

Deletion of the Mouse P450c17 Gene Causes Early Embryonic Lethality

Susanna R. Bair and Synthia H. Mellon*

Department of Obstetrics, Gynecology, and Reproductive Sciences, The Center for Reproductive Sciences, and The Metabolic Research Unit, University of California, San Francisco, San Francisco, California 94143

Received 6 October 2003/Returned for modification 20 November 2003/Accepted 19 March 2004

Dehydroepiandrosterone (DHEA), a 19-carbon precursor of sex steroids, is abundantly produced in the human but not the mouse adrenal. However, mice produce DHEA and DHEA-sulfate (DHEAS) in the fetal brain. DHEA stimulates axonal growth from specific populations of mouse neocortical neurons in vitro, while DHEAS stimulates dendritic growth from those cells. The synthesis of DHEA and sex steroids, but not mouse glucocorticoids and mineralocorticoids, requires P450c17, which catalyzes both 17 α -hydroxylase and 17,20-lyase activities. We hypothesized that P450c17-knockout mice would have disordered sex steroid synthesis and disordered brain DHEA production and thus provide phenotypic clues about the functions of DHEA in mouse brain development. We deleted the mouse P450c17 gene in 127/SvJ mice and obtained several lines of mice from two lines of targeted embryonic stem cells. Heterozygotes were phenotypically and reproductively normal, but in all mouse lines, P450c17^{-/-} zygotes died by embryonic day 7, prior to gastrulation. The cause of this early lethality is unknown, as there is no known function of fetal steroids at embryonic day 7. Immunocytochemistry identified P450c17 in embryonic endoderm in E7 wild-type and heterozygous embryos, but its function in these cells is unknown. Enzyme assays of wild-type embryos showed a rapid rise in 17-hydroxylase activity between E6 and E7 and the presence of C_{17,20}-lyase activity at E7. Treatment of pregnant females with subcutaneous pellets releasing DHEA or 17-OH pregnenolone at a constant rate failed to rescue P450c17^{-/-} fetuses. Treatment of normal pregnant females with pellets releasing pregnenolone or progesterone did not cause fetal demise. These data suggest that steroid products of P450c17 have heretofore-unknown essential functions in early embryonic mouse development.

The synthesis of steroid hormones in the adrenals, gonads, placenta, and brain requires the expression of several steroidogenic enzymes. In all tissues, steroidogenesis is initiated by conversion of cholesterol to pregnenolone by the mitochondrial cholesterol side chain cleavage enzyme, P450_{sc}. Thereafter, the specific steroid that is synthesized by a particular tissue depends upon the differential expression of additional steroidogenic enzymes. The conversion of pregnenolone and progesterone to their 17 α -hydroxylated products and then to either dehydroepiandrosterone (DHEA) or androstenedione, respectively, is mediated by a single microsomal enzyme, P450c17 (33, 34, 48), encoded by a single gene (22, 39). The pattern of P450c17 expression in steroidogenic tissues is species specific: it is expressed in the human adrenal and gonad but not placenta (9, 11, 15, 44), and it is expressed in the rodent gonad and placenta but not adrenal (19, 23, 25).

P450c17 is also expressed in the fetal mouse brain beginning at embryonic day 9.5 (E9.5) (12). At this time, P450c17 is found in cells migrating from the neural crest, and subsequently, P450c17 is found in many cells derived from the neural crest. P450c17 is also expressed in the neocortical subplate, a region that receives thalamic projections, produces signals for cortical projections, and may produce signals for efferent

thalamic projections from the cortex (32, 41). We hypothesized that DHEA, a steroid product of P450c17, may be an endogenous signal in the subplate to target axons coming from this region to specific sites in the developing cortex and showed that DHEA increased axonal outgrowth while DHEA-sulfate (DHEAS) increased dendritic growth (13). DHEA, but not DHEAS, induced other morphological indices of synaptic contacts, increased mRNAs for Tau-1 and type 1 and 2 dopamine receptors (14), mediated increases in intracellular calcium via *N*-methyl-D-aspartic acid receptors (13), protected hippocampal neurons from glutamate toxicity (27), and stimulated neurogenesis in the hippocampus (26). Thus, DHEA may serve as an endogenous neurotrophic agent. However, all these studies have been performed with cultured neuronal cells derived from specific regions of the nervous system at particular times in development. Thus, we sought to determine the function of DHEA and DHEAS in vivo during the development of the nervous system by deleting the gene encoding P450c17 in gene-targeted mice.

MATERIALS AND METHODS

Creation of targeting vector. P450c17 genomic DNA was isolated from a 129/SvJ mouse genomic library (Stratagene, La Jolla, Calif.) with the use of exons 2 to 4 of rat P40c17 cDNA as a probe (30). The 12.5-kb genomic fragment contained ~4 kb of 5'-flanking DNA and extended to the middle of exon 8. The targeting vector was constructed by cloning a 1.8-kb phosphoglycerate kinase (PGK) Neo cassette (43) into the EcoRI/HindIII site of pBluescript SK (Stratagene). The 3' end of the targeting construct consisted of a 2-kb mouse P450c17 fragment, generated by digesting the P450c17 genomic clone with Aval/SstI and blunting the ends with deoxynucleoside triphosphates and DNA polymerase.

* Corresponding author. Mailing address: Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, 513 Parnassus Ave., Box 0556, San Francisco, CA 94143-0556. Phone: (415) 476-5329. Fax: (415) 753-3271. E-mail: mellon@cgl.ucsf.edu.

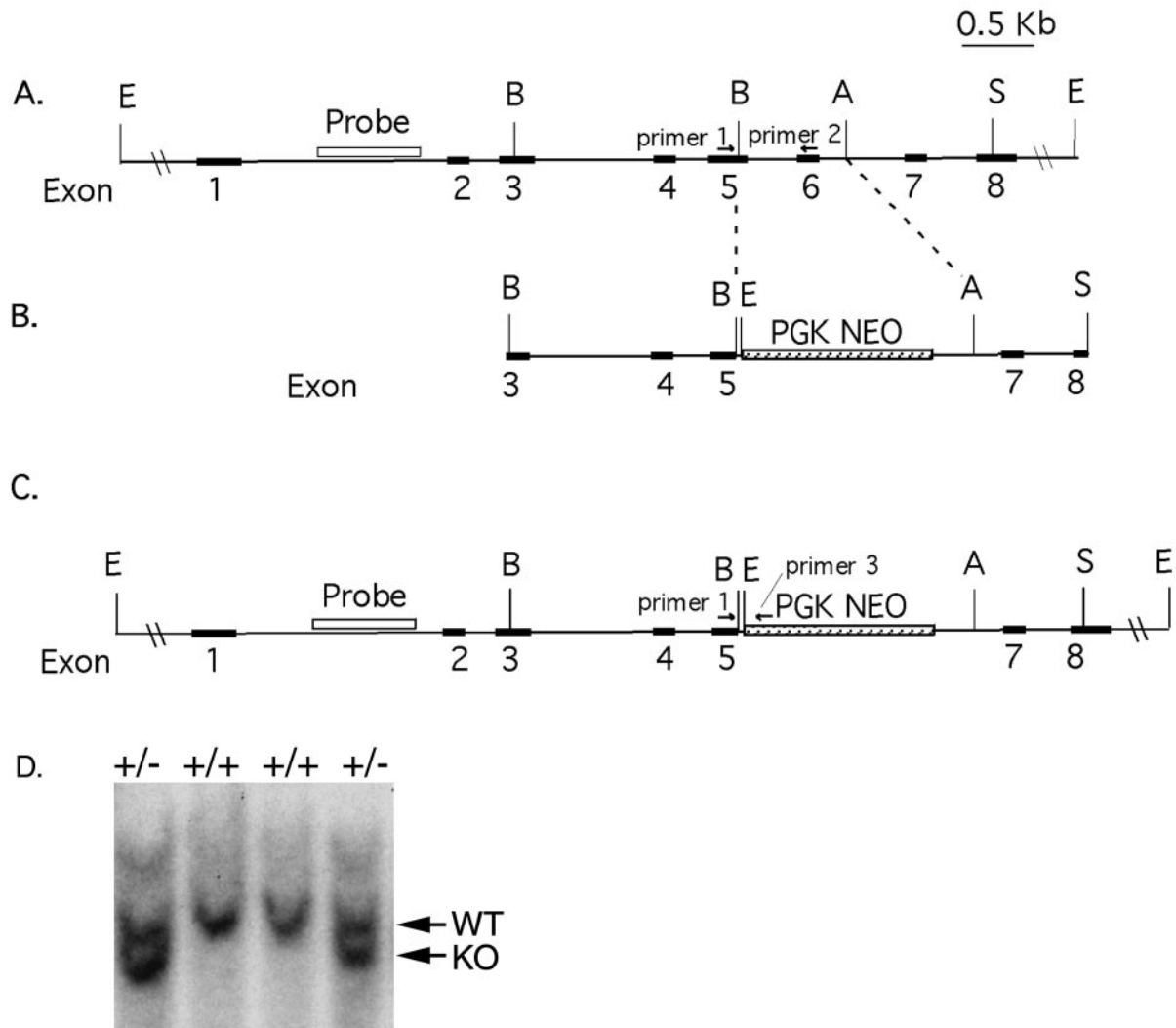


FIG. 1. Targeted disruption of the P450c17 gene. (A) Partial restriction endonuclease map of the murine P450c17 genomic locus showing the locations of the probe used in cloning the genomic fragment and the approximate positions of primers 1 and 2. Restriction endonuclease sites: E, EcoRI; B, BamHI; A, AvaI; S, SacI. (B) P450c17 targeting vector containing the neomycin resistance (NEO) gene under the control of the PGK promoter. (C) Structure of the recombinant P450c17 mutant allele, showing the location of primer 3. (D) Southern blot of DNA prepared from the offspring of a P450c17^{+/-} × P450c17^{+/-} mating. DNA was digested with EcoRI and hybridized to the radiolabeled P450c17 genomic probe shown in panel A. The positions of the wild-type (WT; 10-kb) and targeted (knockout [KO]; 6.5-kb) alleles are indicated with arrows.

This 2-kb mouse P450c17 DNA fragment, from intron 6 through exon 8, was cloned into the HincII site of the PGK Neo pKS construct. The 5' end of the targeting construct consisted of a 1.5-kb DNA fragment of mouse P450c17, from exon 3 to exon 5, generated by digestion of the mouse P450c17 clone with BamHI and blunted with deoxynucleoside triphosphates and DNA polymerase. This fragment was cloned into the PGK Neo-3'c17 plasmid and digested with Ecl136II to generate blunt ends (Fig. 1).

Analysis of transfected mouse ES cells. The targeting vector was linearized with XhoI and electroporated into mouse JM-1 embryonic stem (ES) cells, and mutant ES cell clones were selected in medium containing G418 (200 to 250 μ g/ml). DNA from ES cells and from mouse tails was genotyped by Southern blotting (Fig. 1) or PCR assays. For Southern blots, genomic DNA was digested with EcoRI and analyzed with an 1,142-bp PstI/PstI probe from intron 1 of the P450c17 gene (Fig. 1). The PCR assay for the P450c17-knockout mutation used primer 1, 5' GAATCTCTCTCCAGCCTG 3', and primer 3, 5' CTTGTGTAG CGCCAAGTG 3', which amplify a 270-bp fragment of P450c17 exon 5 into the PGK Neo transgene (Fig. 1). The PCR assay for the wild-type P450c17 gene used primer 1 from P450c17 exon 5 and primer 2, 5' CGGTGTTCTGACTGAAGCC

3', from P450c17 exon 6, which amplify a 490-bp fragment. The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s.

Analysis of gene-targeted mice. Positive ES cell lines 171 and 182 were used to create chimeras by blastocyst injection. Chimeras were mated to wild-type C57BL/6 mice, and the F₁ and F₂ generations were obtained by crossing heterozygotes. All genotyping was done by Southern blot analysis.

Laser catapult microdissection. Sections were stained with hematoxylin-eosin and used to collect embryonic tissue at E7 and E8 by laser catapult microdissection (18). After staining, sections were dehydrated in graded ethanol (95% twice for 5 min each and 100% three times for 5 min each) and cleared in xylene (three times for 5 min each). After air drying for 15 min, laser catapult microdissection was performed under direct microscopic visualization of the embryonic tissue by catapulting selected regions onto a microcentrifuge cap containing mineral oil. To ensure that no maternal tissue was collected, tissue surrounding the embryo was first removed by the laser, and then the remaining embryonic tissue was collected. The P.A.L.M. MicroLaser MicroBeam (P.A.L.M. MicroLaser Technologies AG) was set to the following parameters: 15- μ m laser spot size,

40-mW power, and 3.0-ms duration. The catapulted cells were dissolved immediately in buffer containing 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-Cl (pH 8.5), 0.01% gelatin, 0.45% NP-40, 0.45% Tween 20, and 100 µg of proteinase K/ml and digested at 55°C for 4 h, and proteinase K was inactivated at 95°C for 5 min.

PCR analysis of genomic DNA. Genomic DNA obtained from laser catapult microdissection was analyzed by 40 cycles of PCR, with the use of primer 1 and primer 2 to identify the wild-type allele and primer 1 and primer 3 to identify the knockout allele, as described for analysis of ES cell DNA. The amplified DNA fragment from the knockout allele (270 bp) and the amplified DNA fragment from the wild-type allele (490 bp) were distinguished by agarose gel electrophoresis.

Immunocytochemistry. Embryos from timed matings were removed from the uteri and fixed in their surrounding decidua in 4% paraformaldehyde in phosphate-buffered saline. Fixed tissues were embedded in paraffin, and 4-µm sections were collected onto microscope slides. Slides that contained embryonic tissue were identified by hematoxylin-eosin staining, the adjacent sections were immunostained using antibodies (1:3,000 dilution) to human P450c17 (28), and the corresponding mouse proteins were detected by indirect immunofluorescence, with secondary antibodies coupled to fluorescein isothiocyanate.

P450c17 enzyme activity. P450c17 enzyme activity was assessed as described previously (4) in embryos obtained from timed heterozygous matings. Pregnant mice were killed, and embryos (E7) were removed from the surrounding decidua by using a dissecting microscope, or the decidua containing E6 embryos were used directly without further dissection. Decidua were also used in control incubations. Tissue was sonicated in 50 mM potassium phosphate buffer, pH 7.4, and was used directly. Reaction mixtures contained either [¹⁴C]pregnenolone (New England Nuclear Perkin-Elmer, Boston, Mass.) or 17-OH-[³H]progesterone (American Radiolabeled Chemicals, Inc., St. Louis, Mo.), 1 mM NADPH, and 1 µM cyanoketone to inhibit 3β-hydroxysteroid dehydrogenase activity or 0.1 µM finasteride to inhibit 5α-reductase activity. Tissue was incubated at 37°C for 1 to 4 h, extracted with 2 volumes of isoctane-ethylacetate (1:1, vol/vol), and applied to a silica gel thin-layer chromatography plate. Steroids were separated in a CHCl₃-ethyl acetate (3:1, vol/vol) solvent, and plates were air dried and placed under a phosphorimager screen.

RESULTS

Creation of gene-targeted mice. A targeting vector encompassing a 3.7-kb BamHI/SstI segment of the mouse P450c17 gene extending from exon 3 to exon 8 (Fig. 1A) was built with a 1.8-kb PGK Neo cassette and substituted for a 0.5-kb fragment encompassing part of exon 5 and all of exon 6 (Fig. 1B). The "Ozols peptide" (36) comprises amino acids 346 to 366 in exon 6 (39) and contains amino acids crucial for redox partner binding (5, 10, 21), so that its deletion will destroy all activity. ES cells from 129/SvJ mice were electroporated with the P450c17 targeting vector and selected by growth in medium containing G418. Neomycin-resistant cell lines were assayed for chromosomal integration of the mutated P450c17 gene by Southern blotting, with use of the probe from intron 1 of the P450c17 gene, which lies outside the targeting vector (Fig. 1). Insertion of the PGK Neo cassette inserts a normal EcoRI site, so that the probe detects a 6.5-kb EcoRI fragment in recombinants but a 10-kb fragment in the wild type (Fig. 1C).

Two of five cell lines that contained the mutated P450c17 gene were used for injection into day 4 blastocysts (Table 1), generating 16 chimeric mice. Chimeras were mated to C57BL/6 mice, and over half of the offspring were heterozygous for the P450c17-knockout allele, as indicated by coat color and confirmed by Southern blotting. Of the original 16 chimeric mice, 6 had germ line transmission of the mutated P450c17 allele. All of those mouse lines were mated to C57BL/6 mice to obtain mutant offspring. Heterozygous P450c17^{+/-} mice were phenotypically normal and fertile. These mice were bred to C57BL/6 mice to produce heterozygotes that were mated to generate homozygous P450c17^{-/-}

TABLE 1. Genotypes of embryos from P450c17^{+/-} × P450c17^{+/-} matings

Type	No. of embryos							
	ES cell line 182				ES cell line 171			
	Total	+/+	+/-	-/-	Total	+/+	+/-	-/-
Chimera	4				2			
Chimera × WT ^a	45	23	22	0	32	11	21	0
F ₁ (+/- × +/-)	85	45	40	0	11	5	6	0
F ₂ (+/- × +/-)	65	27	38	0	21	5	16	0
E11.5 embryo	76	46	30	0	19	8	11	0

^a WT, wild type.

mice. We analyzed 182 offspring from two original chimeric mice, derived from two different ES cell lines (Table 1). Southern blotting identified 100 of 182 (55%) heterozygous (P450c17^{+/-}) and 82 of 182 (45%) homozygous wild-type (P450c17^{+/+}) offspring, but no homozygous P450c17^{-/-} live-born offspring were obtained. No mice had difficulty in early postnatal days, and none were lost to early death. As we obtained no mice that contained two copies of the P450c17-knockout allele, we postulated that the P450c17^{-/-} mice experienced embryonic lethality.

To determine the time of embryonic death, timed pregnant mice were killed at E11.5, embryos were removed, and embryonic DNA was analyzed by Southern blotting. Of 95 embryos analyzed, 54 were wild type and 41 were heterozygous for the P450c17-knockout transgene, but no P450c17^{-/-} embryos were identified (Table 1). Thus, P450c17^{-/-} embryos appeared to die before E11.5. To rule out the possibility that no zygotes were formed, we performed timed matings on superovulated mice and obtained E4.5 blastocysts. Analysis of 19 blastocysts by PCR demonstrated the presence of two P450c17^{-/-} zygotes. While this number of knockout blastocysts was less than the 25% expected, only two wild-type blastocysts were identified as well. These low numbers may have been due to maternal uterine tissue contamination of the blastocyst DNA, which would have resulted in the PCR genotyping of the blastocyst as heterozygous. Thus, it appears that P450c17^{-/-} blastocysts are formed, and therefore, the P450c17^{-/-} embryos appeared to die somewhere between E4.5 and E11.5.

Expression of P450c17 in the E7 embryo. There is no established role for P450c17 in the early embryo, and human fetuses harboring null mutations in both P450c17 alleles are not embryonic lethal. Male infants with complete P450c17 deficiency lack embryonic androgen synthesis and are born with female external genitalia and absent Wolffian duct structures and prostate differentiation. Male and female affected infants lack cortisol production and are hypertensive. To determine the location of P450c17 expression during early embryonic development in wild-type mice, we performed timed matings of superovulated female mice, obtained E7.0 embryos, and analyzed these embryos by immunocytochemistry with an antibody to human P450c17 that readily and specifically detects mouse P450c17 (12). P450c17 was detected in the embryonic endoderm of E7.0 embryos (Fig. 2). Thus, P450c17 protein is expressed early in normal mouse embryogenesis.

Analysis of P450c17 enzymatic activity in E7 embryos. To determine whether the immunostainable P450c17 was enzymo-

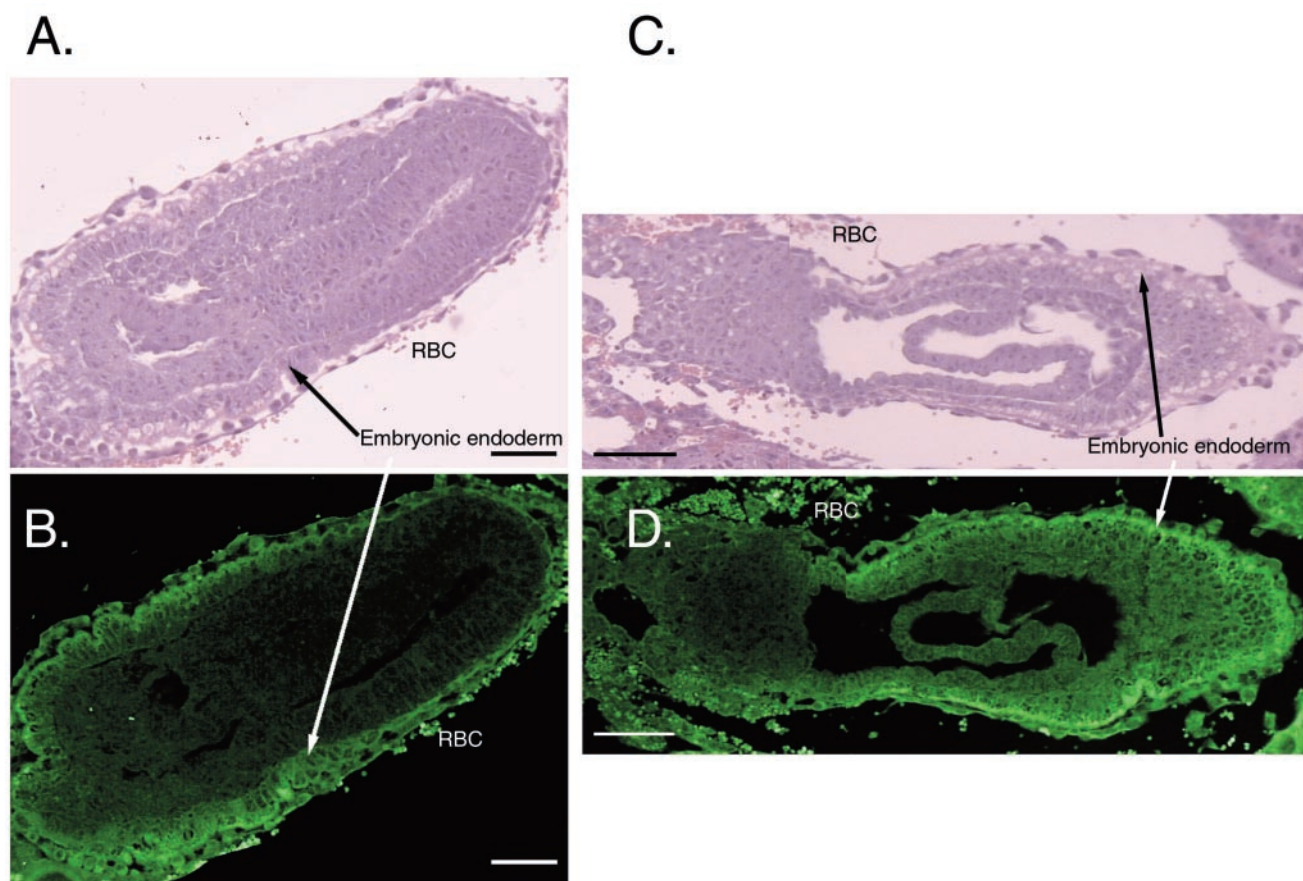


FIG. 2. Expression of the P450c17 gene in the postimplantation mouse embryo. Histological and immunocytochemical analyses were performed on E7.0 mouse embryos. (A and C) Hematoxylin- and eosin-stained sections through two E7 embryos. (B and D) Immunocytochemical analysis of the sections with an antibody to human P450c17. RBC, red blood cells.

logically active and hence had incorporated heme and had access to P450 oxidoreductase, we analyzed wild-type embryos for P450c17 enzymatic activity. Deciduae or embryos from timed matings of normal female mice were incubated with [^{14}C]pregnenolone for 60 to 240 min, and steroidal products were analyzed by thin-layer chromatography (Fig. 3A). Conversion of pregnenolone to 17-OH pregnenolone was barely detectable in wild-type E6 embryos but was substantial in wild-type E7 embryos. Thus, embryos express functional P450c17 protein at very early developmental stages, and P450c17 activity increases dramatically between E6 and E7.

Deciduae or embryos were also incubated with 17-OH-[^3H]progesterone for 1 and 3 h, and steroidal products were analyzed by thin-layer chromatography (Fig. 3B). Conversion of 17-OH progesterone to androstenedione could be seen in E7 embryos by 1 h. $\text{C}_{17,20}$ -lyase activity was not seen when decidual tissue from the same pregnancy was used. Other products were seen in incubations from E7 embryos that were not present in incubations with ovarian microsomal preparations, but these products were not identified.

Analysis of mutant E7 embryos. Analysis of six embryos from one mating of P450c17 $^{+/-}$ mice yielded two embryos lacking P450c17 immunostaining, suggesting that those embryos were P450c17 $^{-/-}$. Other embryos had the expected

P450c17 immunostaining in the embryonic endoderm. To verify the putative P450c17 genotype inferred from immunostaining, we performed laser catapult microdissection. We collected embryonic tissues from the immunostained sections, analyzed their genotype by PCR, and confirmed that those embryos lacking immunodetectable P450c17 protein were P450c17 $^{-/-}$ (Fig. 4). Embryos that were immunopositive for P450c17 were either P450c17 $^{+/-}$ or wild type.

Analysis of E8 embryos. To determine the time of demise of P450c17 $^{-/-}$ embryos, we analyzed E8 embryos derived from P450c17 $^{+/-}$ heterozygous matings. Deciduae containing individual embryos were fixed in paraffin and sectioned. Embryonic tissue devoid of maternal tissue was collected by laser catapult microdissection and genotyped by PCR. From 29 E8 embryos analyzed, we obtained 21 heterozygous embryos (72.4%) and 8 homozygous wild-type embryos (27.6%). No P450c17 $^{-/-}$ embryos were detected. Thus, the P450c17 knockout is early embryonic lethal, with embryos dying at about E7.

Steroid rescue. Because P450c17 $^{-/-}$ mice die at about E7, and because the only known role for P450c17 in the rodent is to produce C_{19} steroids, we attempted to rescue the P450c17 $^{-/-}$ embryos by providing their mothers with the C_{19} steroid DHEA or androstenedione. Administration of 0.1 mM DHEA or androstenedione in the drinking water of heterozygous-

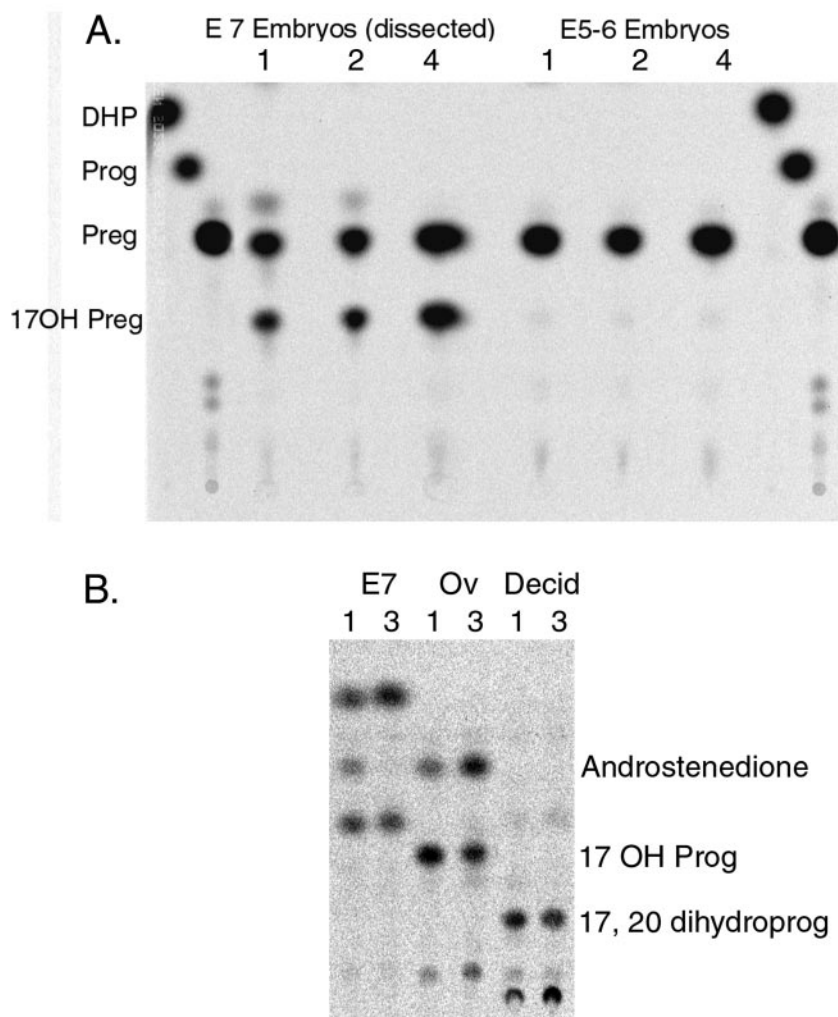


FIG. 3. P450c17 enzyme activities in mouse embryos. (A) 17 α -Hydroxylase activity. E7 embryos from timed pregnancies were removed from the deciduae, or entire E5-E6 decidual tissues containing embryos were incubated with [¹⁴C]pregnenolone, NADPH, and cyanoketone for 1, 2, and 4 h, and the resulting steroids were analyzed by thin-layer chromatography and phosphorimaging in comparison to the migration of known standards. DHP, dihydroprogesterone. (B) C_{17,20}-lyase activity. E7 embryos (*n* = 10) from timed pregnancies were removed from the decidua and incubated with 17-OH-[³H]progesterone, NADPH, and finasteride for 1 and 3 h. Rat ovarian microsomal protein (Ov; 25 μ g) and E7 decidual homogenate (Decid; 25 μ g of protein) were used as controls. The resulting steroids were analyzed by thin-layer chromatography and phosphorimaging in comparison to the migration of known standards.

gous pregnant mice failed to produce any live-born mice that had the knockout genotype. Drinking is intermittent, and DHEA is highly soluble, suggesting a short circulating half-life; hence, we also provided a constant delivery of DHEA by implanting timed-release pellets of DHEA under the skin of timed pregnant heterozygous mice at E4.5 or E3.5. Embryos were analyzed at E9.5 to E11.5 to determine if the DHEA supplementation had rescued any P450c17^{-/-} embryos for 2 to 4 days past their usual time of demise at E7. We used various doses of DHEA in timed-release pellets and analyzed the embryos by Southern blotting or PCR. No dose of DHEA tested was able to rescue P450c17^{-/-} mice at E11 (Table 2). As it was not clear initially if wild-type E7 embryos exhibited the 17,20-lyase activity of P450c17 in addition to the 17 α -hydroxylase activity, we attempted to rescue knockout mice by implanting pellets containing 17-OH pregnenolone at E4.5 and analyzed

embryos at E9.5 to E11.5 as before. Analysis of 18 embryos from pregnant dams treated with 2.5 mg of 17-OH pregnenolone per 21-day release pellet and 10 embryos from pregnant dams treated with 5.0 mg of 17-OH pregnenolone per 21-day release pellet detected no P450c17^{-/-} mice (Table 2). Thus, maternally administered DHEA, androstenedione, and 17-OH pregnenolone could not rescue the lethal P450c17^{-/-} phenotype.

To determine if potential accumulation of steroids that are substrates for P450c17 contributed to the embryonic death of P450c17^{-/-} mice, we implanted high doses of pregnenolone or progesterone (5 mg of steroid/21-day release pellet) into pregnant wild-type C57BL/6 mice at day 4.5 of pregnancy and analyzed embryo number and morphology at E11. Mice treated with either pregnenolone or progesterone had normal-size litters at E11 (eight \pm two embryos/litter in both steroid-

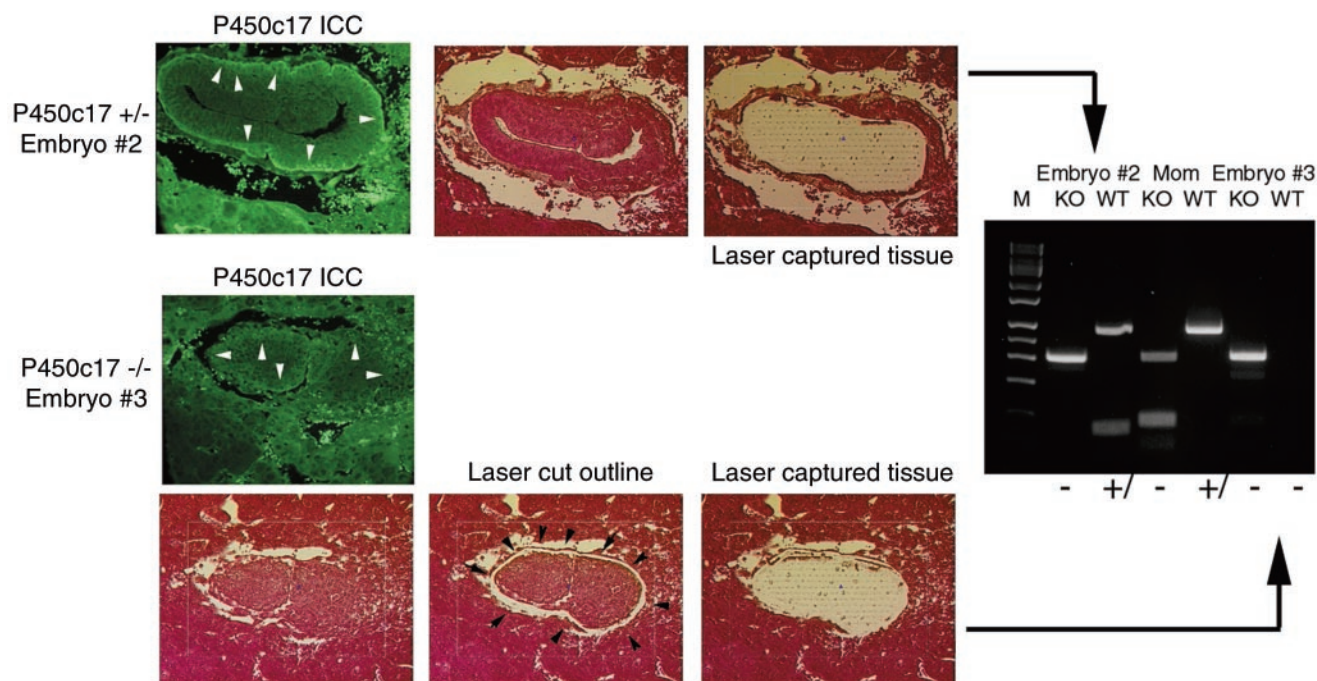


FIG. 4. Laser catapult microdissection of mouse embryo sections. Embryos from timed pregnancies were fixed, sectioned, and analyzed for P450c17 expression by immunocytochemistry (left panels). Embryos that were not immunostained (indicated by white arrowheads), as well as control embryos that were positively immunostained (indicated by black arrowheads), were counterstained with hematoxylin and eosin and collected by laser catapult microdissection (middle panels). Black arrowheads indicate the laser cut outline that was made to ensure that no maternal tissue was collected. DNA was prepared from the tissue and analyzed by PCR amplification (right panels). The size of the wild-type (WT) allele DNA fragment is 500 bp, and the size of the knockout (KO) allele DNA fragment is 270 bp. M, markers.

treated mice versus eight \pm one embryos/litter in untreated or placebo-treated mice) that appeared to be identical in morphology to those of untreated or placebo-treated mice. Thus, the cause of P450c17^{-/-} embryonic death does not appear to be due to the toxicity of pregnenolone or progesterone that may accumulate in P450c17^{-/-} embryos.

DISCUSSION

We created P450c17 gene-knockout mice to study the function of DHEA and DHEAS in the developing rodent nervous system. However, we unexpectedly found that P450c17 is expressed and is catalytically active in the early embryonic endoderm, a site not previously known to express steroido-

genic enzymes, and we found that mice lacking P450c17 die by E7. The embryonic lethality was not due to inadvertent deletion of another gene overlapping the P450c17 locus. Analysis of the mouse genome database indicates that no other genes are located within at least 500 kb of the mouse P450c17 gene.

Steroid hormones are essential for reproduction and for the maintenance of pregnancy, but the sources and roles of these hormones vary dramatically among various mammalian species. Estrogen and progesterone are necessary to establish and sustain pregnancy in mammals (1, 31, 42). Steroid synthesis in pregnancy involves the maternal ovarian corpus luteum, the placenta, and, in some species, the fetus as well. Progesterone is required to suppress uterine contractility and maintain pregnancy (20, 29, 38, 40). In human beings, a luteal-placental shift occurs by the end of the first trimester, and the maternal ovary is no longer necessary after this time (42). In rabbits, no fetal or placental steroidogenesis is needed, as spontaneously occurring knockouts of the gene for P450scc develop normally, survive to term, and die shortly thereafter from adrenal insufficiency (37, 46). In rodents, both maternal ovarian and fetal placental steroidogenesis were thought to be necessary throughout pregnancy (reviewed in reference 8). However, mouse fetal steroidogenesis appears not to be necessary, as P450scc^{-/-}-knockout mice survive to term like the rabbit and die shortly thereafter from adrenal insufficiency (24). We also found that P450scc is expressed in the embryo at E7, in the same region as P450c17 (data not shown). Therefore, if the mouse embryo normally expresses P450scc, and if embryonic and placental P450scc activities are indeed necessary for main-

TABLE 2. Steroid rescue of P450c17^{-/-} embryos^a

Steroid	Dose (mg/21-day release)	Gestational day at pellet implantation	Result
DHEA	2.5	E3.5	No ^{-/-}
	0.025	E3.5	No ^{-/-}
	0.025	E5.5	23 embryos; no ^{-/-}
	0.001	E4.5	10 embryos; no ^{-/-}
	0.01	E4.5	17 embryos; no ^{-/-}
	0.025	E4.5	18 embryos; no ^{-/-}
17-OH-pregnenolone	2.5	E4.5	17 embryos; no ^{-/-}
	2.5	E4.5	18 embryos; no ^{-/-}
	5	E4.5	10 embryos; no ^{-/-}

^a All mice carrying embryos were sacrificed at day E11.5.

tenance of pregnancy, it is possible that P450scc^{-/-} mice might be able to survive by using pregnenolone provided by the mother as the precursor for further steroid hormone production. P450c17 is expressed in several layers of the developing rodent placenta (47), although it is not expressed in the human placenta (6). However, the rodent placenta does not produce C₁₉ steroids before day 11 due to the lack of P450c17 mRNA, protein, and enzyme activity (19, 45, 47). The P450c17-knockout mice die 4 days before this, at a time when the embryo has implanted but may not yet be placentally dependent. Thus, it is unlikely that placental failure due to lack of steroidogenesis is the cause of fetal demise in P450c17-knockout mice.

In addition to placental expression of steroidogenic enzymes, the mouse uterine decidua also expresses P450scc and 3 β -hydroxysteroid dehydrogenase type VI and produces progesterone (2). These data suggest that, during early phases of pregnancy, local progesterone synthesis in the maternal decidua and the trophoblast layers surrounding the embryonic cavity is important for successful implantation and/or maintenance of pregnancy and that local production of progesterone may act as a local immunosuppressant, preventing rejection of the fetus. However, while the decidua may produce progesterone, it is a maternal tissue that would not be affected in the P450c17^{+/-} mothers, and no role for decidual steroids produced by P450c17 has been shown. Indirect evidence suggests that the rabbit, rat, and mouse blastocyst must produce estradiol in order to implant (16, 17). If so, this could explain the embryonic lethality in P450c17^{-/-} mice. However, this mechanism is not generally true for other mammals, and it is not compatible with the survival of P450aro^{-/-} mice to term. Human beings with homozygous defects that destroy all P450c17 protein activity or P450c17 gene expression develop normally and survive to adulthood, having a phenotype of sexual infantilism; sex reversal in 46, XY genetic males; and relatively mild disorders of adrenal steroid production (reviewed in reference 3). Thus, the lethal phenotype of P450c17^{-/-} mice is unexpected and unprecedented, vividly highlighting the profoundly different strategies employed by different mammalian orders in handling the endocrinology of pregnancy and development.

The steroidogenic activity of P450c17 requires two proteins, P450c17 and its obligatory electron transfer partner cytochrome P450 reductase (*Cpr*). *Cpr*^{-/-} mice are also embryonic lethal but not until E9.5 (35), well after the death of P450c17^{-/-} mice. These observations suggest that P450c17 may have another, heretofore unanticipated activity, other than as a P450 enzyme.

The events occurring in the embryo following implantation and prior to gastrulation are now being established. After implantation, the embryo begins to generate an anteroposterior axis. This axis may be formed using cues from the extraembryonic tissues or from the mother. While the location of the primitive streak and subsequent gastrulation of the embryo is crucial for the orientation of both the anteroposterior and dorsoventral axes, signals from the embryonic endoderm prior to gastrulation have been shown to be important for initiation of the anteroposterior axis (reviewed in references 6 and 7). These signals may be initiated by several transcription factors such as *GATA4*, *Otx2*, *lim1*, *HNF-3 β* , and *Hesx1*. Other factors expressed in the embryonic endoderm at ~E6 include *gooseoid*, *nodal*, *cerberus-related 1*, and transforming growth factor β .

GATA4^{-/-}, *lim1*^{-/-}, *Otx2*^{-/-}, and *nodal* embryos fail to gastrulate and die at about E6 to E7. As P450c17 is expressed and is enzymatically active in the embryonic endoderm about this time, its potential steroid products may be additional factors that play a role in the cell movement in the embryonic endoderm and in the generation of the anteroposterior axis of the embryo.

ACKNOWLEDGMENTS

We thank Juanito Meneses for electroporation of ES cells and blastocyst injection and Ron Ferrando and Prescott Woodruff for performing the laser catapult microdissection.

This work was supported from grants from the NIH (HD27970), the NSF (0090995), the March of Dimes, and The Ob, Gyn Research and Education Foundation to S.H.M.

REFERENCES

- Albrecht, E. D., and G. J. Pepe. 1990. Placental steroid hormone biosynthesis in primate pregnancy. *Endocr. Rev.* **11**:124–150.
- Arensburg, J., A. H. Payne, and J. Orly. 1999. Expression of steroidogenic genes in maternal and extraembryonic cells during early pregnancy in mice. *Endocrinology* **140**:5220–5232.
- Auchus, R. J., and M. K. Gupta. 2002. Towards a unifying mechanism for CYP17 mutations that cause isolated 17,20-lyase deficiency. *Endocr. Res.* **28**:443–447.
- Auchus, R. J., T. C. Lee, and W. L. Miller. 1998. Cytochrome b5 augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *J. Biol. Chem.* **273**:3158–3165.
- Auchus, R. J., and W. L. Miller. 1999. Molecular modeling of human P450c17 (17 α -hydroxylase/17,20-lyase): insights into reaction mechanisms and effects of mutations. *Mol. Endocrinol.* **13**:1169–1182.
- Beddington, R. S., and E. J. Robertson. 1998. Anterior patterning in mouse. *Trends Genet.* **14**:277–284.
- Beddington, R. S., and E. J. Robertson. 1999. Axis development and early asymmetry in mammals. *Cell* **96**:195–209.
- Ben-Zimra, M., M. Koler, N. Melamed-Book, J. Arensburg, A. H. Payne, and J. Orly. 2002. Uterine and placental expression of steroidogenic genes during rodent pregnancy. *Mol. Cell. Endocrinol.* **187**:223–231.
- Brentano, S. T., J. Picado-Leonard, S. H. Mellon, C. C. D. Moore, and W. L. Miller. 1990. Tissue-specific, cyclic adenosine 3',5'-monophosphate-induced, and phorbol ester-repressed transcription from the human P450c17 promoter in mouse cells. *Mol. Endocrinol.* **4**:1972–1979.
- Buczko, E., Y. C. Koh, Y. Miyagawa, and M. L. Dufau. 1995. The rat 17 α -hydroxylase-17,20-desmolase (CYP17) active site: computerized homology modeling and site-directed mutagenesis. *J. Steroid Biochem. Mol. Biol.* **52**:209–218.
- Chung, B. C., J. Picado-Leonard, M. Hanu, M. Bienkowski, P. F. Hall, J. E. Shively, and W. L. Miller. 1987. Cytochrome P450c17 (steroid 17 α -hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proc. Natl. Acad. Sci. USA* **84**:407–411.
- Compagnone, N. A., A. Bulfone, J. L. R. Rubenstein, and S. H. Mellon. 1995. Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. *Endocrinology* **136**:5212–5223.
- Compagnone, N. A., and S. H. Mellon. 1998. Dehydroepiandrosterone: a potential signalling molecule for neocortical organization during development. *Proc. Natl. Acad. Sci. USA* **95**:4678–4683.
- Compagnone, N. A., and S. H. Mellon. 2000. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front. Neuroendocrinol.* **21**:1–58.
- DiBlasio, A. M., R. Voutilainen, R. B. Jaffe, and W. L. Miller. 1987. Hormonal regulation of messenger ribonucleic acids for P450scc (cholesterol side-chain cleavage enzyme) and P450c17 (17 α -hydroxylase/17,20-lyase) in cultured human fetal adrenal cells. *J. Clin. Endocrinol. Metab.* **65**:170–175.
- Dickmann, Z., S. K. Dey, and J. S. Gupta. 1975. Steroidogenesis in rabbit preimplantation embryos. *Proc. Natl. Acad. Sci. USA* **72**:298–300.
- Dickmann, Z., J. S. Gupta, and S. K. Dey. 1977. Does "blastocyst estrogen" initiate implantation? *Science* **195**:687–688.
- Dolganov, G. M., P. G. Woodruff, A. A. Novikov, Y. Zhang, R. E. Ferrando, R. Szubin, and J. V. Fahy. 2001. A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na⁺-K⁺-Cl⁻ cotransporter (NKCC1) in asthmatic subjects. *Genome Res.* **11**:1473–1483.
- Durkee, T. J., M. P. McLean, D. B. Hales, A. H. Payne, M. R. Waterman, I. Khan, and G. Gibori. 1992. P450(17 α) and P450SCC gene expression and regulation in the rat placenta. *Endocrinology* **130**:1309–1317.
- Fujii, E., and S. H. Mellon. 2001. Regulation of uterine gamma-aminobutyric acid(A) receptor subunit expression throughout pregnancy. *Endocrinology* **142**:1770–1777.

21. Geller, D. H., R. J. Auchus, B. B. Mendonca, and W. L. Miller. 1997. The genetic and functional basis of isolated 17,20-lyase deficiency. *Nat. Genet.* **17**:201–205.
22. Givens, C., P. Zhang, S. Bair, and S. Mellon. 1994. Transcriptional regulation of rat cytochrome P450c17 expression in mouse Leydig MA-10 and adrenal Y-1 cells: identification of a single protein that mediates both basal and cAMP-induced activities. *DNA Cell Biol.* **13**:1087–1098.
23. Greco, T. L., and A. H. Payne. 1994. Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 β -hydroxysteroid dehydrogenase, P450 17 α -hydroxylase/C17–20 lyase, and P450 aromatase in fetal mouse gonads. *Endocrinology* **135**:262–268.
24. Hu, M. C., N. C. Hsu, N. B. El Hadj, C. I. Pai, H. P. Chu, C. K. Wang, and B. C. Chung. 2002. Steroid deficiency syndromes in mice with targeted disruption of Cyp11a1. *Mol. Endocrinol.* **16**:1943–1950.
25. Johnson, D., and M. Sen. 1990. The cytochrome P450c17 α (17 α -hydroxylase/C17,20 lyase) activity of the junctional zone of the rat placenta. *J. Endocrinol.* **125**:217–224.
26. Karishma, K. K., and J. Herbert. 2002. Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced suppression. *Eur. J. Neurosci.* **16**:445–453.
27. Kimonides, V. G., N. H. Khatibi, C. N. Svendsen, M. V. Sofroniew, and J. Herbert. 1998. Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. *Proc. Natl. Acad. Sci. USA* **95**:1852–1857.
28. Lin, D., S. M. Black, Y. Nagahama, and W. L. Miller. 1993. Steroid 17 α -hydroxylase and 17,20-lyase activities of P450c17: contributions of serine 106 and of P450 reductase. *Endocrinology* **132**:2498–2506.
29. Lofgren, M., J. Holst, and T. Backstrom. 1992. Effects in vitro of progesterone and two 5 α -reduced progestins, 5 α -pregnane-3,20-dione and 5 α -pregnane-3 α -ol-20-one, on contracting human myometrium at term. *Acta Obstet. Gynecol. Scand.* **71**:28–33.
30. Mellon, S. H., and C. Vaisse. 1989. cAMP regulates P450scc gene expression by a cycloheximide-insensitive mechanism in cultured mouse Leydig MA-10 cells. *Proc. Natl. Acad. Sci. USA* **86**:7775–7779.
31. Miller, W. L. 1998. Steroid hormone biosynthesis and actions in the maternal-feto-placental unit. *Clin. Perinatol.* **25**:799–817.
32. Miyashita-Lin, E. M., R. Hevner, K. M. Wassarman, S. Martinez, and J. L. Rubenstein. 1999. Early neocortical regionalization in the absence of thalamic innervation. *Science* **285**:906–909.
33. Nakajin, S., P. F. Hall, and M. Onoda. 1981. Testicular microsomal cytochrome P-450 for C21 steroid side chain cleavage. Spectral and binding studies. *J. Biol. Chem.* **256**:6134–6139.
34. Nakajin, S., J. E. Shively, P. M. Yuan, and P. F. Hall. 1981. Microsomal cytochrome P-450 from neonatal pig testis: two enzymatic activities (17 α -hydroxylase and c17,20-lyase) associated with one protein. *Biochemistry* **20**:4037–4042.
35. Otto, D. M., C. J. Henderson, D. Carrie, M. Davey, T. E. Gundersen, R. Blomhoff, R. H. Adams, C. Tickle, and C. R. Wolf. 2003. Identification of novel roles of the cytochrome P450 system in early embryogenesis: effects on vasculogenesis and retinoic acid homeostasis. *Mol. Cell. Biol.* **23**:6103–6116.
36. Ozols, J., F. S. Heinemann, and E. F. Johnson. 1981. Amino acid sequence of an analogous peptide from two forms of cytochrome P-450. *J. Biol. Chem.* **256**:11405–11408.
37. Pang, S., X. Yang, M. Wang, R. Tissot, M. Nino, J. Manaligod, L. P. Bullock, and J. I. Mason. 1992. Inherited congenital adrenal hyperplasia in the rabbit: absent cholesterol side-chain cleavage cytochrome P450 gene expression. *Endocrinology* **131**:181–186.
38. Perusquia, M., and J. Jasso-Kamel. 2001. Influence of 5 α - and 5 β -reduced progestins on the contractility of isolated human myometrium at term. *Life Sci.* **68**:2933–2944.
39. Picado-Leonard, J., and W. L. Miller. 1987. Cloning and sequence of the human gene for P450c17 (steroid 17 α -hydroxylase/17,20 lyase): similarity to the gene for P450c21. *DNA* **6**:439–448.
40. Pulkkinen, M. O. 1970. Regulation of uterine contractility. *Acta Obstet. Gynecol. Scand.* **1**(Suppl. 1):19–41.
41. Rubenstein, J. L., S. Anderson, L. Shi, E. Miyashita-Lin, A. Bulfone, and R. Hevner. 1999. Genetic control of cortical regionalization and connectivity. *Cereb. Cortex* **9**:524–532.
42. Strauss, J. F., III, F. Martinez, and M. Kiriakidou. 1996. Placental steroid hormone synthesis: unique features and unanswered questions. *Biol. Reprod.* **54**:303–311.
43. Tybulewicz, V. L., M. L. Tremblay, M. E. LaMarca, R. Willemsen, B. K. Stubblefield, S. Winfield, B. Zablocka, E. Sidransky, B. M. Martin, S. P. Huang, et al. 1992. Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature* **357**:407–410.
44. Voutilainen, R., J. Tapanainen, B. Chung, K. J. Matteson, and W. L. Miller. 1986. Hormonal regulation of P450scc (20,22 desmolase) and P450c17 (17 α -hydroxylase/17,20 lyase) in cultured human granulosa cells. *J. Clin. Endocrinol. Metab.* **63**:202–207.
45. Warshaw, M. L., D. C. Johnson, I. Khan, B. Eckstein, and G. Gibori. 1986. Placental secretion of androgens in the rat. *Endocrinology* **119**:2642–2648.
46. Yang, X., K. Iwamoto, M. Wang, J. Artwohl, J. I. Mason, and S. Pang. 1993. Inherited congenital adrenal hyperplasia in the rabbit is caused by a deletion in the gene encoding cytochrome P450 cholesterol side-chain cleavage enzyme. *Endocrinology* **132**:1977–1982.
47. Yokoi, H., Y. Tsuruo, M. Nihira, and K. Ishimura. 1998. Light and electron microscopic immunocytochemistry on the localization of 17 α -hydroxylase/C17,20-lyase (P450c17) in the rat placenta. *J. Med. Investig.* **44**:155–162.
48. Zuber, M. X., E. R. Simpson, and M. R. Waterman. 1986. Expression of bovine 17 α -hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS 1) cells. *Science* **234**:1258–1261.