

# Role of DNA methylation in the tissue-specific expression of the CYP17A1 gene for steroidogenesis in rodents

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## Abstract

The *CYP17A1* gene is the qualitative regulator of steroidogenesis. Depending on the presence or absence of CYP17 activities mineralocorticoids, glucocorticoids or adrenal androgens are produced. The expression of the *CYP17A1* gene is tissue as well as species-specific. In contrast to humans, adrenals of rodents do not express the *CYP17A1* gene and have therefore no P450c17 enzyme for cortisol production, but produce corticosterone. DNA methylation is involved in the tissue-specific silencing of the *CYP17A1* gene in human placental JEG-3 cells. We investigated the role of DNA methylation for the tissue-specific expression of the *CYP17A1* gene in rodents. Rats treated with the methyltransferase inhibitor 5-aza-deoxycytidine excreted the cortisol metabolite tetrahydrocortisol in their urine suggesting that treatment induced CYP17

expression and 17 $\alpha$ -hydroxylase activity through demethylation. Accordingly, bisulfite modification experiments identified a methylated CpG island in the CYP17 promoter in DNA extracted from rat adrenals but not from testes. Both methyltransferase and histone deacetylase inhibitors induced the expression of the *CYP17A1* gene in mouse adrenocortical Y1 cells which normally do not express CYP17, indicating that the expression of the mouse *CYP17A1* gene is epigenetically controlled. The role of DNA methylation for CYP17 expression was further underlined by the finding that a reporter construct driven by the mouse -1041 bp CYP17 promoter was active in Y1 cells, thus excluding the lack of essential transcription factors for CYP17 expression in these adrenal cells.

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## Introduction

The human cytochrome P450c17 enzyme is encoded by the *CYP17A1* gene, which is located on chromosome 10q24.3 (Matteson *et al.* 1986, Picado-Leonard & Miller 1987, Sparkes *et al.* 1991, Fan *et al.* 1992). P450c17 is the qualitative regulator of steroidogenesis in the human adrenals (Miller *et al.* 1997). In its absence, aldosterone is produced in the human adrenal zona glomerulosa (Miller *et al.* 1997). In the presence of the 17 $\alpha$ -hydroxylase activity of P450c17, glucocorticoids are produced in the adrenal zona fasciculata (Miller *et al.* 1997). Androgens (C19 steroids) are produced in the adrenal zona reticularis (Miller *et al.* 1997), in the presence of both 17 $\alpha$ -hydroxylase and 17, 20-lyase activities of P450c17.

In both humans and rodents, the *CYP17A1* gene is regulated hormonally and developmentally (Voutilainen & Miller 1986, Voutilainen *et al.* 1986, Di Blasio *et al.* 1987, Nishihara *et al.* 1988, Keeney *et al.* 1995). Furthermore, the expression of CYP17 is both species- and tissue-specific.

In humans, the *CYP17A1* gene is expressed in the adrenal cortex and the gonads but not in the placenta (Chung *et al.* 1987), while in rodents it is expressed in the gonads and the placenta but not in the adrenals (Namiki *et al.* 1988, Johnson & Sen 1990, Le Goascogne *et al.* 1991, Yamamoto *et al.* 1996). Because the *CYP17A1* gene is not expressed in rodent adrenals, cortisol is not present in measurable concentrations and corticosterone is the main glucocorticoid in these species. In addition, the *CYP17A1* gene is expressed in some non-endocrine organs such as the brain, the heart and kidneys in humans as well as rodents (Compagnone *et al.* 1995, Pezzi *et al.* 2003, Dalla Valle *et al.* 2004).

Till date the precise mechanism/s regulating the tissue- and species-specific expression of the *CYP17A1* gene is unknown. One known means of regulating tissue-specific expression of genes is DNA methylation. DNA methylation is defined as the post-synthetic addition of methyl groups to the 5-position of cytosine in CpG islands within DNA strands (Rodenhiser & Mann 2006). In general, unmethylated regulatory CpG islands are located in tissue-specific expressed

genes and in essential housekeeping genes (Antequera 2003, Rodenhiser & Mann 2006). Methylation of cytosines in such CpG islands leads to the binding of methylated CpG binding domain proteins, transcription repressors and/or histone deacetylases (HDACs), thereby blocking gene transcription. In addition, DNA methylation may block the expression of specific genes by silencing essential transcription factors (Fluck & Miller 2004). DNA methylation has been shown to regulate steroidogenesis. Changes in methylation status of the *CYP17A1* gene in bovine adrenocortical cells were observed during cellular senescence (Hornsby *et al.* 1991). Furthermore, we presented evidence that the *HSD11B2* gene is regulated by DNA methylation, and that rats treated with a methyltransferase inhibitor have increased *HSD11B2* gene expression and higher enzyme activities (Alikhani-Koopaei *et al.* 2004). Accordingly, regulatory CpG islands in the *HSD11B2* gene were identified. Moreover, we showed in previous studies that DNA methylation plays a role in the silencing of the human *CYP17A1* gene in human placental JEG-3 cells (Fluck & Miller 2004). Retrospective analysis of urinary steroid profiles obtained from rats treated with a methyltransferase inhibitor (Alikhani-Koopaei *et al.* 2004) revealed cortisol metabolites that would indicate CYP17 activity in the adrenals. Therefore, in the present study we investigated whether epigenetic factors regulate the species- and tissue-specific expression of the *CYP17A1* gene in rodents. We identified candidate CpG islands in both, the rat and mouse *CYP17A1* genes. Furthermore, we used mouse adrenocortical Y1 cells, which do not express CYP17 endogenously, to investigate whether DNA methylation might be involved in the silencing of the *CYP17A1* gene in the mouse adrenal cortex.

## Materials and Methods

### Materials

5-Aza-2'-deoxycytidine (5-aza-CdR) and trichostatin A (TSA) were purchased from Sigma-Aldrich and dissolved in DMSO. [<sup>3</sup>H]pregnenolone (14 Ci/mmol) was purchased from Perkin Elmer (Boston, MA, USA).

### Animal studies

To assess the effect of 5-aza-CdR treatment on gene expression animal studies have been performed and previously published (Alikhani-Koopaei *et al.* 2004). That study focused on the role of DNA methylation in the expression of the *HSD11B2* gene, but urine of the animals was available for our study. In brief, male Wistar rats weighing 190–210 g were kept in metabolic cages on standard diet for 5 days for adaptation. Thereafter, animals were assigned to one of three different groups: group one, controls, which were injected with PBS once daily for 7 days; group two, low dose 5-aza-CdR treatment consisting of 0.5 mg/kg once a day for

7 days; and group three, high dose 5-aza-CdR treatment consisting of 1 mg/kg 5-aza-CdR. All injections were i.p. Urine of the rats was collected in 24 h portions before and during treatment.

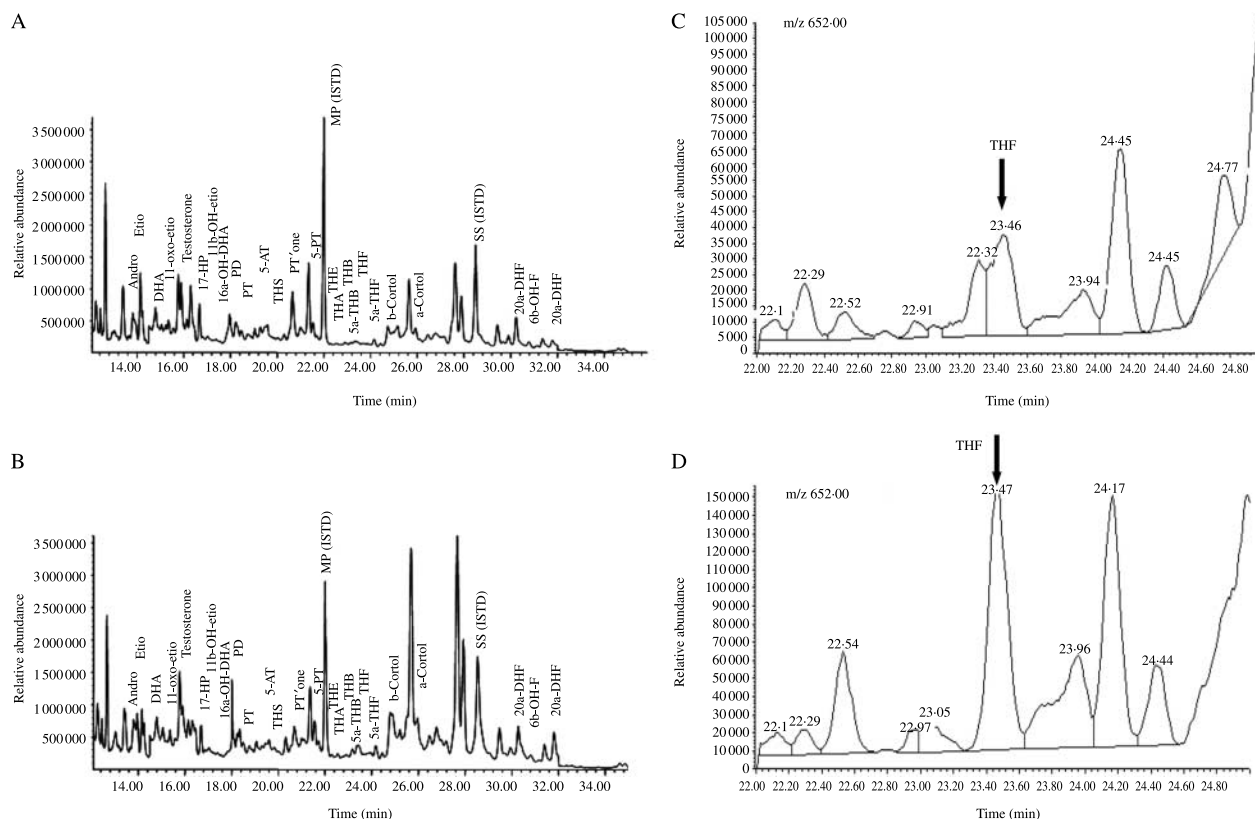
For genomic DNA extraction, we obtained adrenals and testes of normal Wistar rats from our animal facility. Genomic DNA was prepared from purified Leydig cells from adult rat testes after the multi-step isolation method of Klinefelter *et al.* (1988). In brief, this procedure involves the use of testicular perfusion, enzymatic dissociation, centrifugal elutriation, and density percoll gradient centrifugation. After centrifugation, the percoll gradient is divided into a fraction lighter than 1.068 g/ml that contains germ cells, macrophages, and damaged Leydig cells, and a fraction heavier than 1.068 g/ml that contains intact and steroidogenically active Leydig cells. At this stage, the purity of the Leydig cells is >96% as assessed by 3 $\beta$ -hydroxysteroid dehydrogenase staining of the cells. All animal studies were approved by the committee of animal research of the University of Berne, Switzerland.

### Urinary steroid profiling by GC/MS

Total steroids were extracted from urine collections of all rats and analyzed by GC/MS as previously described (Shackleton 1993). Briefly, the derivatives were analyzed on a gas chromatograph (6890N, Agilent Technologies, La Jolla, CA, USA) equipped with a mass selective detector (5973, Agilent Technologies) by selected ion monitoring (SIM) and in a run in the scan mode. Samples were injected at 260 °C and chromatographed during a temperature-programmed run over 35 min on a HP-1/MS column (Agilent). Forty-one different steroid metabolites were investigated (Fig. 1), among them 20 were cortisol metabolites. In the SIM mode, one characteristic ion was chosen for each steroid of interest, i.e. mass/charge (*m/z*) 652 was chosen for tetrahydrocortisol (THF), *m/z* 564 for tetrahydrocorticosterone (THB) and 5-THB and *m/z* 490 for tetrahydrodehydrocorticosterone. The standard stigmaterol was used for calculating steroid concentrations. Medroxyprogesterone was used as a recovery standard to control for loss of steroids during sample preparation.

### Bioinformatics

Rat (accession number X69816) and mouse (accession number AY594330) *CYP17A1* genes were analyzed for CpG islands using the CpG Island Searcher program (<http://cpgislands.usc.edu/cpg.aspx>). The CpG island was defined as a DNA sequence of 200 bp with a calculated percentage of CpGs of more than 50% and a calculated versus expected CpG distribution higher than 0.6. To assess identities between human, mouse and rat *CYP17A1* genes, we performed sequence alignments using the Graph Align program ([http://darwin.nmsu.edu/cgi-bin/graph\\_align.cgi](http://darwin.nmsu.edu/cgi-bin/graph_align.cgi)). To recognize putative binding sites for transcription factors (*cis*-elements), we used the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>).



**Figure 1** Cortisol metabolites in urine of rats treated with the methyltransferase inhibitor 5-aza-CdR and controls. Rats were treated with 1 mg/kg 5-aza-CdR for 7 days. Urine was collected every 24 h. Control rats were injected with saline. (A) and (B) Picture of total ion current (scan mode) after GC/MS analysis of urine of a control animal (A) and a treated animal (B). Standards stigmasterol (SS) and medroxyprogesterone (MP), as well as several other steroids are named. (C) and (D) GC/MS chromatograms showing the selected ion monitoring (SIM mode) of ion  $m/z$  652 for THF measurement in urine of a representative control rat (C) and a rat treated with 1 mg/kg 5-aza-CdR for 3 days (D). A summary of the data is given in Table 2. Andro, androsterone; Etio, etiocholanolone; DHA, dehydroepiandrosterone; 11-oxo-etio, 11-oxo-etiocholanolone; 17-HP, 17-hydroxypregnanolone; 11b-OH-etio, 11b-hydroxy-etiocholanolone; 16a-OH-DHA, 16a-hydroxy-dehydroepiandrosterone; PD, pregnanediol; PT, pregnetriol; 5-AT, 5-androstene-3 $\beta$ ,16 $\alpha$ ,17b-triol; THS, tetrahydro-11-deoxycortisol; PT,one, 11-oxo-pregnanetriol; 5-PT, 5-pregnene-3 $\beta$ ,16 $\alpha$ ,17b-triol; THE, tetrahydrocortisone; THA, tetrahydrodehydrocorticosterone; THB, tetrahydrocorticosterone; 5a-THB, 5a-tetrahydrocorticosterone; 5a-THF, 5a-tetrahydrocortisol; b-cortol,  $\beta$ -cortol; a-cortol,  $\alpha$ -cortol; 20a-DHE, 20 $\alpha$ -dihydrocortisone; 6b-OH-F, 6b-hydroxycortisol; 20a-DHF, 20 $\alpha$ -dihydrocortisol.

#### Extraction of DNA, RNA and reverse transcription

Genomic DNA from rat organs and mouse cell lines was extracted using either DNA mini kit (Qiagen) or Nucleospin DNA/RNA kit (Macherey Nagel, Oensingen, Switzerland). Total RNA was extracted and reverse-transcribed into cDNA using the Improm RNA Transcriptase kit (Promega) and random primers (0.5/1  $\mu$ g RNA).

#### Methylation sensitive endonuclease cleavage

For methylation sensitive cleavage, the restriction enzymes *Ava*I, *Nhe*I (Promega) and *Dra*III (Roche) were used. Reactions were incubated at 37 °C for 72 h replacing enzymes every 24 h. DNA was purified with the PCR purification kit (Macherey Nagel). Target DNA segments were amplified by PCR using specific primers (A–C, Table 1)

for 28–32 cycles. Rat  $\beta$ -actin served as control. Cleavage was assessed semi-quantitatively comparing amplified DNA levels of specifically digested adrenal and testes samples against mock digested control DNA and  $\beta$ -actin after agarose gel electrophoresis. Three independent experiments were performed.

#### PCR

DNA samples were amplified by Go-TAQ polymerase (Promega) and specific primers (Table 1) in a final volume of 25–50  $\mu$ l. PCR conditions were 45 s at 95 °C, 45 s at 50–60 °C, 45 s at 72 °C for 28–35 cycles. Aliquots of PCR products were separated by electrophoresis on a 1.5–2% agarose gel, visualized by ethidium bromide staining, and detected on an Alpha Imager 3400 (Alpha Innotech, San Leandro, CA, USA).

**Table 1** List of oligonucleotides

Name	Sequence	NCBI accession number (5' start site for S) (3' for AS)
Mouse_CYP17 (PCR)	S: 5'-CATATCCTTGTACGGTGG-3' AS: 5'-ACCAGCCAGATCGGTTTATG-3'	AY594330 (8081) AY594330 (9299)
Mouse_β-actin	S: 5'-TGCGTGACATCAAAGAGAAG-3' AS: 5'-TATGGAATCCTGTGGCAG-3'	NM_007393 (705) NM_007393 (901)
Mouse_CYP17 (cloning) <sup>a</sup>	S: 5'- <b>ATCGGTACCT</b> GAGTATTGGCATTGCGTCC-3' AS: 5'- <b>ATCAAGCTT</b> TGGAGTGGAGAAGTAC-3'	AY594330 (3410) AY594330 (4451)
A <sup>b</sup>	S: 5'-CATGTACGGAGGCACACTCT-3' AS: 5'-AGTGGTGGGAATCAAACCTCG-3'	X69816 (673) X69816 (838)
B <sup>b</sup>	S: 5'-ATGCACGAGCCTTTACCTG-3' AS: 5'-AGTGGTGGGAATCAAACCTCG-3'	X69816 (653) X69816 (838)
C <sup>b</sup>	S: 5'-GCCGAACACCAACTTTCAAT-3' AS: 5'-TAGCCCACTTTGCTCCTCAG-3'	X69816 (5847) X69816 (6036)
Rat_β-actin	S: 5'-AAAGGTGACCAATGCTGGAG-3' AS: 5'-GCTCTATCCTGGCCTCA-3'	V01217 (2627) V01217 (3050)
Bs rat_big	S: 5'-TGATAATTGAAATGTTATTTTGTGG-3' AS: 5'-AATAAATTATTTGTTATGTTATT-3'	X69816 (563) X69816 (958)
Bs rat_small	S: 5'-CTCTGGAATTAGAGTTATAGAAAATT-3'	X69816 (778)

<sup>a</sup>Nucleotides added for restriction sites KpnI/HindIII are given in bold.

<sup>b</sup>Primers for PCR amplification of specific, CpG islands containing regions in the rat *CYP17A1* gene (see also Fig. 3A).

### Bisulfite sequencing

Genomic DNA of rat organs and of purified rat Leydig cells (10 µg) were fragmented by sonication (Branson Ultrasonics, Danbury, CT, USA) for 45 s. DNA was denatured for 30 min at 42 °C with 5 µl 3 M NaOH, before adding 510 µl sodium bisulfite (40·5%), 30 µl 10 mM hydroxyquinone and water to a total volume of 610 µl per reaction. The reaction was incubated for 16 h at 55 °C. DNA was purified using the PCR purification kit (Macherey Nagel), desulfonated with 5 µl 3 M NaOH for 30 min at 37 °C and neutralized by 5 µl 3 M ammonium acetate. After purification, DNA was amplified by nested PCR with Go-TAQ polymerase, two forward and one reverse primer, which were designed for the area between -1030 and -635 in the rat CYP17 promoter encompassing two CpG islands (Table 1). The cytosines were modified in the sequences of the primers as shown in Table 1 (Bs rat\_bigS, Bs rat\_bigAS and Bs rat\_small). The PCR fragments were purified and cloned using the pGEM T-Easy Topo cloning kit (Promega). To assess the level of DNA methylation, seven clones of PCR products obtained from rat adrenals, testes and Leydig cells were sequenced (Microsynth, Balgach, Switzerland).

### Cell culture

Mouse adrenocortical Y1 cells were purchased from the American Type Culture Collection (ATCC CRL-2128). Mouse testicular Leydig MA-10 cells were kindly provided by Prof. W L Miller. Standard medium for Y1 cells is DMEM/Ham's F12 (Gibco BRL Invitrogen) supplemented with 7·5% horse serum, 2% FCS and antibiotics (10 U/ml penicillin/10 µg/ml streptomycin). Standard medium for

MA-10 cells is Waymouth (Sigma-Aldrich) supplemented with 15% horse serum and antibiotics (all from Gibco BRL Invitrogen). For MA-10 cells, culture dishes were pre-coated with 0·1% gelatin (Sigma-Aldrich).

### Treatment of cells with 5-aza-CdR and TSA, and assessment of steroidogenesis

Y1 cells were grown at a density of 800 000 cells per 100 mm dish. One microgram 5-aza-CdR was added for 72 h. Medium containing 5-aza-CdR was replaced every 24 h. TSA (100 nM) was added to the cells for the last 24 h. Since 5-aza-CdR and TSA were dissolved in DMSO, cells treated with solvent DMSO served as control. To assess CYP17 expression, RNA was extracted and RT-PCR performed as described above using specific mouse CYP17 primers (Table 1).

### Plasmid constructs and luciferase reporter assays

The construct containing the mouse -1041 bp CYP17 promoter was built with the primers given in Table 1. The PCR fragment was cloned into the pGL3 basic vector using KpnI and HindIII restriction sites in front of the luciferase gene. For transient transfections, MA-10 and Y1 cells were subcultured into 24-well plates at a density of 60 000 cells/well. Transient transfection of cells was carried out overnight with calcium phosphate including the pGL3 vector either empty, or the -1041 bp CYP17 promoter reporter construct (0·8 µg/well). Thereafter, cells were lysed and assayed for dual-luciferase activities as described by the manufacturer (Promega). Co-transfection with 50 ng/well Renilla

**Table 2** Concentrations (ng/24 h) of urinary tetrahydrometabolites (THF) of cortisol from untreated and treated rats (1 mg/kg 5-aza-CdR)

	Baseline	Day 3	Day 5	
Treated rats (n=3)	22±0.7	153.3±33.8	296.3±9.5	<i>P</i> <0.05*
Control rats (n=3)	20.7±1.1	18.3±2.2	21.7±3.8	

THF concentrations are given as mean±S.E.M. \**P* value for treated versus control rats.

luciferase reporter (pRL-TK) was used to control transfection efficiency. Three independent experiments were performed in duplicate.

### Data analysis

Quantitative data represent the mean of at least three independent experiments. Experimental variation is given as S.E.M. statistical analysis was carried out using Prism 4.00 (GraphPad software Inc., SanDiego, CA, USA). Either non-parametric Mann-Whitney test or normality test (Kolmogorov-Smirnov) followed by one-way ANOVA test were applied. Significance was assumed for *P*≤0.05.

## Results

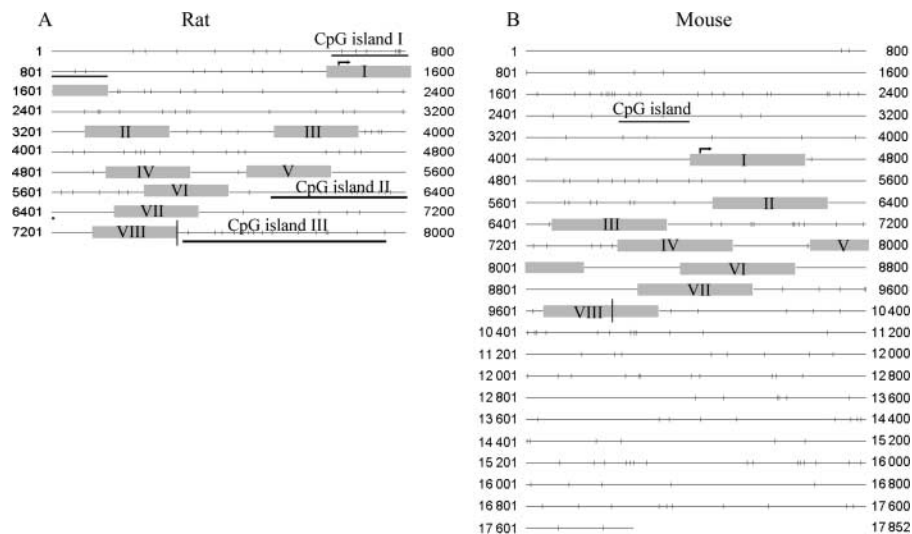
### Rats treated with the methyltransferase inhibitor 5-aza-CdR excrete cortisol metabolites in the urine

Hypothesizing that methylation might control CYP17 expression and thus cortisol production, we analyzed the

urine of rats treated with the methyltransferase inhibitor 5-aza-CdR for THF and corticosterone excretion. These urine samples were available for retrospective analysis from a previous project studying methylation of the rat *HSD11B2* gene (Alikhani-Koopaei *et al.* 2004). Using a very sensitive GC/MS method a total of 41 steroid metabolites were assessed (Fig. 1A and B). Between urine steroid profiles of 5-aza-CdR treated and control rats, we only found a substantial change in THF levels. Neither basal THF nor THB levels (controls 149±8 ng/24 h, treated 156.3±37.8 ng/24 h) differed between the two groups. Basal THF levels were low in all animals (Fig. 1 and Table 2). However, when rats were treated with 5-aza-CdR for 5 days, THF increased about 14-fold compared to controls (Fig. 1 and Table 2), while THB increased only 1.5-fold (controls on day 5, 287.3±42.8 ng/24 h; treated animals on day 5, 427.7±81.1 ng/24 h). Animals treated for 7 days had similar values (data not shown). These results suggested that 5-aza-CdR treatment induced CYP17 expression for cortisol production in rats.

### CpG islands as possible methylation targets in the CYP17A1 genes of rats and mice

The human *CYP17A1* gene shares 75 and 67% nucleotide sequence identity in the coding regions with the rat and mouse *CYP17A1* genes respectively. However, promoter nucleotide sequence identity of the 1500 nucleotides upstream of the transcription start site is ~45% between humans and rodents and ~68% between rats and mice. Furthermore, rats and mice share ~80% nucleotide sequence identity in their coding regions. To study whether the



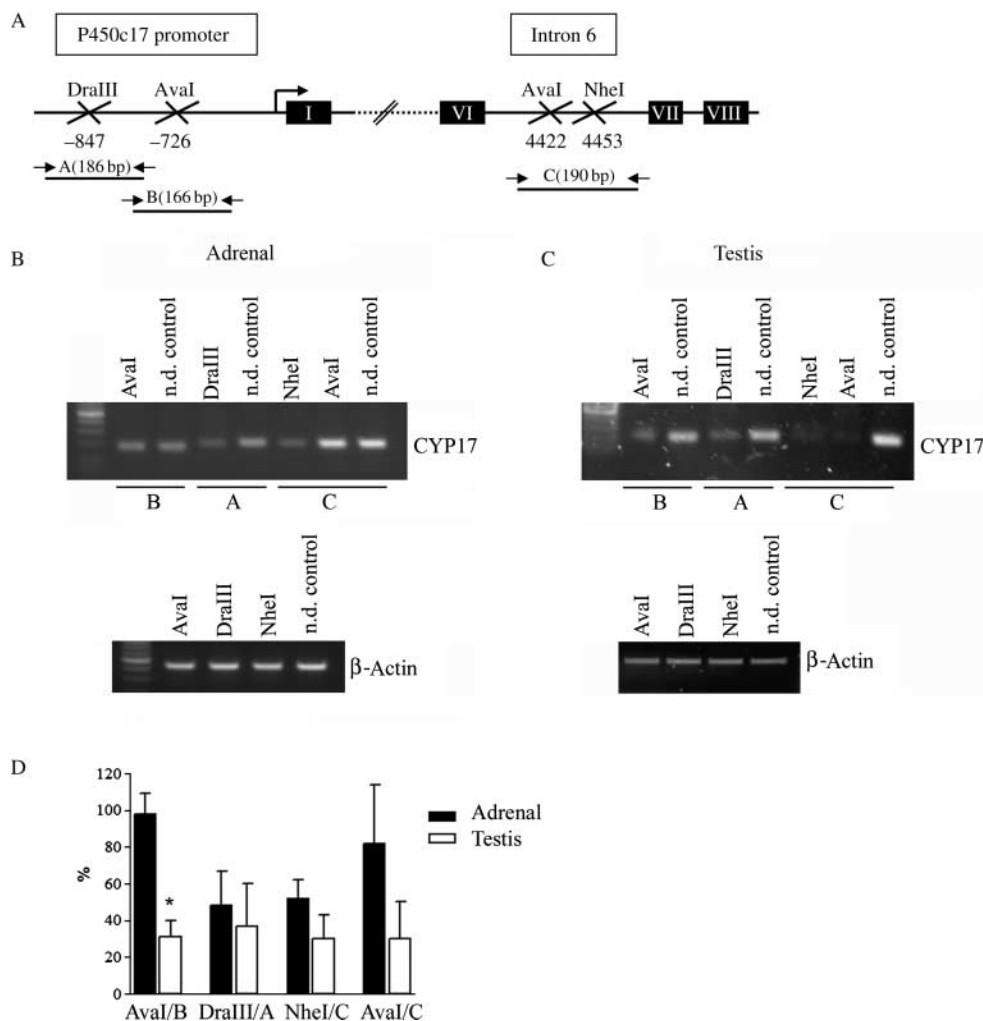
**Figure 2** CpG islands as possible methylation targets in the *CYP17A1* genes of rat and mouse. Both rat and mouse *CYP17A1* genes are composed of eight exons. *In silico* analysis of the genomic DNA of the rat *CYP17A1* gene revealed three CpG islands i.e. in the promoter region (−874/−661), in intron 6 (4608/4844) and in the 3' UTR (5896/6273) (A). In the genomic DNA of the mouse *CYP17A1* gene, only one single CpG island was identified in the promoter region (B).

*CYP17A1* gene in rodents is epigenetically regulated, we searched for candidate sites for DNA methylation by computational analysis of the mouse and rat *CYP17A1* genes using a CpG island searcher. Three candidate islands i.e. in the promoter region (−905/−692), in the intron 6 region (4577/4813) and in the 3′ UTR (5856/6242) were identified for the rat *CYP17A1* gene (Fig. 2A), while only one candidate island was identified in the promoter region (−2791/−2644) for the mouse *CYP17A1* gene (Fig. 2B). So, despite similarities between the mouse and rat *CYP17A1* genes, the identified CpG islands are differently located.

Thus, *in silico* analysis suggested that mouse and rat *CYP17A1* genes harbor CpG islands that may be regulated by DNA methylation.

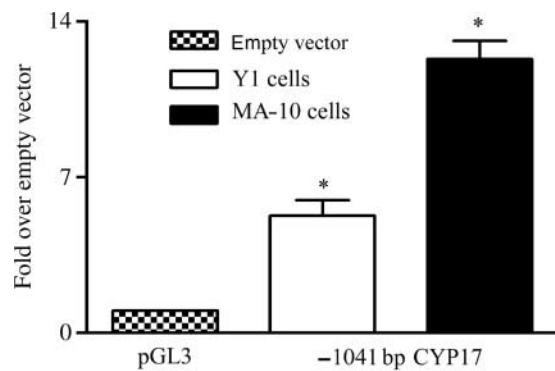
#### Targeted islands of methylation in the rat *CYP17A1* gene

To identify regions in the rat *CYP17A1* gene regulated by methylation, we investigated candidate areas by using methylation sensitive restriction endonucleases. Three CpG islands were found within the rat *CYP17A1* gene (Fig. 2A) which might be targets for digestion by the methylation



**Figure 3** Identification of methylation targeted CpG islands in the rat *CYP17A1* gene. (A) Scheme of the studied CpG islands in the rat *CYP17A1* gene. (B) and (C) Methylation analysis using methylation sensitive endonuclease digests. Genomic DNA was extracted from adrenal glands and testes of normal rats. Methylation sensitive restriction sites Aval, DraIII within the promoter region and Aval, NheI within the intron six of the *CYP17A1* gene were analyzed. Representative agarose gels are shown for the CYP17 DNA analysis of adrenal (B) versus testis (C). (D) Quantitative analysis of three experiments revealed more PCR products for both Aval sites when analyzing the adrenal DNA in comparison to the testis DNA, thus suggesting methylated CpG islands in the rat adrenals. Quantitative data are given as mean  $\pm$  S.E.M. \* $P \leq 0.05$ .





**Figure 6** Activity of a mouse *CYP17A1* promoter reporter construct in testicular Leydig MA-10 and adrenal Y1 cells. Cells were transiently transfected with a mouse -1041 bp *CYP17* promoter luciferase reporter vector. PRL-TK served as internal control for transfection efficiency; transfection of the empty vector served as control for the specificity of the experiment. All experiments were performed in duplicates. Data represent the mean  $\pm$  S.E.M. of three independent experiments. \* $P \leq 0.05$ .

*CYP17A1* gene in Y1 cells. Cells were treated either with 5-aza-CdR and/or with TSA and the expression of the *CYP17A1* gene was analyzed by RT-PCR. We found little expression of the *CYP17A1* gene in Y1 cells after 5-aza-CdR or TSA treatments alone (Fig. 5). However, after treatment with both 5-aza-CdR and TSA, *CYP17* was clearly expressed when compared with the endogenous *CYP17A1* gene expression in mouse testicular Leydig MA-10 cells. Therefore, both DNA methylation and histone deacetylation may be involved in the silencing of the *CYP17A1* gene in mouse adrenocortical Y1 cells.

In addition, we tried to assess the activities of the microsomal *CYP17* protein of Y1 cells after both 5-aza-CdR and TSA treatment. To assess possible *CYP17* activities we incubated cells with radiolabeled precursor pregnenolone and looked for conversion to 17OH-pregnenolone or DHEA by thin layer chromatography. Unfortunately, we were not able to detect steroid conversion unambiguously (data not shown). Also detection of *CYP17* protein by western blot failed after treatment of Y1 cells with 5-aza-CdR and/or TSA.

#### Activity of a mouse *CYP17* promoter reporter construct in MA-10 and Y1 cells

Several transcription factors have been identified which regulate the expression of the mouse *CYP17A1* gene (Youngblood & Payne 1992, Busygina *et al.* 2005). DNA methylation may silence the expression of the *CYP17A1* gene directly, or silence essential transcription factors, or both. This has been shown for the human *CYP17A1* gene in human placental JEG-3 cells (Fluck & Miller 2004). We transfected adrenal Y1 cells with a mouse -1041 bp *CYP17* promoter reporter construct in order to test whether the lack of *CYP17* expression was due to missing transcription factors.

Since transiently transfected reporter constructs are not regulated by methylation, such a promoter construct is expected to be active in the presence of all required transcriptional regulators. We found that the *CYP17* promoter construct was active in Y1 cells, indicating that transcription factors which are essential for *CYP17* expression are endogenously expressed in Y1 cells (Fig. 6). However, activity of the construct was clearly higher in MA-10 cells which need high *CYP17* activity for testosterone production. These data underline a role for DNA methylation and histone deacetylation in the tissue-specific silencing of the *CYP17A1* gene in rodents.

## Discussion

In this study, we show that the tissue- and species-specific expression of the *CYP17A1* gene is regulated by DNA methylation in rodents. Not only the expression of the *CYP17A1* gene, but also the expression of several other genes that are involved in steroidogenesis, is regulated in a tissue-specific fashion i.e. *CYP19A1* and *HSD11B2* (George & Wilson 1980, Hu *et al.* 1999, Alikhani-Koopaei *et al.* 2004). Tissue-specific expression of genes may be controlled epigenetically (Rodenhiser & Mann 2006). In previous work we showed that the human *CYP17A1* gene, which is not expressed in the placenta and in human placental JEG-3 cells may be induced by treatment with a methyltransferase inhibitor (Fluck & Miller 2004). Others showed that the *CYP19A1* gene is expressed in chicken fibroblasts after treatment with both methyltransferase and HDAC inhibitors (Leshin 1985). Similarly, treatment with methyltransferase inhibitor enhances the transcription of the *HSD11B2* gene (Alikhani-Koopaei *et al.* 2004).

Rats treated with the methyltransferase inhibitor 5-aza-CdR excreted cortisol metabolites in their urine suggesting an effect on *CYP17A1* gene expression as 17 $\alpha$ -hydroxylase activity of the *CYP17* protein is essential for cortisol production. Presence of CpG islands in the DNA sequences of both the rat and mouse *CYP17A1* genes indicated a role for DNA methylation.

Rodent adrenals produce both cortisol and corticosterone for a limited period during fetal life indicating the presence of 17 $\alpha$ -hydroxylase activity (Keeney *et al.* 1995). However, during late gestation, *CYP17A1* gene expression stops in the rodent adrenals. The loss of *CYP17A1* gene expression coincides with an increase in aldosterone production and morphologically with differentiation of the zona glomerulosa in the rodent adrenal cortex (Salmenpera & Kahri 1976, Dalle *et al.* 1978, Keeney *et al.* 1995). Recently, the production of androstenedione, cortisol and 17 $\alpha$ -hydroxyprogesterone, which requires P450c17 activities, has been observed in rat adrenals at postnatal 16–20 days (before the development of the gonads), which coincided with the peak in the *CYP17* mRNA level in adrenal tissue from those animals (Pignatelli *et al.* 2006). However, no *CYP17A1* gene



expression was found after 25–60 days of life. In contrast to the rodent, a constant level of CYP17 is expressed in the adult human adrenal cortex throughout life. Accordingly, the 17 $\alpha$ -hydroxylase activity of P450c17 and cortisol production are constant throughout life (Rainey *et al.* 2002). By contrast, the 17,20-lyase activity of P450c17 is low after birth, increases during adrenarche at around 6–8 years and then decreases after 30 years (Rainey *et al.* 2002).

DNA methylation is used in biological systems to specifically repress the expression of genes. Furthermore, changes in normal DNA methylation occur with age and in tumorigenesis (Bird 2002). Performing methylation studies using methylation sensitive restriction enzymes, we found candidate regions for methylation of the rat *CYP17A1* gene in adrenal versus testis tissue. Bisulfite modification confirmed the presence of a methylated region within the rat CYP17 promoter in adrenal tissue. We did not study adrenal tissue after treatment of rats with 5aza-CdR. Of course, analysis of CYP17 methylation status in adrenals of rats treated with 5aza-CdR compared to controls would have provided best evidence for the role of methylation in the regulation of this gene. However, from previous experiments we know that such treatment is quite toxic for the animals that we decided for a different approach. For our studies, we chose rat testis as the control tissue in which the *CYP17A1* gene is expressed and therefore (most likely) not methylated. However, we found that even in the testis the *CYP17A1* gene was not unambiguously unmethylated. Only the analysis of genomic DNA of purified rat Leydig cells revealed a (mostly) unmethylated *CYP17A1* gene. Therefore, we think that the finding in the total testis is explained by the fact that the *CYP17A1* gene is expressed only in testicular Leydig cells which comprise only 2–7% of the testicular volume (Mori & Christensen 1980).

From our rat experiments we had weak evidence that tissue-specific expression of the *CYP17A1* gene in the adrenal cortex may be controlled by DNA methylation. Therefore, we wanted to further investigate the role of methylation for *CYP17A1* gene expression in rodents in a cellular system. Although our *in silico* analysis revealed marked differences in the CpG island mapping between rat and mouse (Fig. 2), we used mouse adrenocortical Y1 cells for this purpose as no such rat cell line is available. Like rat and mouse adrenals, Y1 cells do not express the *CYP17A1* gene endogenously. Both methyltransferase and HDAC inhibitors induced the expression of the *CYP17A1* gene in adrenocortical Y1 cells. The effect of the methyltransferase inhibitor was clearly stronger in the presence of a HDAC inhibitor. Therefore, DNA methylation of the *CYP17A1* gene in Y1 cells repressed the transcription at least in part by recruiting the HDAC. However, although being able to detect *CYP17A1* gene expression after demethylation and/or acetylation, we were not able to detect CYP17 activities. This might be due to toxicity of the applied chemicals or because other essential factors for P450c17 enzyme activities were missing. Y1 cells are derived from a tumor of an adult

male LAF<sub>1</sub> (C57L X A/HeJ) mouse. DNA methylation has been shown to play a role in the silencing of the *CYP21* gene in these cells (Szyf *et al.* 1990). This has been demonstrated by experiments using methylation-sensitive restriction endonuclease digest targeting the *CYP21* gene in these cells. Further confirmation derives from siRNA experiments. Expression of an antisense construct targeting DNA methyltransferase in Y1 cells restored *CYP21* gene expression (MacLeod & Szyf 1995). Altered DNA methylation patterns are known mechanism/s of tumorigenesis in many types of cancer (Bird 2002, Rodenhiser & Mann 2006). However, the *CYP17A1* gene is also not expressed in the normal mouse adrenal cortex. Therefore, our findings that DNA methylation controls CYP17 expression of both mouse adrenocortical Y1 cells and rat tissues suggest that epigenetic factors are involved in the tissue-specific expression of the *CYP17A1* gene in rodents.

DNA methylation may repress gene transcription by blocking the binding of essential transcription factors. To assess which transcription factors bind to the methylated region of the rat and mouse *CYP17A1* genes, we performed *in silico* studies. For the mouse CYP17 promoter region (–2791/–2644), we found possible binding of the activating protein-1 (AP-1), c-myc, v-myc, hepatocyte nuclear factor 4, chicken ovalbumin upstream promoter transcription factor (COUP-TF) and heat shock factor. The effect of most of these transcription factors on *CYP17A1* gene expression is unknown. Only AP-1 and COUP-TF are thought to regulate the transcription of genes involved in steroidogenesis. Binding of AP-1 transcription factors to the rat StAR, *CYP11B1* and CYP17 promoters transactivate transcription (Nason *et al.* 1992, Mukai *et al.* 1998, Sandhoff *et al.* 1998). Binding of COUP-TF to both bovine and rat *CYP17A1* gene promoters repress CYP17 transcription (Zhang & Mellon 1997). However, it remains unclear whether these factors are essential for the regulation of the mouse *CYP17A1* gene as the 5' regulatory region of the mouse *CYP17A1* gene has been investigated only as far as –1021 bp upstream of the transcription start site (Youngblood & Payne 1992, Busygina *et al.* 2005).

By contrast, *in silico* investigations for the rat *CYP17A1* gene promoter revealed no binding sites for transcription factors within the methylated island at –905/–692. For the rat *CYP17A1* gene, the promoter and its regulatory transcription factors have been studied only up to –440 bp upstream of the transcription start site (Givens *et al.* 1994, Zhang & Mellon 1996, 1997, Lin *et al.* 2001). Whether essential regulatory *cis*-elements exist further upstream is unknown.

In summary we suggest that DNA methylation is involved in the tissue-specific expression of the *CYP17A1* gene in both rat and mouse. Although the tissue-specific pattern of the CYP17 expression differs fundamentally between humans and rodents, regulatory mechanism/s controlling tissue-specific CYP17 expression seem to be similar between both species.

## Declaration of interest

None of the authors has anything to declare.

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## Author contribution statement

Elika Missaghian: experimental design, most experimental work and analysis, writing. Petra Kempná: experimental design and analysis. Andrea Hirsch: some experimental work. Bernhard Dick, Rasoul Alikhani-Koopaei and Brigitte M Frey: rat experiments and steroid urine analysis, data discussion. Bernard Jégou: purified rat Leydig cells. Primus E Mullis: data discussion. Christa E Flück: idea, experimental design, work analysis and overall supervision, writing.

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## References

Alikhani-Koopaei R, Fouladkou F, Frey FJ & Frey BM 2004 Epigenetic regulation of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 expression. *Journal of Clinical Investigation* **114** 1146–1157.

Antequera F 2003 Structure, function, evolution of CpG island promoters. *Cellular and Molecular Life Sciences* **60** 1647–1658.

Bird A 2002 DNA methylation patterns, epigenetic memory. *Genes and Development* **16** 6–21.

Di Blasio AM, Voutilainen R, Jaffe RB & Miller WL 1987 Hormonal regulation of messenger ribonucleic acids for P450<sub>sc</sub> (cholesterol side-chain cleavage enzyme) and P450c17 (17 $\alpha$ -hydroxylase/17,20-lyase) in cultured human fetal adrenal cells. *Journal of Clinical Endocrinology and Metabolism* **65** 170–175.

Busygina TV, Vasiliev GV, Kilmova NV, Ignatieva EV & Osadchuk AV 2005 Binding sites for transcription factor SF-1 in promoter regions of genes encoding mouse steroidogenesis enzymes 3 $\beta$ HSDI and P450c17. *Biochemistry* **70** 1152–1156.

Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE & Miller WL 1987 Cytochrome P450c17 (steroid 17 $\alpha$ -hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *PNAS* **84** 407–411.

Compagnone NA, Bulfone A, Rubenstein JL & Mellon SH 1995 Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. *Endocrinology* **136** 5212–5223.

Dalla Valle L, Toffolo V, Vianello S, Belvedere P & Colombo L 2004 Expression of cytochrome P450c17 and other steroid-converting enzymes in the rat kidney throughout the life-span. *Journal of Steroid Biochemistry and Molecular Biology* **91** 49–58.

Dalle M, Giry J, Gay M & Delost P 1978 Perinatal changes in plasma and adrenal corticosterone and aldosterone concentrations in the mouse. *Journal of Endocrinology* **76** 303–309.

Fan YS, Sasi R, Lee C, Winter JS, Waterman MR & Lin CC 1992 Localization of the human CYP17 gene (cytochrome P450(17 $\alpha$ )) to 10q24.3 by fluorescence *in situ* hybridization and simultaneous chromosome banding. *Genomics* **14** 1110–1111.

Fluck CE & Miller WL 2004 GATA-4 and GATA-6 modulate tissue-specific transcription of the human gene for P450c17 by direct interaction with Sp1. *Molecular Endocrinology* **18** 1144–1157.

George FW & Wilson JD 1980 Pathogenesis of the henny feathering trait in the Sebright bantam chicken. Increased conversion of androgen to estrogen in skin. *Journal of Clinical Investigation* **66** 57–65.

Givens CR, Zhang P, Bair SR & Mellon SH 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 and Cell Biology* **13** 1087–1098.

Le Goascogne C, Sananes N, Guezou M, Takemori S, Kominami S, Baulieu EE & Robel P 1991 Immunoreactive cytochrome P-450(17 $\alpha$ ) in rat and guinea-pig gonads, adrenal glands and brain. *Journal of Reproduction and Fertility* **93** 609–622.

Hornsby PJ, Yang LQ, Raju SG & Cheng CY 1991 Changes in gene expression and DNA methylation in adrenocortical cells senescing in culture. *Mutation Research* **256** 105–113.

Hu MC, Chou SJ, Huang YY, Hsu NC, Li H & Chung BC 1999 Tissue-specific, hormonal, and developmental regulation of SCC-LacZ expression in transgenic mice leads to adrenocortical zone characterization. *Endocrinology* **140** 5609–5618.

Johnson DC & Sen M 1990 The cytochrome P450(17)  $\alpha$  (17 $\alpha$ -hydroxylase/C17,20-lyase) activity of the junctional zone of the rat placenta. *Journal of Endocrinology* **125** 217–224.

Keeney DS, Jenkins CM & Waterman MR 1995 Developmentally regulated expression of adrenal 17 $\alpha$ -hydroxylase cytochrome P450 in the mouse embryo. *Endocrinology* **136** 4872–4879.

Klinefelter GR, Hall PF & Ewing LL 1988 Effect of luteinising hormone deprivation *in situ* on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biology of Reproduction* **36** 769–783.

Kurosumi M, Ishimura K, Fujita H & Osawa Y 1985 Immunocytochemical localization of aromatase in rat testis. *Histochemistry* **83** 401–404.

Lejeune H, Sanchez P & Saez JM 1998 Enhancement of long-term testosterone secretion and steroidogenic enzyme expression in human Leydig cells by co-culture with human Sertoli cell-enriched preparations. *International Journal of Andrology* **21** 129–140.

Leshin M 1985 5-Azacytidine and sodium butyrate induce expression of aromatase in fibroblasts from chickens carrying the henny feathering trait but not from wild-type chickens. *PNAS* **82** 3005–3009.

Lin CJ, Martens JW & Miller WL 2001 NF-1C, Sp1, and Sp3 are essential for transcription of the human gene for P450c17 (steroid 17 $\alpha$ -hydroxylase/17,20 lyase) in human adrenal NCI-H295A cells. *Molecular Endocrinology* **15** 1277–1293.

MacLeod AR & Szyf M 1995 Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. *Journal of Biological Chemistry* **270** 8037–8043.

Matteson KJ, Picado-Leonard J, Chung BC, Mohandas TK & Miller WL 1986 Assignment of the gene for adrenal P450c17 (steroid 17 $\alpha$ -hydroxylase/17,20 lyase) to human chromosome 10. *Journal of Clinical Endocrinology and Metabolism* **63** 789–791.

Miller WL, Auchus RJ & Geller DH 1997 The regulation of 17,20 lyase activity. *Steroids* **62** 133–142.

Mori H & Christensen AK 1980 Morphometric analysis of Leydig cells in the normal rat testis. *Journal of Cell Biology* **84** 340–354.

Mukai K, Mitani F, Agake R & Ishimura Y 1998 Adrenocorticotrophic hormone stimulates CYP11B1 gene transcription through a mechanism involving AP-1 factors. *European Journal of Biochemistry* **256** 190–200.

Namiki M, Kitamura M, Buczko E & Dufau ML 1988 Rat testis P-450(17 $\alpha$ ) cDNA: the deduced amino acid sequence, expression and secondary structural configuration. *Biochemical and Biophysical Research Communications* **157** 705–712.

Nason TF, Han XG & Hall PF 1992 Cyclic AMP regulates expression of the rat gene for steroid 17 $\alpha$ -hydroxylase/C17-20 lyase P-450 (CYP17) in rat Leydig cells. *Biochimica et Biophysica Acta* **1171** 73–80.

- Nishihara M, Winters CA, Buzko E, Waterman MR & Dufau ML 1988 Hormonal regulation of rat Leydig cell cytochrome P450c17 mRNA levels and characterization of a partial length rat P450c17 cDNA. *Biochemical and Biophysical Research Communications* **154** 151–158.
- Pezzi V, Mathis JM, Rainey WE & Carr BR 2003 Profiling transcript levels for steroidogenic enzymes in fetal tissues. *Journal of Steroid Biochemistry and Molecular Biology* **87** 181–189.
- Picado-Leonard J & Miller WL 1987 Cloning and sequence of the human gene for P450c17 (steroid 17  $\alpha$ -hydroxylase/17,20 lyase): similarity with the gene for P450c21. *DNA* **6** 439–448.
- Pignatelli D, Xiao F, Gouveia AM, Ferreira JG & Vinson GP 2006 Adrenarche in the rat. *Journal of Endocrinology* **191** 301–308.
- Rainey WE, Carr BR, Sasano H, Suzuki T & Mason JI 2002 Dissecting human adrenal androgen production. *Trends in Endocrinology and Metabolism* **13** 234–239.
- Rodenhiser D & Mann M 2006 Epigenetics and human disease: translating basic biology into clinical applications. *Canadian Medical Association Journal* **174** 341–348.
- Salmenpera M & Kahri AI 1976 Corticosterone, 18-OH-deoxycorticosterone, deoxycorticosterone and aldosterone secretion in tissue culture of foetal rat adrenals in the presence and the absence of ACTH. *Acta Endocrinologica* **83** 781–793.
- Sandhoff TW, Hales DB, Hales KH & McLean MP 1998 Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* **139** 4820–4831.
- Shackleton CH 1993 Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. *Journal of Steroid Biochemistry and Molecular Biology* **45** 127–140.
- Sparkes RS, Klisak I & Miller WL 1991 Regional mapping of genes encoding human steroidogenic enzymes: P450scc to 15q23–q24, adrenodoxin to 11q22; adrenodoxin reductase to 17q24–q25; and P450c17 to 10q24–q25. *DNA and Cell Biology* **10** 359–365.
- Szyf M, Milstone DS, Schimmer BP, Parker KL & Seidman JG 1990 Cis modification of the steroid 21-hydroxylase gene prevents its expression in the Y1 mouse adrenocortical tumor cell line. *Molecular Endocrinology* **4** 1144–1152.
- Voutilainen R & Miller WL 1986 Developmental expression of genes for the steroidogenic enzymes P450scc (20,22-desmolase), P450c17 (17 $\alpha$ -hydroxylase/17,20-lyase), and P450c21 (21-hydroxylase) in the human fetus. *Journal of Clinical Endocrinology and Metabolism* **63** 1145–1150.
- Voutilainen R, Tapanainen J, Chung BC, Matteson KJ & Miller WL 1986 Hormonal regulation of P450scc (20,22-desmolase) and P450c17 (17 $\alpha$ -hydroxylase/17,20-lyase) in cultured human granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **63** 202–207.
- Yamamoto T, Chapman BM, Johnson DC, Givens CR, Mellon SH & Soares MJ 1996 Cytochrome P450 17 $\alpha$ -hydroxylase gene expression in differentiating rat trophoblast cells. *Journal of Endocrinology* **150** 161–168.
- Youngblood GL & Payne AH 1992 Isolation and characterization of the mouse P450 17  $\alpha$ -hydroxylase/C17-20-lyase gene (CYP17): transcriptional regulation of the gene by cyclic adenosine 3',5'-monophosphate in MA-10 Leydig cells. *Molecular Endocrinology* **6** 927–934.
- Zhang P & Mellon SH 1996 The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3',5'-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17 $\alpha$ -hydroxylase/c17-20 lyase). *Molecular Endocrinology* **10** 147–158.
- Zhang P & Mellon SH 1997 Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. *Molecular Endocrinology* **11** 891–904.

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