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# The bovine genome contains three differentially methylated paralogous copies of the P450c17 encoding gene (*CYP17A1*)

Jens Vanselow *, Rainer Fürbass

*Leibniz Institute for Farm Animal Biology (FBN), Research Unit Molecular Biology, 18196 Dummerstorf, Germany*

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ABSTRACT

*CYP17A1* encodes the key enzyme of androgen biosynthesis, P450c17. The gene is expressed in a number of steroidogenic tissues among them testis, ovary, placenta and adrenal gland. The proper analysis of *CYP17A1* expression and of epigenetic parameters however, is hampered by the presence of more than one copy of the gene within the bovine genome. Therefore, as a prerequisite for future studies we characterized these copies and analyzed their promoter methylation and expression profiles in different tissues. DNA methylation levels were determined by bisulfite modification, amplification, cloning and sequencing. Transcription was analyzed by RT-PCR. From bovine genomic DNA three different *CYP17A1* promoter sequences could be amplified with a sequence similarity of 94.8%, 95.6% and 98.7%. Based on these sequences we could reconstruct, by in silico analysis, the promoter regions and eight potentially coding exons of two loci, *CYP17A1a* and *CYP17A1b*, and the promoter region and truncated first exon of a third locus, *CYP17A1x*. By using locus-specific primers, only transcripts of *CYP17A1a*, but not of *CYP17A1b* could be detected in testis, epididymis, theca, corpus luteum, placental cotyledons, adrenal gland and preoptic brain area. Methylation analysis revealed that only the *CYP17A1a* promoter was hypo-methylated in the tested P450c*17* active tissues, whereas both other copies showed higher levels of methylation. From these data we conclude that the bovine genome contains three paralogous copies of the *CYP17A1* gene, of which two (*CYP17A1b* and *CYP17A1x*) might be silenced by epigenetic modification (promoter methylation).



## 1. Introduction

Androgens are produced by a number of steroidogenic glands among them testis and adrenal gland. But also in female reproductive organs as placenta and ovary, large amounts of androgens are produced, mainly as precursors for oestrogen biosynthesis. Steroid 17-alpha-hydroxylase/17,20 lyase (P450c17) catalyzes the final step of androgen biosynthesis and is encoded by the *CYP17A1* gene. In the ovary this gene is almost exclusively expressed in the theca [1]. It shows a peak of expression in large dominant follicles and a steep decline of expression after the LH surge [4,15,21].

Several lines of evidence demonstrate that epigenetic mechanisms as DNA methylation, histone modification and chromatin modulation are essentially involved in gene regulation. In vertebrates methylation of cytosines (C) that occurs mainly in the context of CpG dinucleotides, plays an important role in the local organization of the chromatin and thus the long-term regulation of gene expression via epigenetic mechanisms. In recent years, it became evident that the transcriptional silencing that is associated with DNA methylation is involved in the protection against intragenomic parasites [22] and in carcinogenesis [11]. But also essential regulatory processes during mammalian development as genomic imprinting or X-chromosome inactivation [13] are closely associated with differential methylation of CpG-rich regions called CpG islands. However, the potential role of DNA methylation in cell type- or differentiation-specific gene expression or in the regulation of CpG-poor promoters is less well established. In the ovine and bovine it was demonstrated that promoters of the *CYP19A1* gene that encodes the key enzyme of oestrogen biosynthesis, aromatase cytochrome P450, as well as the distal promoter of the oxytocin gene show expression correlated DNA methylation and chromatin structures in placenta and ovary, respectively [7,8,12,18]. In recent publications we investigated the cell type-specific promoter methylation and expression levels of *CYP11A*1 (encoding cytochrome P450 cholesterol side-chain cleavage enzyme), *HSD3B1* (encoding 3-β-hydroxysteroid dehydrogenase) and *CYP19A1* in bovine follicles before and after the LH surge and after luteinization [15,17,19]. We found that silencing of *CYP19A1* during luteinization is associated with de novo methylation of its proximal promoter 2. Reliable methylation data on the *CYP17A1* gene are not available yet because highly similar paralogous copies within the bovine genome impeded the analysis.

* Corresponding author. Fax: +49 38208 68702.
*E-mail address:* vanselow@fbn-dummerstorf.de (J. Vanselow).

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The present study was therefore aimed to elucidate and characterize the different copies of *CYP17A1* in the bovine genome and to collect first comparative data connecting expression with promoter methylation. The putative role of these different *CYP17A1* paralogs has to be clarified for the interpretation of expression and methylation data of this gene.

## 2. Materials and methods

### 2.1. Tissue collection

Theca samples from large dominant follicles were collected from ($n = 6$) cycling cows as described earlier [15,19]. Adrenal gland ($n = 3$ animals), liver ($n = 6$), testis ($n = 7$), mammary gland ($n = 5$) and spleen ($n = 1$), as well as hippocampal ($n = 2$), hypothalamic ($n = 1$) and preoptic ($n = 1$) brain areas were obtained from a local slaughterhouse. Placentomes and corpora lutea were collected from ($n = 6$) cows between day 90–160 of pregnancy after slaughter. Cotyledons (foetal portions of placentomes) were manually separated from caruncles (maternal portion). All tissues were immediately transferred to RNAlater solution (Qiagen, Hilden, Germany), incubated at 4 °C overnight and were then transferred to −20 °C for long-term storage until DNA and/or RNA preparation.

### 2.2. RNA preparation and RT-PCR

Total RNA was prepared with the RNeasy Mini Kit (Qiagen) from small tissue chunks conserved in RNAlater. Primers for cDNA synthesis and amplification of different transcripts were designed according to published mRNA sequences (Table 1). For cDNA synthesis, RNA was reverse transcribed in a 25 µl reaction volume using M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Mannheim, Germany) with gene-specific primers. cDNA was amplified with *Taq* DNA Polymerase (#EPTQA, MP Biochemicals, Eschwege, Germany) under the following cycling conditions: preincubation at 95 °C for 5 min, 30 cycles denaturation at 95 °C for 1 min, annealing at 63 °C for 1 min, and extension at 70 °C for 2 min. This was followed by 10 min at 70 °C. Primers used for amplification are shown in Table 1. PCR products were analyzed by electrophoresis on a 3% ethidium bromide agarose gel. As positive controls and to test primer specificity, PCR products with binding sites for primer pairs 4a/5a (*CYP17A1a* mRNA) and 4b/5b (predicted *CYP17A1b* mRNA), respectively, were generated and cloned into pGEM-T plasmids (Promega, Heidelberg, Germany).

### 2.3. DNA isolation, bisulfite modification, amplification, cloning and sequencing

Genomic DNA was isolated with the QIAamp DNA Kit (Qiagen). The EZ DNA Methylation-Gold™ Kit (Zymo Research, HISS Diagnostics, Freiburg, Germany) was used to modify the DNA with bisulfite. Subsequent PCR was performed using HotStarTaq Plus (Qiagen) under the following cycling conditions: preincubation at 95 °C for 5 min, 45 cycles denaturation at 95 °C for 1 min 15 s, annealing at 53 °C for 1 min 15 s, and extension at 72 °C for 2 min. This was followed by 10 min at 72 °C. Primers used for amplification of modified DNA are shown in Table 1.

PCR products from modified DNA were subsequently cloned and sequenced. The efficiency of conversion of unmethylated C to U was greater than 99%, as described in a previous study [17]. For methylation analysis, the percentage of methylation (at each CpG) was determined from 9 to 24 clones per individual sample. The tissue-specific means and standard errors of means were then calculated from ($n = 6$) theca and ($n = 3$) adrenal gland, cotyledon and liver samples, each.

## 3. Results

### 3.1. Amplification of modified genomic DNA and reconstruction of three distinct CYP17A1 loci

Preliminary attempts to record the tissue-specific methylation profiles of the *CYP17A1* promoter with the bisulfite direct sequencing method (described in [17]) yielded sequences with single nucleotide mutations at various non CpG positions (not shown). This indicated that the amplicons consisted of a mix of different molecules. In order to collect data from individual molecules, we re-sequenced the PCR products after cloning. For this, more than 500 individual clones were sequenced and evaluated. This analysis revealed that three distinct loci had been amplified with the same primer pair. The corresponding sequences could be clearly distinguished due to distinct diagnostic single nucleotide mutations. These three different sequences were found in each of the animals with a relative frequency distribution of 30.5 ± 2.2%, 34.8 ± 1.4% and 34.7 ± 2.4% (mean ± SEM, $n = 15$). The corresponding genes/genomic loci were named *CYP17A1a*, *CYP17A1b* and *CYP17A1x* throughout the present study. By BLAST search (Basic Local Alignment Search Tool) of the NCBI bovine genomic database (National Center for Biotechnology Information) we identified two contigs assigned to bovine chromosome 26. Contig NW_001503027 included loci identical to two of the amplified promoter regions. The first locus assigned by NCBI as "*CYP17A1*" (Gene symbol) with GeneID: 281739, corresponded to *CYP17A1a*. The second, not yet assigned locus was found about 12 kb downstream from *CYP17A1a* at the very end of the contig. Therefore, only the promoter and a part of the first exon were included in the contig. This new, so far undescribed locus is named *CYP17A1x* throughout the present study. The second contig, NC_007327, also contained *CYP17A1a*, and about 500 kb downstream, *CYP17A1b*, which was also assigned by NCBI as "*CYP17A1*", however, with GeneID 782561. This contig, however, did not include *CYP17A1x*, as expected. Because both contigs still included large portions of unspecified sequences and gaps,

**Table 1**
Primers used for RT-PCR and amplification of bisulfite modified genomic DNA.

| Gene/mRNA | Name | Sequence | nt | Accession no. |
|---|---|---|---|---|
| *RT-PCR* | | | | |
| *CYP17A1a*/*CYP17A1b* | 5 (rt) | GGCCACGATCCACTTTATCACAGA | | NM_174304 |
| *CYP17A1a* | 4a (for) | GAAAAGATGAAGGGTTGTGTTCAAAC | | NM_174304 |
| | 5a (rev) | CGAAGATGTCCCCTATAGTAGCG | 215 | NM_174304 |
| *CYP17A1b* | 4b (for) | GAAAAGATGAAGGGTTGTGTTCAAAT | | XM_0011251231 |
| | 5b (rev) | CGAAGATGTCCCCTATGGTAGCA | 215 | XM_0011251231 |
| *PCR of mod. DNA* | | | | |
| *CYP17A1a*/*CYP17A1b* | for | TATATTTAAAATGGTAAATTTTATGTTATG | 462 | NW_001503027 |
| *CYP17A1x* | rev | ACTTCTTTCAAAAACTAACTCCACAC | | NW_001494358 |

for, forward primer; nt, length of PCR product in nucleotides; rev, reverse primer; rt, primer for reverse transcription.