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Expression of cytochrome P450scc mRNA and protein in the rat kidney from birth to adulthood

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Abstract

The expression of cytochrome P450scc, encoded by the CYP11A gene, was investigated in the rat kidney from birth to adulthood. In the male and female rat kidneys, the corresponding mRNA was detected by semi-quantitative reverse transcriptase and polymerase chain reaction (RT-PCR) analysis with specific primers, resulting in higher levels of expression during the first 15 days from birth.

RT-PCR and sequence analysis showed that the P450scc mRNA coding region was the same for both kidney and testis, whereas 5′-RACE analysis (rapid amplification of cDNA ends) demonstrated that the renal transcription utilizes a distal transcription start site (TSS) located 76 b upstream of that used in ovarian and testicular P450scc mRNA expression, which is placed 43 b upstream of the first ATG. The 5′-UTR sequence of renal P450scc cDNA exactly matched the contiguous upstream untranslated region of the gene, suggesting that alternative splicing was not involved in the synthesis of this transcript.

Northern hybridization detected a specific transcript only in the newborn male, but not in adult rat kidney, confirming the higher levels of expression in the first days of the rat's life. Positive immunodetections of cytochrome P450scc were found in renal cortical distal tubules and the results were confirmed by Western blotting analysis.

As demonstrated by semi-quantitative RT-PCR, the male kidney also expresses the messengers corresponding to the steroidogenic acute regulatory (StAR) and steroidogenic factor 1 (SF-1) proteins, which are normally required for steroidogenesis in steroidogenic tissues, such as gonads and adrenal cortex. These studies suggest that the rat kidney has the capability for local steroid hormone production, although the physiological significance of the pregnenolone eventually produced remains to be established.

Keywords: Cytochrome P450scc; Extraglandular steroidogenesis; Rat kidney

1. Introduction

The biosynthesis of steroid hormones is catalyzed by at least five different forms of cytochrome P450 and some hydroxysteroid dehydrogenases (HSD). The cleavage of the cholesterol side-chain to form pregnenolone is the first and rate-limiting step in the steroid hormone biosynthetic pathway in all steroidogenic tissues. This reaction occurs in the inner mitochondrial membrane and requires the mitochondrial cholesterol side-chain cleavage enzyme system formed by ferrodoxin reductase, ferrodoxin and cytochrome P450scc [1]. The first two enzymes act as intermediates in the transfer of electrons from NADPH to P450scc and are distributed in the mitochondria of both steroidogenic and non-steroidogenic tissues, including kidney [2]. The latter enzyme utilizes three pairs of electrons to catalyze three separate mono-oxygenations at a single active site: the first at the 22R position of cholesterol, the second at the 20α position, while the third causes the scission of the C20–C22 bond, yielding pregnenolone and isocaproic acid [1]. Transfer of cholesterol substrate into the inner mitochondrial membrane, where the cytochrome P450scc resides, is facilitated by the steroidogenic acute regulatory (StAR) protein [3], that is essential for efficient adrenal and gonadal steroidogenesis.

Southern blotting analysis of rat genomic DNA suggests the presence of a single P450scc gene (CYP11A) [4], as in humans [1], where the gene spans at least 20 kb and is divided into 9 exons.

The nuclear gene coding P450scc was regarded as being expressed specifically in steroidogenic endocrine cells [4]. An extraglandular occurrence of this enzyme, however, has been demonstrated, in the past, in rat submandibular glands [5], that were shown to convert acetate into cholesterol, which was further transformed into pregnenolone. More recently, the expression of P450scc has been detected in rat brain oligodendrocytes: pregnenolone formed from

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cholesterol is converted into 5α-pregnane neurosteroids acting on GABAergic neurons [6]. Similarly, Iwahashi et al. [7] demonstrated P450scc activity selectively localized in the myelinated region of the rat cortical white matter. In addition, P450scc expression has been detected in the human and mouse skins [8–10], in the mouse hindgut [10], in the human heart [11] and in the human pancreas [12].

The mammalian kidney shares the same embryonic origin from intermediate mesoderm with the steroidogenic cells of the gonads and mammalian cortex. Available information, however, attributes only complementary steroidogenic capabilities to this organ, including: terminal activation of 25-hydroxycholecalciferol by 1α-hydroxylation; low levels of 21-hydroxylase activity [13], probably involved in 11-deoxycorticosterone formation from circulating pregnenes; activities of the enzymes 3β-hydroxysteroid dehydrogenase (HSD)/Δ^5–Δ^4 isomerase (3β-HSD) [14,15], steroid 17α-hydroxylase/C-17,20 lyase and 20α-HSD in the kidney of aged rats [15] and in the human kidney [16].

Although the kidney is not considered a steroidogenic tissue, small amounts of P450scc mRNA were detected in the rat kidney [2] and P450scc immunoreactivity was found in the kidney of 15.5- and 19.5-day-old embryos [17]. Therefore, we have investigated whether this organ could be the site of autonomous steroidogenesis at some stages of the rat life cycle.

2. Materials and methods

2.1. Tissue preparation and RNA extraction

Samples of kidney were collected from 0-, 7-, 15-, 30- and 60-day-old Wistar strain male and female rats. Ovary, adrenal, testis and spleen were taken from adult rats. The animals were killed by cervical dislocation after CO_2-induced asphyxia. Tissues were immediately dissected out, frozen in liquid nitrogen and stored at −80 °C until analyzed. Total RNA was extracted using the commercial product RNAzolB (Celbio, Italy) according to the manufacturer's instructions. The RNA samples were kept at −80 °C until use.

2.2. Primers

Specific oligonucleotide primers (Table 1) were selected from the published coding sequences of rat ovary P450scc, StAR, steroidogenic factor 1 (SF-1) and 18*S* rRNA. All primers utilized, except 18*S* rRNA, were designed to span known introns, revealing any contaminating genomic DNA as larger sized PCR fragments.

2.3. Reverse transcriptase and polymerase chain reaction (RT-PCR)

To analyze the possibility of renal P450scc mRNA expression, total RNA (2 μg) extracted from the kidney of adult male rats was reverse-transcribed into single-stranded cDNA by incubation with 25 U of MMLV reverse transcriptase and 10 U of RNase inhibitor (Applied Biosystems, Italy) in a 20 μl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP and 1.25 μM SCC2 primer for 60 min at 42 °C.

Total single-stranded cDNA obtained by RT was amplified by PCR in a 100 μl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of SCC2 and SCC1 primers and 1.25 U of *Taq* DNA polymerase (Applied Biosystems). Amplification conditions were: 95 °C for 45 s (DNA denaturation), 60 °C for 30 s (annealing), and 72 °C for 40 s (extension) for 40 cycles. The extension phase of the last cycle was prolonged by 10 min.

Moreover, after the first positive result, total RNA from the kidney of male adult rats was reverse-transcribed and amplified by PCR with two more sets of primers encompassing

Table 1
Primers used in RT-PCR and 5′- and 3′-RACE analyses

Primer		Sequences	Position[a]	GenBank no.
SCC1	Sense	5′-GGGGACATCAGCGATGACCTATT-3′	600 → 622	J05156
SCC2	Antisense	5′-GGGGTGGAGTCTCAGTGTCTCCTT-3′	1178 → 1155	J05156
SCC3	Sense	5′-GTGGCAGTCGTGGGGACAGTATG-3′	1 → 23	J05156
SCC4	Antisense	5′-GAGCATGGGAACACTGGTGTGGA–3′	757 → 736	J05156
SCC5	Sense	5′-CACGACCTCCATGACTCTGCAAT-3′	998 → 1020	J05156
SCC6	Antisense	5′-CTTGGTCCCCACATCACGGATAC-3′	1499 → 1477	J05156
SCC7	Sense	5′-AATCCAGCTTCTTTCCCA-3′	1285 → 1302	J05156
SCC8	Sense	5′-CCGTGATGTGGGGACCAAG-3′	1477 → 1499	J05156
SCC9	Antisense	5′-TAATGGATTCTGTGTGTG-3′	241 → 224	J05156
SCC10	Antisense	5′-GTTTATCCAACCATTGTCACCA-3′	197 → 176	J05156
StAR1	Sense	5′-CCAGGAGCTGTCCTACATCCAG-3′	266 → 287	NM_031558
StAR2	Antisense	5′-TACTACCCCTCTCGTTGTCCT-3′	1011 → 991	NM_031558
SF-1-1	Sense	5′-GGTGTCGGGCTACCACTACGGG-3′	755 → 777	D42152
SF-1-2	Antisense	5′-TGAAGCCATTGGCCCGAATCTG-3′	127 → 106	D42153
18S-1	Sense	5′-TCGAACGTCTGCCCTATCAACT-3′	347 → 368	V01270
18S-2	Antisense	5′-AGACTTGCCCTCCAATGGATC-3′	611 → 591	V01270

[a] Nucleotide position in the reported sequence.

Table 2
Target gene, PCR primers and conditions used in the semi-quantitative RT-PCR analyses

Target gene	Primers	Annealing (°C)	Extension time (s)	PCR cycle (*n*)
P450scc	SCC1–SCC2	60	30	38
StAR	StAR1–StAR2	60	30	38
SF-1	SF-1-1–SF-1-2	60	20	38
18*S* RNA	18S-1–18S-2	60	20	14

at the end the region −20/+1479 nt of P450scc cDNA (SCC3 and SCC4; SCC5 and SCC6).

To perform a semi-quantitative evaluation of RT-PCR products, we used total RNA from kidney tissues of newborn and 7-, 15-, 30- and 60-day-old rats of both sexes and from ovary of adult rats as a positive control. Decreasing amounts (1 μg, 100 and 10 ng) of RNA from all tissues were simultaneously subjected to RT-PCR with the SCC1 and SCC2 pair of primers, as described above. The analysis was performed twice. An aliquot (1/10) of the products was run in 1% agarose gel and stained with ethidium bromide; the fluorescence was then compared under UV light.

In the following experiments, another approach to a semi-quantitative evaluation of RT-PCR products was adopted and used to analyze the P450scc, StAR, SF-1 and 18*S* rRNA expression in total RNA extracted from male kidney tissues of newborn and 7-, 15-, 30- and 60-day-old rats. Ovary and testis were used as positive controls and spleen as a negative control. In this case, in preliminary experiments, each primer set was used to amplify equal amounts of cDNA derived from the testis, a tissue with high levels of specific messenger, for 10–40 cycles, and, based on these analyses, a predetermined number of cycles was chosen for each primer set to maintain product accumulation in the linear range. Primer sets and parameters of the reactions are listed in Table 2. The subsequent analyses were performed with different quantities (ranging from 200 ng for steroidogenic tissues to 1 μg for renal and splenic tissues) of total RNA. For these analyses, we used the SuperScript One-Step RT-PCR System (Invitrogen, Italy), in which all components for RT-PCR are mixed in one tube and reverse transcription is automatically followed by PCR cycling without any additional steps. Quantification was performed by measuring the relative intensity of the band stained by ethidium bromide, after agarose gel electrophoresis, using the Quantity One Quantitation software (Bio-Rad, Italy). The analyses were performed twice. For each RT-PCR, a negative control was prepared by using all reagents except RNA solution, that was substituted with an equivalent volume of sterile water to check for cross-contamination.

2.4. 3′- and 5′-RACE analyses of cytochrome P450scc mRNA

Determination of the 5′- and 3′-ends was performed by the so-called cap-finder (CF) cDNA approach introduced by Clontech [18] and following the indications of Franz et al. [19]. First-strand cDNA was synthesized by incubating 2 μg of total RNA extracted from 7- and 60-day-old male rat kidneys and adult rat testis in a 25 μl reaction volume containing 200 U of Superscript II reverse transcriptase (Invitrogen), 50 mM Tris–HCl (pH 8.3), 8 mM $MgCl_2$, 1 mM of dNTPs, 10 μM DTT and 1 μM oligodT primer (oligodT*Sal*IA: 5′-CTG CGC CAG AAT TGG CAG GTC CAG $(T)_{25}$G/A/C-3′) at 48 °C for 1 h. The tagging of the 3′-end of the first-strand cDNA is achieved by addition of the CF primer (CFB1: 5′-GAG AGA ACG CGT GAC GAG AGA CTG ACA GGG GGG GGA/T/C-3′) to the reaction mixture [19]. Following reverse transcription, the first-strand cDNA has known sequences at both the 5′- and 3′-terminal ends and could be used directly in 5′- and 3′-RACE (rapid amplification of cDNA ends) PCR reactions.

For 3′-RACE reaction, 2 μl of the first-strand mixture were added to 50 μl of the PCR buffer containing 200 μM of dNTPs, 2 mM $MgCl_2$, 0.2 μM of the anchor primer (oligodT*Sal*IB: 5′-CTG CGC CAG AAT TGG CAG GTC GAC-3′), 2.5 U of Biotherm *Taq* polymerase (Società Chimici Italiani, Italy), and the gene-specific SCC7 primer. The first-round PCR product was diluted 1:100 and used as a template for the second round of PCR amplifications using the SCC8 primer and the PCR anchor primer. The amplification procedure consisted of 2 min at 95 °C followed by a touch-down PCR reaction with annealing temperatures decreasing from 66 °C to 58 °C over 16 cycles and the final 24 cycles maintained at 58 °C. The extension phase of the last cycle was prolonged by 10 min. The resultant amplicon was purified from the sliced gel band and directly sequenced. All gene-specific oligonucleotide primers used for the 3′-RACE analysis are listed in Table 1.

The site of transcription initiation was determined by 5′-RACE analysis. This was carried out as described for the 3′-RACE using the gene-specific primer SCC9 and the anchor primer CFB2 (5′-GAG AGA ACG CGT GAC GAG AGA CTG ACA G-3′) in the first PCR reaction. The cDNA obtained was further amplified by a second PCR using the gene-specific SCC10 primer and the anchor primer. The extension phase of the last cycle was prolonged by 10 min. The touch-down PCR conditions and the following steps were the same as for 3′-RACE. All gene-specific oligonucleotide primers used for the 5′-RACE analysis are listed in Table 1.

2.5. Nucleotide sequencing

Sequencing was performed on double-stranded DNA using the ABI PRISM Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems). Electrophoresis of sequencing reactions was completed on the ABI PRISM model 377, version 2.1.1 automated sequencer. The homology searches were carried out using the Basic Blast program version 2.0 at http://www.ncbi.nlm.nih.gov/BLAST/, whereas the alignment was performed using the ClustalW program at http://www2.ebi.ac.uk/clustalw/.

2.6. Northern blotting analysis of cytochrome P450scc

Poly(A)$^+$-enriched RNAs (1 μg) were obtained, using the NucleoTrap mRNA Kit (M-Medical, Italy), from testis, ovary and adrenal of adult rats and from kidneys of newborn and 2-month-old male rats, according to the manufacturer's instructions. The RNAs were electrophoresed through 1.1% formaldehyde-denaturing gel, blotted onto a nylon membrane (Hybond-N$^+$, Amersham Pharmacia, Italy) and baked at 80 °C for 2 h. The DIG-labeled RNA Molecular Weight Marker II (Roche Applied Science, Italy) was used as a size standard. The membranes were hybridized overnight at 68 °C with a 502 nt, single-stranded DIG-labeled cDNA probe encompassing the +978/+1479 coding region of rat ovarian P450scc mRNA in 5× SSC containing 50% formamide, 0.02% SDS, 0.1% lauroylsarcosine, 1% blocking reagent and 100 μg/ml of transfer RNA. After incubation with an anti-DIG antibody, signals were detected using a DIG-nucleotide detection kit (Roche Applied Science) according to the manufacturer's instructions. The signals were revealed by exposing the membranes to an X-ray film for 1 h. After stripping, the nylon membrane was rehybridized with a 207 bp, double-stranded DIG-labeled cDNA probe spanning the +831/+1037 coding region of rat β-actin mRNA, as a control for mRNA integrity. The analysis was performed three times.

2.7. Western immunoblot analysis with an anti-P450scc antibody

The kidney, testis and spleen from male rats were minced and homogenized in a Ultra–turrax homogenizer at 4 °C in the homogenization buffer (100 mM potassium phosphate buffer, pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 4 mM $MgCl_2$, and 2 μg/ml each of leupeptin, pepstatin A and aprotin. After the removal of nuclei and debris by three centrifugations at 1000 × *g* for 15 min at 4 °C, the mitochondrial fractions were pelted by centrifugation at 10,000 × *g* for 45 min at 4 °C. The mitochondrial fractions were suspended in the same buffer and the centrifugation was repeated. Finally, the mitochondrial pellets were suspended in a 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 20% glycerol, 0.5 mM phenylmethylsulfonylfluoride, and 2 μg/ml each of leupeptin, pepstatin A and aprotin.

The presence of cytochrome P450scc in the rat kidney was examined by Western immunoblot analysis with an antibody against amino acids 421–441 of rat P450scc (Chemicon, Italy). The testis and the spleen were used as a positive and a negative control tissue, respectively. Proteins were measured by the BCA protein assay kit (Pierce, Celbio, Italy) with BSA as a standard. Forty μg of renal and splenic mitochondrial proteins and 2–4 μg of corresponding testicular proteins were loaded onto 10% SDS-PAGE (Nupage, Invitrogen, Italy) and transferred onto a nitrocellulose membranes (Roche Applied Science). After an overnight blocking step at 4 °C with 5% BSA in TBS-T buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20), the filter was hybridized with 1/3,000 dilution of the antibody against rat P450scc in TBS-T buffer for 2 h at room temperature. The filter was then washed (3 × 5 min; 1 × 15 min) with TBS-T and further incubated for 1 h at room temperature with 1/35,000 dilution of goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Pierce) in TBS-T. The membrane was washed as described above and HRP activity was detected using the SuperSignal West-Dura Chemiluminescent kit (Pierce) according to the manufacturer's protocol.

2.8. Immunohistochemical analysis with an anti-P450scc antibody

Rat kidneys of newborn and 7- and 60-day-old rats were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3–5 h. Paraplast embedding and sectioning at 4 μm were performed according to standard procedures. Sections were deparaffinized and then endogenous peroxidase activity was eliminated from the sections by incubation with 3% H_2O_2 in absolute methanol for 30 min at room temperature. After blocking of non-specific binding components with 5% normal goat serum and 1% BSA in PBS containing 0.3% Triton X-100 for 1 h at room temperature, the primary antibody (anti-P450scc, 1/800) was overlaid upon each section for 16 h at 4 °C. Sections were subsequently incubated with biotinylated goat anti-rabbit IgG antibody for 30 min, followed by exposure to ExtrAvidin-Peroxidase (Sigma, Italy) for 30 min at room temperature. Immunoreactivity was detected by immersing the tissue sections in AEC solution (Sigma). Haematoxylin was used as a nuclear counterstain. The specificity of immunolabeling was tested by omitting the primary antibody. Sections of ovary, adrenal and testis were used as positive controls.

3. Results

3.1. RT-PCR of cytochrome P450scc

Three fragments of the expected size were consistently detected on agarose gel after RT-PCR with total RNA extracted from male adult kidney and performed with 3 sets of primers encompassing the region −20/+1479 nt of P450scc cDNA (SCC1–SCC2; SCC3–SCC4; SCC5–SCC6). Sequencing in both directions showed complete identity between renal and ovarian amplificates, confirming the existence of a single gene encoding P450scc mRNA in the rat and the possible expression of this cytochrome in the rat kidney.

To characterize the expression profile of this gene in the kidney throughout the rat lifespan, we analyzed the levels of P450scc mRNA by a semi-quantitative RT-PCR approach based on the use of different quantities of total

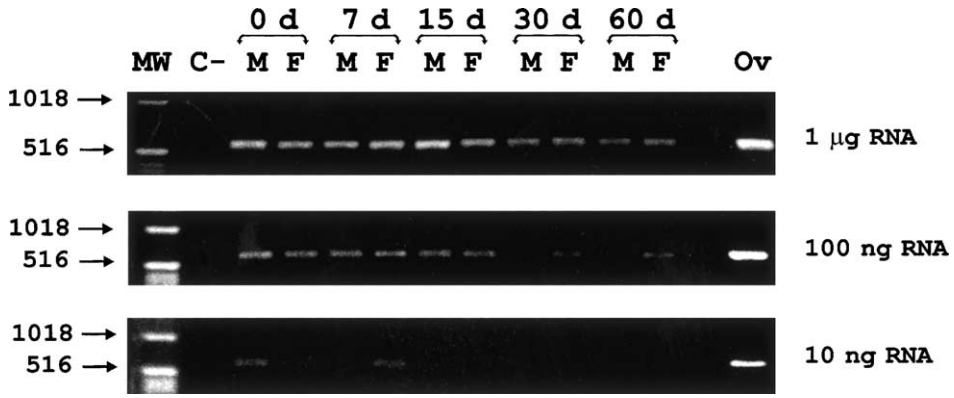

Fig. 1. Representative expression analysis of P450scc mRNA in kidney tissues of newborn and 7-, 15-, 30- and 60-day-old male (M) and female (F) rats and in ovary (Ov) of adult rats, as determined by semi-quantitative RT-PCR performed with different quantities of total RNA: 1 μg, 100 and 10 ng. Amplifications were repeated twice using independently extracted RNA samples from different animals, and indicated the same results. MW: molecular weight; C−: negative control (water).

RNA and the SCC1 and SCC2 pair of primers. Starting with 1 μg of total RNA, both male and female renal tissues at all ages gave a positive signal for P450scc mRNA in the RT-PCR analysis, as evidenced by an amplified fragment of the expected size (579 nt) on agarose gel (Fig. 1). P450scc expression was clearly higher in the ovary, used as a positive control, compared to the renal samples and, among the latter, the highest amplifications were obtained with both male and female samples up to 15 days from birth. This result was confirmed with the amplifications performed with 100 ng of total RNA, as a clear but weak signal is evident in the samples corresponding to the first 15 days, whereas a very faint signal was obtained with RNA from 30- and 75-day-old female rats. Moreover, with 10 ng of total RNA, a very faint signal was obtained only with male and female newborns and with 7-day-old female rats.

These results were further substantiated by the use of another semi-quantitative RT-PCR protocol, in which we analyzed the expression profiles not only of cytochrome P450scc, but also of the StAR and SF-1 proteins that are necessary for pregnenolone production in the gonads and cortical cells of the adrenal. In this case, a predetermined number of cycles was chosen for each primer set to maintain product accumulation in the linear range of PCR amplification. The amplifications of 18*S* rRNA indicated that the quality and quantity of the RNA preparations was similar for all tissues.

With this approach too, the highest signals for renal P450scc mRNA transcription were obtained with RNA samples extracted from animals up to 15 days from birth. The transcript concentration was approximately 200-fold lower then that measured with ovary from adult rats (random days of the cycle) and approximately 100-fold lower than in the testis (Fig. 2, Panel A). The higher expression of P450scc in the ovary than in the testis was also confirmed by the Northern blotting analysis. No amplification was obtained with RNA extracted from male spleen that was used as a negative control.

As regards the rat kidney StAR expression, we initially performed an RT-PCR analysis with total RNA extracted from male adult rats. This sample gave a positive signal, as evidenced by an amplified fragment of the expected size on agarose gel. Fragment identity was confirmed by sequencing. The relative abundance of this transcript in kidney was then examined at different ages. The renal transcripts concentration was approximately 100-fold lower than that in the ovary and approximately 50-fold lower than in the testis (Fig. 2, Panel B). Differently from the P450scc, the StAR analysis gave a positive result with the spleen, although the level of expression was lower than that measured with renal samples.

Finally, we analyzed the expression of SF-1, previously demonstrated in our laboratory at the messenger level in the rat kidney [20]. The analysis shows that all renal samples were positive without any difference between age in terms of transcript abundance. Moreover, a transcript of the expected size was detected also in the spleen (Fig. 2, Panel C).

3.2. 3′- and 5′-RACE analyses

In order to verify if the renal P450scc mRNA corresponds to the messenger transcribed in steroidogenic tissues not only inside the coding region but also in the 3′- and 5′-UTRs, we performed both 3′ and 5′-RACE analyses with RNA extracted from 7- and 60-day-old male rats. The analyses were also carried out using RNA extracted from adult testis as a positive control. In the 3′-RACE analysis, both the renal samples gave an amplified fragment of the same length as did the testis (Fig. 3, Panel A). These fragments were sequenced and found to be identical to that obtained with testis 3′-RACE analysis and those reported in GenBank for rat P450scc cDNA. This result demonstrates the use, in both the testis and the kidney, of the same polyadenylation signal located 155 b after the stop codon.

The 5′-RACE analysis was performed with two different gene-specific 3′-primers to clearly identify the transcription

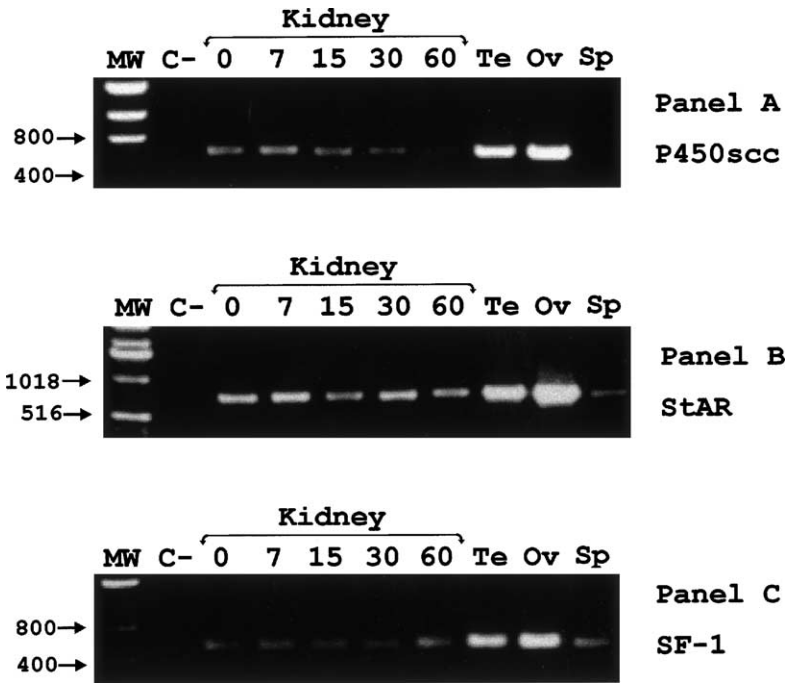

Fig. 2. Representative expression analysis of P450scc (Panel A), StAR (Panel B) and SF-1 (Panel C) mRNAs in kidney tissues of newborn and 7-, 15-, 30- and 60-day-old male rats and in testis (Te), ovary (Ov) and spleen (Sp) of adult rats, as determined by semi-quantitative RT-PCR in duplicate, using independently extracted RNA samples from different animals with the same results. MW: molecular weight; C−: negative control (water).

start site (TSS) used in kidney. We obtained two different amplification fragments with the longer one expressed in both kidney samples (Fig. 3, Panel B). The fragments were sequenced and aligned with the corresponding promoter region of the CYP11A gene (GenBank accession no. M63125). In the 5′-UTR of kidney P450scc mRNA, we found a distal TSS located 76 b upstream of that used in the testicular mRNA, which is placed 43 b upstream from the translation initiator ATG [21,22]. The 5′-UTR sequence of kidney P450scc cDNA exactly matched the contiguous upstream untranslated region of the gene, suggesting that alternative splicing was not involved in the synthesis of this transcript.

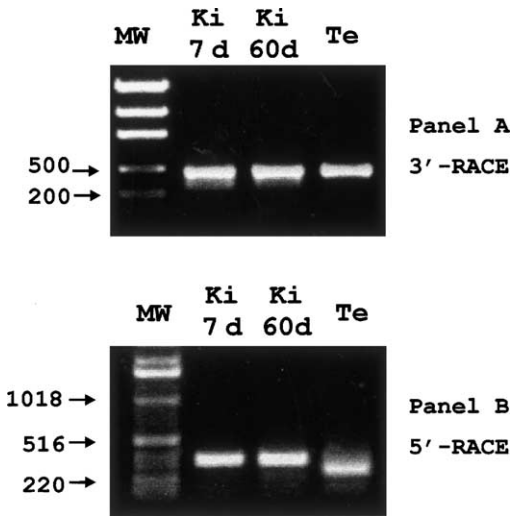

Fig. 3. (Panel A) Agarose gel showing products from 3′-RACE analysis of P450scc mRNA from 7- and 60-day-old rat kidneys (Ki) and from adult rat testis (Te). A single amplified fragment was obtained with all the samples analyzed and subsequently sequenced. (Panel B) Agarose gel showing products from 5′-RACE analysis of P450scc mRNA from 7- and 60-day-old rat kidneys (Ki) and from adult rat testis (Te). Fragments of different sizes were obtained with kidney and testis RNAs. All fragments were directly sequenced. MW: molecular weight.

3.3. Northern blotting

P450scc mRNA expression in kidney and steroidogenic tissues was examined by Northern blotting with poly(A)+ enriched RNA using a single-stranded P450scc cDNA probe. Northern blot analysis revealed a single message size of approximately 2 kb in all steroidogenic tissues, such as adrenal, ovary, and testis, whereas a positive signal for P450scc mRNA was obtained with kidney tissue from newborn but not adult rats (Fig. 4). All samples were positive for β-actin mRNA. The analysis was performed thrice, changing the position of the kidney sample loading to avoid possible contamination with highly positive samples as adrenal.

3.4. Western blotting

The presence of the P450scc enzyme was assessed in the mitochondrial extract from kidney samples by immunoblot analysis using a commercial antibody. As illustrated in

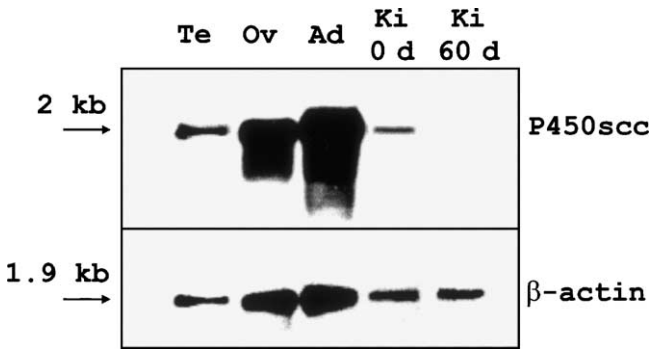

Fig. 4. *Upper panel:* A representative Northern blot analysis of poly(A)+-enriched RNAs extracted from adult rat testis (Te), ovary (Ov), adult female adrenal (Ad), male newborn (Ki 0 d) and adult male rat kidneys (Ki 60 d) with a rat DIG-labeled cDNA P450scc probe. *Lower panel:* Northern blot analysis of the same samples performed with a rat DIG-labeled cDNA β-actin probe.

Fig. 5, a protein band derived from testis as well as kidney samples from all ages, except 60-day-old rats, was recognized by the antibody. The intensity of the immunoreaction in all positive kidney samples was markedly lower than in the testis, also considering that for testis samples only 2 μg (TeA) or 4 μg (TeB) of mitochondrial protein were loaded instead of 40 μg. A higher expression of cytochrome P450scc was found in samples from newborn and 7-day-old animals. Moreover, the protein could not be detected in spleen samples, confirming the negative result obtained with RT-PCR analysis. The inferred molecular weight of the P450scc band was approximately 54 kDa, judging from its electrophoretic mobility.

3.5. Immunohistochemistry

Immunohistochemistry was used to investigate the expression of P450scc enzyme in different regions of the rat kidney. The analysis was performed with sections of kidney together with sections of adrenal, testis and ovary as positive controls. Positive staining for P450scc was obtained mainly in the renal cortex where, on the basis of cell morphology, immunoreactive segments were identified as the distal convoluted tubules. The study was performed with sections obtained from newborn, 7- and 60-day-old male rats, but a clear signal was obtained only with the first two ages (Fig. 6). The immunoreactivity was localized on Leydig cells in the testis, corpus luteum and theca cells in the ovary and cortical adrenal as expected, confirming the specificity of the antibody used.

4. Discussion

The present data shows that the CYP11A gene is expressed both at the messenger and protein levels in the rat kidney, thus confirming the preliminary reports concerning the expression of small amounts of P450scc mRNA in the rat kidney [2] and P450scc immunoreactivity in the kidney of 15.5- and 19.5-day-old embryos [17].

The finding that renal P450scc cDNA, inside the coding region, is identical to the testicular one supports the assumption of a single CYP11A gene in the rat genome, as previously demonstrated by McMasters et al. [4] by Southern blotting analysis.

The levels of P450scc mRNA expression in renal tissues are considerable lower than those in the classical steroidogenic tissues, suggesting a local, paracrine function rather than an endocrine one. Although the physiological significance of steroid production in this organ remains unknown, both semi-quantitative approaches, together with the results obtained by Northern hybridization and Western blotting, suggest a regulated timing of P450scc expression, as the highest levels where found up to 15 days from birth. Differences between sexes were negligible.

By means of 3′- and 5′-RACE analyses, we found that P450scc expression in the rat kidney utilizes the same polyadenylation site as the testis, whereas a different TSS located 76 b upstream of that used in testicular P450scc mRNA, which is placed 43 b upstream of the first ATG, is used in the renal P450scc transcription. No indication for the use of this alternative TSS was obtained with Northern analysis, but this negative result could be explained by the insufficient size difference between the testicular and renal transcripts (only 76 b). This result is very similar to that previously reported for the P450c17 expression in rat extraglandular tissues, where we demonstrated the use of three

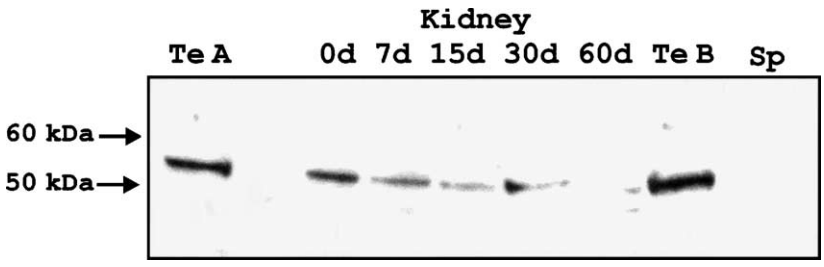

Fig. 5. Western immunoblot analysis of P450scc-like protein in the kidneys of newborn and 7-, 15-, 30- and 60-day-old rats. Each lane contained 40 μg of mitochondrial proteins of the respective tissues, except testis for which 2 μg (TeA) and 4 μg (TeB) of proteins were loaded. Electrophoresis was performed on 10% polyacrylamide gels. Testis and spleen (Sp) of adult rats were used as positive and negative controls, respectively.

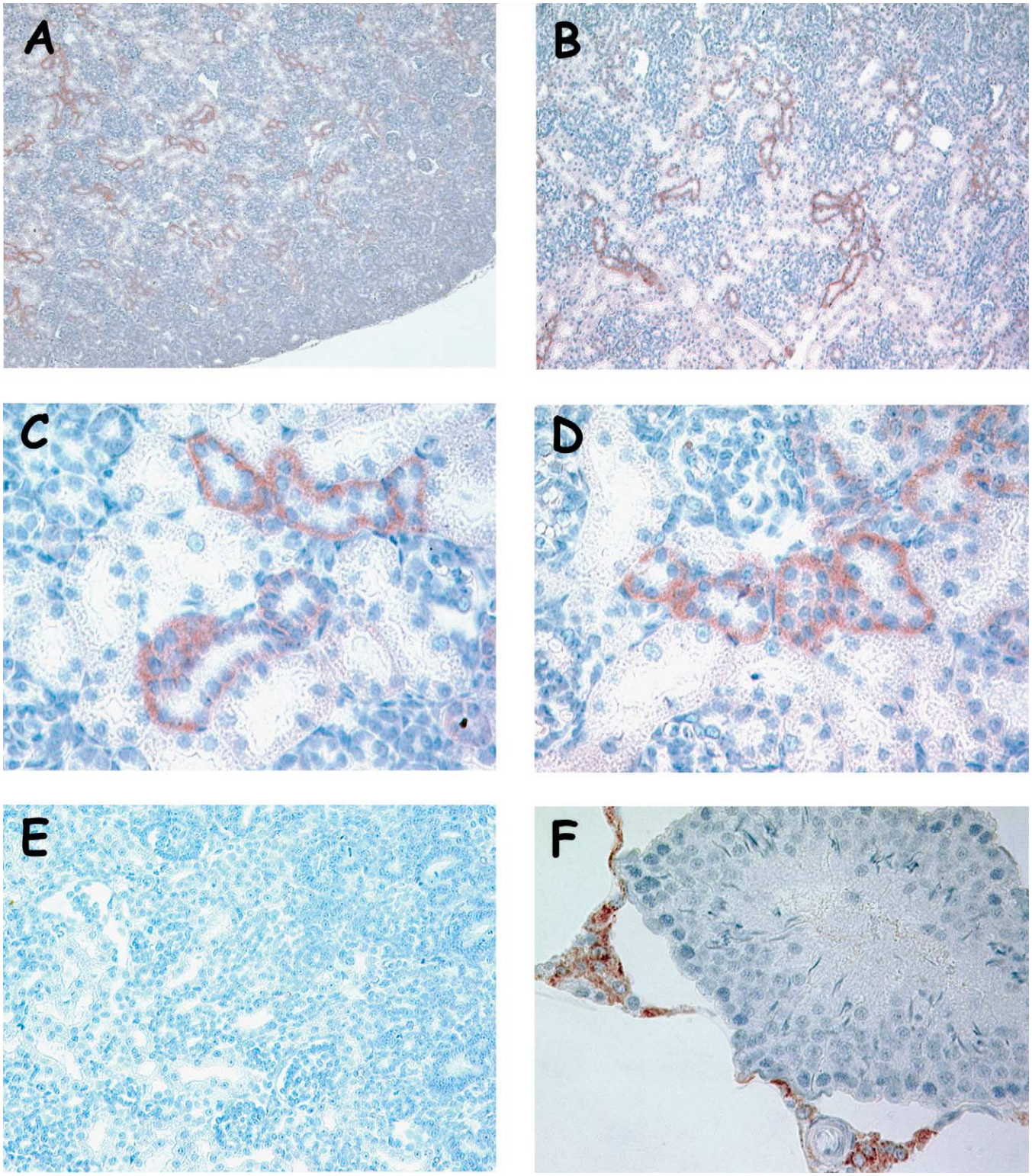

Fig. 6. Immunohistochemical staining for cytochrome P450scc in kidney tissues of newborn (C and D) and 7-day-old rats (A and B). Sections were exposed to an antiserum against rat P450scc. A positive immunoresponse was localized in the cytoplasm of distal tubule cells (orange precipitate). (E) Nonimmune control section; (F) testicular positive control section. *Magnification:* (A, B and E) 70×; (C, D and F) 280×.

different TSS, two of which expressed only in the liver and gastrointestinal tract, suggestive of a tissue-specific regulation of steroidogenic enzyme expression in non-endocrine tissues [20].

We also analyzed the expression of SF-1 by semi-quantitative RT-PCR. This tissue-specific transcription factor, implicated as a key regulator of steroid hydroxylase gene expression in adrenal and gonadal tissues, has been demonstrated also in the rat and mouse pituitaries, hypothalamus [23] and human spleen [24]. It is assumed to play important roles in the process of steroidogenic as well as non-steroidogenic tissue differentiation, as the tissues expressing this factor showed severe developmental defects after the SF-1 gene disruption [24]. In the human spleen, the SF-1 is expressed in the endothelial cells of the splenic sinus and pulp vein, where its expression is lower than that of the adrenal, but greater than or equal to that of the gonads [25]. The transcription of SF-1 in the spleen has been demonstrated also in the frog, *Rana rugosa* [26]. In the present paper, SF-1 was transcribed in the spleen of adult rats less intensely than in the gonads, but comparably to the kidney. Because, the rat spleen does not express P450scc, as well as P450c17 [27,28] and P450arom (Dalla Valle et al., in preparation), it is evident that, in this case, it cannot be correlated with steroid hydroxylase gene expression, but probably with hematopoietic/immune cell proliferation and/or differentiation as proposed by Ramayya et al. [25] and Morohashi [24].

In the kidney, SF-1 appears to be transcribed throughout the animal's life. The fact that Yamamoto et al. [29] were unable to detect SF-1 mRNA in the rat kidney by RT-PCR could be explained by differences in protocols (35 cycles instead of the 38 used in this work, for example). The co-expression of cytochrome P450scc and SF-1 in rat renal tissue suggests a possible role of this transcription factor in the regulation of CYP11A gene expression. A similar function has been suggested by Patel et al. [30] in the human skin, where the activity of cytochrome P450scc has recently been demonstrated [9]. On the other hand, the expression of cytochrome P450scc does not require SF-1 in the primitive gut of the mouse embryo [10] and in the human placenta [31], indicating that this transcription factor is not obligatory for the expression of cytochrome P450 steroid hydroxylases in extraglandular and trophoblastic tissues.

Our data show that the rat kidney expresses also the messenger of StAR, the protein delivering cholesterol to P450scc within the mitochondria. This protein has been previously demonstrated in human renal distal tubules [32,33], where its expression has been correlated with the mitochondrial 1α-hydroxylation of vitamin D, a terminal biosynthetic step. However, the co-expression of SF-1, P450scc and StAR in the rat kidney suggests instead a possible classical implication of these proteins in the regulation of overall hormonal steroidogenesis. Interestingly, cytochrome P450scc appears to be expressed in the distal tubules of the rat kidney, as does StAR in the human kidney.

At the protein level, the presence of cytochrome P450scc in the rat kidney was revealed by Western blotting analysis and immunohistochemistry. Conclusive evidence of the corresponding enzymatic activity, however, is still lacking because incubations of rat kidney mitochondria with [H^3]cholesterol or with 22R- and 25-hydroxycholesterol followed by pregnenolone radioimmunoassay resulted in activity only two-three times higher than that of the boiled tissue. Nevertheless, this weak activity declined after incubation with aminoglutethimide, an inhibitor of cytochrome P450scc (data not shown). Probably, this low level of activity is due to the fact that P450scc is restricted to the renal distal tubules, that represent only a fraction of the whole kidney, from which incubated mitochondria were extracted. A possible dilution effect was also invoked to explain the failure to observe the conversion of cholesterol to pregnenolone in the rat brain, while positive results were obtained with cell cultures of oligodendrocytes, the cells in which the expression of P450scc was detected by immunohistochemistry [34]. Analogously, a definite demonstration of P450scc activity in the rat kidney might be achieved using a cell line of distal tubular cells.

The capability of the rat kidney to convert pregnenolone to progesterone, a reaction catalyzed by the enzyme complex 3β-HSD/Δ^5–Δ^4 isomerase has been previously demonstrated [14,15]. In this regard, it is of interest the recent demonstration of the expression of the progesterone receptor in epithelial cells of the distal tubules of the human kidney [35]. Thus, it may be speculated that epithelial cells of the distal tubules of the rat kidney might be able to respond in a paracrine and/or autocrine way to progesterone produced in situ.

The fact that P450scc is more expressed during the first 2 weeks of postnatal life correlates well with the pattern of rat kidney development which continues up to 20 days after birth [36]. Among the regulatory factors involved in this process, vascular endothelial growth factor (VEGF) appears to be essential for glomerulogenesis in the mouse [37]. Significantly, VEGF mRNA was reported to be specifically induced by progesterone in human breast cancer cells [38]. Thus, a similar role of renal progesterone might be speculated in young rats.

Renal progesterone may exert other functions in adult rats as well. In the rabbit, progesterone significantly increased Ca^{2+} and decreased Na^+ uptakes by the luminal membranes of distal tubule cells and seemed to influence electrolyte transport through a nongenomic mechanism [39]. Notably, a putative membrane progesterone receptor has been identified in the rat and found to be expressed in the distal convoluted tubules [40].

On the other hand, it is possible that progesterone is not the only or main active steroid produced by the rat kidney, as its conversion to androgens has been previously established [15].

In conclusion, in the rat, the CYP11A gene is expressed, at the messenger and protein levels, not only in

the steroidogenic endocrines and the brain, but also in the kidney, where the CYP17 gene and other steroidogenic enzymes are also co-expressed (Dalla Valle et al., in preparation). It is suggested that the rat kidney might be capable of postnatal autonomous steroidogenesis which could actually be a continuation of that occurring during the fetal phase, as indicated by P450scc immunoreactivity in rat fetuses [17]. Although its functional significance remains unknown, an involvement of nephrosteroids in the regulation of kidney development and differentiation seems likely.

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References

[1] W.L. Miller, Molecular biology of steroid hormone synthesis, Endocr. Rev. 9 (3) (1988) 295–318.
[2] S.H. Mellon, J.A. Kushner, C. Vaisse, Expression and regulation of adrenodoxin and P450scc mRNA in rodent tissues, DNA Cell Biol. 10 (5) (1991) 339–347.
[3] D.M. Stocco, B.J. Clark, Regulation of the acute production of steroids in steroidogenic cells, Endocr. Rev. 17 (3) (1996) 221–244.
[4] K.M. McMasters, L.A. Dickson, R.V. Shamy, K. Robischon, G.J. Macdonald, W.R. Moyle, Rat cholesterol side-chain cleavage enzyme (P-450scc): use of a cDNA probe to study the hormonal regulation of P-450scc mRNA levels in ovarian granulosa cells, Gene 57 (1) (1987) 1–9.
[5] J.M. Rosner, J.C. Macome, D.P. Cardinali, In vitro biosynthesis of sterols and steroids by rat submaxillary glands, Endocrinology 85 (6) (1969) 1000–1003.
[6] I. Jung-Testas, Z.Y. Hu, E.E. Baulieu, P. Robel, Neurosteroids: biosynthesis of pregnenolone and progestrone in primary cultures of rat glial cells, Endocrinology 125 (4) (1989) 2083–2091.
[7] K. Iwahashi, H.S. Ozaki, M. Tsubaki, J.-I. Ohnishi, Y. Takeuchi, Y. Ichikawa, Studies of the immunohistochemical and biochemical localization of the cytochrome P450-scc-linked monooxigenase system in the adult rat brain, Biochem. Biophys. Acta 1035 (2) (1990) 182–189.
[8] A. Slominski, G. Ermak, M. Mihm, ACTH receptor, CYP11A1, CYP17 and CYP21A2 genes are expressed in skin, J. Clin. Endocrinol. Metab. 81 (7) (1996) 2746–2749.
[9] D. Thiboutot, S. Jabara, J.M. McAllister, A. Sivarajah, K. Gilliland, Z. Cong, G. Clawson, Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1), J. Invest. Dermatol. 120 (6) (2003) 905–914.
[10] D.S. Keeney, Y. Ikeda, M.R. Waterman, K.L. Parker, Cholesterol side-chain cleavage cytochrome P450 gene expression in the primitive gut of the mouse embryo does not require steroidogenic factor 1, Mol. Endocrinol. 9 (8) (1995) 1091–1098.
[11] K.M. Kayes-Wandover, P.C. White, Steroidogenic enzyme gene expression in the human heart, J. Clin. Endocrinol. Metab. 85 (7) (2000) 2519–2525.
[12] A. Morales, A. Cuellar, J. Ramirez, F. Vilchis, V. Diaz-Sanchez, Synthesis of steroids in pancreas: evidence of cytochrome P-450scc activity, Pancreas 19 (1) (1999) 39–44.
[13] S. Lajic, L. Eidsmo, M. Holst, Steroid 21-hydroxylase in the kidney: demonstration of levels of messenger RNA which correlate with the level of activity, J. Steroid Biochem. Mol. Biol. 52 (2) (1995) 181–186.
[14] F. Labrie, J. Simard, V. Luu-The, G. Pelletier, A. Belanger, Y. Lachance, H.F. Zhao, C. Labrie, N. Breton, Y. De Launoit, M. Dumont, E. Dupont, E. Rhéaume, C. Martel, J. Couët, C. Trudel, Structure and tissue-specific expression of 3ß-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase genes in human and rat classical and peripheral steroidogenic tissues, J. Steroid Biochem. Mol. Biol. 41 (3-8) (1992) 421–435.
[15] L. Dalla Valle, P. Belvedere, C. Simontacchi, L. Colombo, Extraglandular hormonal steroidogenesis in aged rats, J. Steroid Biol. Mol. 43 (1992) 1095–1098.
[16] M. Quinkler, C. Bumke-Vogt, B. Meyer, V. Bähr, W. Oelkers, S. Diederich, The human kidney is a progesterone-metabolizing and androgen-producing organ, J. Clin. Endocrinol. Metab. 88 (6) (2003) 2803–2809.
[17] N.A. Compagnone, A. Bulfone, J.L. Rubenstein, S.H. Mellon, Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis, Endocrinology 136 (6) (1995) 2689–2696.
[18] CapFinder PCR cDNA Synthesis Kit, Clontechniques 11 (1996) 2–3.
[19] O. Franz, I. Bruchhaus, T. Roeder, Verification of differential gene transcription using virtual northern blotting, Nucleic Acids Res. 27 (11) (1999) e3.
[20] L. Dalla Valle, S. Vianello, P. Belvedere, L. Colombo, Rat cytochrome P450c17 gene transcription is initiated at different start sites in extraglandular and glandular tissues, J. Steroid Biochem. Mol. Biol. 82 (2002) 377–384.
[21] R.B. Oonk, K.L. Parker, J.L. Gibson, J.S. Richards, Rat cholesterol side-chain cleavage cytochrome P-450 (P-450scc) gene. Structure and regulation by cAMP in vitro, J. Biol. Chem. 265 (36) (1990) 22392–22401.
[22] P. Zhang, H. Rodriguez, S.H. Mellon, Transcriptional regulation of P450scc gene expression in neural and steroidogenic cells: implications for regulation of neurosteroidogenesis, Mol. Endocrinol. 9 (11) (1995) 1571–1582.
[23] H.A. Ingraham, D.S. Lala, Y. Ikeda, X. Luo, W.H. Shen, M.W. Nachtigal, R. Abbud, J.H. Nilson, K.L. Parker, The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis, Genes Dev. 8 (19) (1994) 2302–2312.
[24] K.-I. Morohashi, Gonadal and extragonadal functions of Ad4BP/SF-1: developmental aspects, Trends Endocrinol. Metab. 10 (5) (1999) 169–173.
[25] M.S. Ramayya, J. Zhou, T. Kino, J.H. Segars, C.A. Bondy, G.P. Chrousos, Steroidogenic factor 1 messenger ribonucleic acid expression in steroidogenic and nonsteroidogenic human tissues: Northern blot and in situ hybridization studies, J. Clin. Endocrinol. Metab. 82 (6) (1997) 1799–1806.
[26] K. Kawano, I. Miura, K. Morohashi, M. Takase, M. Nakamura, Molecular cloning and expression of the SF-1/Ad4BP gene in the frog, *Rana rugosa*, Gene 222 (2) (1998) 169–176.
[27] L. Dalla Valle, J. Couet, J. Simard, P. Belvedere, C. Simontacchi, F. Labrie, L. Colombo, Occurence of cytochrome P-450c17 and androgen biosynthesis in the oesophagus and gastrointestinal tract of the rat, Mol. Cell. Endocrinol. 111 (1) (1995) 83–92.
[28] S. Vianello, M.R. Waterman, L. Dalla Valle, L. Colombo, Developmentally regulated expression and activity of 17α-hydroxylase/C-17,20 lyase cytochrome P450 (P450c17) in rat liver, Endocrinology 138 (1997) 3166–3174.
[29] T. Yamamoto, B.M. Chapman, J.W. Clemens, J.R. Richards, M.J. Soares, Analysis of cytochrome P-450 side-chain cleavage gene promoter activation during trophoblast cell differentiation, Mol. Cell Endocrinol. 113 (2) (1995) 183–194.
[30] M.V. Patel, I.A. McKay, J.M. Burrin, Transcriptional regulators of steroidogenesis, DAX-1 and SF-1, are expressed in human skin, J. Invest. Dermatol. 117 (6) (2001) 1559–1565.

[31] N. Huang, W.L. Miller, Cloning of factors related to HIV-inducible LBP proteins that regulate steroidogenic factor-1-independent human placental transcription of the cholesterol side-chain cleavage enzyme, P450scc, J. Biol. Chem. 275 (4) (2000) 2852–2858.
[32] A. Gradi, R. Tang-Wai, H.M. McBride, L. Chu Lee, G.C. Shore, J. Pelletier, The human steroidogenic acute regulatory (StAR) gene is expressed in the urogenital system and encodes a mitochondrial polypeptide, Biochim. Biophys. Acta 1258 (3) (1995) 228–233.
[33] S.E. Pollack, E.E. Furth, C.B. Kallen, F. Arakane, M. Kiriakidou, K.F. Kozarsky, J.F. Strauss III, Localization of the steroidogenic acute regulatory protein in human tissues, J. Clin. Endocrinol. Metab. 82 (12) (1997) 4243–4251.
[34] E.E. Baulieu, Neurosteroids: a novel function of the brain, Psychoneuroendocrinology 23 (8) (1998) 963–987.
[35] C. Bumke-Vogt, V. Bahr, S. Diederich, S.M. Herrmann, I. Anagnostopoulos, W. Oelkers, M. Quinkler, Expression of the progesterone receptor and progesterone- metabolising enzymes in the female and male human kidney, J. Endocrinol. 175 (2) (2002) 349–364.
[36] M.G. Marquez, I. Cabrera, D.J. Serrano, N. Sterin-Speziale, Cell proliferation and morphometric changes in the rat kidney during postnatal development, Anat. Embryol. (Berl.) 205 (5–6) (2002) 431–440.
[37] Y. Kitamoto, H. Tokunaga, K. Tomita, Vascular endothelial growth factor is an essential molecule for mouse kidney development: glomerulogenesis and nephrogenesis, J. Clin. Invest. 99 (10) (1997) 2351–2357.
[38] S.M. Hyder, L. Murthy, G.M. Stancel, Progestin regulation of vascular endothelial growth factor in human breast cancer cells, Cancer Res. 58 (3) (1998) 392–395.
[39] M.G. Brunette, M. Leclerc, Renal action of progesterone: effect on calcium reabsorption, Mol. Cell. Endocrinol. 194 (1–2) (2002) 183–190.
[40] F.S. Raza, H. Takemori, H. Tojo, M. Okamoto, G.P. Vinson, Identification of the rat adrenal zona fasciculata/reticularis specific protein, inner zone antigen (IZAg), as the putative membrane progesterone receptor, Eur. J. Biochem. 268 (7) (2001) 2141–2147.