA gels were UV irradiated, and the electro-eluted DNA-protein complexes were resolved on SDS-PAGE (*Materials and Methods*). Standard molecular mass markers (250, 148, 90, 60, and 42 kDa) served to calculate the estimated size of three labeled protein-DNA complexes of 90, 105, and 130 kDa (*italicized characters*). Where indicated, unlabeled SCC1 or mutated SCC1 DNA (see M3 in Fig. 2A) was added to the binding mixture at a 25-fold molar excess.

Functional studies showed that coexpression of the AP-2 isoforms with the rat P450scc promoter (–378/+8 CAT) in a heterologous HEK293 cell system results in a marked activation of transcription. Such activity requires an intact AP-2 recognition site present in the promoter. As predicted, this AP-2 site is nested (*underlined*) within the previously perceived PNF element (sense 5'-TAGCCTTGAGCT) and fulfills the requirements for specific binding of the AP-2 isoforms  $\alpha$  and  $\gamma$  to a consensus sequence,  ${}^G/C$ CCNN ${}^A/C$  ${}^G/A$ G ${}^G/C$  ${}^T$  (57).

The identification of AP-2 proteins as regulators of P450scc expression in the placenta is consistent with PNF characteristics and previously reported notions about the role of AP-2 in the placenta. First, the AP-2 gene family encodes for 48- to 52-kDa proteins that bind to DNA in dimers (59–63). This can explain the slow migration of the PNF-SCC1 complex in EMSA, when compared with SF-1 that binds SCC1 as a monomer. Second, AP-2 isoforms are expressed in the placenta where they regulate transcription of genes essential for placental development (58) and endocrine functions. Examples are the genes encod-



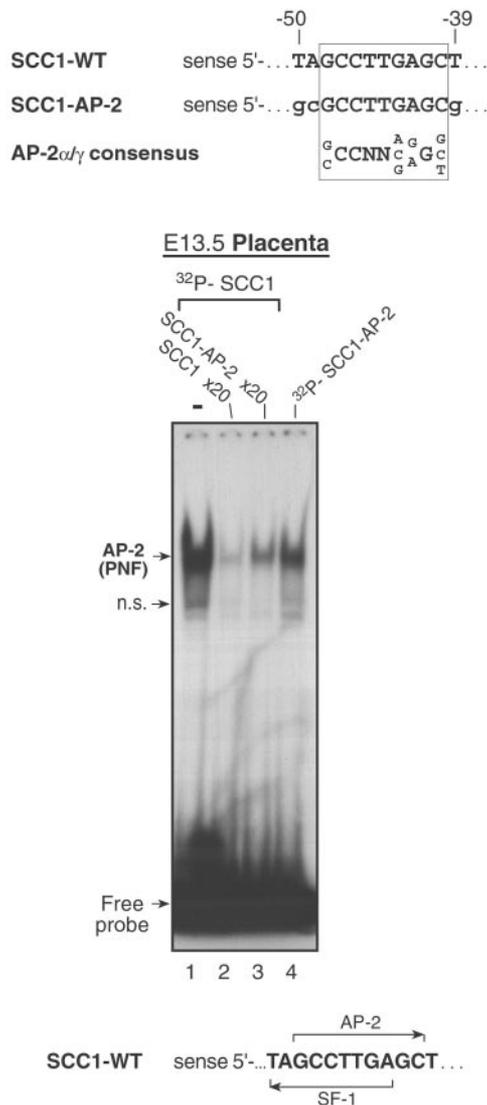
**Fig. 10.** Antisera Identify PNF as Isoforms of AP-2

EMSAs were conducted in the absence (–) or presence of specific antisera to AP-2 isoforms (anti- $\alpha$ , anti- $\gamma$ ), or nonspecific control antiserum ( $\alpha$ -c-Jun). Tissue and cell extracts were used from human term placenta (h-Placenta), JAR cells, and mouse E9.5 giant trophoblast cells (E9.5 giants). Two radiolabeled probes, *i.e.* human SCC1 (h-SCC1, *Materials and Methods*) or rat SCC1 (r-SCC1), were used. *Arrows* denote AP-2 (PNF)-containing complexes and a nonspecific complex (n.s.).

ing both human CG (hCG) subunits (64–66), the hCG receptor (67), and placental lactogens in humans and sheep (68, 69). Most relevant to our studies is the fact that hCG expression levels increase concomitantly with the dual onset of P450scc and AP-2 expression during the differentiation of cytotrophoblasts to syncytiotrophoblasts in first trimester human placenta (64, 70).

Strongly supportive of our findings, a recent study by Peng and Payne (71) showed that AP-2 is one of three factors involved in the transcription of placenta-specific isoform VI of the murine  $3\beta$ -HSD gene. Thus, the involvement of AP-2 in the regulation of  $3\beta$ -HSD VI and P450scc can explain the coordinated spatio-temporal expression of both genes observed during the second half of mouse pregnancy (22). Lastly, the third gene product involved in progesterone synthesis is StAR. Because the human placenta does not express StAR (72), it was proposed that the placental MLN64 protein replaces the role of StAR function in this organ (73). Current studies in our laboratory show that AP-2 is also implicated in the regulation of the human MLN64 promoter, which is strongly activated in primary giant trophoblast cells (Sher, N., and J. Orly, unpublished results). Collectively, these observations suggest that all the genes required for synthesis of steroid hormones in the human placenta are commonly regulated by AP-2.

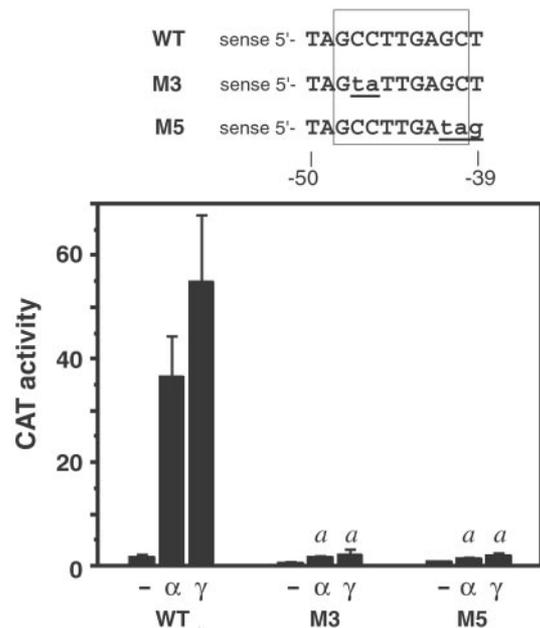
The fact that the AP-2 element-dependent activation of the P450scc promoter in the giant trophoblasts was cAMP inducible is in agreement with the well



**Fig. 11.** Site-Specific Mutations Define the AP-2 Binding Element in SCC1

*Upper panel.* Shown is a consensus binding element for AP-2 $\alpha$  and AP-2 $\gamma$  (boxed) (57), a sense orientation of the WT SCC1 region (SCC1-WT) including the PNF binding site (**bold uppercase letters**), and SCC1-AP-2 in which the flanking bases (**bold lowercase letters**) of PNF site were mutated so that a putative AP-2 binding element remained intact. *Lower panel.* Lanes 1–3, shows E13.5 placental extracts incubated with  $^{32}$ P-SCC1 probe and molar excess ( $\times 20$ ) of unlabeled SCC1 or SCC1-AP-2 oligonucleotides. Lane 4 shows complex formation of AP-2 with  $^{32}$ P-SCC1-AP-2. Arrows denote AP-2 (PNF) complex and a nonspecific complex (n.s.).

established data that cAMP and protein kinase A signaling are involved in AP-2-dependent promotion of transcription (66, 74, 75). Especially relevant to our findings are studies showing that cAMP signaling activates AP-2-dependent transcription of hCG $\beta$  and hCG $\alpha$  in JEG-3 cells and primary villous trophoblasts (65, 76). Therefore, it is conceivable that P450scc expression in murine giant cells is also mediated by a



**Fig. 12.** Recombinant AP-2 $\alpha$  and AP-2 $\gamma$  Transactivate the P450scc Promoter

*Upper panel* depicts the sense orientation of WT and two mutations (M3 and M5) of the AP-2 binding site (boxed) in the -378/+8 CAT construct of the rat P450scc promoter (see Fig. 6). Mutated bases are shown in underlined lowercase characters. *Lower panel.* CAT constructs WT, M3, and M5 were coexpressed in HEK293 cells (PEI transfected) with either AP-2 $\alpha$  ( $\alpha$ ) or AP-2 $\gamma$  ( $\gamma$ ) expression plasmids. Control transfections (-) did not include AP-2 expression vectors. After a 24-h incubation, the promoter activity was determined by use of 15  $\mu$ g protein and a 2-h CAT assay. Results are presented (mean  $\pm$  SE) as percent of  $^{14}$ C-chloramphenicol converted to its acetylated products. Triplicate transfections were performed for each construct. *a* denotes  $P < 0.01$  when compared with the counterpart activities of the WT construct.

cAMP/AP-2 signaling pathway triggered by hormonal cues yet to be discovered.

The pursuit of tissue-specific transcription factors regulating P450scc expression in the placenta started earlier on, when several investigators focused on the -155/-131 region of human P450scc, a region that was sufficient to confer transcription activation when positioned in a minimal heterologous promoter tested in JEG-3 cells (32, 41). Later on, this DNA element was shown to bind several factors, not necessarily of placental origin (77, 78). Other investigators used rat choriocarcinoma Rcho-1 cells as a model to study the regulation of rat P450scc (38). In contrast to our findings with similar promoter constructs, stable or transient transfections of Rcho-1 cells with a series of promoter segments showed that the proximal -379/+37 region was hardly active at all. Instead, more distal sequences of this promoter (-639/-428) were implicated in the developmental activation of the P450scc gene during differentiation in culture. No further identification of particular *cis*-acting elements required for activation of the P450scc promoter in the Rcho-1 cells was ever reported. Collectively, the

above studies imply that the regulatory mechanism of P450scc expression in the placenta requires *cis*-elements that are distinct from those that control the expression of the gene in the adrenal cortex and gonads. Our findings do not support this conclusion. We show herein that AP-2, described as a helix-span-helix transcription factor (79), and SF-1, which belongs to the zinc-finger nuclear receptor family, both bind to largely overlapping DNA sequences (Fig. 11). Therefore, it seems that transcription of P450scc presents a unique example of flexible adaptation of transcription machinery, by which a single modular sequence element is compatible to interact with different tissue-specific factors: AP-2 in the ephemeral placenta and SF-1 expressed in the fetus and adult steroidogenic tissues.

Interestingly, Pena *et al.* (80) were the first to suggest that AP-2 is involved in the regulation of ovine P450scc gene expression. These studies, using human JEG-3 cells, showed that AP-2 does not bind directly to the promoter but rather enables transactivation of the basal transcription machinery by protein-protein interactions with Sp1. In this regard, the present study shows that anti-Sp1 serum, which readily ablates Sp1 binding to other genes (54), did not affect the AP-2 binding to SCC1 (Fig. 1), suggesting that AP-2 binds directly to the promoter and Sp1 is not involved in this complex formation. Also, we did not observe Sp1 binding to the rat SCC2 oligonucleotide (Fig. 4), which is somewhat homologous to the OF3 region of the ovine gene, shown to be involved in transcriptional activation by Sp1 (80).

It is possible that AP-2 is not the sole regulator of P450scc transcription in the placenta. *In situ* hybridization and ribonuclease protection assays have shown that onset of AP-2 $\gamma$  expression in the mouse uterus commences on d 6.5 p.c. (58), whereas migrating giant trophoblast cells do not express P450scc before d 8 (21). Also, AP-2 $\gamma$  is expressed in all cells of the placenta (58), while we have shown that giant trophoblasts are certainly the only placental cell type expressing P450scc (21, 22). Lastly, whereas AP-2 expression is sustained through d 14.5 of pregnancy (Ref. 58 and our results), P450scc expression ceases as of d 11.5 (20, 22). These differences in the patterns of P450scc and AP-2 $\gamma$  expression predict that, although AP-2 may be essential for control of the P450scc gene in the mouse trophoblast cells, additional positive and negative regulatory proteins are likely to be involved in the spatio-temporal control of this gene during placentation. Such regulatory devices remain to be resolved in future studies.

## MATERIALS AND METHODS

### Materials

Acetyl-coenzyme A, aprotinin, benzamide, 8-Bromo-cAMP,  $\beta$ -glycerophosphate, DNase I, estradiol (E<sub>2</sub>), hydrocortisone, insulin, NaF, pepstatin, phenyl-methylsulfonylfluoride (PMSF),

poly(dI-dC), propylene glycol, protease inhibitor cocktail, proteinase K, sodium ortho-vanadate, and transferrin were obtained from Sigma (St. Louis, MO). Polyethyleneimine (PEI), molecular mass 25 kDa, was purchased from Aldrich (Sheboygan, WI). BSA was purchased from ICN Biochemicals, Inc. (Cleveland, OH). Tissue culture media were obtained from Life Technologies, Inc. (Paisley, UK) and included DMEM mixed with Ham's F-12 medium (no. 32500-019), DMEM (no. 52100 013), Ham's F-12 (no. 21700-026), and fetal bovine serum (no. 10106-169). Lipofectamine (no. 18324-012) was also obtained from the latter source. Ovine FSH (NIDDK-oFSH-20) was kindly provided by NIDDK's National Hormone and Pituitary Program and A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). PMSG was obtained from Vetimex (Bladel, The Netherlands) and hCG from Organon (Oss, The Netherlands). Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). Collagenase-dispase was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Anti SF-1 IgG was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-AP-2 $\gamma$  IgG (sc-897x) and monoclonal antibody to AP-2 $\alpha$  (sc-12726x), anti-c-Jun (sc44x), and anti-Sp1 (sc-059x) IgGs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Oligonucleotides and PCR primers were synthesized by Genset Oligos (Paris, France) or Sigma-Genosys (Cambridgeshire, UK).

### Animals

Female Sprague Dawley rats (21 d old) and C57 $\times$ BALB/c F<sub>1</sub> mice (6–8 wk) were obtained from Harlan Laboratories (Jerusalem, Israel) and maintained under a schedule of 16 h light, 8 h dark with food and water *ad libitum*. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols had the approval of the Institutional Committee on Animal Care and Use, The Alexander Silverman Institute of Life Sciences, The Hebrew University of Jerusalem. Pregnant female mice were obtained by hormonal hyperstimulation as previously described (21). Noon time on the day after mating was considered d 0.5 p.c., or E0.5. Rat ovarian granulosa cells were prepared from estradiol-primed animals as described previously (81).

### Tissue Extracts

Cell and tissue extracts for EMSA were obtained as described previously (54). Briefly, cells were homogenized using a Dounce homogenizer in 3–4 volumes of buffer A [400 mM KCl; 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4; 10% glycerol; 1 mM EDTA; 1 mM dithiothreitol (DTT); 5 mM NaF; 1 mM sodium ortho-vanadate; 5 mg/ml aprotinin; 2 mM pepstatin; 1 mM PMSF; and 1% vol/vol protease inhibitor cocktail], and the protein slurry was freeze-thawed three times in liquid nitrogen and a 37 C bath. Finally, the cell lysates were centrifuged for 3 min at 14,000  $\times$  g, and the protein content in the supernatants was determined by a modified Bradford assay (82). Extracts were kept at –70 C until use. Nuclear extracts were obtained as previously described (83).

### EMSA Studies

Protein extract (10–30  $\mu$ g) was incubated with 2–5 ng of double-stranded DNA previously labeled by a fill-in reaction using Klenow fragment (Promega Corp., Madison, WI) and [ $\alpha$ -<sup>32</sup>P]-dCTP (Amersham Pharmacia Biotech, Little Chalfont, UK). Binding assay was performed using a final volume of 30  $\mu$ l containing 100 mM KCl, 15 mM Tris-HCl (pH 7.5), 10 mM DTT, 1 mM EDTA, 12% glycerol, and 0.75–4.5  $\mu$ g poly(dI-dC). After incubation for 35 min at room temperature, the binding products were resolved on a native prerun polyacrylamide gel (5%) using TBE running buffer (50 mM Tris; 50 mM boric acid; and 10 mM EDTA, pH 8.3). When competition experiments were conducted, the protein extract was added last to the

reaction mixture. When antibodies were used for detection of a given protein-DNA complex, the reaction cocktail without the probe was preincubated with 2–8  $\mu$ g of the antibody for 25 min at room temperature/4 C before addition of the labeled DNA. The dried gels were analyzed using a FLA-3000 Bio-Imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). Gels were also exposed to Super RX medical x-ray film (Fuji Photo Film Co., Ltd.) for 2–24 h at  $-70$  C and developed by an X-Omat processor (Curix 60, Agfa, Munchen, Germany).

### Oligonucleotide Probes

Sequences of the SCC1 probe and its mutants M1–M6 are shown in Fig. 2; sequence of SCC1-AP2 is shown in Fig. 11; other oligonucleotide probes (sense strand), including overhanging restriction site sequences (*lowercase*) were:

SCC1-DR0, 5'-gatcGCTCCTCTCTTGACC TTGACCTA-GTTAccta;

human SCC1, 5'-gatcGTATGGCCTTGAGCTGGTccta (45);

SCC2, 5'-gatcGGAGGGGGGAGGTCAACACTCCATCA-ccta;

COUP-TF, 5'-gatcTTAGGGGTCAAAGGTCAAATGGAccta (48);

GCNF, 5'-gatcTCGAACATTTAGGTCAAGGTCAAGTCCccta (49);

RXR/RAR, 5'-agctGTCACAGGTACAGGTACAGGTCA-CAGTTCAAGCTccta (50);

NGRE, 5'-agcttGAGTTTTAAAAGGTCAATGCTCAATTTccta (51);

cAMP response element, 5'-gctcagcCCTTGGCTGACGT-CAGAGGccta (52);

Estrogen response element, 5'-gatcACGGGT AGAGGT-CACTGTGACCTCACCCGccta (53);

Sp1, 5'-gatcCGATCG GGGCGGGGCGAGCccta (54);

CEBP/ $\beta$ , 5'-CTGCAGGATGAGGCAATC ATTCATCCT-ccta (54).

### UV Cross-Linking

For UV cross-linking studies we labeled a 50 mer probe with [ $\alpha$ - $^{32}$ P]-dCTP by a fill-in reaction, using the  $-71/-22$  region of the rat *P450scc* antisense strand (5'-ACTTATAACCACTA-ACTAGCTCAAGGCTAA GAGAGGAGCTGATGGAGTGT) as template and the  $-71/-54$  of the sense strand as a primer (5'-ACACTCCATCAGTCTCTC). Pooled EMSA reactions (120  $\mu$ l each, details above) were resolved in single lanes of 5% polyacrylamide gel. After electrophoresis, the gel was placed under a UV source (254 nm, UVC 515, Ultra-LUM Inc., Carson, CA) and irradiated for 10 min. The wet gel was then exposed (15 min) to a PhosphorImager screen and the resulting pattern of the gel radioactivity assisted in the excision of slices containing the protein-DNA complexes. These gel slices were placed in dialysis bags and electroeluted under 100 V for 1 h. The eluates were concentrated by miniconcentrators (Fugisep, Intersip, Filtration systems, Wokingham, UK) and analyzed by 10% SDS-PAGE (84). Dried gels were exposed to hypersensitive film (Kodak Biomax MS, Eastman Kodak Co., Rochester NY) for 6 d at  $-80$  C. Molecular mass of the protein-DNA complexes was determined by standard markers (Multimark, Novex, San Diego, CA).

### Expression Plasmids

Plasmids AP2 $\alpha$ /pcDNA3.1(+) and AP2 $\gamma$ /pcDNA3.1 were kindly provided by Ronald J. Weigel (Stanford, CA).

### Promoter-Reporter Constructs

The  $-378/+8$  region of the rat *P450scc* (33) was cloned by a PCR-based approach (85) using rat genomic DNA (86) as

template. 5'-*Hind*III and 3'-*Xba*I cloning sites (*lowercase*) were included in addition to *ggcc* in the forward (5'-ggc-caagcttGAGTTATAAAGGGCCTGGGGAC) and the reverse (5'-ggcctctagaGTGTCTCTGCCCAAACCTCCA) primers, respectively. Mutations of SCC1 were obtained by use of a larger reverse primer ( $-60/+8$ , 5'-ggcctctagaGTGTCTCT-GCCCAAACCTCCAGAGCCACACTTATA-ACCACTAAGTCAATACTAAGAGAGGAGC) in which point mutations M3 and M5 were introduced as shown in Figs. 5 and 6. The PCR products were digested with *Hind*III and *Xba*I before ligation into promoter-less pCAT-Basic vector (Promega Corp.).

Cloning of  $-378/+8$  *P450scc* into pEGFP-1 vector (CLONTECH Laboratories, Inc., Palo Alto, CA) was performed after *Sma*I and *Hind*III digest of the vector, followed by 5'-dephosphorylation of the DNA by calf intestinal alkaline phosphatase (Promega Corp.). The various  $-378/+8$  CAT constructs were digested by *Xba*I and filled in by use of Klenow fragment. After precipitation, the digested plasmids were further digested by *Hind*III, and ligation (overnight at 16 C) was performed in the presence of a 10-fold molar excess of insert over vector DNA. All the generated plasmids were sequenced before use.

### Cell Cultures

Giant trophoblast cells were prepared from E9.5 mouse uteri. To this end, uterine myometrium layers were removed from individual implantation sites, and the trophoblast layers were separated from the overlying decidua by a gentle scraping of longitudinally cut half-sites. The giant cells (20 mg tissue/ml) were enzymatically dispersed by a modification of a previous procedure (24) using serum-free DMEM-F12 medium containing 10 mg/ml BSA, 4 mg/ml collagenase-dispase, and 20  $\mu$ g/ml DNase I. After 15 min incubation at 37 C, the cells were suspended by pipetting. This process was repeated twice before the enzymes were washed with serum-containing medium (10%), and the cell pellet was collected after a 2-min centrifugation at  $200 \times g$ . Cells were suspended in DMEM/F-12 growth medium containing 10% fetal bovine serum, 180 U/ml penicillin (Teva, Petach-Tikva, Israel), 0.24 mg/ml streptomycin (Sigma), and 25 mM HEPES (pH 7.4) and seeded (60–100 cells) into individual wells of a 24-well plate (Nunc, Copenhagen, Denmark). Cultures were maintained at 37 C in a humidified incubator, 95% air and 5% CO<sub>2</sub>.

Human choriocarcinoma JAR cells were grown in the same growth medium specified for the primary giant cells. HEK293 cells were grown in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and the same antibiotics as in the primary giant cell medium.

Primary rat ovarian granulosa cells were prepared from E<sub>2</sub>-primed animals and grown in serum-free cultures exactly as described before (81).

### Transfections

**Lipofectamine.** On the morning after seeding, giant cells in each well were transfected with 250 ng DNA and lipofectamine reagent in serum-free medium according to the basic protocol provided by the manufacturer. Six hours after onset of transfection, the medium was replaced by a standard serum-supplemented culture medium, as specified above. On the next morning, the DNA was washed and fresh serum-free medium was added before a 8-Br-cAMP treatment (0.5 mM) commencing 5 h later. CAT analyses were performed after a 24-h incubation with or without 8-Br-cAMP. Cells expressing GFP were monitored by live confocal microscopy as of d 4 after transfection.

**Electroporation.** Transfection of granulosa cell suspension ( $4 \times 10^5$  cells/0.8 ml) was performed by electroporation (54, 81) in the presence of  $-378/+8$  CAT constructs (20  $\mu$ g DNA), and the cells were seeded into four wells, each con-

taining 0.5 ml medium (24-well plate). Extracts for CAT activity were made after a 6-h incubation of the cells with FSH (100 ng/ml).

**Calcium Phosphate (Ca-Pi).** Twenty-four hours before transfection, JAR cells were harvested and reseeded in a 24-well plate at 50% confluence (0.5 ml/well). Transfection by Ca-Pi was conducted according to standard procedures (41) using 2.5  $\mu$ g DNA per well. After 20 h incubation with the DNA precipitate, cells were washed two to three times and further incubated with fresh growth medium for an additional 24 h before harvesting and CAT analysis.

**PEI.** Twenty-four hours before transfection, HEK293 cells were harvested and reseeded in a six-well plate at 50% confluence. On the day of transfection, DNA was diluted in 150 mM NaCl (2  $\mu$ g/ml). Concomitantly, PEI was diluted in 150 mM NaCl to a final concentration of 2 mM. After a 10-min incubation at room temperature, both solutions were mixed (1:1 vol/vol ratio) and allowed to stand for another 15 min. During this incubation, the cell culture medium was changed to a serum- and antibiotics-free DMEM (2 ml/well). DNA-PEI mixtures were added to cell cultures (0.5 ml/well) and were allowed there for 5 h. Afterward, cells were washed and replaced with regular medium for another 24 h, after which cells were harvested for CAT assay.

### Confocal Microscopy

To document the activity of -378/+8GFP constructs, giant cells were seeded onto a glass slide (12 mm diameter) that were hand glued at the bottom of a 10-mm hole drilled at the center of a 35-mm tissue culture dish (Falcon 3001, Becton Dickinson and Co., Plymouth, UK). These dishes allowed scanning of live cells by use of an inverted microscope attached to a 1024 confocal workstation (Bio-Rad Laboratories, Inc., Hercules, CA). After the indicated treatments, cells were viewed using a 40 $\times$  oil immersion objective (numerical aperture 1.3). The excitation wavelength was 488 nm, and the emission of GFP was collected by use of a 525  $\pm$  20 nm filter. All cells were scanned using same scanning conditions (laser power, gain of photo multiplier tube, iris size, and zoom). The acquired images were processed using Image Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD) and displayed in pseudocolor images equivalent to 50–255 gray levels (scale provided in Fig. 5).

### CAT Assay

After the indicated treatments, cell lysates were prepared and CAT activity was analyzed as previously described (54, 81). If not otherwise indicated, data are presented as percent of [<sup>14</sup>C]-chloramphenicol (Amersham International, Little Chalfont, UK) converted to its acetylated products (per protein and time of assay). Each figure presents either the mean  $\pm$  SE of several independent transfections or depicts a distribution-free presentation to accommodate the large variation of the CAT activities observed in the primary giant cell cultures.

### Purification of Human PNF

Purification of hPNF was conducted by a three-step procedure: 1) Human term placental cotyledons (200 g) were extracted in EMSA buffer A as described above (*Tissue Extracts*). Ammonium sulfate (dry form) was added to the resulting extract to attain 30% saturation. After chilling for 30 min at 4 C, the suspension was centrifuged at 15,000  $\times$  g for 30 min at 4 C, and the pellet was redissolved in 20 ml of buffer B [25 mM HEPES (pH 7.9), 10% (vol/vol) glycerol, 0.1 M KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 mM benzamidine, 1 mM sodium ortho-vanadate, 1 mM NaF, and 5 mM  $\beta$ -glycerophosphate (87)]. 2) The placental proteins were diluted in 200 ml of buffer C [10 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 0.1 M

KCl, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, 2.5  $\mu$ M NaF, 0.5 mM sodium ortho-vanadate, 5 mg/ml aprotinin, 0.5 mM PMSF, 2.5 mM  $\beta$ -glycerophosphate, and 0.5 mM benzamidine] and 50 ml of heparin-Sepharose 6 beads (fast flow, Amersham Pharmacia Biotech, Uppsala, Sweden) were added. The slurry was stirred at 4 C for 2 h, and bound proteins were eluted by buffer C containing a linear gradient of 0.1–1.5 M KCl. Eluted fractions were tested for binding activity by EMSA. 3) Final purification of PNF was obtained by the use of a DNA affinity column constructed by binding of biotinylated SCC1 to streptavidin agarose beads (Sigma). Active fractions of the heparin column (550–650 mM KCl) were pooled, and the buffer was adjusted to attain the composition of EMSA reaction cocktail (see above). Nonspecific double-stranded DNA (SCC1-DR0, 65  $\mu$ g/ml) was added, and the proteins were allowed to adsorb to the SCC1 resin (0.4 ml) for 3 h at 4 C. Readsorption of unbound proteins was repeated (overnight) with a fresh aliquot of DNA resin. The DNA resin was stepwise washed with buffer D (glycerol 15%; 1 mM EDTA; 1 mM DTT; 0.1% Triton X-100; 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) containing 150 and 300 mM KCl, and PNF activity was eluted by 650 mM of KCl in buffer D. The latter active fraction was repurified on SCC1 resin using less SCC1-DR0 DNA (33 ng/ml) before it was precipitated by adding deoxycholate (200  $\mu$ g/ml, 30 min at 4 C) and then cold trichloroacetic acid (10%). After an overnight incubation at 4 C, the protein suspension was centrifuged at 14,000 rpm for 15 min at 4 C, and the pellet was dissolved in SDS-PAGE sample buffer. The ability of the purified protein to bind SCC1 was confirmed by Southwestern analysis performed as described previously (88) using 10% of the protein content. The rest of the purified protein was resolved by electrophoresis on a 7.5% minigel and stained by SimplyBlue SafeStain (Invitrogen, Groningen, The Netherlands, catalog no. LC6060). The stained band was excised, and its protein content was reduced (10 mM DTT) and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate. Each gel piece was then treated with 50% acetonitrile in 10 mM ammonium bicarbonate to remove the stain from the protein, and dried. After rehydration with 10 mM ammonium bicarbonate containing trypsin (0.1 g), the protein band was incubated overnight at 37 C, and the resulting peptides were recovered with 60% acetonitrile with 0.1% trifluoroacetate.

The tryptic peptides were resolved by reverse-phase chromatography on 0.1  $\times$  300-mm fused silica capillaries (internal diameter 100  $\mu$ m, J&W Science, Folsom, CA), hand-filled with porous R2 (Roche Molecular Biochemicals). The peptides were eluted using an 80-min linear gradient of 5–95% acetonitrile with 0.1% acetic acid in water at a flow rate of about 1  $\mu$ l/min. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA). Mass spectrometry was performed in the positive ion mode using repetitively full mass spectrometric scan followed by collision-induced dissociation of the most dominant ion selected from the first mass spectrometry scan. The mass spectrometry data were compared with simulated proteolysis and collision-induced dissociation of the proteins in the nonredundant database (NCBI) using Sequest software (Eng, J., and J. Yates, University of Washington, and Finnigan MAT).

### Data Presentation and Statistical Analysis

CAT activities were normalized per protein and time of assay, and the data are presented as percent of the amount of [<sup>14</sup>C]-chloramphenicol converted to the acetylated products. Results of CAT assays performed in granulosa cells, JAR, and HEK293 cells are presented as the mean  $\pm$  SD of population of several independent transfections as indicated in each figure. Student's unpaired two-tailed *t* test was performed using Excel '97 (Microsoft Corp., Redmond, WA) statistical analysis functions. Differences between the activities of the indicated constructs were considered statistically

significant at  $P < 0.05$ . Regarding the E9.5 cells, results of each individual experiment were presented whereas medians and confidence horns were used as statistical parameters (89).

### Acknowledgments

We thank Dr. N. Melamed-Book of this institute for assistance with confocal microscopy. We are grateful to Dr. D. Melloul of Hadassah Medical Center, Dr. M. Lebendiker of the Protein Purification Unit, Wolfson Center for Applied Structural Biology, this institute, T. Ziv and A. Admon from the Smoler Protein Center of the Israel Institute of Technology, for their critical help during the purification and sequencing of human AP-2. We are also grateful to Dr. R. J. Weigel and Dr. L. A. McPherson (Stanford University) for providing the AP-2 $\alpha$  and AP-2 $\gamma$  expression plasmids. We thank Dr. U. Motro, of this institute, for assistance with some of the statistical analyses, and N. Sher for helpful discussions of this manuscript.

Received February 4, 2002. Accepted April 18, 2002.

Address all correspondence and requests for reprints to: Dr. Joseph Orly, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: orly@vms.huji.ac.il.

This work was supported by The Israel Science Foundation (Grant 672/00) and United States-Israel Binational Foundation (Grant 1999315).

### REFERENCES

1. Finn CA, Martin L 1974 The control of implantation. *J Reprod Fertil* 39:195–206
2. Psychoyos A 1973 Hormonal control of ovoiimplantation. *Vitam Horm* 31:201–256
3. Csapo AI, Pulkkinen MO, Wiest WG 1973 Effects of luteectomy and progesterone replacement therapy in early pregnant patients. *Am J Obstet Gynecol* 115:759–765
4. Pepe GJ, Rothchild I 1973 Metabolic clearance rate of progesterone: comparison between ovariectomized, pregnant, pseudopregnant and deciduoma-bearing pseudopregnant rats. *Endocrinology* 93:1200–1205
5. Pepe GJ, Albrecht ED 1995 Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr Rev* 16:608–648
6. Strauss III JF, Martinez F, Kiriakidou M 1996 Placenta steroid hormone synthesis: unique features and unanswered questions. *Biol Reprod* 54:303–311
7. Albrecht ED, Pepe GJ 1990 Placental steroid hormone biosynthesis in primate pregnancy. *Endocr Rev* 11:124–150
8. Hall PF 1984 Cellular organization for steroidogenesis. *Int Rev Cytol* 86:53–95
9. Miller WL 1988 Molecular biology of steroid hormone synthesis. *Endocr Rev* 9:295–317
10. Bain PA, Yoo M, Clarke T, Hammond SH, Payne AH 1991 Multiple forms of mouse  $\beta$  3-hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4 isomerase and differential expression in gonads, adrenal glands, liver, and kidneys of both sexes. *Proc Natl Acad Sci USA* 88:8870–8874
11. Carlone DL, Richards JS 1997 Functional interactions, phosphorylation, and levels of 3',5'-cyclic adenosine monophosphate-regulatory element binding protein and steroidogenic factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. *Mol Endocrinol* 11:292–304
12. Simpson ER, Davis SR 2001 Minireview: aromatase and the regulation of estrogen biosynthesis—some new perspectives. *Endocrinology* 142:4589–4594
13. Tang OS, Thong KJ, Baird DT 2001 Second trimester medical abortion with mifepristone and gemeprost: a review of 956 cases. *Contraception* 64:29–32
14. Whittaker PG, Stewart MO, Taylor A, Lind T 1989 Some endocrinological events associated with early pregnancy failure. *Br J Obstet Gynaecol* 96:1207–1214
15. Mitchell SN, Smith SK 1992 The effect of progesterone and human interferon  $\alpha$ -2 on the release of PGF $2\alpha$  and PGE from epithelial cells of human proliferative endometrium. *Prostaglandins* 44:457–470
16. Adachi S, Oku M 1995 The regulation of oxytocin receptor expression in human myometrial monolayer culture. *J Smooth Muscle Res* 31:175–187
17. Zhao K, Kuperman L, Geimonen E, Andersen J 1996 Progesterone represses human connexin43 gene expression similarly in primary cultures of myometrial and uterine leiomyoma cells. *Biol Reprod* 54:607–615
18. Csapo AI, Wiest WG 1969 An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. *Endocrinology* 85:735–746
19. Sherman MI 1983 Endocrinology of rodent trophoblast cells. In: Loke YW, Whyte A, eds. *Biology of trophoblast*. Amsterdam: Elsevier Science Publishers B.V.; 401–467
20. Durkee TJ, McLean MP, Hales DB, Payne AH, Waterman MR, Khan I, Gibori G 1992 P450(17 $\alpha$ ) and P450SCC gene expression and regulation in the rat placenta. *Endocrinology* 130:1309–1317
21. Schiff R, Arensburg J, Itin A, Keshet E, Orly J 1993 Expression and cellular localization of uterine side-chain cleavage cytochrome P450 messenger ribonucleic acid during early pregnancy in mice. *Endocrinology* 133:529–537
22. Arensburg J, Payne AH, Orly J 1999 Expression of steroidogenic genes in maternal and extraembryonic cells during early pregnancy in mice. *Endocrinology* 140:5220–5232
23. Siiteri PK, Febres F, Clemens LE, Chang RJ, Gondos B, Stites D 1977 Progesterone and maintenance of pregnancy: is progesterone nature's immunosuppressant? *Ann NY Acad Sci* 286:384–397
24. Salomon DS, Sherman MI 1975 The biosynthesis of progesterone by cultured mouse midgestation trophoblast cells. *Dev Biol* 47:394–406
25. Abbaszade IG, Arensburg J, Park CH, Kasa VJ, Orly J, Payne AH 1997 Isolation of a new mouse  $\beta$ 3-hydroxysteroid dehydrogenase isoform,  $\beta$ 3-HSD VI, expressed during early pregnancy. *Endocrinology* 138:1392–1399
26. Simpson ER, Waterman MR 1988 Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annu Rev Physiol* 50:427–440
27. Richards JS, Hedin L 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. *Annu Rev Physiol* 50:441–463
28. Waterman MR 1994 Biochemical diversity of cAMP-dependent transcription of steroid hydroxylase genes in the adrenal cortex. *J Biol Chem* 269:27783–27786
29. Lala DS, Rice DA, Parker KL 1992 Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol Endocrinol* 6:1249–1258
30. Morohashi K, Honda S, Inomata Y, Handa H, Omura T 1992 A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450 s. *J Biol Chem* 267:17913–17919
31. Rice DA, Kirkman MS, Aitken LD, Mouw AR, Schimmer BP, Parker KL 1990 Analysis of the promoter region of the gene encoding mouse cholesterol side-chain cleavage enzyme. *J Biol Chem* 265:11713–11720
32. Hum DW, Staels B, Black SM, Miller WL 1993 Basal transcriptional activity and cyclic adenosine 3',5'-mono-

- phosphate responsiveness of the human cytochrome P450scc promoter transfected into MA-10 Leydig cells. *Endocrinology* 132:546–552
33. Clemens JW, Lala DS, Parker KL, Richards JS 1994 Steroidogenic factor-1 binding and transcriptional activity of the cholesterol side-chain cleavage promoter in rat granulosa cells. *Endocrinology* 134:1499–1508
  34. Chau YM, Crawford PA, Woodson KG, Polish JA, Olson LM, Sadovsky Y 1997 Role of steroidogenic-factor 1 in basal and 3',5'-cyclic adenosine monophosphate-mediated regulation of cytochrome P450 side-chain cleavage enzyme in the mouse. *Biol Reprod* 57:765–771
  35. Luo X, Ikeda Y, Parker KL 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* 77:481–490
  36. Luo X, Ikeda Y, Schlosser DA, Parker KL 1995 Steroidogenic factor 1 is the essential transcript of the mouse Ftz-F1 gene. *Mol Endocrinol* 9:1233–1239
  37. Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J 1995 Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc Natl Acad Sci USA* 92:10939–10943
  38. Yamamoto T, Chapman BM, Clemens JW, Richards JS, Soares MJ 1995 Analysis of cytochrome P-450 side-chain cleavage gene promoter activation during trophoblast cell differentiation. *Mol Cell Endocrinol* 113:183–194
  39. Ahlgren R, Simpson ER, Waterman MR, Lund J 1990 Characterization of the promoter/regulatory region of the bovine CYP11A (P-450 scc) gene. Basal and cAMP-dependent expression. *J Biol Chem* 265:3313–3319
  40. Momoi K, Waterman MR, Simpson ER, Zanger UM 1992 3',5'-cyclic adenosine monophosphate-dependent transcription of the CYP11A (cholesterol side chain cleavage cytochrome P450) gene involves a DNA response element containing a putative binding site for transcription factor Sp1. *Mol Endocrinol* 6:1682–1690
  41. Moore CC, Hum DW, Miller WL 1992 Identification of positive and negative placenta-specific basal elements and a cyclic adenosine 3',5'-monophosphate response element in the human gene for P450scc. *Mol Endocrinol* 6:2045–2058
  42. Oonk RB, Parker KL, Gibson JL, Richards JS 1990 Rat cholesterol side-chain cleavage cytochrome P-450 (P-450 scc) gene. Structure and regulation by cAMP *in vitro*. *J Biol Chem* 265:22392–22401
  43. Urban RJ, Shupnik MA, Bodenbun YH 1994 Insulin-like growth factor-I increases expression of the porcine P-450 cholesterol side chain cleavage gene through a GC-rich domain. *J Biol Chem* 269:25761–25769
  44. Pestell RG, Hammond VE, Crawford RJ 1993 Molecular cloning and characterization of the cyclic AMP-responsive ovine CYP11A1 (cholesterol side-chain cleavage) gene promoter: DNase 1 protection of conserved consensus elements. *J Mol Endocrinol* 10:297–311
  45. Morohashi K, Sogawa K, Omura T, Fujii-Kuriyama Y 1987 Gene structure of human cytochrome P-450(SCC), cholesterol desmolase. *J Biochem (Tokyo)* 101:879–887
  46. Takayama K, Morohashi K, Honda S, Hara N, Omura T 1994 Contribution of Ad4BP, a steroidogenic cell-specific transcription factor, to regulation of the human CYP11A and bovine CYP11B genes through their distal promoters. *J Biochem (Tokyo)* 116:193–203
  47. Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP, Parker KL 1993 Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. *Mol Endocrinol* 7:852–860
  48. Cooney AJ, Tsai SY, O'Malley BW, Tsai MJ 1992 Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTC A response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol Cell Biol* 12:4153–4163
  49. Chen F, Cooney AJ, Wang Y, Law SW, O'Malley BW 1994 Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. *Mol Endocrinol* 8:1434–1444
  50. Mangelsdorf DJ, Umesono K, Kliewer SA, Borgmeyer U, Ong ES, Evans RM 1991 A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* 66:555–561
  51. Wilson TE, Fahrner TJ, Milbrandt J 1993 The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Mol Cell Biol* 13:5794–5804
  52. Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF 1991 The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature [published erratum appears in *DNA Cell Biol* 1991 Jun;10(5):397–398]. *DNA Cell Biol* 10:1–14
  53. Vanacker JM, Pettersson K, Gustafsson JA, Laudet V 1999 Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER)  $\alpha$ , but not by ER $\beta$ . *EMBO J* 18:4270–4279
  54. Silverman E, Eimerl S, Orly J 1999 CCAAT enhancer-binding protein beta and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells. *J Biol Chem* 274:17987–17996
  55. Wu C, Wilson S, Walker B, Dawid I, Paisley T, Zimarino V, Ueda H 1987 Purification and properties of *Drosophila* heat shock activator protein. *Science* 238:1247–1253
  56. Williams T, Admon A, Luscher B, Tjian R 1988 Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev* 2:1557–1569
  57. McPherson LA, Weigel RJ 1999 AP2 $\alpha$  and AP2 $\gamma$ : a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs. *Nucleic Acids Res* 27:4040–4049
  58. Shi D, Kellems RE 1998 Transcription factor AP-2  $\gamma$  regulates murine adenosine deaminase gene expression during placental development. *J Biol Chem* 273:27331–27338
  59. Mitchell PJ, Wang C, Tjian R 1987 Positive and negative regulation of transcription *in vitro*: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50:847–861
  60. Luscher B, Mitchell PJ, Williams T, Tjian R 1989 Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. *Genes Dev* 3:1507–1517
  61. Moser M, Imhof A, Pscherer A, Bauer R, Amselgruber W, Sinowatz F, Hofstadter F, Schule R, Buettner R 1995 Cloning and characterization of a second AP-2 transcription factor: AP-2  $\beta$ . *Development* 121:2779–2788
  62. Oulad-Abdelghani M, Bouillet P, Chazaud C, Dolle P, Chambon P 1996 AP-2.2: a novel AP-2-related transcription factor induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Exp Cell Res* 225:338–347
  63. Cheng C, Ying K, Xu M, Zhao W, Zhou Z, Huang Y, Wang W, Xu J, Zeng L, Xie Y, Mao Y 2002 Cloning and characterization of a novel human transcription factor AP-2 $\beta$  like gene (TFAP2BL1). *Int J Biochem Cell Biol* 34:78–86
  64. Johnson W, Albanese C, Handwerker S, Williams T, Pestell RG, Jameson JL 1997 Regulation of the human chorionic gonadotropin  $\alpha$ - and  $\beta$ -subunit promoters by AP-2. *J Biol Chem* 272:15405–15412
  65. Johnson W, Jameson JL 1999 AP-2 (activating protein 2) and Sp1 (selective promoter factor 1) regulatory elements play distinct roles in the control of basal activity

- and cyclic adenosine 3',5'-monophosphate responsiveness of the human chorionic gonadotropin- $\beta$  promoter. *Mol Endocrinol* 13:1963–1975
66. LiCalsi C, Christophe S, Steger DJ, Buescher M, Fischer W, Mellon PL 2000 AP-2 family members regulate basal and cAMP-induced expression of human chorionic gonadotropin. *Nucleic Acids Res* 28:1036–1043
  67. Hu YL, Lei ZM, Rao CV 1996 *cis*-Acting elements and *trans*-acting proteins in the transcription of chorionic gonadotropin/luteinizing hormone receptor gene in human choriocarcinoma cells and placenta. *Endocrinology* 137:3897–3905
  68. Richardson BD, Langland RA, Bachurski CJ, Richards RG, Kessler CA, Cheng YH, Handwerger S 2000 Activator protein-2 regulates human placental lactogen gene expression. *Mol Cell Endocrinol* 160:183–192
  69. Limesand SW, Anthony RV 2001 Novel activator protein-2 $\alpha$  splice-variants function as transactivators of the ovine placental lactogen gene. *Eur J Biochem* 268:2390–2401
  70. Martinez F, Kiriakidou M, Strauss III JF 1997 Structural and functional changes in mitochondria associated with trophoblast differentiation: methods to isolate enriched preparations of syncytiotrophoblast mitochondria. *Endocrinology* 138:2172–2183
  71. Peng L, Payne AH 2001 AP-2 $\gamma$  and the homeodomain protein distal-less 3 are required for placental-specific expression of the murine 3 $\beta$ -hydroxysteroid dehydrogenase VI gene. *J Biol Chem* 277:7945–7954
  72. Sugawara T, Holt JA, Driscoll D, Strauss III JF, Lin D, Miller WL, Patterson D, Clancy KP, Hart IM, Clark BJ 1995 Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. *Proc Natl Acad Sci USA* 92:4778–4782
  73. Watari H, Arakane F, Moog LC, Kallen CB, Tomasetto C, Gerton GL, Rio MC, Baker ME, Strauss III JF 1997 MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc Natl Acad Sci USA* 94:8462–8467
  74. Imagawa M, Chiu R, Karin M 1987 Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* 51:251–260
  75. Garcia MA, Campillos M, Marina A, Valdivieso F, Vazquez J 1999 Transcription factor AP-2 activity is modulated by protein kinase A-mediated phosphorylation. *FEBS Lett* 444:27–31
  76. Knofler M, Saleh L, Bauer S, Vasicek R, Griesinger G, Strohmer H, Helmer H, Husslein P 2000 Promoter elements and transcription factors involved in differentiation-dependent human chorionic gonadotropin- $\alpha$  messenger ribonucleic acid expression of term villous trophoblasts. *Endocrinology* 141:3737–3748
  77. Hum DW, Aza-Blanc P, Miller WL 1995 Characterization of placental transcriptional activation of the human gene for P450scc. *DNA Cell Biol* 14:451–463
  78. Huang N, Miller WL 2000 Cloning of factors related to HIV-inducible LBP proteins that regulate steroidogenic factor-1-independent human placental transcription of the cholesterol side-chain cleavage enzyme, P450scc. *J Biol Chem* 275:2852–2858
  79. Williams T, Tjian R 1991 Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins. *Science* 251:1067–1071
  80. Pena P, Reutens AT, Albanese C, D'Amico M, Watanabe G, Donner A, Shu IW, Williams T, Pestell RG 1999 Activator protein-2 mediates transcriptional activation of the CYP11A1 gene by interaction with Sp1 rather than binding to DNA. *Mol Endocrinol* 13:1402–1416
  81. Orly J, Clemens JW, Singer O, Richards JS 1996 Effects of hormones and protein kinase inhibitors on expression of steroidogenic enzyme promoters in electroporated primary rat granulosa cells. *Biol Reprod* 54:208–218
  82. Zor T, Selinger Z 1996 Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem* 236:302–308
  83. Kurten RC, Levy LO, Shey J, Durica JM, Richards JS 1992 Identification and characterization of the GC-rich and cyclic adenosine 3',5'-monophosphate (cAMP)-inducible promoter of the type II  $\beta$  cAMP-dependent protein kinase regulatory subunit gene. *Mol Endocrinol* 6:536–550
  84. Ronen FT, Timberg R, King SR, Hales KH, Hales DB, Stocco DM, Orly J 1998 Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* 139:303–315
  85. Orly J, Rei Z, Greenberg NM, Richards JS 1994 Tyrosine kinase inhibitor AG18 arrests follicle-stimulating hormone-induced granulosa cell differentiation: use of reverse transcriptase-polymerase chain reaction assay for multiple messenger ribonucleic acids. *Endocrinology* 134:2336–2346
  86. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds. 1988 *Current protocols in molecular biology*. 1st ed. New York: Green Publishing Associates/Wiley-Interscience
  87. Marshak S, Totary H, Cerasi E, Melloul D 1996 Purification of the  $\beta$ -cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. *Proc Natl Acad Sci USA* 93:15057–15062
  88. Staudt LM, Clerc RG, Singh H, LeBowitz JH, Sharp PA, Baltimore D 1988 Cloning of a lymphoid-specific cDNA encoding a protein binding the regulatory octamer DNA motif. *Science* 241:577–580
  89. Mood AM, Graybill FA 1963 *An introduction to the theory of statistics*. 2nd ed. New York: McGraw-Hill

