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

Elika Missaghian1,*, Petra Kemprá1, Bernhard Dick2, Andrea Hirsch1, Rasoul Alikhani-Koupaei2, Bernard Jéou3, Primus E Mullis1, Brigitte M Frey2 and Christa E Flück1

1Pediatric Endocrinology and Diabetology, University Children's Hospital Bern and 2Department of Nephrology and Hypertension, Inselspital, University of Bern, Freiburgstrasse 15, Room G3 812, CH-3010 Bern, Switzerland
3Inserm, U625, GERHM, IFR140, Université de Rennes 1, Campus de Beaulieu, Rennes Cedex F-35042, France
*(Elika Missaghian has previously published under the name Elika Samandari)

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 17α-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.


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 17α-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 17α-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. [3H]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 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 steriodogenically active Leydig cells. At this stage, the purity of the Leydig cells is >96% as assessed by 3β-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 stigmasterol 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). To recognize putative binding sites for transcription factors (cis-elements), we used the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html).
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 μg RNA).

Methylation sensitive endonuclease cleavage

For methylation sensitive cleavage, the restriction enzymes *AvaI*, *NheI* (Promega) and *DraIII* (Roche) were used. Reactions were incubated at 37 °C for 24 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 β-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 β-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 μl. 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>NCBI accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse_CYP17 (PCR)</td>
<td>S: 5'-CATATCTTGTGTCACCGTG-3'&lt;br&gt;AS: 5'-ACCAGCGATGCTGGTTATG-3'&lt;br&gt;Mouse β-actin</td>
<td>AY594330 (8081)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-TGGTGACATCAAGAG-3'&lt;br&gt;AS: 5'-TATTGATCCTCAGGGATC-3'&lt;br&gt;Mouse_CYP17 (cloning)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AY594330 (9299)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-ATGGTGGGAACTCATACGTC-3'&lt;br&gt;G: 5'-ATGACCAAGCTTCCAGT-3'&lt;br&gt;A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NM_007393 (705)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-TTGGAATGTTATTTTGTGG-3'&lt;br&gt;C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NM_007393 (901)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-AGGACCACCTCTTTTTACG-3'&lt;br&gt;Rat β-actin</td>
<td>AY594330 (3410)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-AAAAAGTGCACCAGCTGGAG-3'&lt;br&gt;A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>X69816 (673)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-AGTGGTGGAATTAGAGTTATAGAAAATT-3'&lt;br&gt;G&lt;sup&gt;b&lt;/sup&gt;</td>
<td>X69816 (838)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-ATGCCGCCACCAACGTCCATG-3'&lt;br&gt;Rat β-actin</td>
<td>X69816 (653)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-ATTGCAAGCAGCTTTTACG-3'&lt;br&gt;Bs rat_big</td>
<td>X69816 (635)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-TGTAATATGGATGTTATTTGTGG-3'&lt;br&gt;Bs rat_bigAS</td>
<td>X69816 (6036)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-AAATATTATATATTGTGTATATAT-3'&lt;br&gt;Bs rat_small</td>
<td>X69816 (958)</td>
</tr>
<tr>
<td></td>
<td>S: 5'-CTCTGGAATAGCTTATAGAAAATT-3'&lt;br&gt;Bs rat_small</td>
<td>X69816 (778)</td>
</tr>
</tbody>
</table>

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

<sup>b</sup>Primer 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 (Micsyntoch, 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 luciferase served as control.
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., San Diego, 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

| Table 2 Concentrations (ng/24 h) of urinary tetrahydrometabolites (THF) of cortisol from untreated and treated rats (1 mg/kg 5-aza-CdR) |
|---|---|---|---|
| Treated rats (n=3) | Baseline | Day 3 | Day 5 |
| 22 ± 0·7 | 153·3 ± 33·8 | 296·3 ± 9·5 |
| 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.

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, DralI within the promoter region and Aval, Nhel 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 ± S.E.M. *P<0.05.
sensitive restriction enzymes AvaI, DraIII and NheI (Fig. 3A). Genomic DNA extracted from testes and adrenals was incubated with these restriction enzymes and targeted DNA regions were studied by PCR. Experimental design foresees that methylated DNA regions may not be cleaved by methylation sensitive restriction enzymes; thus, amplification of such regions should yield products A, B and C (Fig. 3A). By contrast, in the absence of DNA methylation DNA may be cleaved and none or markedly less PCR products should be obtained. Non-digested DNA served as control of DNA quality and \textit{\textbeta}-actin as PCR control for specificity and quantitative analysis. PCR amplification of adrenal samples after digest gave a higher yield for both AvaI fragments B and C than testes samples (Fig. 3B and C); but the difference was only significant for the AvaI site included in the promoter region (Fig. 3D) suggesting that this site might be protected from digestion through DNA methylation. By contrast, the DraIII and NheI sites yielded less PCR products A and C for both adrenals and testes, suggesting that these sites were cleaved and thus, unmethylated.

It is known that methylation of CpG islands within promoter regions is likely to regulate the transcription of genes. Thus, the results from our first experiments prompted us to further investigate the promoter region of the rat \textit{CYP17A1} gene harboring the AvaI site by bisulfite modification and sequencing. After bisulfite treatment of genomic DNA extracted from adrenals, the cytosines within the CpG island of AvaI remained unchanged suggesting cytosine methylation (Fig. 4). By contrast, all other non-CpG dinucleotide cytosines appeared as thymidines, assessing complete bisulfite modification. These data were confirmed for seven different clones generated from rat adrenals. For the rat testis, the same analysis revealed that in one out of seven clones the cytosine within the AvaI CpG region appeared as thymidine, consistent with the absence of DNA methylation. Because the expression of the \textit{CYP17A1} gene is restricted to Leydig cells which make barely 3% of the volume of a testis (Kurosumi et al. 1985, Lejeune et al. 1998), we repeated the experiment with genomic DNA extracted from purified rat Leydig cells (>96%). Consistent with our hypothesis we then found that five out of seven clones showed thymidine conversion of the cytosine in the AvaI CpG island (Fig. 4).

Mouse adrenocortical Y1 cells express \textit{CYP17A1} after treatment with 5-aza-CdR and TSA

Similar to rats, mice do not express the \textit{CYP17A1} gene in their adrenal cortex. Our studies in rats suggested that the \textit{CYP17A1} gene may be regulated by methylation. DNA methylation of genes may recruit the binding of the HDAC, which will deacetylate histones thereby blocking transcription. Hence, we assessed the effect of DNA methylation and/or histone deacetylation on the expression of the \textit{CYP17A1} gene in mouse adrenocortical Y1 cells after treatment with 5-aza-CdR and TSA. Y1 cells were treated with 1 \textmu M 5-aza-CdR and/or 100 nM TSA or DMSO (solvent) for 72 h. Expression of the \textit{CYP17A1} gene was studied by RT-PCR (35 cycles). Expression of the \textit{CYP17A1} gene in mouse testicular Leydig MA-10 cells served as positive control and \textit{\textbeta}-actin served as internal control. Results are given from a representative experiment, which was confirmed six times independently.
in independent experiments. CYP17 expression was due to missing transcription factors.

We transfected Y1 cells 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 ± S.E.M. of three independent experiments. *P ≤ 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, Alkhami-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 (Alkhami-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 17α-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 17α-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 (Salmempera & Kahr 1976, Dalle et al. 1978, Keeney et al. 1995). Recently, the production of androstenedione, cortisol and 17α-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 17α-
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 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 LAF1 (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 methyla-
tion 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
epigeneric 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
to 2791/−2644, we found possible binding of the activating
protein-1 (AP-1), c-myb, v-myb, 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
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 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,
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.

Funding

This work was supported by grants from the Swiss National Science Foundation: 3100A0-102153 to B M F and 320000-116299 to C E F.

Author contribution statement


Acknowledgements

We thank Prof. Walter L Miller at the University of California San Francisco for providing MA-10 cells and Isabelle Dorval-Coffeck for her contribution to the preparation of purified rat Leydig cells. We thank Gaby Hofer for technical support throughout this work and acknowledge Dr Chantal Cripe-Mamie for helping in the final preparation of the manuscript.

References


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.


Leshin M 1985 5-Aza-2′-deoxyadenosine inhibits the expression of aromatase in fibroblasts from chickens carrying the henny feathering trait but not from wild-type chickens. PNAS 82 3005–3009.


www.endocrinology-journals.org


Sparkes RS, Klisak I & Miller WL 1991 Regional mapping of genes encoding human steroidogenic enzymes: P450ccc to 15q23-q24, adrenodoxin to 11q22; adrenodoxin reductase to 17q24-q25; and P450c17 to 10q24-q25. *DNA and Cell Biology* **10** 389–365.

Szyf M, Mahtone 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.


Zhang P & Mellon SH 1996 The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3′,5′-monophosphate-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17α-hydroxylase/c17-20 lyase. *Molecular Endocrinology* **10** 147–158.


**Received in final form 15 April 2009**

**Accepted 29 April 2009**

**Made available online as an Accepted Preprint 29 April 2009**