

Cytochrome P450 17 α Hydroxylase/17,20 Lyase (CYP17) Function in Cholesterol Biosynthesis: Identification of Squalene Monooxygenase (Epoxidase) Activity Associated with CYP17 in Leydig Cells

Ying Liu, Zhi-Xing Yao, and Vassilios Papadopoulos

Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D.C. 20057

Cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) is a microsomal enzyme catalyzing two distinct activities, 17 α -hydroxylase and 17,20-lyase, essential for the biosynthesis of adrenal and gonadal steroids. CYP17 is a potent oxidant, it is present in liver and nonsteroidogenic tissues, and it has been suggested to have catalytic properties distinct to its function in steroid metabolism. To identify CYP17 functions distinct of its 17 α -hydroxylase/17,20-lyase activity, we used MA-10 mouse tumor Leydig cells known to be defective in 17 α -hydroxylase/17,20-lyase activity. A CYP17 knocked down MA-10 clone (MA-10^{CYP17KD}) was generated by homologous recombination and its steroidogenic capacity was compared with wild-type cells (MA-10^{wt}). Although no differences in cell morphology and proliferation rates were observed between these cells, the human chorionic gonadotropin-induced progesterone formation and *de novo* synthesis of steroids were dramatically reduced in MA-10^{CYP17KD} cells; their steroidogenic ability could be rescued in part by transfecting CYP17 DNA into the cells. Knocking down CYP17 mRNA by RNA interference yielded similar results. How-

ever, no significant difference was observed in the steroidogenic ability of cells treated with 22R-hydroxycholesterol, which suggested a defect in cholesterol biosynthesis. Incubation of MA-10^{CYP17KD} cells with ¹⁴C-labeled squalene resulted in the formation of reduced amounts of radiolabeled cholesterol compared with MA-10^{wt} cells. In addition, treatment of MA-10^{CYP17KD} cells with various cholesterol substrates indicated that unlike squalene, addition of squalene epoxide, lanosterol, zymosterol, and desmosterol could rescue the hormone-induced progesterone formation. Further *in vitro* studies demonstrated that expression of mouse CYP17 in bacteria resulted in the expression of squalene monooxygenase activity. In conclusion, these studies suggest that CYP17, in addition to its 17 α -hydroxylase/17,20-lyase activity, critical in androgen formation, also expresses a secondary activity, squalene monooxygenase (epoxidase), of a well-established enzyme involved in cholesterol biosynthesis, which may become critical under certain conditions. (*Molecular Endocrinology* 19: 1918–1931, 2005)

STEROIDS ARE SYNTHESIZED from cholesterol by a series of enzymatic reactions mediated both by cytochrome P450 and non-P450 enzymes. The steroidogenic cytochrome P450 enzymes are found as single genes in multiple species, but can mediate mul-

multiple enzymatic steps (1–4). Conversely, the noncytochrome P450 enzymes, such as 3 β -HSD (hydroxysteroid dehydrogenase), 17 β HSD, and 3 α HSD, are found as multiple genes encoding different proteins (1–4). As shown in Fig. 1, steroidogenesis begins with the substrate cholesterol, synthesized via a number of enzymatic reactions from acetate, including squalene monooxygenase (epoxidizing). The cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) is a microsomal enzyme that catalyzes two distinct activities, 17 α -hydroxylase and 17,20-lyase, essential for the biosynthesis of glucocorticoids and sex steroid precursors (1–4). More specifically, CYP17 mediates 17 α -hydroxylation of pregnenolone or progesterone to yield 17 α -OH pregnenolone or 17 α -OH progesterone and also mediates cleavage of the c17,20 bond of these compounds to yield dehydroepiandrosterone (DHEA) or androstenedione. Thus, microsomal cytochrome

First Published Online March 10, 2005

Abbreviations: BrdU, 5-Bromo-2'-deoxyuridine; CYP17, cytochrome P450 17 α hydroxylase/17,20 lyase; DHEA, dehydroepiandrosterone; hCG, human chorionic gonadotropin; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HSD, hydroxysteroid dehydrogenase; IPTG, isopropyl-1-thiol- β -D-galactopyranoside; MVA, mevalonolactone; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NCBI, National Center for Biotechnology Information; PKA, protein kinase A; siRNA, short interfering RNA; TLC, thin-layer chromatography.

***Molecular Endocrinology* is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.**

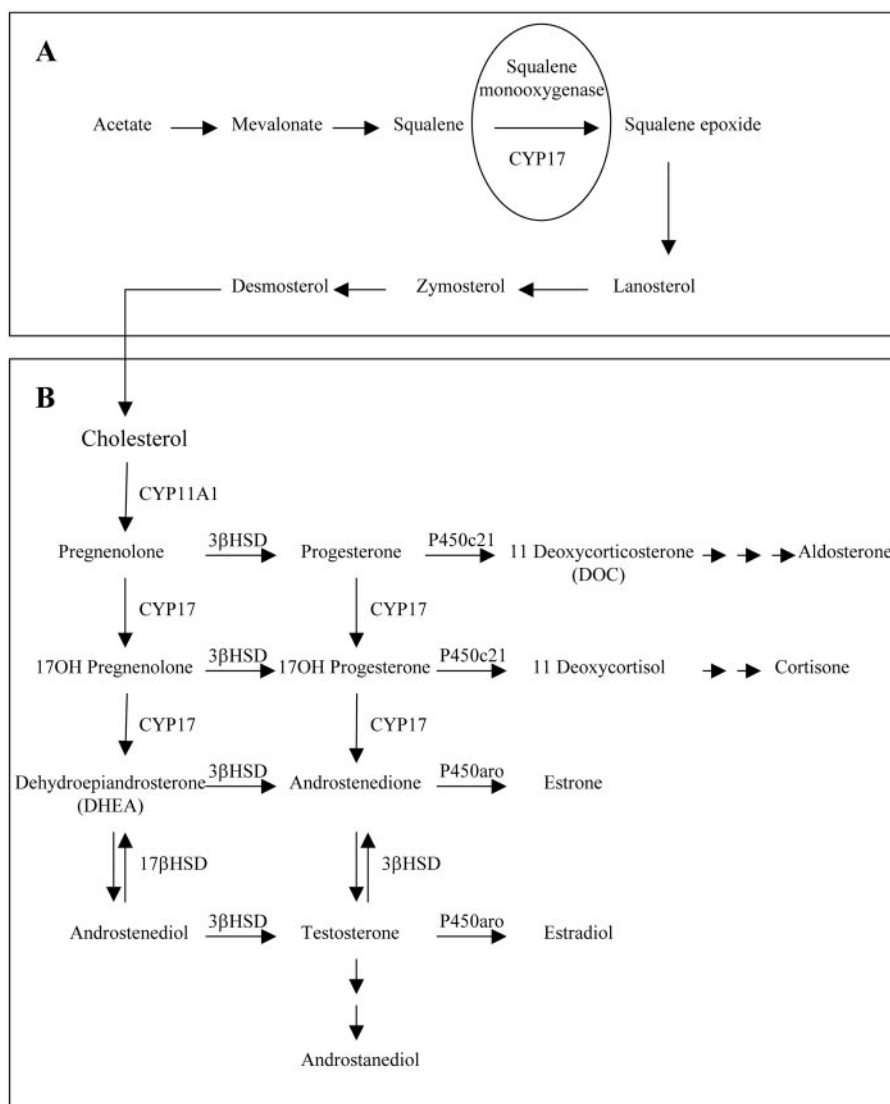


Fig. 1. Cholesterol Biosynthesis (A) and Steroidogenic Pathway (B)

Each *arrow* represents one or more enzymatic steps in cholesterol and steroid biosynthesis. The names of the participating enzymes are shown: squalene monooxygenase catalyzes the formation of squalene epoxide from squalene; P450scc (CYP11A1; mitochondrial cholesterol side-chain cleavage enzyme) mediates 20 α hydroxylation, 22 hydroxylation and scission of the c20, 22 bond; 3 β HSD mediates both 3 β HSD and Δ 5- Δ 4 isomerase activities; CYP17 (microsomal 17 hydroxylase) mediates c17 hydroxylation of pregnenolone and progesterone to yield 17OH pregnenolone or 17OH progesterone, and scission of the c17, 20 bond of the respective 17-hydroxylated steroids (c17, 20 lysis reaction) to yield DHEA and androstenedione (1, 3).

CYP17 is believed to be involved in the formation of four of the main steroid hormones, namely, testosterone, estradiol, cortisol, and DEHA (1–4).

Deficiency of CYP17, normally present in both the gonads and adrenal cortex, leads to impaired production of cortisol, androgens and estrogens, with accompanying overproduction of mineralocorticoids, in particular 11-deoxycorticosterone (5). Consequently, affected females (46XX) have hypertension and absence of sexual development producing primary amenorrhea, whereas affected males (46XY) are also hypertensive and have female external genitalia (6). Despite the increased levels of corticosterone and 11-

deoxycorticosterone, suppressed levels of aldosterone are observed in most patients with 17 α -hydroxylase deficiency, although in some cases elevated aldosterone levels were reported (7).

CYP17 was originally thought to be present exclusively in the steroidogenic cells of the rodent gonads (1). Later on CYP17 mRNA, protein and/or activity were found in other tissues in the rat, including liver (8, 9), brain (10, 11), stomach, duodenum, and kidney (12). In humans, CYP17 was found, in addition to the gonadal tissues, in the adrenal and other nongonadal tissues (13). A tissue-wide distribution of this enzyme in other species, such as zebrafish, has been also

shown (14). Although in rodents the role of this enzyme in the nongonadal tissues is not clear, a comparison of CYP17 protein expression and activity in immature rat liver vs. the gonads, indicated that the total amount of steroid metabolites produced by the liver CYP17 could be greater than that produced by the gonadal CYP17 (9). This finding suggested that CYP17 might play a role in synthesis of liver steroid hormones distinct to androgens and estrogens.

In agreement with its widespread localization, CYP17 is a potent oxidant, and other catalytic properties for the enzyme have been suggested (15). Although almost all of today's knowledge on CYP17 stems from work performed with the gonadal enzyme, a recent detailed analysis by Lieberman and Warne (15) of existing experimental evidence bearing on the role of 17-hydroxylase suggests that it may be only an assumption to maintain that a C21-steroid, *e.g.* pregnenolone (or progesterone), is a uniquely specific substrate (15) for the enzyme. The later review together with additional experimental data indicating that cytochrome P450 enzymes may exist in multiple conformations and that each of two or more conformers may contain multiple substrate binding sites (16), and the finding that CYP17 is present in nonsteroidogenic tissues, suggested that CYP17 may serve other critical cell functions.

MA-10 cells are a clonal strain of mouse Leydig tumor cells adapted to continuous culture (17). MA-10 mouse Leydig tumor cells synthesize progesterone as the major final steroid product after stimulation by human chorionic gonadotropin (hCG) (17). Although these cells contain CYP17 mRNA (18), they are deficient in 17 α -hydroxylase/17,20-lyase CYP17 activity (17, 19, 20). Thus, MA-10 Leydig tumor cells represent an ideal *in vitro* cell system to study the 17 α -hydroxylase/17,20-lyase-independent functions of CYP17 in steroidogenesis. We report herein that in Leydig cells, CYP17, in addition to its 17 α -hydroxylase/17,20-lyase activity, critical in androgen formation, is associated with the squalene monooxygenase (epoxidase) activity critical in cholesterol biosynthesis.

RESULTS

CYP17 Gene Knockdown in MA-10 Cells by Gene Targeting

A CYP17 knocked down MA-10 clone (MA-10^{CYP17KD}) was generated by homologous recombination and its steroidogenic capacity was compared with wild-type cells (MA-10^{wt}). Using CYP17 cDNA [Ref. 21 National Center for Biotechnology Information (NCBI) GenBank accession no. NM_007809] as a probe, we screened a mouse bacterial artificial chromosome library and isolated a clone containing CYP17 genomic DNA (120 kb). The CYP17 gene was sequenced and shown to include eight exons (Fig. 2A). The sequence of the entire CYP17 gene sequence was deposited in the

NCBI GenBank (accession no. AY594330). A targeting vector was constructed with the neomycin resistance (*neo*) gene (2.0 kb) used to replace the entire (exons and introns, 8.2 kb) CYP17 as well as 5' and 3' DNA fragments (>3 kb) (Fig. 2B). The rationale for replacing the entire gene instead of interrupting one of the exons was to avoid potential recombination with sequences homologous to the remaining genomic sequences of the CYP17 gene. The targeted allele was generated by homologous recombination (Fig. 2C). The *KpnI* linearized targeting vector was transfected into MA-10 wild-type cells, and G418-resistant positive clones were selected. The integration of the CYP17 deletion in the MA-10 selected clones was evaluated by Southern blot (Fig. 2D). Hybridization of the genomic DNA digested with *XbaI* and *Clal* with the designed 3' probe resulted in the identification of a 7.0-kb fragment in the MA-10 wild-type genomic DNA and 7.0- and 3.7-kb fragments in the MA-10 cells containing a CYP17-deleted allele. The generated MA-10 CYP17 knockdown (MA-10^{CYP17KD}) clone was maintained as a stable cell line.

CYP17 Gene Knockdown Does Not Affect Cell Morphology and Proliferation

Figure 3, A and B, demonstrates that no morphological differences were observed between the MA-10^{wt} and MA-10^{CYP17KD} cells. Moreover, there were no differences between the rates of MA-10^{wt} and MA-10^{CYP17KD} cell proliferation, examined by 5-bromo-2'-deoxyuridine (BrdU) incorporation (Fig. 3C).

CYP17 Gene Knockdown Reduces hCG-Stimulated Progesterone Formation

Immunoblot analysis using anti-CYP17 antiserum (22) demonstrated the presence of two immunoreactive proteins of 48–50 kDa and 28–30 kDa (Fig. 4). CYP17 immunoreactive protein levels were reduced in MA-10^{CYP17KD} compared with MA-10^{wt} cells (Fig. 4). Real-time quantitative PCR analysis indicated that CYP17 mRNA levels were reduced by 50% ($P < 0.001$) in MA-10^{CYP17KD} compared with MA-10^{wt} cells (Fig. 5A). Treatment of MA-10^{wt} cells with increasing concentrations of hCG for 2 h resulted in a dose-dependent increase in progesterone formation (Fig. 5B). However, treatment of MA-10^{CYP17KD} cells with increasing concentrations of hCG for 2 h resulted in 90% and 76% reduced progesterone formation in the presence of 10 and 50 ng/ml hCG, respectively (Fig. 4B), suggesting that CYP17 may play a role in progesterone biosynthesis. This reduced steroid formation by MA-10^{CYP17KD} in response to hCG could be rescued by transfecting the cells with the pZeoSV2(-) vector containing mouse CYP17 cDNA (Fig. 5B).

MA-10^{wt} and MA-10^{CYP17KD} cells treated with 10 μ M and 20 μ M 22(R)-hydroxycholesterol for 2 h produced equal amounts of progesterone (Fig. 5C), indicating that cholesterol metabolism to progesterone was not

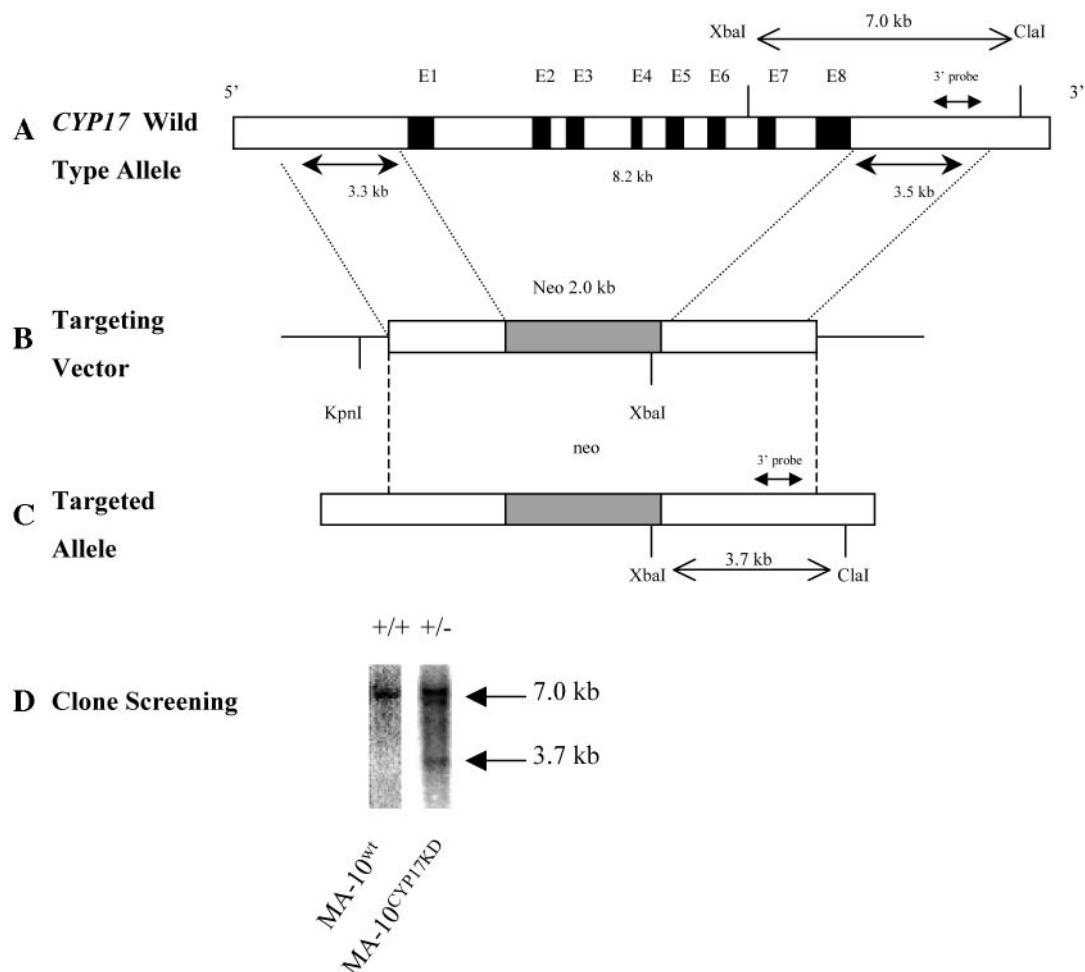


Fig. 2. Strategy Used to Knock Down CYP17 Gene in MA-10 Mouse Tumor Leydig Cells via Gene Targeting

Schematic representation of (A) the genomic CYP17 wild-type locus, (B) the targeting vector, (C) the targeted locus showing exons (E1–E8), *KpnI* (to linearize the targeting vector), *Clal* and *XbaI* (to generate the 7-kb fragment in wild-type and 3.7 kb in the targeted gene) restriction sites, and the position of 3' probe. D, Southern blot analysis of MA-10 wild-type cells and MA-10 cells transfected with the targeting gene. Genomic DNA was purified and digested with *Clal* and *XbaI* and hybridized with ³²P-labeled 3' probe. The generated 7.0- and 3.7-kb fragments in the wild-type (+) and targeted gene (–), respectively, are shown.

affected by knocking down the *CYP17* gene in MA-10 cells. Considering the critical role of cAMP-dependent protein kinase A (PKA) in mediating the effects of peptide hormones in steroidogenesis (1–4) and on CYP17 activity (23), we also examined whether PKA activity was altered in MA-10^{CYP17KD} compared with MA-10^{wt} cells. No difference in the hCG-stimulated PKA activity between the two cells lines was observed (data not shown).

RNA interference methodology was also used to specifically suppress CYP17 mRNA expression by complementary double-stranded RNA. Real-time quantitative PCR measurement of CYP17 mRNA in control and MA-10 cells treated with short interfering RNA (siRNA) for CYP17 (MA-10^{si-CYP17KD} cells) demonstrated suppression of CYP17 mRNA levels by 50% compared with wild-type cells ($P < 0.001$; Fig. 6A). Treatment of MA-10^{si-CYP17KD} cells with increasing concentrations of hCG for 2 h resulted in 40% and

50% reduction of progesterone formation in the presence of 10 and 50 ng/ml hCG, respectively (Fig. 6B), suggesting again that *CYP17* may play a role in progesterone biosynthesis. As with the MA-10^{CYP17KD} cells, MA-10^{si-CYP17KD} cells treated with 10 μ M and 20 μ M 22(R)-hydroxycholesterol for 2 h produced equal amounts of progesterone to MA-10^{wt} cells (Fig. 6C)

De Novo Synthesis of Steroids Is Reduced in MA-10^{CYP17KD} Cells

To investigate the effect of *CYP17* on the *de novo* synthesis of steroids by MA-10^{wt} and MA-10^{CYP17KD} cells, we blocked endogenous cholesterol synthesis with the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor lovastatin. Cells were then incubated with the radiolabeled cholesterol precursor [³H]mevalonactone. HPLC separation of the samples and steroid identification using standards,

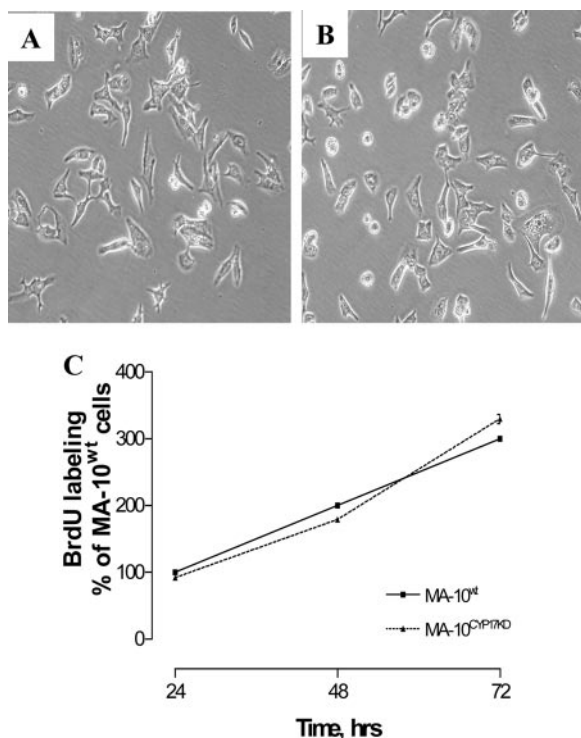


Fig. 3. Effect of *CYP17* Gene Knockdown on Cell Morphology and Proliferation

Phase contrast microscopy of MA-10^{wt} (A) and MA-10^{CYP17KD} (B) cells. C, Cell proliferation assay. Cells were plated in 96-well plates (10^4 cells/well) and cell proliferation was determined after 24, 48, and 72 h by BrdU incorporation. Results shown are means \pm SEM ($n = 6$) of the percentage of absorbance in MA-10^{CYP17KD} cells compared with MA-10^{wt} cells, which was set as 100% in each day.

indicated that the MA-10^{CYP17KD} cells synthesize much less pregnenolone, progesterone and DHEA (Fig. 7B), compared with MA-10^{wt} cells (Fig. 7A). Similar results were obtained using various thin layer chromatography separation methods (data not shown).

CYP17 Is Associated with the Squalene Monooxygenase (Epoxidase) Activity

To study the role of CYP17 in cholesterol biosynthesis MA-10^{wt} and MA-10^{CYP17KD} cells were incubated with ¹⁴C-labeled squalene and a radiolabeled steroid co-migrating with radiolabeled cholesterol was isolated by thin-layer chromatography (TLC). In agreement with the data presented earlier (Figs. 5 and 6), radiolabeled cholesterol synthesis from squalene was 50% lower in MA-10^{CYP17KD} compared with MA-10^{wt} cells (Fig. 8A).

To determine which step in cholesterol biosynthesis was affected by knocking down *CYP17* gene and mRNA, we added substrates of the various metabolic steps leading to cholesterol formation. A 30- μ M concentration of squalene, squalene epoxide, lanosterol, zymosterol, and desmosterol were added onto MA-10^{wt} and MA-10^{CYP17KD} cells for 3 h followed by 2 h of

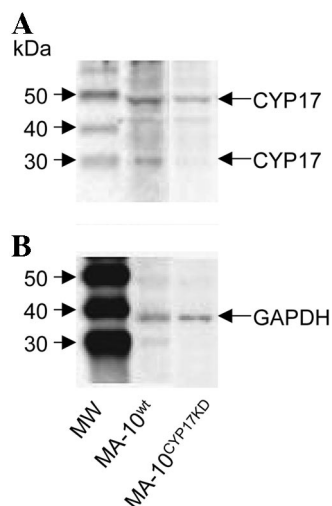


Fig. 4. Immunoblot Analysis of CYP17 Expression

Protein extracts from MA-10^{wt} and MA-10^{CYP17KD} cells were separated by SDS-PAGE on a 10% gel, electrotransferred to nitrocellulose membranes, and submitted to immunoblotting using an anti-CYP17 antiserum (A). Anti-GAPDH was used as loading control (B). MW, Molecular weight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

treatment with saturating concentrations (50 ng/ml) of hCG. Figure 8B shows that, in contrast to squalene, addition of squalene epoxide, lanosterol, zymosterol and desmosterol rescued the hCG-induced progesterone formation in MA-10^{CYP17KD} cells, suggesting that CYP17 was associated with squalene monooxygenase activity (formerly known as squalene epoxidase).

To demonstrate that CYP17 protein was responsible for the squalene epoxidation reaction seen, mouse CYP17 cDNA was inserted into the histidine tag containing pET15b vector (24) and transfected into TOP10 *Escherichia coli* cells. Isopropyl-1-thiol- β -D-galactopyranoside (IPTG, 1 mM) together with 5-aminolevulinic acid (1 mM) and riboflavin were used to induce the expression of the recombinant mouse CYP17 and examine the expression of CYP17 and squalene monooxygenase activities. Recombinant CYP17 was further purified by affinity chromatography using nickel-nitrilotriacetic acid His-bind resin. Proteins in fractions from the purification steps were separated by SDS-PAGE followed by Coomassie blue staining (Fig. 9A). Two major bands of 48–50 kDa and 28–30 kDa were eluted (fraction 4) and recognized by the CYP17 antiserum used (Fig. 9B).

Bacteria where CYP17 expression was induced as described above were used to examine their ability to metabolize radiolabeled progesterone and squalene. ³H-Progesterone was incubated with bacteria together and substrate loss was determined by TLC separation. Figure 9C shows that, as expected, mouse CYP17 catalyzes progesterone metabolism. Figure 9D shows that CYP17 expressing bacteria induce the me-

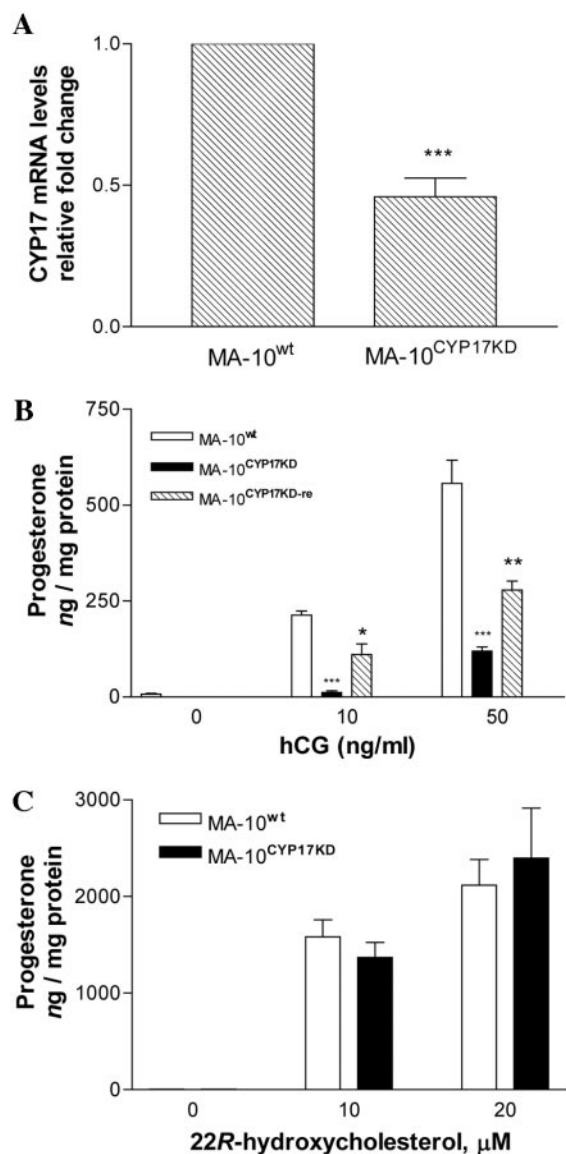


Fig. 5. Steroidogenesis in CYP17 Knockdown Cells

A, Real-time quantitative RT-PCR used to compare the content of CYP17 transcripts in MA-10^{wt} and MA-10^{CYP17KD} cells. The relative fold change was defined as the transcript level of CYP17 in MA-10^{CYP17KD} cells divided by that in MA-10^{wt} that was set as 1. Representative data (means \pm SEM) from three independent experiments (each experiment performed in triplicates) are shown. B, hCG-induced progesterone formation in MA-10^{wt}, MA-10^{CYP17KD}, and MA-10^{CYP17KD-re} cells. Cells were treated for 2 h with the indicated concentration of hCG. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. C, 22R-hydroxycholesterol-supported progesterone formation in MA-10^{wt} and MA-10^{CYP17KD} cells. Cells were treated for 2 h with the indicated concentration of 22R-hydroxycholesterol. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. Data shown are means \pm SEM from an experiment performed in triplicates. Similar results were obtained in two other independent experiments. Statistics were performed by comparing the results obtained between MA-10^{wt} and MA-10^{CYP17KD} or MA-10^{CYP17KD-re} cells run in parallel experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

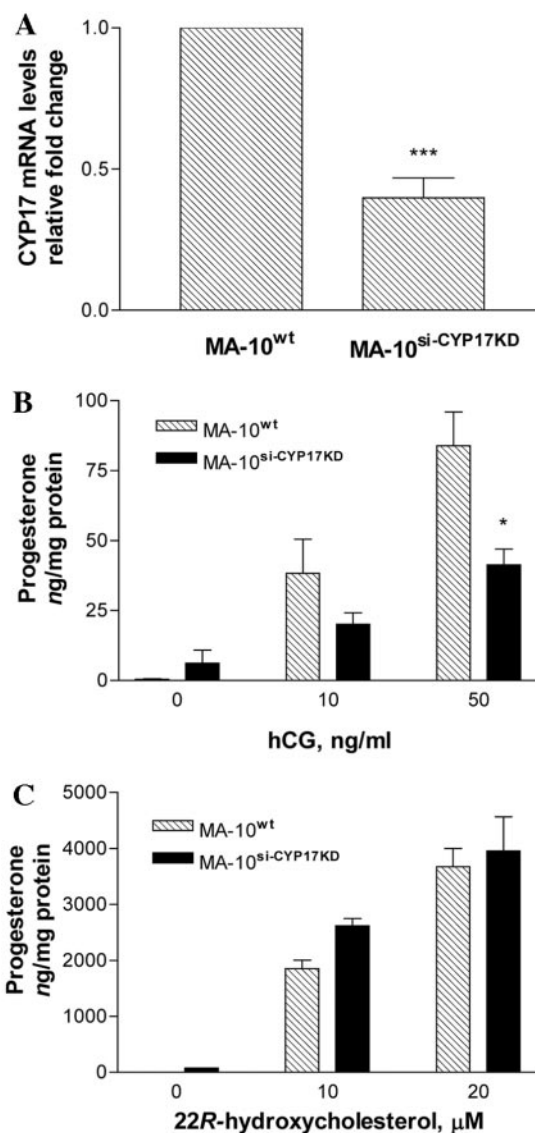


Fig. 6. Effect of CYP17 siRNA on Progesterone Formation

CYP17 siRNA was prepared and transfected into MA-10 wild-type cells as described in *Materials and Methods*. A, Real-time quantitative RT-PCR used to compare the content of CYP17 transcripts in MA-10^{wt} and MA-10^{si-CYP17KD} cells. The relative fold change was defined as the transcript level of CYP17 in MA-10^{si-CYP17KD} cells divided by that in MA-10^{wt} that was set as 1. Representative data (means \pm SEM) from three independent experiments (each experiment performed in triplicates) are shown. B, hCG-induced progesterone formation in MA-10^{wt} and MA-10^{si-CYP17KD} cells. Cells were treated for 2 h with the indicated concentration of hCG. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. C, 22R-hydroxycholesterol-supported progesterone formation in MA-10^{wt} and MA-10^{si-CYP17KD} cells. Cells were treated for 2 h with the indicated concentration of 22R-hydroxycholesterol. At the end of the incubation progesterone was measured in the media by RIA and cells were collected to determine protein levels. Data shown are means \pm SEM from an experiment performed in triplicates. Similar results were obtained in two other independent experiments. Statistics were performed by comparing the results obtained between MA-10^{wt} and MA-10^{si-CYP17KD} cells run in parallel experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

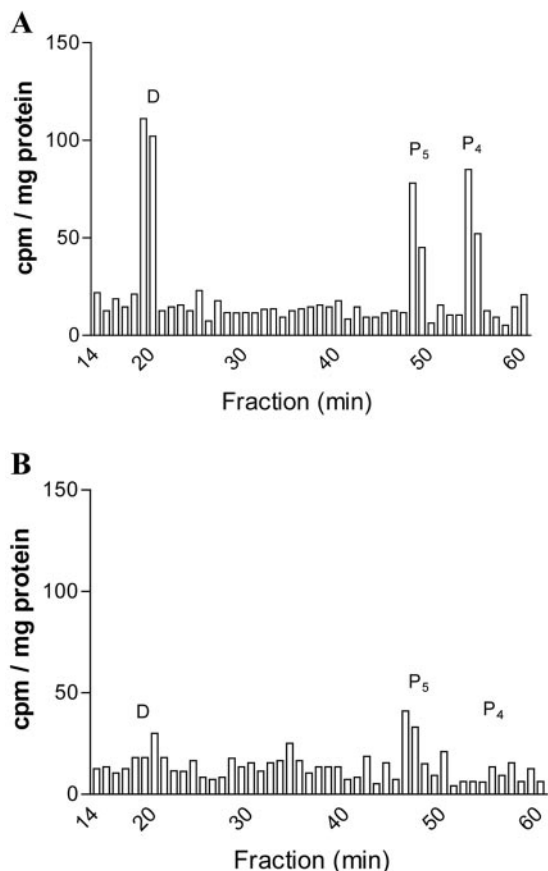


Fig. 7. *De Novo* Synthesis of Steroids in MA-10^{CYP17KD} Cells
MA-10^{wt} and MA-10^{CYP17KD} cells were treated with lovastatin and incubated with [³H]mevalonactone for 1 h as described in *Materials and Methods*. Extracts were separated by HPLC, and fractions were collected every minute. Radioactivity was quantified by liquid scintillation spectrometry. A, MA-10^{wt}; B, MA-10^{CYP17KD}. Mevalonactone elutes with the solvent front (fractions collected at 5–6 min). D, DHEA; P₅, pregnenolone; P₄, progesterone.

tabolism of ¹⁴C radiolabeled squalene, determined as described (25), in a substrate- and protein-dependent manner. Figure 9E shows that control bacteria and bacteria transformed with control pET15b vector failed to metabolize squalene.

The Squalene Monooxygenase Activity Is Distinct to 17 α -Hydroxylase/17,20-Lyase Activity Associated with CYP17

Figure 10A shows that the 17 α -hydroxylase/17,20-lyase activity inhibitor SU10603 (26) inhibited in a dose-dependent manner the hCG-induced testosterone formation by normal rat Leydig cells (ANOVA; ***, $P < 0.001$). However, this compound failed to inhibit progesterone formation by MA-10 Leydig cells (Fig. 10B), suggesting that 17 α -hydroxylase/17,20-lyase activity is distinct to the squalene monooxygenase activity associated with CYP17. These data were further confirmed in *in vitro* studies using bacteria ex-

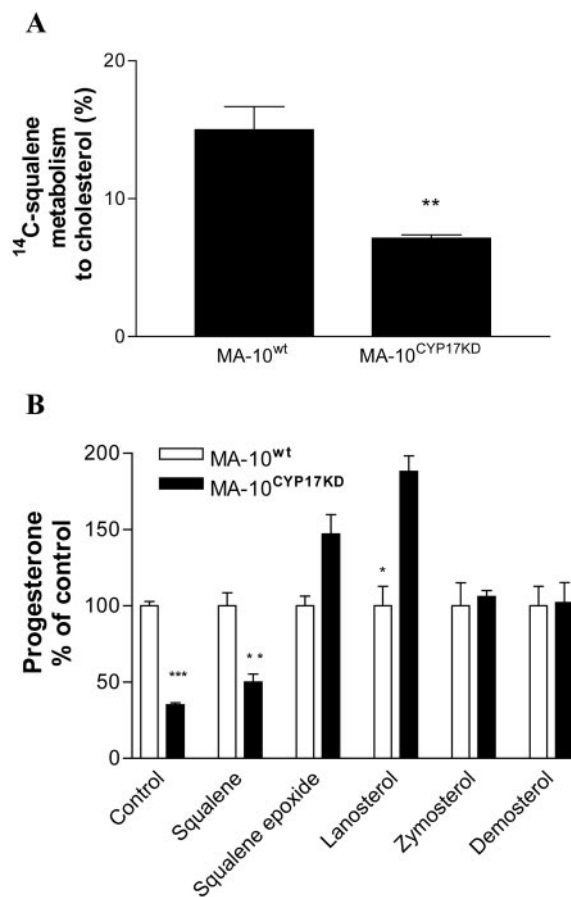


Fig. 8. CYP17 Expression Is Associated with Squalene Monooxygenase Activity

A, Cholesterol biosynthesis in MA-10^{wt} and MA-10^{CYP17KD} cells was determined by adding ¹⁴C-labeled squalene (0.05 μ Ci) substrate. Organic extracts were separated onto C18 silica gel TLC plates and cholesterol fractions were collected as described in *Materials and Methods*. Radioactivity was measured by via liquid scintillation spectroscopy. The percent of cholesterol formation from squalene was determined. Results shown are means \pm SEM from three independent experiments performed in triplicates. B, Progesterone formation by MA-10^{wt} and MA-10^{CYP17KD} cells incubated in the presence of various cholesterol synthesis substrates. Cells cultured in 96-well plates were incubated with 30 μ M of the cholesterol squalene, squalene epoxide, lanosterol, zymosterol, or demosterol for 3 h at 37 C. Cells were then treated with 50 ng/ml hCG for another 2 h at 37 C. Progesterone produced was measured by RIA and cells were used to measure total cell protein levels as described in *Materials and Methods*. Results shown are represented as percent of progesterone formation in MA-10^{CYP17KD} compared with MA-10^{wt} cells and they are means \pm SEM from three independent experiments performed in triplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

pressing CYP17. Figure 10C shows that SU10603 inhibited the 17 α -hydroxylase/17,20-lyase activity expressed by recombinant CYP17. However, this compound failed to inhibit the squalene monooxygen-

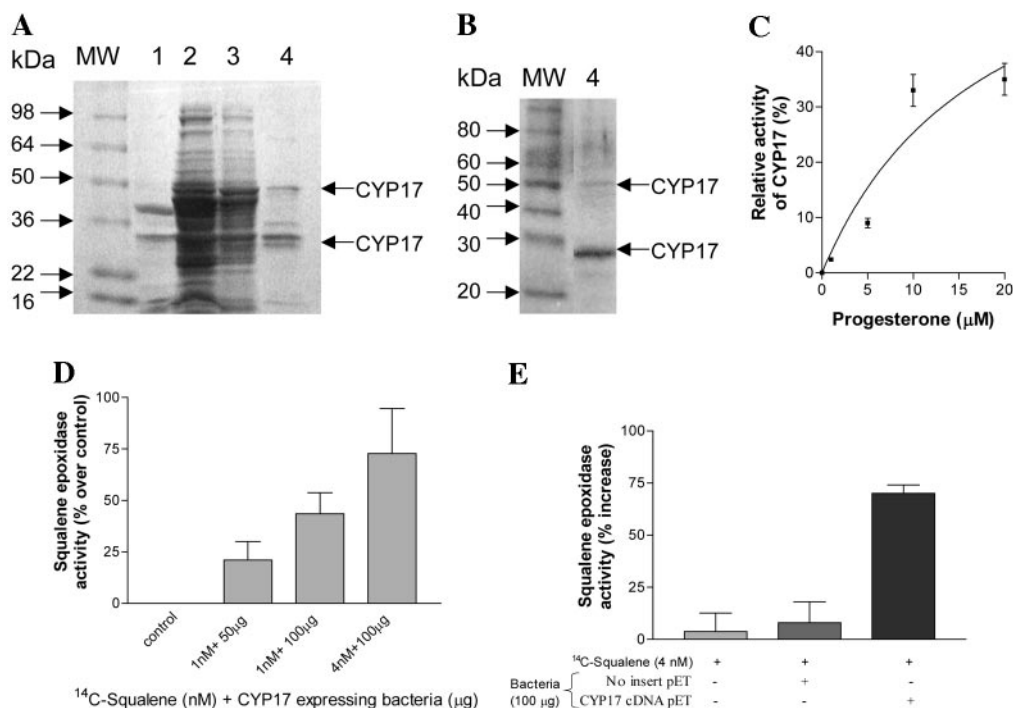


Fig. 9. Expression of Mouse CYP17 in *E. coli* Induces the Expression of 17 α -Hydroxylase/17,20-Lyase and Squalene Monooxygenase Activities

A, Coomassie blue staining of proteins from the various steps of the isolation of recombinant CYP17 expressed in *E. coli*. IPTG-induced bacteria containing the CYP17 expression vector (pET15bCYP17) were lysed and purified by nickel affinity chromatography. MW, Molecular weight standards; 1, bacterial lysates; 2, column flow-through fraction; 3, first column wash fraction; 4, eluate. B, Immunoblot analysis of the eluted proteins using anti-CYP17 antiserum. C, CYP17 17 α -hydroxylase/17,20-lyase activity determined by progesterone conversion. The reaction between ^3H -progesterone and CYP17 expressed in *E. coli* was performed and determined as described in *Materials and Methods*. D and E, CYP17 squalene monooxygenase activity determined by squalene conversion. The reaction between ^{14}C -squalene and recombinant CYP17 expressed in bacteria (D) or control bacteria transformed with the pET15b vector alone (E) were performed and determined as described in *Materials and Methods*. Data shown in C–E are means \pm SEM from one representative experiment performed in triplicates. Similar results were obtained in four independent experiments.

ase activity expressed by the recombinant enzyme in bacteria (Fig. 10D).

DISCUSSION

CYP17 enzyme mediates the 17 α -hydroxylation of pregnenolone or progesterone to yield 17 α -OH pregnenolone or 17 α -OH progesterone and the cleavage of the C-17, 20 bonds of these compounds to yield the androgens DHEA or androstenedione in rodent gonads and androgens and corticosteroids in human gonads and adrenals (Fig. 1B). Although the function of CYP17 in gonadal and adrenal steroids is unquestionable, our interest in the function of this enzyme stemmed from our findings on the mechanisms underlying DHEA formation in brain. The levels of DHEA in brain are distinct from those in plasma, and its function as neuroactive steroid at the γ -amino-butyric acid and N-methyl-D-aspartate receptor level, as well as at other brain functions, has been well established (3, 27). We initially reported that rat tumor glioma cells,

which do not contain the enzyme P450c17, were able to produce DHEA through an alternative P450c17-independent pathway (18). This pathway involved the formation of a DHEA precursor where both C-17 and C-20 are oxygenated (18). This pathway was also found in MA-10 Leydig tumor cells (18), rat brain glia (28), human brain glia cell lines (29), bovine brain (30), and human brain postmortem specimens (31), suggesting that this is not a species- and tissue-specific process. These studies led us to attempt generating a CYP17 knockout mouse model with the hope that this animal model will be able to solve the question of whether an alternative pathway of DHEA formation exists. The results from these studies indicated that CYP17 deletion caused a primary phenotype (infertility) probably not due to the anticipated androgen imbalance and a secondary phenotype (change in sexual behavior) due to the androgen imbalance (32). These results unveiled that CYP17, in addition to its role in androgen formation, is present in germ cells where it is required for fertility, an unexpected and surprising finding further suggesting that CYP17 exerts functions

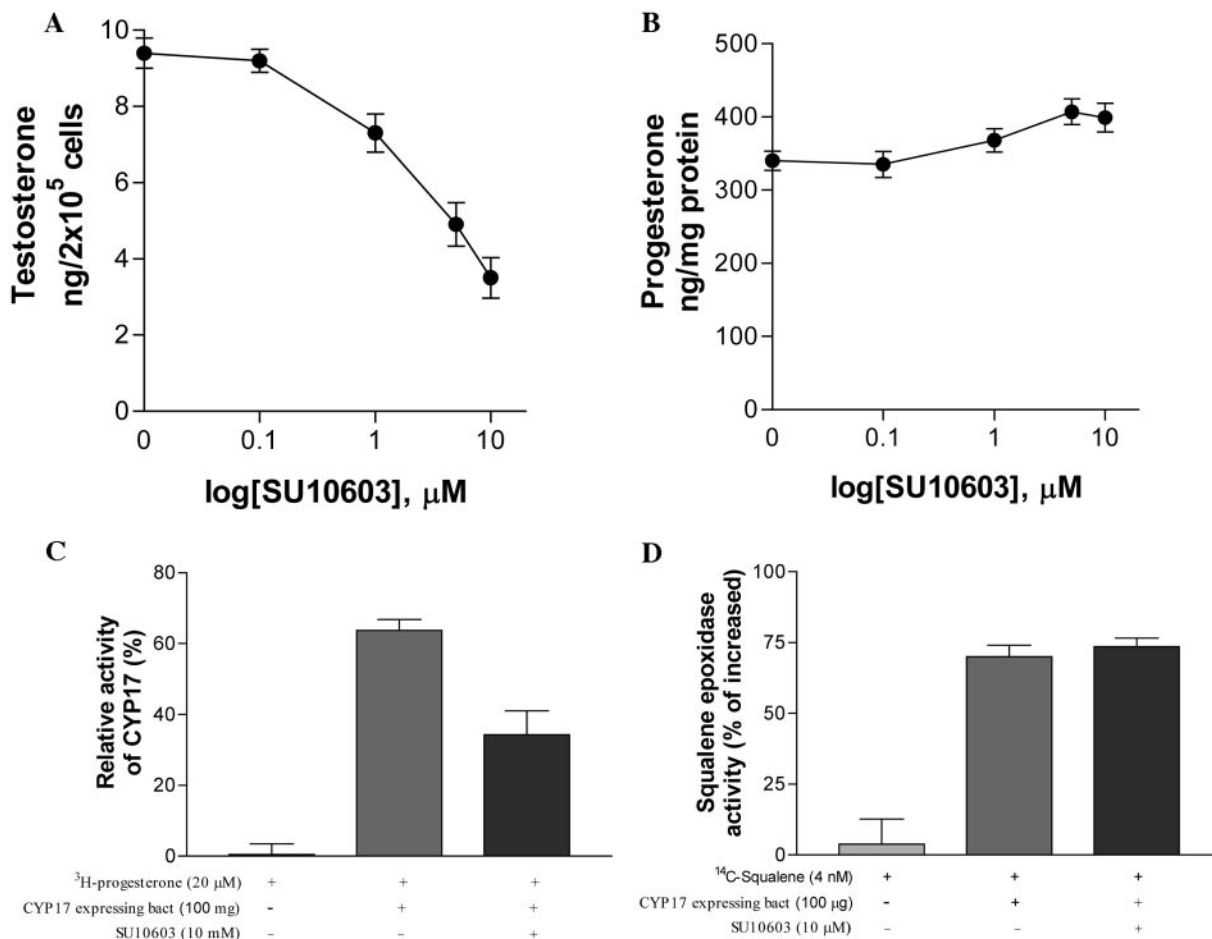


Fig. 10. The Squalene Monooxygenase Activity Is Distinct to 17α -Hydroxylase/ $17,20$ -Lyase Activity Associated with CYP17

Purified rat Leydig cells (A) and MA-10 mouse Leydig cells (B) were treated for 2 h with hCG (50 ng/ml) in the presence or absence of the indicated concentrations of SU10603. The levels of testosterone and progesterone were measured by RIA in rat Leydig cells and MA-10 cells, respectively. CYP17 17α -hydroxylase/ $17,20$ -lyase activity, determined by ^3H -progesterone conversion (C) and CYP17 squalene monooxygenase activity, determined by ^{14}C -squalene conversion (D) in *E. coli* expressing or not CYP17 were determined in the presence or absence of SU10603, as described in *Materials and Methods*. Results shown are means \pm SEM from three independent experiments performed in triplicates.

distinct to 17α -hydroxylase/ $17,20$ -lyase enzymatic activities (32).

To investigate 17α -hydroxylase/ $17,20$ -lyase-independent functions of CYP17, we used MA-10 mouse tumor Leydig cells known to be defective in 17α -hydroxylase/ $17,20$ -lyase activity (19, 20) and where we were unable to show the presence of immunoreactive CYP17 by immunocytochemistry (19). A CYP17 knocked down MA-10 clone (MA-10^{CYP17KD}) was generated by homologous recombination and its steroidogenic capacity was compared with wild-type cells (MA-10^{wt}). The results obtained demonstrated that the expected 3.7-kb restriction fragment was present in the MA-10^{CYP17KD} mutant cells, indicating that the CYP17 gene was altered/interrupted. Like many permanent cell lines, MA-10 cells are polyploid. Southern blot analysis demonstrated that only part of the CYP17 DNA was interrupted because the bands found in MA-10^{wt} cells were also present in the MA-10^{CYP17KD}

cells. Despite this partial interruption of the CYP17 gene, immunoblot analysis and real-time quantitative PCR analysis indicated that CYP17 protein and mRNA levels were reduced by approximately 50 and 75%, respectively, in MA-10^{CYP17KD} compared with MA-10^{wt} cells and CYP17 gene knockdown reduced by 50% the hCG-stimulated progesterone formation.

In contrast to previous studies where we failed to show by immunocytochemistry the presence of CYP17 immunoreactivity in MA-10 cells (19), immunoblot analysis of MA-10 extracts, performed with the same antiserum, demonstrated the presence of two immunoreactive proteins of approximately 30 and 50 kDa. Although CYP17 has been shown to be a 50-kDa protein, the presence of a lower molecular mass immunoreactive form in rat Leydig cells was previously shown by Hales *et al.* (22). The finding that expression of the histidine-tagged mouse CYP17 cDNA in bacteria followed by nickel affinity purification of the expressed proteins results in the isolation

of both the 50- and 30-kDa proteins recognized by the CYP17 antiserum, suggests that the 30-kDa protein is indeed encoded by the CYP17 cDNA, and it is either a degradation product or a truncated form of the 50-kDa enzyme.

There were no morphological differences and differences in the rates of cell proliferation between the MA-10^{wt} and MA-10^{CYP17KD} cells. It was interesting, although not surprising, that inactivation of part of the gene was associated with this dramatic reduction of the gene function. Such phenomena were previously reported in various cell types, including steroidogenic cells (33–35). It was, however, surprising the finding that MA-10^{CYP17KD} cells produced dramatically reduced amounts of progesterone, the well-established precursor of CYP17 enzyme, in response to hCG treatment, suggesting that CYP17 may play a role in progesterone biosynthesis rather than metabolism. This reduced progesterone formation by MA-10^{CYP17KD} cells in response to hCG could be rescued by transfecting the cells with the CYP17 cDNA, indicating that the reduction seen was indeed due to the inhibition of CYP17 expression.

Parallel studies, where CYP17 mRNA expression was suppressed by 50% using complementary silencing RNAs, led to a 50% suppression of the hCG-stimulated progesterone formation, further demonstrated that the reduced progesterone biosynthesis in response to hCG in MA-10^{CYP17KD} cells was not due to nonspecific effects introduced by the gene targeting procedure. MA-10^{wt}, MA-10^{CYP17KD}, and MA-10^{si-CYP17KD} cells treated with the hydro-soluble cholesterol 22R-hydroxycholesterol, which is able to freely cross membranes acting directly on the P450_{scc}, produced equal amounts of progesterone, suggesting that cholesterol metabolism to progesterone was not affected by knocking down the CYP17 gene in MA-10 cells. Moreover, the finding that PKA activity was similar in all three cell lines suggested that the LH receptor-activated cAMP-dependent signal transduction pathway, critical in steroidogenesis and CYP17 activity (23) was not affected by knocking down the expression of CYP17.

Incubation of MA-10^{CYP17KD} cells depleted of endogenous cholesterol, using the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin, with radiolabeled mevalonate further demonstrated that MA-10^{CYP17KD} cells synthesize much less pregnenolone and progesterone compared with MA-10^{wt} cells. These findings suggest that CYP17 might be involved in cholesterol biosynthesis. The formation of DHEA by MA-10 cells was a surprising finding because these cells are not known to form androgens and are devoid of CYP17 activity (17–20). We previously reported that MA-10 cells could form DHEA using as a precursor a steroid oxygenated at C17 and C20 that could be a cholesterol metabolite (18). In that case, decreased cholesterol synthesis would result in reduced formation of DHEA, via a CYP17-independent pathway. The reduced biosynthesis of cholesterol in MA-10^{CYP17KD} compared with MA-10^{wt} cells was demonstrated by incubating the cells with radiolabeled

squalene and measuring the formation of reduced amounts of radiolabeled cholesterol. Interestingly, cholesterol synthesis from squalene was 50% lower in MA-10^{CYP17KD}, compared with MA-10^{wt} cells, in agreement with the 50% reduction of CYP17 mRNA levels in MA-10^{CYP17KD} cells, suggesting that the dramatic decrease in progesterone formation seen in MA-10^{CYP17KD} cells may be due to the amplification of the response to hCG. This inhibition was localized at the level of squalene monooxygenase (EC 1.14.99.7) enzyme activity (formerly known as squalene epoxidase; Ref. 36) because, in contrast to squalene, addition of squalene epoxide, lanosterol, zymosterol, and desmosterol rescued the hCG-induced progesterone formation in MA-10^{CYP17KD} cells. To confirm these data, mouse CYP17 was expressed in bacteria that were incubated with radiolabeled progesterone or squalene, substrates of the 17 α -hydroxylase/17,20-lyase and squalene monooxygenase activities, respectively. Because *Escherichia coli* contains both reduced nicotinamide adenine dinucleotide phosphate (NADPH)-flavodoxin reductase and flavodoxin that serve as an electron-transfer system for microsomal cytochrome P450s, including CYP17 (37), expression of mouse CYP17 in these cells resulted in progesterone metabolism. These data are in agreement with similar studies in *E. coli* performed using bovine and human CYP17 (37–39). The finding that expression of mouse CYP17 in *E. coli* could also result in the metabolism of radiolabeled squalene in a substrate- and protein-dependent manner clearly indicates that CYP17 itself expresses squalene monooxygenase enzymatic activity.

Squalene monooxygenase (epoxidase) is a flavin-requiring, noncytochrome P450 oxidase that catalyzes the conversion of squalene to (3S)-2,3-oxidosqualene (Refs. 40–42; and <http://www.uky.edu/pharmacy/ps/porter>), the first oxygenation step in steroid biosynthesis (42). It has been suggested that squalene monooxygenase is one of the rate-limiting steps in sterol biosynthesis, and it is a target for the development of therapeutics for hypercholesterolemia (43, 44). In neural tissue, this enzyme is critical for cholesterol synthesis used in myelin membrane formation, and it has been identified as the site of inhibition of tellurium-containing compounds that cause peripheral neuropathy (<http://www.uky.edu/pharmacy/ps/porter>).

Despite the observation that CYP17 expresses squalene monooxygenase activity and the fact that both proteins are strong oxidases, these two proteins are distinct. First, CYP17 is a cytochrome P450, whereas squalene monooxygenase is not (Refs. 40–42; and <http://www.uky.edu/pharmacy/ps/porter>) and second, a search through NCBI gene and protein banks failed to identify the presence of any sequence homology either at the nucleotide or amino acid levels, between CYP17 and squalene monooxygenase (NCBI, accession nos. NM_007809 vs. NM_009270 and NP_031835 vs. NP_033296). Moreover, using the 17 α -hydroxylase/17,20-lyase enzymatic activity inhibitor SU10603, we were able to demonstrate that the squalene monooxygenase activity is distinct to 17 α -hydroxylase/17,20-

lyase activity associated with CYP17. This was based on the observations that SU10603 1) inhibited the hCG-stimulated testosterone formation by normal rat Leydig cells, known to express an active CYP17 enzyme, as well as the 17 α -hydroxylase/17,20-lyase activity expressed by recombinant CYP17 expressed in bacteria, and 2) failed to inhibit progesterone formation by MA-10 Leydig cells, devoid of CYP17 activity, and squalene metabolism by the recombinant enzyme.

In clinical cases, deficiency of CYP17 leads to impaired production of cortisol, androgens and estrogens, with accompanying overproduction of mineralocorticoids, leading to male pseudohermaphroditism (XY individuals) and/or absence of pubertal development (6, 7). However, no peripheral neuropathy has been reported in these individuals. These observations suggest that the squalene monooxygenase activity of CYP17 is secondary to its 17 α -hydroxylase/17,20-lyase activity and to the endogenous squalene monooxygenase enzyme. Nevertheless, considering the widespread distribution of the enzyme in nongonadal tissues, such as liver, and nonsteroidogenic tissues indicates that the squalene monooxygenase function of CYP17 may be involved in cholesterol formation either as a secondary or an alternative pathway. This hypothesis is supported by the findings that CYP17 might play a role in synthesis of liver steroid hormones distinct to androgens and estrogens (9). Moreover, our recent observations that CYP17 is present in germ cells, where it is required for germ cell function, sperm formation, and fertility (32), suggest that a function associated with cholesterol synthesis might be at the origin of the morphological defects seen in CYP17 sperm.

In conclusion, the studies presented herein confirm the Lieberman and Warne proposal (15) that CYP17 is a potent oxidant that has other catalytic properties distinct to its 17 α -hydroxylase/17,20-lyase activity and identifies squalene monooxygenase activity, critical in cholesterol biosynthesis, as one of its associated enzymatic activities. Evidently, additional *in vitro* and *in vivo* studies are required to further validate these findings and identify their physiological significance.

MATERIALS AND METHODS

Materials

[1,2,6,7-³H(N)] progesterone (specific activity, 92 Ci/mmol) and (RS)-[5-³H]mevalonolactone (MVA; 27.8 Ci/mmol) were obtained from NEN/DuPont (Boston, MA). [1,5,9,14,20,24-¹⁴C]-squalene (200 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). α -³²P-deoxy-CTP (3000 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Trilostane was a gift from Stegram Pharmaceuticals (Sussex, UK) and SU-10603 was a gift from CIBA-GEIGY (Suffern, NY). Antiprogestosterone and antitestosterone antisera were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Cell culture supplies were purchased from Invitrogen (Carlsbad, CA), and cell culture plasticware was from Corning (Corning, NY). Primers were synthesized by Biosynthesis Inc. (Lewisville, TX). PCR supplies were from CLONTECH (San Diego, CA), plasmid purification and DNA extraction kits were from QIAGEN (Valencia, CA). All restriction enzymes used were supplied by New England Biolabs

(Beverly, MA). Squalene, lanosterol, and desmosterol were obtained from Sigma (St. Louis, MO). Squalene epoxide was purchased from American Radiolabeled Chemicals, Inc. and zymosterol from Steraloids Inc. (Newport, RI). Organic solvents were of HPLC grade purchased from Fluka (New York, NY) and Fisher Scientific (Pittsburgh, PA).

CYP17 Gene Sequencing

Using CYP17 cDNA (21, NCBI GenBank accession no. NM007809) as a probe, a mouse bacterial artificial chromosome library was screened and a clone containing the CYP17 genomic DNA (120 kb) was isolated (Genome Systems Inc., St. Louis, MO). Nucleotide sequencing was performed using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Gel electrophoresis and computer analyses were performed at Lombardi Cancer Center sequencing facility (Georgetown University). The CYP17 gene sequence obtained was deposited in the NCBI GenBank (accession no. AY594330).

Construction of Targeting Vectors

The various isolated DNA fragments were subcloned into pCR2.1 (Invitrogen), pBlueScript (KS+) (Stratagene, La Jolla, CA) and pGT-N29 (New England Biolabs) vectors. The 3.3-kb 5' and 3.5-kb 3' DNA CYP17 fragments were constructed together with neomycin resistance (*neo*) gene. Figure 2 details the procedure used for the construction of targeting vectors.

Cell Transfection and Clone Screening

MA-10 mouse Leydig tumor cells were maintained in DMEM/F-12 medium with 5% fetal bovine serum, 2.5% horse serum, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 C and 4% CO₂ in 95% air. The knockdown gene-targeting construct containing the *neo* gene as a selection marker (8.2-kb fragment of CYP17 gene replaced by 2.0 kb *neo* gene) was transfected into MA-10 cells by electroporation. Briefly, 40 μ g of the targeting vector was linearized by *Kpn*I and suspended together with 10⁷ MA-10 mouse Leydig cells in 0.8 ml of DMEM/F-12 medium. Cells were electroporated at 220 V, 950 μ F and 19 ms in the Gene Pulsar II System (Bio-Rad, Hercules, CA). Neomycin-resistant clones were selected in the presence of 200 μ g/ml G418. Homologous recombination was identified by genomic Southern blot hybridization performed as previously described (32, 33).

Real-Time Quantitative RT-PCR

Total RNA from MA-10^{wt} and MA-10^{CYP17KD} cells was isolated using the RNeasy B reagent (Tel-Test, Inc., Friendswood, TX). Reverse transcription and real-time PCR were performed using the TaqMan reagent (Applied Biosystems). An Applied Biosystems Prism 7700 Sequence Detection System was used with the default thermal cycling program (95 C for 10 min followed by 40 cycles of 95 C, 15 sec, 60 C, 1 min). 18S rRNA was used as endogenous reference. The primers (in exon 5) used for Q-PCR amplification of CYP17 were designed by Primer Express (Applied Biosystems) and they were: sense: 5'-AAGGCCAGGACCCAAGTGT-3', antisense: 5'-CCACCGTGACAAGGATATGCT-3'.

siRNA Preparation and Transfection

siRNAs corresponding to CYP17 gene were designed as recommended by Silencer siRNA Construction Kit (Ambion Inc., Austin, TX), and the templates were chemically synthe-

sized by Biosynthesis Inc. The following pair gene-specific sequences were used: antisense si-CYP17 oligonucleotide template: 5'-AACTGCAGTGATTGTCTGGTCACTGTCTC-3' and sense si-CYP17 oligonucleotide template: 5'-AATGACCGACAATCACTGCAGCCTGTCTC-3'. siRNA preparation and purification were performed according to the manufacturer's instruction (Ambion, Inc). Transfections of siRNA for endogenous gene targeting were carried out using the Oligofectamine Reagent (Invitrogen). A 200-nM siRNA concentration was transfected into MA-10 cells. After incubation for 4 h at 37 °C in a CO₂ incubator, growth medium containing three times the normal concentration of serum was added to the cells. Real-time quantitative RT-PCR, to assess CYP17 mRNA levels, and RIA to determine progesterone formation by MA-10^{si-CYP17KD} compared with MA-10^{wt} cells, were performed 48–72 h after transfection.

Transfection of CYP17 cDNA into MA-10^{CYP17KD} Cells

CYP17 mouse cDNA (1.8 kb) (21) was prepared by RT-PCR using the following pair of primers: sense (upstream –295) 5'-ccatagcgcagaagccccaggaga-3' and antisense (downstream 1540): 5'-acgtagattcgctcttaggtgct-3'. The generated cDNA was inserted into pZeoSV2(–) vector (Zeocin⁺) (Invitrogen) and used to transfect MA-10^{CYP17KD} cells using Lipofectamine 2000 (Invitrogen). Cells were selected by their ability to resist to Zeocin (400 µg/ml media) for 7 d. Zeocin-resistant cells (MA-10^{CYP17KD-re}) were seeded in 96-well plates and their ability to form progesterone in response to hCG was monitored by RIA.

Measurement of Progesterone Formation

MA-10^{wt}, MA-10^{CYP17KD}, and MA-10^{CYP17KD} cells transfected with either CYP17 cDNA (MA-10^{CYP17KD-re}) or CYP17 siRNA (MA-10^{si-CYP17KD}) were washed with serum-free medium and treated with the indicated concentrations of hCG or 22(R)-hydroxycholesterol for 2 h at 37 °C. In search of the metabolic step in cholesterol synthesis affected by CYP17, 30 µM of the substrates squalene, squalene epoxide, lanosterol, zymosterol or desmosterol were added in cultured MA-10^{wt} and MA-10^{CYP17KD} cells. After a 3-h incubation at 37 °C, 50 ng/ml hCG were added for an additional 2 h treatment. Progesterone formed was measured by RIA (45).

De Novo Steroid Synthesis

Steroid synthesis experiments were performed with MA-10^{wt} and MA-10^{CYP17KD} cells at 60–80% confluence. To determine the ability of the cells to synthesize *de novo* steroids, cells were incubated at 37 °C and 4% CO₂ with specific steroidal substrates as previously described (46), with some modification. In brief, endogenous cholesterol synthesis was blocked by incubating cells with 20 µM lovastatin, a competitive nonsteroidal inhibitor of hydroxymethylglutaryl-CoA reductase, in serum-free medium for 1 h. Cells were subsequently incubated with (RS)-[5-³H] mevalonactone (MVA, 1.5 µCi/ml) and 10 µM unlabeled MVA. One hour later, media and cells were combined and homogenates were prepared on ice. Homogenates were extracted twice with 4 ml of diethyl-ether/ethyl acetate (1:1, vol:vol) and evaporated. Extracts were reconstituted in 100% methanol and separated on a Beckman Gold HPLC system, using a 126 NM solvent module, 166 NM detector and a Beckman Coulter (4.6 mm × 25 cm) column (Beckman Coulter, Inc., Fullerton, CA). Fractions eluted with 50% acetonitrile were collected and radioactivity present in the fractions was measured by liquid scintillation spectrometry. Radiolabeled steroids were identified by their respective retention times compared with radiolabeled standards (retention time of MVA = 6 min, DHEA = 19 min, pregnenolone = 49 min, progesterone = 53 min). In previous

experiments, steroids in these fractions were identified as pregnenolone, progesterone, and DHEA, respectively by gas chromatography-mass spectrometry (18).

Cholesterol Biosynthesis

MA-10^{wt} and MA-10^{CYP17KD} cells were seeded into six-well plates and used at 60–80% confluency. Fresh serum-free medium was then added together with ¹⁴C-labeled squalene (0.05 µCi). After a 4-h incubation, cells and media were extracted with diethyl-ether/ethyl acetate (1:1, vol:vol). The organic phase was collected and evaporated to dryness. The dried residues were resuspended in ethyl ether and run on C18 silica gel TLC plates (Whatman, Inc., Clifton, NJ) with a mobile phase of methylene chloride/ethyl acetate (97:3, vol:vol) (29). ¹⁴C-labeled standards for squalene and cholesterol were run on each plate. Fractions were collected from the plate and radioactivity was measured by liquid scintillation counting.

Immunoblot Analysis

Protein extracts from MA-10^{wt}, MA-10^{CYP17KD}, or transformed bacteria were used for immunoblot analysis performed as previously described (47). Anti-CYP17 antiserum was a gift from Drs. D. B. Hales (University of Illinois, Chicago, IL) and A. Payne (Stanford University, Stanford, CA) and anti-GAPDH antiserum, used as a loading control, was from Trevigen (Gaithersburg, MD). Immunoreactive proteins were detected using an enhanced chemiluminescence western blot detection kit (Amersham Biosciences).

Expression of Recombinant CYP17 in Bacteria and Purification of the Recombinant Protein

The pET system (Novagen, Madison, WI) was used to express the mouse CYP17 recombinant protein (24). The pET-15b (containing a histidine tag) vector was modified by constructing in an oligo DNA fragment (5'-CATATGGCGGCCGCTTAATTAACCGGGTTCGAC ACTAGTGGATCC-3') containing two cloning sites (*NotI*-*SalI*) between the multiple cloning sites (*NdeI*-*BamHI*). The CYP17 cDNA insert containing the full-length coding sequence as well as the *NotI* and *SalI* site extensions at the 5' and 3' ends was generated by PCR using the following primers: sense 5'-ATGGCGGCCGCAATGTGGGAACCTTGTGGGTCT-3', antisense 5'-CGGGT-CGACCTCTAGGTGCTAACCTCAAC-3', respectively. The CYP17 fragment was inserted into the modified pET15b vector and linearized with *NotI* and *SalI* downstream of the T7lac promoter. The CYP17 expression vector was used to transform the TOP10 *E. coli* strain where the expression of the recombinant CYP17 protein was induced by exposure of the cells to 1 mM IPTG, 1 mM 5-aminolevulinic acid, and riboflavin for 40 h at 30 °C. Control experiments were performed using the TOP10 *E. coli* bacterial strain alone or transformed with the pET15b vector. Purification of recombinant mouse CYP17 was performed using the BugBuster Ni-NTA His-Bind Purification Kit (Novagen). CYP17 protein expression was monitored by 4–20% SDS-PAGE protein separation followed either by Coomassie Blue staining (24) or electrotransferring to nitrocellulose membranes and immunoblot analyses performed as described (47).

Assay for CYP17 17 α -Hydroxylase/17,20-Lyase Activity in Bacteria

Measurement of CYP17 activities in bacteria extracts was performed as described by Ehmer *et al.* (38) and Imai *et al.* (39). In brief, ³H-progesterone was added in 400 µl of 0.1 M sodium phosphate buffer (pH 7.4) containing 12.5 mM glu-

cose, 1 mM NADPH, in the presence or absence of SU10603 (10 μ M), and the solution was preincubated at 37 C for 2 min. The reaction was initiated by the addition of 100 μ l of thawed bacterial suspension and the samples were incubated for 45 min at 37 C. The reaction was stopped by heating the tubes at 95 C for 5 min. Steroids were extracted in 600 μ l of cold ethylacetate for 5 min. After centrifugation at 3000 \times g, 400 μ l of supernatant were removed and evaporated to dryness, and the extracted steroids were dissolved with 100 μ l of acetone and separated on TLC (Whatman Silica Gel 60A). The solvent system used was chloroform:ethylacetate (80:20). The location of separated steroids were determined by UV light (254 nm) and visualized and analyzed by phosphoimaging using the Cyclone Storage phosphor system (PerkinElmer, Boston, MA). CYP17 activity was determined by quantifying the loss of the substrate progesterone.

Assay for CYP17 Squalene Monooxygenase Activity in Bacteria

The CYP17 squalene monooxygenase activity was determined on the basis of the procedure described by Singh *et al.* (25) with minor modifications. In brief, 100 μ l bacterial suspension, 30 μ M flavin adenine dinucleotide, 14 C-squalene, and 10 μ g of phosphatidylglycerol were added in 200 μ l of 20 mM Tris-HCl buffer (pH 7.4) in the presence of 1 mM EDTA, and in the presence or absence of SU10603 (10 μ M). The reaction was initiated by the addition of 1 mM NADPH and the mixture was incubated for 60 min at 37 C. The reaction was stopped by the addition of 0.5 ml of 10% KOH in methanol and the incubation volume was brought to 1 ml with water. Samples were capped and after saponification for 60 min at 80 C the neutral lipids were extracted with 3 ml of petroleum ether. The solvent was removed under evaporative centrifugation and the lipids were resuspended in 50 μ l of the petroleum ether and spotted onto silica thin-layer plates. Lipids were separated in 5% ethyl acetate in hexane and visualized and analyzed by phosphoimaging using the Cyclone Storage Phosphor screen. CYP17 squalene monooxygenase activity was determined by quantifying the loss of the substrate squalene.

Isolation of Rat Leydig Cells

Testicular interstitial cells were prepared by collagenase dissociation (48) of testes obtained from adult Sprague Dawley (300 g) rats. This preparation contained 20–30% 3 β HSD-positive cells (Leydig cells). Leydig cells were further purified using discontinuous Percoll gradient centrifugation as previously described (48). The preparations obtained contained 75–85% Leydig cells as shown by the histochemical staining for 3 β HSD. When purified rat Leydig cells were used, 50,000 cells per 500 μ l of serum-free media were incubated in borosilicate culture tubes at 32 C. At the end of the incubation time period the cell media were saved, centrifuged at 1500 \times g for 10 min, and stored at –20 C until use. Testosterone formation by the cells was determined by RIA, as previously described (45).

Cell Proliferation Assay

MA-10^{wt} and MA-10^{CYP17KD} cells were plated in 96-well plates at a concentration of 10⁴ cells/well. After seeding in the plate for 24, 48, and 72 h, cell proliferation was analyzed by measuring the amount of BrdU incorporation determined by the BrdU ELISA (Roche Molecular Biochemicals, Indianapolis, IN).

Miscellaneous

Cell protein content was determined according to the method of Bradford (49) using BSA as standard. Statistical analysis of

the data, expressed as mean \pm SEM, was performed by ANOVA followed by the Student-Newman-Keul's test using the Instat (version 3.0) package from GraphPad (San Diego, CA).

Acknowledgments

The authors would like to thank Dr. M. Ascoli (University of Iowa) for the gift of the MA-10 cell line, Drs. D. B. Hales (University of Illinois, Chicago, IL) and A. H. Payne (Stanford University, Stanford, CA) for the gift of the CYP17 antiserum, Stegram Pharmaceuticals (Sussex, UK) for the gift of trilostane, and CIBA-Geigy (Basel, Switzerland) for the gift of SU 10603.

Received July 5, 2004. Accepted March 2, 2005.

Address all correspondence and requests for reprints to: Vassilios Papadopoulos, Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, 3900 Reservoir Road Northwest, Washington, D.C. 20057. E-mail: papadopv@georgetown.edu.

This work was supported by Grant IBN-0110711 from the National Sciences Foundation.

REFERENCES

- Hall PF 1986 Cytochromes P450 and regulation of steroid synthesis. *Steroids* 48:131–196
- Miller WL 2002 Androgen biosynthesis from cholesterol to DHEA. *Mol Cell Endocrinol* 198:7–14
- Mellon SH, Griffin LD 2002 Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol Metab* 13:35–43
- Rainey WE, Carr BC, Sasano H, Suzuki T, Mason JI 2002 Dissecting human adrenal androgen production. *Trends Endocrinol Metabol* 13:234–239
- Auchus RJ 2001 The genetics, pathophysiology, and management of human deficiencies of P450c17. *Endocrinol Metab Clin North Am* 30:101–119
- Katsumata N, Satoh M, Mikami A, Mikami S, Nagashima-Miyokawa A, Sato N, Yokoyama S, Tanaka T 2001 New compound heterozygous mutation in the CYP17 gene in a 46, XY girl with 17 α -hydroxylase/17,20-lyase deficiency. *Horm Res* 55:141–146
- Yanase T 1995 17 α -Hydroxylase/17,20-lyase defects. *J Steroid Biochem Mol Biol* 53:153–157
- Vianello S, Waterman MR, Dalla Valle L, Colombo L 1997 Developmentally regulated expression and activity of 17 α -hydroxylase/C-17,20-lyase cytochrome P450 in rat liver. *Endocrinology* 138:3166–3174
- Katagiri M, Tatsuta K, Imaoka S, Funae Y, Honma K, Matsuo N, Yokoi H, Ishimura K, Ishibashi F, Kagawa N 1998 Evidence that immature rat liver is capable of participating in steroidogenesis by expressing 17 α -hydroxylase/17,20-lyase P450c17. *J Steroid Biochem Mol Biol* 64:121–128
- Compagnone NA, Bulfone A, Rubenstein JL, Mellon SH 1995 Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. *Endocrinology* 136:5212–5223
- Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, Mukai H, Morrison JH, Janssen WG, Kominami S, Harada N, Kimoto T, Kawato S 2004 Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017 α and P450 aromatase localized in neurons. *Proc Natl Acad Sci USA* 101:865–870
- Dalla Valle L, Vianello S, Belvedere P, Colombo L 2002 Rat cytochrome P450c17 gene transcription is initiated at different start sites in extraglandular and glandular tissues. *J Steroid Biochem Mol Biol* 82:377–384

13. Pezzi V, Mathis JM, Rainey WE, Carr BR 2003 Profiling transcript levels for steroidogenic enzymes in fetal tissues. *J Steroid Biochem Mol Biol* 87:181–189
14. Wang Y, Ge W 2004 Cloning of zebrafish ovarian P450c17 (CYP17, 17 α -hydroxylase/17, 20-lyase) and characterization of its expression in gonadal and extra-gonadal tissues. *Gen Comp Endocrinol* 135:241–249
15. Lieberman S, Warne PA 2001 17-Hydroxylase: an evaluation of the present view of its catalytic role in steroidogenesis. *J Steroid Biochem Mol Biol* 78:299–312
16. Atkins WM, Wang RW, Lu AYH 2001 Allosteric behaviour in cytochrome P450-dependent in vitro drug-drug interactions: a prospective based on conformational dynamics. *Chem Res Toxicol* 14:338–347
17. Ascoli M 1981 Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* 108:88–95
18. Cascio C, Prasad VVK, Lin YY, Lieberman S, Papadopoulos V 1998 Detection of P450c17-independent pathways for dehydroepiandrosterone (DHEA) biosynthesis in brain glial tumor cells. *Proc Natl Acad Sci USA* 95:2862–2867
19. Cascio C, Guarneri P, Li H, Brown RC, Amri H, Boujrad N, Kotoula M, Vidic B, Drieu K, Papadopoulos V 1999 Peripheral-type benzodiazepine receptor. Role in the regulation of steroid and neurosteroid biosynthesis. In: Baulieu EE, Robel P, Schumacher M. *Neurosteroids: a new regulatory function in the central nervous system*. Contemporary endocrinology. Totowa, NJ: The Humana Press Inc.; 75–96
20. Ascoli M, Segaloff DL 1989 Regulation of the differentiated functions of Leydig tumor cells by epidermal growth factor. *Ann NY Acad Sci* 564:99–115
21. Youngblood GL, Sartorius C, Taylor BA, Payne AH 1991 Isolation, characterization, and chromosomal mapping of mouse P450 17 α -hydroxylase/c17–20 lyase. *Genomics* 10: 270–275
22. Hales DB, Sha L, Payne AH 1987 Testosterone inhibits cAMP-induced de novo synthesis of Leydig cell cytochrome P-45017 α by an androgen receptor-mediated mechanism. *J Biol Chem* 262:11200–11206
23. Löhr JB, Kühn-Velten WN 1997 Protein phosphorylation changes ligand-binding efficiency of cytochrome P450c17 (CYP17) and accelerates its proteolytic degradation: putative relevance for hormonal regulation of CYP17 activity. *Biochem Biophys Res Commun* 231:403–408
24. Li H, Papadopoulos V 1998 Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. *Endocrinology* 139: 4991–4997
25. Singh DK, Mokashi V, Elmore CL, Porter TD 2003 Phosphorylation of supernatant protein factor enhances its ability to stimulate microsomal squalene monooxygenase. *J Biol Chem* 278:5646–5651
26. Gower DB 1974 Modifiers of steroid-hormone metabolism: a review of their chemistry, biochemistry and clinical applications. *J Steroid Biochem* 5:501–523
27. Paul SM, Purdy RH 1992 Neuroactive steroids. *FASEB J* 6:2311–2322
28. Cascio C, Brown RC, Liu Y, Han Z, Hales DB, Papadopoulos V 2000 Pathways of dehydroepiandrosterone formation in developing rat brain glia. *J Steroid Biochem Mol Biol* 75:177–186
29. Brown RC, Cascio C, Papadopoulos V 2000 Pathways of neurosteroid biosynthesis in cell lines from human brain: regulation of dehydroepiandrosterone formation by oxidative stress and β -amyloid peptide. *J Neurochem* 74:847–859
30. Brown RC, Liu Y, Papadopoulos V 2002 Brain dehydroepiandrosterone: biosynthesis and regulation. In: Morfin R, ed. *DHEA and brain*. London: Taylor and Francis; 129–146
31. Brown RC, Han Z, Cascio C, Papadopoulos V 2003 Oxidative stress mediated dehydroepiandrosterone formation in Alzheimer's disease pathology. *Neurobiol Aging* 24:57–65
32. Liu Y, Yao Z-X, Bendavid C, Borgmeyer C, Han Z, Cavalli LR, Chan W-Y, Folmer J, Zirkin BR, Haddad BR, Gallicano GI, Papadopoulos V 12 May 2005 Haploinsufficiency of cytochrome P450 17 α -hydroxylase/17,20 lyase (CYP17) causes infertility in male mice. *Mol Endocrinol* 10.1210/me.2004-0418
33. Papadopoulos V, Amri H, Boujrad N, Li H, Vidic B, Garnier M 1997 Targeted disruption of the peripheral-type benzodiazepine receptor gene inhibits steroidogenesis in the R2C Leydig tumor cell line. *J Biol Chem* 272:32129–32135
34. Accili D, Taylor SI 1991 Targeted inactivation of the insulin receptor gene in mouse 3T3–L1 fibroblasts via homologous recombination. *Proc Natl Acad Sci USA* 88:4708–4712
35. Altuvia S, Stein WD, Goldenberg S, Kane SE, Pastan I, Gottesman MM 1993 Targeted disruption of the mouse *mdr1b* gene reveals that steroid hormones enhance *mdr* gene expression. *J Biol Chem* 268:27127–27132
36. Yamamoto S, Bloch K 1970 Studies on squalene epoxidase of rat liver. *J Biol Chem* 245:1670–1674
37. Jenkins CM, Waterman MR 1998 NADPH-flavodoxin reductase and flavodoxin from *Escherichia coli*: characteristics as a soluble microsomal P450 reductase. *Biochemistry* 37:6106–6113
38. Ehmer PB, Jose J, Hartmann RW 2000 Development of simple and rapid assay for the evaluation of inhibitors of human 17 α -hydroxylase-C_{17,20}-lyase (P450c17) by coexpression of P450c17 with NADPH-cytochrome-P450-reductase in *Escherichia coli*. *J Steroid Biochem Mol Biol* 75:57–63
39. Imai T, Globberman H, Gertner JM, Kagawa N, Waterman MR 1993 Expression and purification of functional human 12 α -hydroxylase/17,20-lyase (P450c17) in *Escherichia coli*. *J Biol Chem* 268:19681–19689
40. Tai HH, Bloch K 1972 Squalene epoxidase of rat liver. *J Biol Chem* 247:3767–3773
41. Sakakibara J, Watanabe R, Kanai Y, Ono T 1995 Molecular cloning and expression of the rat squalene epoxidase. *J Biol Chem* 270:17–20
42. Ono T 2002 The first step of oxygenation in cholesterol biosynthesis. *Biochem Biophys Res Commun* 292:1283–1288
43. Horie M, Tsuchiya Y, Hayashi M, Iida Y, Iwasawa Y, Nagata Y, Sawasaki Y, Fukuzumi H, Kitani K, Kamei T 1990 NB-598: a potent competitive inhibitor of squalene epoxidase. *J Biol Chem* 265:18075–18078
44. Chugh A, Ray A, Gupta JB 2003 Squalene epoxidase as hypocholesterolemic drug target. *Prog Lipid Res* 42:37–50
45. Papadopoulos V, Mukhin AG, Costa E, Krueger KE 1990 The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. *J Biol Chem* 265: 3772–3779
46. Guarneri P, Papadopoulos V, Pan B, Costa E 1992 Regulation of pregnenolone synthesis in C6–2B glioma cells by 4'-chlorodiazepam. *Proc Natl Acad Sci USA* 89:5118–5122
47. Li H, Degenhardt B, Tobin D, Yao Z, Tasken K, Papadopoulos V 2001 Identification, localization, and function in steroidogenesis of PAP7: a peripheral-type benzodiazepine receptor-and PKA (R1 α)-associated protein. *Mol Endocrinol* 15:2211–2228
48. Papadopoulos V, Carreau S, Drosowsky MA 1985 Effects of phorbol ester and phospholipase C on LH-stimulated steroidogenesis in purified rat Leydig cells. *FEBS Lett* 188:312–316
49. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254