cDNA Cloning, Genomic Structure and Expression Analysis of the Bovine Lanosterol 14α-Demethylase (CYP51) in Gonads

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Meiosis activating sterol (MAS), the intermediate of cholesterol biosynthesis, is an important substance to stimulate oocytes maturation in FSH-induced signal transduction pathway. Lanosterol 14α-demethylase (CYP51) converts lanosterol to MAS. Although MAS is firstly isolated from bovine testis, the information about bovine *CYP51* gene and its expression is little. In present studies, the cDNA cloning, genomic structure, chromosomal mapping, and expression patterns of bovine *CYP51* were demonstrated. The cDNA coding bovine CYP51 contains a 1509 bp open reading frame and a 1119 bp 3′ untranslated region. And the bovine *CYP51* genen includes 10 exons and spans about 17 kb. Screening the cattle RH_{5000} panel bovine *CYP51* is mapped to chromosome 4 (0cR). The sequenced promoter region is TATA-less and contains several highly conserved regulatory elements, such as GC-box, cAMP-responsive elements (CRE), sterol regulatory element (SRE) which is important fragment for its transcription. No evidence of processed pseudogenes is found using long PCR and Southern blot. Northern blot analysis reveals that an approximately 2.7 kb mRNA is expressed in all the examined bovine tissues, while a 1.8 kb mRNA is found only in the mature bovine testis where the MAS is accumulated. Immunochemistry analysis shows that leydig cells express the highest level of the CYP51 protein in testis. Among different stages follicles it is localized primarily to the oocytes with the level varying slightly. Granulosa cells of primordial, primary and secondary follicles show background staining. While granulosa cells facing the antrum and cumulus granulosa cells of antral follicles show considerably heavier staining. The highest level is expressed in corpus lutea. These data indicate a stage- and cell type-specific expression of CYP51 protein in bovine oogenesis.

Key words lanosterol 14α-demethylase; CYP51; meiosis activating sterol; bovine

Sterol biosynthesis is an essential metabolic pathway in all eukaryotic organisms. Lanosterol 14α-demethylase (CYP51) is a cytochrome P450 enzyme of the sterol biosynthesis pathway belonging to the *CYP51* gene family which is the most evolutionarily conserved member of the CYP superfamily and the only cytochrome P450 family present in all kingdoms of biology.[1,2] CYP51 protein catalyzes the oxidative removal of the 14α-methyl group (C32) of lanosterol and 24-methylene-24,25-dihydrolanosterol in yeast and fungi, lanosterol and 24,25-dihydrolanosterol in mammals, and obtusifoliol in plants.

In mammalian gonads, CYP51 enzyme is not only important to the synthesis of cholesterol, but also has a role in overproducing meiosis activating sterol (MAS), an intermediate of the postsqualene part of cholesterol biosynthesis, including follicular fluid meiosis activating sterol (FF-MAS) and testis meiosis activating sterol (T-MAS) accumulating mainly in ovaries and testis respectively. MAS was proved to have the ability to trigger resumption of oocytes meiosis,[3] which can promote not only the nuclear but also plasma maturation and preimplantation development of mouse oocytes and fertilized oocytes *in vitro*.[4,5] Our lab recently have proved MAS also can promote the formation of follicle in fetal mouse ovaries (Xie, unpublished, 2005). CYP51 protein is highly expressed in mammalian testis leydig cell and round, elongating spermatids.[6,7] It resides in the endoplasmatic reticulum of most cells and also in acrosomal membranes of spermatids where transport through the Golgi apparatus is suggested.[8] But the role of MAS in the spermatogenesis is not well known.

The mammalian CYP51 cDNA and the gene have previously been characterized in mouse,[9] rat,[10] human,[11,12] and pig.[13] CYP51 mRNAs are widely expressed in those mammalian tissues with the highest level in haploid male germ cells spermatids. Two different types of CYP51 transcripts somatic transcripts and additional testis-specific transcripts are observed for differential polyadenylation site usage. *CYP51* genes have several common characteristics: ten exons and nine introns, highly conserved exon/intron borders and proximal promoter structures.[14] The promoter region is TATA-less and contains several highly conserved regulatory elements, such as GC-box, cAMP-responsive elements (CRE), sterol regulatory element (SRE), suggesting an evolutionary conserved mechanism of CYP51 transcriptional regulation in mammals. In somatic cells CYP51 expression is regulated by sterol regulatory element binding proteins (SREBPs),[14] while the germ-cell-specific cAMP/CREMτ-dependent upregulation of CYP51 expression might contribute to increase the production of MAS in testis.[14,15]

Although T-MAS was first found and purified from bovine testis, the information of bovine CYP51 is little. In the present study, we characterized the bovine *CYP51* gene and its expression and discussed the probable role of CYP51 and MAS in reproduction.

MATERIALS AND METHODS

RNA Extraction Bovine tissues were collected from a local abattoir. All tissues were snap frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted. Total RNA from bovine tissues was extracted using TRIzol™ Reagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) according to manufacturer's instructions. RNA concentrations were calculated based on the absorbance at

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260 nm. RNA quality was confirmed by electrophoresis on agarose-formaldehyde gels.

5′ and 3′-RACE Cloning of the Bovine cDNA Encoding CYP51 BLASTN search of the bovine EST database was performed to identify bovine cDNA clones homologous to mouse CYP51 cDNA. A UniGene Bt.621 was found and used to design 3′-RACE and 5′-RACE PCR primers. According to the SMART RACE cDNA amplication Kit user manual (Clontech, Inc., U.S.A.), total RNA(2 μg), purified from liver with TRIzol, was used to synthesize 3′-RACE-Ready cDNA using reverse primer 10 pmol 3′-CDS. Following first strand synthesis, PCR and nested PCR was carried out using GSP1+UPM, GSP2+NUP with conditions showed in Table 2. 5′-RACE-Ready cDNA synthesis was performed with primers 10 pmol 5′-CDS and 10 pmol SMARTII A oligo. PCR was carried out using GSPR and UPM as conditions showed in Table 2. The PCR products were cloned into the pGEM-T-easy vector (Promega, Madison, WI, U.S.A.) and then subjected to sequence analysis.

Northern Blot Analysis The Northern blot was performed to determine the length and the relative abundance of CYP51 mRNAs in bovine tissues using DIG Northern Starter Kit (Roche Applied Science, Mannheim, Germany). The bovine CYP51 5′-RACE products were cloned into the pSPT18 vector after cut with *Eco*RI and *Pst*I. The vector was linearized with *Hin*dIII and used as a template for the *in vitro* transcription by T7 RNA polymerase. Total RNA (20 μg) was denatured in formamide, separated in denaturing agarose gel (1.2% agarose/2.2 M formaldehyde), and blotted onto a Nylon membranes positively charged (Roche). The membrane was hybridized at 68 °C overnight with the DIG-RNA probe specific for bovine CYP51 and then washed with high-stringency (68 °C 0.1×SSC, 0.1% SDS). After immunological detection, the membrane was exposed to X-ray film for 0.5 min.

Genomic DNA Isolation and Southern Blot Analysis Genomic DNA was isolated from bovine liver using Proteinase K and Phenol method. The DNA was dissolved with TE and measured the concentration by absorbance at 260 nm. Southern blots containing 10 μg/lane of *Eco*RI, *Bam*HI, *Hin*dIII-digested genomic DNA were hybridized at 42 °C overnight with the bovine CYP51 probe the same as in the Northern blot. Then the blot was washed with 0.1×SSC, 0.1% SDS at 65 °C. Finally the membrane was exposed to X-ray film for 3 min.

Immunochemistry Bovine testis and ovary were immediately fixed in Bouins solution, dehydrated, and embedded in paraffin. Briefly, sections (8 μm) were cut and mounted onto 3-aminopropyltriethoxysilane-coated slides, deparaffinized, and rehydrated. Nonspecific binding was blocked in 10% normal horse serum in PBS for 1 h. The sections were incubated with rabbit anti-human CYP51 (donated from Waterman) in 10% horse serum overnight at 4 °C. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG followed by an avidin-alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA, U.S.A.). In some sections, rabbit anti-human CYP51 was replaced with normal rabbit IgG as a negative control. The sections were counterstained with hematoxylin and mounted. The degree of staining was assessed subjectively by blinded examination of the slides by two investigators.

RESULTS

Identification of Bovine CYP51 and Sequence Analysis With a combination of bioinformatics tools, PCR cloning, and 3′ and 5′ Rapid Amplification of cDNA Ends (RACE) analysis, we have obtained the complete bovine CYP51 cDNA, which is about 2.7 kb (genebank ID 73253833), including an open reading frame of 1509 and 1119 bp of 3′ untranslated sequence with two typical (AATAAA) and one modified (ATTAAA) polyadenylation signal sequences (Fig. 1). The first ATG triplet in the cDNA sequence was in frame, but 6 bp downstream to other known mammalian CYP51 initial codon. The predicted amino acid sequence was also similar in length and composition to those of other known mam-

Fig. 1. 3′-URT of Bovine CYP51 cDNA

Two typical (AATAAA) and one modified (ATTAAA) polyadenylation signal are highlighted by underlining, and another polyadenylation signal (AATGAA) is denoted by wave line that was used to generate the short mRNA. Arrows indicated the polyadenylated site of short transcript. The stop codon were shown in bold.

Fig. 2. Alignment of Human, Pig, Bovine, Rabbit, Rat and Mouse CYP51 Proteins

Predicted substrate recognition sites (SRS1—6) are framed. A heme binding region is denoted by dot-frame and the iron-binding cysteine is shown by arrowheads.

malian CYP51s. And the sequence identity was high ranging typically from 88 to 93% within the mammalian CYP51 family. Six putative substrate recognition sites (SRSs) and a iron-binding cysteine are highly conserved reflecting the basic function of CYP51 for sterol biosynthesis[16] (Fig. 2).

Genomic Analysis and Chromosomal Mapping of Bovine CYP51 Upon AC108175, a contig of bovine genomic working draft which is aligned best to *CYP51 cDNA*, two pairs of primers BMF1, BMR1 and BMF2, BMR2 were designed (Table 2) and obtained two specific fragments, 7.8 and 8.2 kb respectively, by long PCR. The partial sequenced fragments are identical to that of AC108175. Then exon/intron boundaries were estimated by aligning bovine *CYP51* cDNA with genomic DNA of AC108175. The result showed that the bovine *CYP51* gene is composed of ten exons and nine introns, spanning approximately 17 kb of the genome. The size of each exon and intron and the sequence across each exon/intron boundary are listed in Table 1. To analysis the putative regulatory sequences of bovine *CYP51*, a genomic fragment plus flanking regions was obtained with primer B5F and B5R and completely sequenced (Fig. 3). The promoter region is TATA-less and contains several highly conserved regulatory elements: cAMP-responsive elements CRE1, CRE2 and CRE3, a sterol regulatory element SRE1, two GC-box, a GCAAT sequence (potential nuclear factor Y-binding site), a downstream promoter element (DPE), and three highly conserved regions with unknown function (DBE1, DBE2, DBE3). The 949-bp region of the bovine *CYP51*, including the proximal promoter and the first exon is 62.8% GC-rich. This region forms two CpG islands with the (Obs/Exp) CpG ratio of 1.03 and 0.88 respectively. But the major transcription start sites require further study.

Long PCR produced only one specific fragment with primers BMF1+BMR1, BMF2+BMR2. No additional short band was found (not shown). Furthermore, Southern blot of *Eco*RI, *Hin*dIII, *Bam*HI-digested bovine genomic DNA revealed only one band (Fig. 4). If there are any intronless pseudogenes in bovine as found in human and rat, more than two bands should be obtained on Southern Blot. These results suggest there is no *CYP51* pseudogene in bovine.

We further refined the chromosomal location of bovine *CYP51* by screening the cattle RH_{5000} panel (http://cagst.animal.uiuc.edu/RHmap2004/index.html). The result demonstrated that *CYP51* was linked with EST marker AW261176 on chromosome 4 (0cR). The EST is the 3′ boundary of the bovine *CYP51*. The human *CYP51* gene[11] was located at chromosome 7q21.2—21.3, a region syntenic with bovine chromosome 4.[17] Furthermore, the bovine *CYP51* gene is linkage with a maker gene AKAP9 as arrangement in human chromosome 7q21—22.

Table 1. Intron/Exon Organization of the Bovine *CYP51* Gene

Exon	5′ Donor	Intro size	3′ Acceptor	Exon
1	gactggggcg/**GT**ACGTGCAC	1823	TCTTTTGC**AG**/aaaagtccac	2
2	atatgagaag/**GT**AAGTGTTA	2300	CTCTCTTC**AG**/tatggacctg	3
3	gcctaataca/**GT**AGGTGACG	1223	TATTTTGA**AG**/gttttcttgg	4
4	ggagaaaaaa/**GT**AAGCACAA	1598	TTCCCTCT**AG**/atttgtttga	5
5	ctagtttcag/**GT**ATGGACGA	881	TGAATTAC**AG**/acgcagggac	6
6	ctacttacaa/**GT**AAGAGCTC	390	CATTCTGT**AG**/ggatgggcgt	7
7	ttatgaccag/**GT**TTGTGGGA	1392	CTCTTTTT**AG**/ctcaaggatc	8
8	aactcctctg/**GT**GAGTCTCC	799	TCACCTGC**AG**/actgtggcag	9
9	tttggagctg/**GT**AAGATGAT	3406	TCTTTTAT**AG**/ggcgtcatcg	10

Table 2. Primer Sequences Used for PCR Reaction

Forward primers	Reverse primers	PCR condition
GSP1: 5′ CTG GTC TAC CTG TTC CGC CTC 3′	UPM	94 °C 2 min 1 cycle; 94 °C 15 s, 62 °C 15 s, 72 °C 2 min 30 cycles; 72 °C 3 min 1 cycle
GSP2: 5′ AAA GTT GGG GAG AAA GTG GAG 3′	NUP	94 °C 2 min 1 cycle; 94 °C 15 s, 62 °C 15 s, 72 °C 2 min 30 cycles; 72 °C 3 min 1 cycle
UPM	GSPR: 5′ CTC TCT GCG TTT CTG GAT TGC CTT G 3′	94 °C 2 min 1 cycle; 94 °C 15 s, 72 °C 1.5 min 3 cycles; 94 °C 15 s, 68 °C 15 s, 72 °C 1 min 27 cycles; 72 °C 3 min 1 cycle
BMF1: 5′ GCT GAC GCC ACA TAA GCC GAG AT 3′	BMR1: 5′ AGC CAA GAT AGA TGC CAC GGA GGT 3′	94 °C 2 min 1 cycle; 94 °C 10 s, 68 °C 15 s, 72 °C 1 min 27 cycles; 72 °C 3 min 1 cycle
BMF2: 5′ GAA ATC AGA AGT CAA CTC AAC GAG AAG G 3′	BMR1: 5′ AGC CAA GAT AGA TGC CAC GGA GGT 3′	94 °C 2 min 1 cycle; 94 °C 15 s, 68 °C 15 s, 72 °C 1 min 27 cycles; 72 °C 3 min 1 cycle
B5F: 5′ AAC CAC GAG TTG TTA CCT CTA TCA TTG 3′	BMR2: 5′ ACA CTG CCC AAT AAA TGA ATG AAT AAC C 3′	94 °C 2 min 1 cycle; 94 °C 15 s, 68 °C 15 s, 72 °C 1 min 27 cycles; 72 °C 3 min 1 cycle

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-788 AACCACGAGTTGTTACCTCT ATCATTGCATATGAATACTG GCCTGGTTTTATTCGAGGTG AGATGGGAATGACCCCAGCG
-698 TCCCAACCTTCAGGGTGTAT AAGGCGTCTTTCACAATCTC CCTTTATCGCGGATATTTGG AACTCTCCGACCGCCCGGGA
-618 CGCGGCCCCCGAACGGGTTC AGGTGCGGCGCCTTTCAGAG CCCAGGAAGGGGGCGGCGCC CCAGCCACCCGCCCTGCTGA
-538 CGCCACATAAGCCGAGATCA CCTCAGCCGCGCGCGGTGCA ATCACCGAACGCGCGCCGCC GCCGCCGGCACGCCGCAACC
-458 CTAGCCCTGAAATCCTTCCG CCCGTCCCATTACAGTGTTG TGTGCTGGCGCCCCGCACCC AGGACACACAGGCTCGGGTG
-378 GCCCGCGATTCTTAGGGGTG ACCTCCTCTTAAGCCTATGT TTCTCTTCTGTCTCTGCACT CCCCGTGCCACAGGTGCAAC
-298 CTGCTTCTTCAGGGCCTCTC TCTCTCGGCGCAAGGAGACT TCGGCATTGGGATGCTTTTA AGGGGCGTGGCCAGAGGCAG
-218 GTCCCGCCCTATTCTGTGAC GTATAGGGTGGTGTGCGAAA GAGGCGGAGTTATAGGGGGT GGGGGGTGGGGCTGGGCTTA
-138 GTAGGAGACCTGCTGGCCCG GCCACCTCCTAACACCCATC TGCCAGCTTCTGTTCCTCGG TGGATTCGGAGGAGCCGCGG
 -58 CGACCTCGGCCTCCAACGTC TCTGGCAAATTGACGCGGGG GTGGCGGCCGGGATAGTGAT GCTGGACTTACTGCAGGCGG
  23 GCGGGTCAGTGCTGGGGCAG GCGATGGAGCAAGTAACGGG CGGCAACCTCGCATCCATGC TGCTCATCGCCTGCGCCTTC
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Fig. 3. Proximal Promoter of the Bovine *CYP51* Gene

GC-box; CRE, cAMP-responsive element; SRE, sterol regulatory element; GCAAT sequence; DPE, downstream promoter element; DBE are indicated by box, double underline, wave underline, underline, dotted underline, broken underline, respectively. The nucleotides of the bovine *CYP51* gene are negatively numbered from the initiation codon shown in bold.

Expression Pattern of CYP51 mRNA in Various Tissues The expression patterns of CYP51 mRNA species in liver, kidney, lung, spleen, heart, testis, ovary of bovine were investigated by Northern blot. In all tissues examined, 2.7 kb CYP51 mRNA was observed (Fig. 5). A high level of CYP51 mRNA was observed in the testis, liver, lung and kidney, and a lower level of CYP51 mRNA was expressed in the heart, spleen and ovary. In the mature bovine testis, 1.8 kb CYP51 mRNA was observed together with the 2.7 kb mRNA, but not in the newborn bovine testis. The 5′-RACE from liver and testis produce the identical specific band, and sequence of the 3′-RACE products from testis revealed that a polyadenylation signal (AATGAA) located at 59 nucleotides downstream of the stop codon (Fig. 1) was used to generate the 1.8 kb mRNA. The overlapping region of the 3′-RACE products from liver and testis is identical which suggest 1.8 kb mRNA expressed in testis is a transcript from the same gene as in liver.

Immunochemistry. Localization of the CYP51 Protein in Bovine Testis It was established previously that the polyclonal antihuman CYP51 antibody recognizes specifically the bovine CYP51 protein.[8] Using this antibody, the highest level of immunodetectable CYP51 protein was found in Leydig cells of sexually matured bovine testis (Fig. 6A). While in round and elongating spermatids the signal was weak. Only background CYP51 levels were found in spermatogonia and spermatocytes. The specificity of staining was evaluated by control testis sections in which the primary anti-

body was replaced with nonimmune serum. And there was no residual staining in testicular tubules and Leydig cells (Fig. 6A′).

Localization of the CYP51 Protein in Bovine Ovaries Using the same antibody as in the testis studies, CYP51 protein was localized primarily to the oocytes. The staining was heaviest in oocytes of primordial and primary follicles (Fig. 6C) and reduced slightly with oocyte growth. Granulosa cells

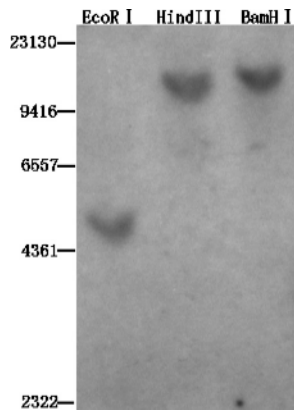

Fig. 4. Southern Blots of Bovine Genomic DNA with CYP51 Probe

Genomic DNA was digested with *Eco*RI, *Bam*HI, *Hin*dIII and the fragment was about 5.1, 11, 11.7 kb length respectively.

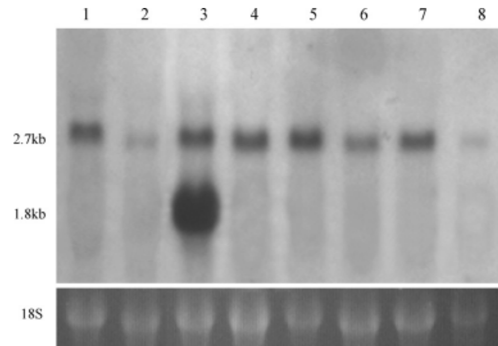

Fig. 5. Northern Blots of Bovine RNA with CYP51 Probes

A blot containing 20 μg/lane of total RNA was hybridized with the DIG-RNA bovine CYP51. lane 1, liver; lane 2, ovary; lane 3, testis (mature); lane 4, testis (newborn); lane 5, lung; lane 6, spleen; lane 7, kidney; lane 8, heart.

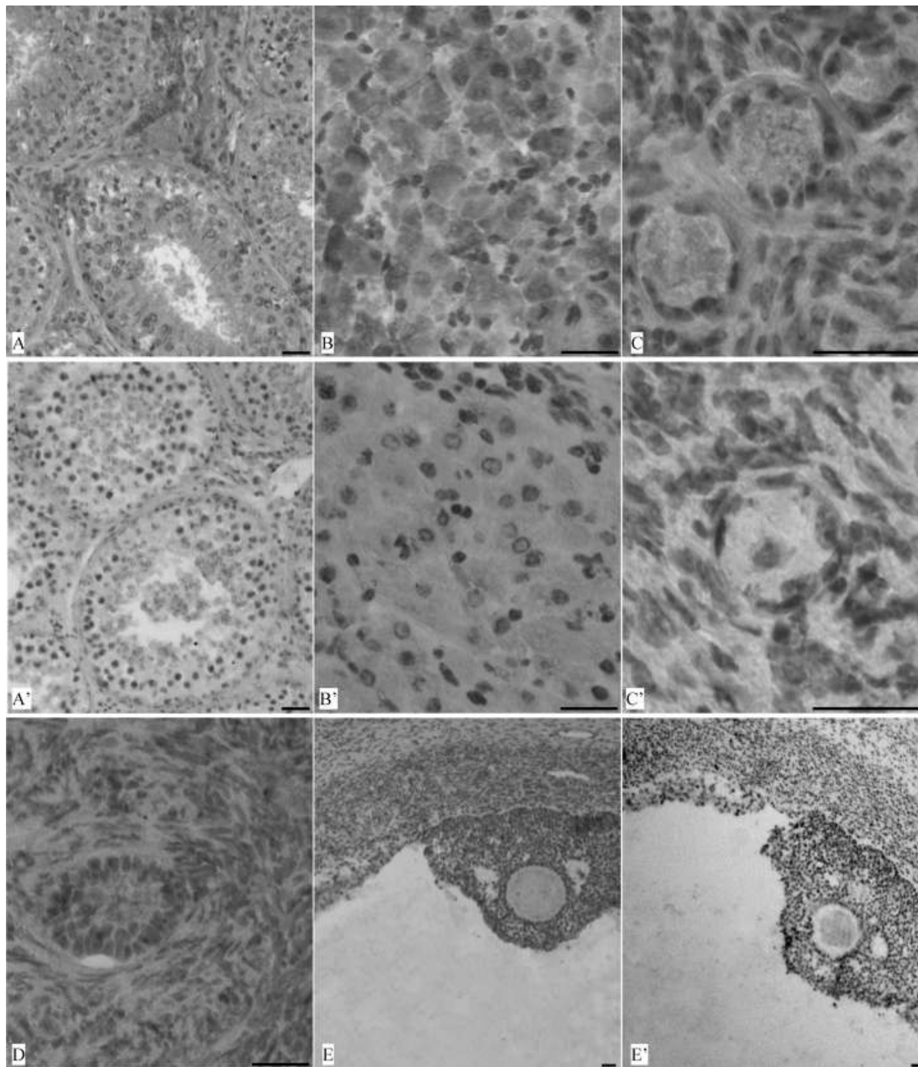

Fig. 6. Immunolocalization of CYP51 Protein in Bovine Glands Staining with the Anti-human CYP51 Antibody: (A) Testis; (B) Luteal; (C) Primordial and Primary Follicle; (D) Secondary Follicle; (E) Graafian Follicle; (A′, B′, C′, E′) the Corresponding Control Reaction with Normal Rabbit Serum

Bar, 50 μm.

of primordial, primary and secondary follicles showed background staining (Figs. 6C, D). While in antral follicles, granulosa cells facing the antrum and cumulus granulosa cells showed considerably heavier staining (Fig. 6E). The expression of CYP51 reached highest level in corpus lutea (Fig. 6B).

DISCUSSION

In this study, the molecular cloning, genomic structure, chromosomal mapping, and expression of bovine CYP51 were demonstrated. We characterized the full-length cDNA of bovine CYP51. But the 5′-RACE did not reached the most 5′ end of the cDNA[18] that would belong to truncated messages amplified. The first in-frame initial codon is 6bp downstream to other known mammalian CYP51 initial codon. Rozman D. *et al.* (2005) thought not the first but the next initial codon was used to translation. Because the 17 amino acid shorter proteins translated from next ATG can explain the 3 kDa size difference between the human and bovine CYP51 proteins, and the first ATG is lack of the Kozak sequence. But the thought should be to verify with further experiment.

We found a 2.7 kb long somatic transcript and a 1.8 kb long testis specific transcript. Aligning 3′-RACE sequence of 2.7 and 1.8 kb mRNA shows that the somatic transcript may use the last modified polyadenylation signal (ATTAAA), while testis specific transcript use the upper polyadenylation signal (AATGAA) locating at 59 nucleotides downstream of the stop codon (Fig. 1) to generate the 1.8 kb mRNA. Maybe the lost of about 900 bp 3′-UTR sequence could increase the stability or translation efficiency of CYP51 mRNA.

Alignment CYP51 cDNA with the contig genome working draft of GenBank Accession No. AC108175, showed that the gene consist of 10 exons (Table 1) and span approximately 17 kb. The insertion sites of the introns in the bovine *CYP51* gene were completely identical with those in mouse,[9] rat,[10] human,[11] and pig.[13] Additionally, the proximal promoter region is also highly conserved. Positions and sequences of major DNA regulatory elements in the *CYP51* proximal promoter are nearly identical in the known mammalian species (Fig. 3): GC box, CRE, SRE, GCAAT sequence, DPE and DBE.[1] Sequences of DPE and SRE1 elements are 100% identical. The common regulation elements show that the *CYP51* gene has a highly conserved mechanism of transcriptional regulation of the *CYP51* gene in mammalian species and is highly conserved during evolution due to its housekeeping role in the major sterol biosynthesis in eukaryotes.[3]

Intronless *CYP51* pseudogenes have been observed in human[11] and rat,[10] while in the present study, no *CYP51* pseudogenes was observed in bovine using long PCR with genomic DNA, for only one specific fragment was obtained. Furthermore, Southern blot (Fig. 4) of *Eco*RI, *Hin*dIII, *Bam*HI-digested bovine genomic DNA revealed only one band. These results suggest there is no *CYP51* pseudogene in bovine.

Only one somatic CYP51 transcript (2.7 kb) in bovine tissues was demonstrated by Northern blot (Fig. 5), while in rats, there are three different somatic CYP51 mRNAs (3.1, 2.7, 2.3 kb).[10] In contrast to the low level of CYP51 mRNA in pig kidney and lung,[13] a high level of the CYP51 mRNA was expressed in that of bovine (Fig. 5). These facts suggest that there is species and tissue difference in the expression of the CYP51 gene. Additional the long transcript, a short transcript (1.8 kb) was found only in the mature bovine testis (Fig. 5) where the MAS is accumulated.[3] Meanwhile, it has been reported that the MAS becomes high along with the rat and stallion testis development.[19,20] These data all suggest the short transcript seems to contribute to the overproduction of MAS in testis. But the function of MAS as to meiosis in male germ cells remains unclear.

Expression of the CYP51 protein in bovine testis showed leydig cells express high levels as well as that in rat,[6] mouse,[7] pig, human and marmoset (Majdič *et al.*, and Rozman, unpublished). The highest expression in all mammalian leydig cells of testis may be related to its function of the large amount synthesis of cholesterol and hormone. It also may contribute the overproduction testis MAS. The functional CYP51 protein is detected also in acrosomal membranes of bull ejaculated sperm using immunoblot.[8] But our result demonstrated background levels of CYP51 protein in spermatids. The divergence may be immunoblot is more sensitive than immunochemistry. Perhaps, the CYP51 protein activity in spermatids is so high that background level is enough for its physiological role.

The expression of the CYP51 protein in the ovary remains a matter of investigation. Using a full-length anti human CYP51 antibody, CYP51 protein was localized primarily to the bovine oocytes in all developing stages. The staining was heaviest in oocytes of primordial and primary follicles and reduced slightly with the oocytes growth. Granulosa cells of primordial, primary and secondary follicles only showed the background staining (Figs. 6C, D). But with the appearance of antral, granulosa cells facing the antrum and cumulus granulosa cells showed considerably heavier staining (Fig. 6E). Using the same antibody, CYP51 protein was detected in the mouse primary oocytes while only background levels in oocytes of secondary and Graafian follicles. Granulosa cells in all developing stages showed background level of expression.[7] But in eCG stimulated mouse, our lab showed a similar results to that of eCG stimulated rats[21] that a high expression of the CYP51 protein in primordial and primary oocytes, and reduced but detectable staining in more mature oocyte stages. Granulosa cells in primordial and primary follicles showed background level of expression and become higher in Graafian follicles. In addition, Yamashita *et al.* (2001) found CYP51 immunostained in theca, granulosa, and cumulus cells, but not in oocytes of the mature follicles.[22] In pig granulosa cells of Graafian follicles CYP51 protein was detected either. From the location of CYP51 in ovaries of bovine, mouse, pig, rat, although the antibody, treatment and experimental condition are different, we obtained a similar expression pattern: (i) a high expression of the CYP51 protein in primordial and primary oocytes; (ii) Granulosa cells showed background level of expression in primordial and primary follicles; (iii) a reduced or background level expression of CYP51 protein was found in Graafian follicles oocytes; (iv) CYP51 protein expression was increased in granulosa cells and cumulus cells with the progress of Graafian follicles formation. According to the expression pattern, we proposed CYP51 protein in oocytes probable only responsible for itself growth. Because cholesterol is an important membrane constituent, and the rapid proliferation

of the primary oocytes would require higher endogenous cholesterol production. But at the time of antrum formation the oocytes growth is almost complete, so the expression of CYP51 is reduced from high level in primary oocytes to background level in Graafian follicles oocytes (Figs. 6C—E). Corresponding, the CYP51 protein in granulosa cells may be related with the accumulation of MAS in Graafian follicle fluid, because the expression is upregulated in granulosa cells of Graafian follicles (Figs. 6C—E). The proposal was supported by recent study that LH and AY-9944 (an inhibitor of Delta14-reductase) can increased the accumulation of FF-MAS in follicles and COCs after 8 h culture, but they were unable to detect the accumulation of FF-MAS in DOs.[23] In our result the expression of CYP51 reached highest level in corpus lutea (Fig. 6). It may accord with the physiological role of corpus lutea that produce large amounts of progesterone.[22]

The present study describes the cloning and gene structure of bovine *CYP51*, and demonstrates the tissue difference in expression of CYP51 mRNA, especially a testis specific shorter mRNA. We also demonstrate the protein location in gonads, and propose the granulosa cells may mainly contribute to the accumulation of MAS. But the proposal is needed further detailed studies to conform. And conditional knockout or RAN interference technics are hopeful methods to illustrate the *in vivo* roles of MAS in gametogenesis.

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