Identification, Sequence Analysis and mRNA Tissue Distribution of the Bovine Sterol Transporters ABCG5 and ABCG8

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ABSTRACT

The family of ATP-binding cassette (ABC) transporters consists of several transmembrane proteins that use ATP hydrolysis as an energy source for the transport of a variety of substances through cellular membranes. Two members of this family, ABCG5 and ABCG8, are implicated in the intestinal absorption and biliar excretion of sterols. Cholesterol content in milk is highly variable among species, breeds, and individuals of the same species, but a potential application of these genes in lipid homeostasis in the mammary gland has never been addressed. In the present work, expression of *ABCG5* and *ABCG8* in the bovine was demonstrated for the first time and characterized by quantitative PCR. The entire coding region and promoter area were sequenced and screened for motifs involved in lipid homeostasis. Both ABCG5 and ABCG8 presented a high level of length and sequence identity with other mammalian species. In the intergenic promoter region, 2 GATA boxes, a liver receptor homolog-1 response element, and a nuclear factor-kappaB response element, important factors in other lipid regulatory processes, were identified. As expected, high expression levels of both *ABCG5* and *ABCG8* were present in liver and digestive tract samples, and interestingly, in the mammary gland, opening new avenues for further investigation about their potential role in lipid trafficking and excretion during lactation.

Key words: ATP-binding cassette transporter, mammary gland, cholesterol

INTRODUCTION

The ATP-binding cassette (ABC) transporters are membrane proteins that transport a wide variety of compounds, including ions, peptides, sugars, and lipids, against concentration gradients, at the cost of ATP energy (Klein et al., 1999). The ABC transporter proteins form one of the largest families known, with more than

2,000 distinct ABC genes present in various current databases. The human genome contains 49 ABC genes, 16 of which have a known function and 14 of which are associated with genetic diseases (Stefkova et al., 2004).

Three members of the ABC transporter family play an important role in cholesterol homeostasis: ABCA1, ABCG5, and ABCG8. These were linked to the study of rare human genetic diseases: mutations in *ABCA1* were found to be the cause for Tangier disease and high-density lipoprotein deficiency (Brooks-Wilson et al., 1999; Albrecht et al., 2004), whereas *ABCG5/8* were mutated in sitosterolemia patients (Berge et al., 2000). These diseases are characterized by abnormal accumulation of sterols in blood and tissues. In addition, common polymorphic variations of these genes described in humans have a significant effect on blood lipid concentrations in the general population (Hubacek et al., 2001; Weggemans et al., 2002).

The basic structure of the transport complex includes, in most cases, 2 nucleotide-binding domains (NBD), and 2 transmembrane modules. The ABCG5 and ABCG8 proteins are members of the G subfamily of ABC transporters. The proteins of this subfamily are half-transporters containing only 1 transmembrane module and 1 NBD, so they have to dimerize to form a functional transporter. The ABCG5 and ABCG8 proteins constitute a functional transport complex that transports sterols through cell membranes. They are very specifically expressed in the organism, and have been described to be present only in intestine and liver cells, where they regulate the absorption of diet sterols and their excretion in the bile, respectively (Yu et al., 2002; Mutch et al., 2004).

In all mammalian species studied to date, *ABCG5* and *ABCG8* are located on the same chromosome in a head-to-head orientation, sharing an intergenic region with a high degree of evolutionary conservation. This intergenic region has been consistently demonstrated to exhibit a bidirectional promoter function, leading to the simultaneous expression of both genes in response to the same stimuli (Remaley et al., 2002; Freeman et al., 2004).

Digestion in ruminants presents important physiological and anatomical differences compared with other

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mammalian species. It is the net result of a sequence of processes that occur in different segments of the gastrointestinal tract, including fermentation in the reticulum-rumen, acid hydrolysis and degradation in the abomasum and small intestine, and secondary fermentation in the cecum and large intestine (Merchen et al., 1997). Many studies have reported benefits in digestion and milk composition of cows after supplementation or nutrient substitution with vegetable oils (Petit, 2003; Scholljegerdes et al., 2004) focusing only on their fatty acid composition, but not on the sterol components that are abundant in the bovine diet.

Cholesterol content in milk (mg of cholesterol per 100 g of fat) presents important variation among breeds and individuals within breed. It is influenced by a wide range of physiological and environmental determinants, including diet (Precht, 2001).

Because milk is one of the principal sources of cholesterol in the modern diet, population studies such as the Seven Countries Study (Menotti et al., 1999) included the incidence of milk consumption in the analysis of cardiovascular disease determinants. A significant positive correlation exists among intake of dairy products, blood lipid concentrations, and long-term cardiovascular disease mortality (Kushi et al., 1995). Milk cholesterol is derived principally from serum cholesterol, and only a minor part is thought to be synthesized in the mammary gland (Long et al., 1980). No cholesterol-transporting protein, however, has been previously described to regulate these processes in farm animals.

Although *ABCG5* and *ABCG8* have been sequenced and characterized in some mammalian species, such as *Mus musculus* (Lu et al., 2002), *Rattus norvegicus* (Yu et al., 2003a), and *Homo sapiens* (Lu et al., 2001), their expression and function has never been investigated in any ruminant or dairy animal. In the present study, the nucleotide sequences of bovine *ABCG5* and *ABCG8* transporters were determined and their expression demonstrated in various tissues, with major interest on mRNA abundance in the mammary gland.

MATERIALS AND METHODS

RNA Tissue Bank and Reverse Transcription

A bovine tissue bank comprising 18 tissues was obtained after slaughter of lactating Holstein-Friesian cows without previous history of disease or drug treatment. After tissue homogenization (Ultra-Turrax T8, IKA-Werke GmbH, Staufen, Germany), total RNA was isolated using the TriFast method (peqGold TriFast, Peqlab, Erlangen, Germany) following the manufacturer's instructions. For leukocyte RNA extraction, the RNeasy Midi Kit (Qiagen GmbH, Hilden, Germany) was used as previously described (Albrecht et al., 2004).

The RNA was quantified at 260 nm in a spectrophotometer (BioPhotometer, Eppendorf, Germany), at an optical density 260/280 ratio of 1.7 to 2.0 for all samples.

Synthesis of the first strand cDNA was performed using 1 µg of total RNA and 200 U of MMLV-reverse transcription (Promega, Mannheim, Germany). The reverse transcription reaction was carried out according to the manufacturer in a 20-µL reaction volume in a gradient cyler (Biometra, Göttingen, Germany), and was achieved by successive incubations at 25°C for 10 min and 42°C for 50 min, finishing with enzyme inactivation at 90°C for 2 min.

PCR and Sequence Analysis

The cDNA, stored at -20°C, served as a template for PCR. To screen for evolutionary conserved sequences within the coding regions, gene sequences from rat, mouse, and human were blasted and compared with the Basic Local Alignment Search Tool (BLAST) from HUSAR Bioinformatics Laboratory (DKFZ, Heidelberg, Germany). Primers were designed using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Rozen and Skaletsky, 2000). The 2.0- and 2.1-kb coding regions of *ABCG5* and *ABCG8*, respectively, were obtained by amplification and sequencing of 5 overlapping cDNA fragments (0.3 to 0.9 kb), using combinations of the primers listed in Table 1. For amplification of the promoter region, primers were designed as described for other species (Remaley et al., 2002). Briefly, a forward primer in exon 1 of *ABCG8* was used in combination with a reverse primer in exon 1 of *ABCG5*, thus amplifying the region between both genes (see Table 1 for primer sequences).

The PCR reactions were performed in a PCR thermocycler (Biometra) and contained 150 ng of liver cDNA, 10× PCR reaction buffer (200 mM Tris HCl, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1.0% Triton X-100, and 1 mg/mL nuclease-free BSA), 0.8 µM of both forward and reverse primer (Metabion, Martinsried, Germany), 4.0 mM dNTP (ABgene, Germany), and 1.25 U of the proofreading enzyme Pfu Polymerase (Promega). The PCR products were subjected to gel electrophoresis in 1.5% agarose gels containing ethidium bromide (0.5 µg/mL). The DNA fragments were extracted using Wizard SV Gel and PCR Clean-Up System (Promega) and commercially sequenced (Medigenomix, Germany) from both strands.

Protein sequence alignment and promoter analysis were performed using HUSAR Bioinformatics Laboratory software (<http://genome.dkfz-heidelberg.de/biounit/>) and Prosite (<http://kr.expasy.org/prosite/>), respectively.

Table 1. Primers used for the amplification of ABCG5 and ABCG8 coding and promoter regions

ATP-binding cassette transporter	Forward primer	Reverse primer	Product size (bp)
ABCG5	5'-CCGCTGGGAAGTCCTGAG-3'	5'-AGCTCCCTAAGATGCACATGA-3'	371
	5'-CCTCAAAGATGTCTCCTTGAC-3'	5'-GCAGTCATGCAGTCCAG-3'	463
	5'-GTCATGCTGTTGATGAGCC-3'	5'-CCAAGTAGCACAAGGGCTTAG-3'	905
	5'-GCGACCAGGAGAGTCAGG-3'	5'-GACCCGCTTAGTCACAATTTC-3'	824
ABCG8	5'-GCCTCCAGGACAGCTTGTC-3'	5'-GGATTCCTGGGTCCACAG-3'	631
	5'-CGCGTGGGCAACATCTAC-3'	5'-ATGATGACGTAGACACAGTGCTCA-3'	896
	5'-CCTGGATGTCATCTCCAAAT-3'	5'-AATTGTTTCAGTTAGCTTTTGA-3'	784

Real-Time PCR

Quantitative reverse transcription PCR of *ABCG5* and *ABCG8* mRNA in bovine samples was carried out using LightCycler DNA Master SYBR Green technology (Roche Diagnostics, Mannheim, Germany). For each gene, primer pairs were designed (Table 2) covering exon boundaries to avoid amplification of genomic DNA. The PCR reactions were performed in a final volume of 10 μ L, using 1 μ L of the LC FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 pmol of each primer, 3 mM MgCl₂, and 1 μ L of cDNA. Before amplification, an initial denaturation step at 95°C was performed, ensuring activation of the polymerase and complete denaturation of the cDNA. All PCR reactions were performed with 40 cycles; product-specific PCR cycle conditions are given in Table 3. To each amplification cycle, a fourth segment with an elevated temperature fluorescence acquisition point was added to remove non-specific signals before SYBR Green I quantification. Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification.

Data were analyzed using the second derivative maximum method described in the LightCycler Relative Quantification Software. All runs included a negative cDNA control consisting of PCR-grade water, and each sample was measured in duplicate. The *ABCG5* and *ABCG8* mRNA values were expressed relative to the mean of 4 housekeeping genes (*18S*, *ubiquitin*, *glyceral-*

dehyde-3-phosphate dehydrogenase, and β -actin; see Table 2 for primer sequences) and calculated as n-fold expression relative to that in liver.

RESULTS AND DISCUSSION

Complete sequences of *ABCG5* and *ABCG8* coding regions were obtained by overlapping PCRs and published in GenBank (<http://www.ncbi.nlm.nih.gov>) under the accession numbers NM_001024547 (*ABCG5*) and NM_001024663 (*ABCG8*). The bovine *ABCG5* and *ABCG8* genes were predicted to encode for 2 proteins of 652 and 674 AA, respectively. The nucleotide sequences were highly comparable with previous data in other mammalian species, such as *Mus musculus* (mouse), *Rattus norvegicus* (rat), and *Homo sapiens* (human), showing a very high degree of homology in protein sequence and length. In Figures 1 and 2, alignments of both proteins with their human, mouse, and rat homologues are shown. Bovine *ABCG5* presented 80, 76, and 75% identity compared with human, mouse, and rat homologues, respectively. Similar values were obtained when blasting bovine *ABCG8* (77% with human, 76% with mouse, and 75% with rat).

All known ABC transporters share the NBD structure, responsible for ATP binding and hydrolysis. They contain different highly conserved sequences within their structure: a signature motif, found in all ABC transporters from bacteria to human (Higgins, 1992), and the Walker A and B motifs, present in all ATP-

Table 2. Primers used for quantitative reverse transcription-PCR measurements

Gene ¹	Forward primer	Reverse primer	Product size, bp
ABCG5	5'-AGCTCAGGCTCAGGGAAAAC-3'	5'-GTCGCTCTGCAGGACGTAG-3'	188
ABCG8	5'-ATAGGGAGCTCAGGTTGTGG-3'	5'-TCGTCCACCCTTTGTGCG-3'	260
GAPDH	5'-GTCTTCACTACCATGGAGAAGG-3'	5'-TCATGGATGACCTTGCCAG-3'	197
β -Actin	5'-AACTCCATCATGAAGTGTGACG-3'	5'-GATCCACATCTGCTGGAAGG-3'	214
Ubiquitin	5'-AGATCCAGCATAAGGAAGGCAT-3'	5'-GCTCCACCTCCAGGGTGAT-3'	198
18S	5'-AAGTCTTTGGGTTCCGGG-3'	5'-GGACATCTAAGGGCATCACA-3'	365

¹ABCG5 and ABCG8 = ATP-binding cassette transporters, G5 and G8; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Table 3. Conditions (temperature and time) for quantitative reverse transcription-PCR

Gene ¹	Denaturation		Primer annealing		Elongation		Fluorescence acquisition	
	°C	s	°C	s	°C	s	°C	s
ABCG5	95	15	60	10	72	25	85	3
ABCG8	95	15	55	10	72	20	85	3
GAPDH	95	15	58	10	72	20	72	3
β -Actin	95	15	62	10	72	25	85	3
Ubiquitin	95	15	60	10	72	20	83	3
18S	95	15	62	10	72	25	85	3

¹ABCG5 and ABCG8 = ATP-binding cassette transporters, G5 and G8; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

binding proteins (Walker et al., 1982). As expected, in the bovine *ABCG5* and *ABCG8* genes, a high level of evolutionary conservation was found in these regions. The NBD is predicted to be localized between AA 50 and 291 and between AA 47 and 311 in *ABCG5* and *ABCG8*, respectively (underlined in Figures 1 and 2). The characteristic signature and Walker A and B motifs were identified in both proteins (sequence in bold type in Figures 1 and 2).

Expression of *ABCG5* and *ABCG8* must be regulated precisely and simultaneously. Half-transporters must form homodimers or heterodimers with other ABC transporter proteins to constitute a functional transport system. Therefore, simultaneous expression and co-localization of both transcripts have been observed (Freeman et al., 2004), which seems to be mandatory for their biological function. In addition, posttranscriptional processing of both proteins and transport to their functional location is dependent on the presence of both transcripts (Graf et al., 2003).

The *ABCG5* and *ABCG8* genes are located contiguously on the same chromosome in a head-to-head orientation, sharing an intergenic promoter region (Berge et al., 2000). Transcription of both proteins occurs simultaneously and according to the same stimuli. For that reason, special interest was put on the sequencing and characterization of the bovine intergenic promoter region (Figure 3). In the bovine *ABCG5/8* cluster, the region between the start codons of both genes comprises 430 bp. Similar to the coding regions, a high degree of conservation was found comparing the bovine nucleotide sequences with other previously reported mammalian species (Kok et al., 2003; Yu et al., 2003b). Therefore, it is highly likely that regulation of expression of bovine *ABCG5/G8* genes occur as characterized before. Within the bovine *ABCG5/G8* intergenic region, response elements for transcription enhancer factor 1, liver receptor homolog-1, and nuclear factor-kappaB along with 2 GATA boxes were identified. The transcription enhancer factor 1 family members are important stimulator elements in genes related to cardiac muscle

differentiation (Mahoney et al., 2005). No function in lipid-related genes, however, has been reported to date, although this element is highly conserved in the *ABCG5/8* promoter region during evolution. Important data arise from the existence of 2 GATA boxes and liver receptor homolog-1 and nuclear factor-kappaB response elements. The GATA boxes are present in adipocyte precursor cells and control their transition to mature adipocytes by transcriptional regulation of genes involved (Tong et al., 2005). The liver receptor homolog-1 was reported previously (Freeman et al., 2004) to stimulate activity of the *ABCG5/8* promoter, hypothesizing that it was a key regulator of a number of genes involved in excretion of sterols from liver and intestine. The nuclear factor-kappaB is another widely studied response element because of its crucial role in the regulation of many atherosclerosis-related genes (Israelian-Konarakis and Reaven, 2005). Presence of these regulatory elements on the *ABCG5/8* promoter region underlines the importance of these genes in cholesterol homeostasis, because their expression is regulated coordinately with other genes involved in this process.

In addition to identification and sequence analysis of the *ABCG5* and *ABCG8* genes, the specificity of their expression was studied in a bovine tissue bank. After conventional PCR and agarose gel electrophoresis, the same specific tissue distribution was observed for both genes (presented for *ABCG5* in Figure 4A). High-intensity bands are present in cDNA samples from liver and colon, and bands of lesser intensity appeared in cDNA samples from abomasum, jejunum, lymphatic nodes, mammary gland, leukocytes, and placenta. Results were negative for the remaining tissue bank samples as well as for the negative control (water).

These results were confirmed by quantitative PCR (Figures 4B and 4C). Similar tissue-specific distribution and highly comparable specific expression between both genes were obtained. For both *ABCG5* and *ABCG8*, a high level of expression was found in liver and colon samples, with an approximately 10-fold expression compared with other positive tissues. Among the posi-

	1							70
cow	<u>MGELPFLTLG</u>	ESSELQVNRS	<u>SQCSLEEVPA</u>	KP...RHSLG	IFDASYSVSH	HVGFWNDIAS	RRQKWTRQIL	
human	<u>MGDLSSLTPG</u>	GSMGLQVNRG	<u>SQSSLEGAPA</u>	TAPE.PHSLG	ILHASYSVSH	RVRPWWDITS	CRQKWTRQIL	
mouse	<u>MGELPFLSPE</u>	GARGPHINRG	<u>SLSSLEQGSV</u>	TGTEARHSLG	VLHVSYSVSN	RVGPWWNIKS	CQQKWDRQIL	
rat	<u>MSELPFLSPE</u>	GARGPHNNRG	<u>SQSSLEEGSV</u>	TGSEARHSLG	VLNVSFVSVN	RVGPWWNIKS	CQQKWDRKIL	
								140
cow	KDVSLSVESG	<u>QIMCILGSSG</u>	<u>SGKTTLLDAM</u>	SGRLRRTGTL	LGEVFNQOE	LHREQQDFCF	SYVLQSDTLL	
human	KDVSLYVESG	<u>QIMCILGSSG</u>	<u>SGKTTLLDAM</u>	SGRLGRAGTF	LGEVYVNGRA	LRREQQDFCF	SYVLQSDTLL	
mouse	KDVSLYIESG	<u>QIMCILGSSG</u>	<u>SGKTTLLDAI</u>	SGRLRRTGTL	EGEVFNQCE	LRRDQFQDFCF	SYVLQSDVFL	
rat	KDVSLYIESG	<u>QTMCILGSSG</u>	<u>SGKTTLLDAI</u>	SGRLRRTGTL	EGEVFNQCE	LRRDQFQDFCF	SYLLQSDVFL	
		Walker A						210
cow	SNLTVRETLN	YTALLAIRRG	SQSFFQKKVE	AVMTELSLSH	VADQLIGNYS	FGGISHGERR	<u>RVSIAAQLLQ</u>	
human	SSLTVRETLH	YTALLAIRRG	NPGSFQKKVE	AVMAELSLSH	VADRLIGNYS	LGGISTGERR	<u>RVSIAAQLLQ</u>	
mouse	SSLTVRETLR	YTAMLALCRS	SADFYNNKVE	AVMTELSLSH	VADQMIGSYN	FGGISSGERR	<u>RVSIAAQLLQ</u>	
rat	SSLTVRETLR	YTAMLALRSS	SADFYDKKVE	AVLTELSLSH	VADQMIGNYN	FGGISSGERR	<u>RVSIAAQLLQ</u>	
						Signature		280
cow	<u>DPKVMFLDEP</u>	TTGLDWLTAN	QIVVLLAELA	RRDRIVILT	HQPRSELFQL	FDKIAILSCG	ELVFCGTPVE	
human	<u>DPKVMFLDEP</u>	TTGLDCMTAN	QIVVLLVELA	RRNRIVVLT	HQPRSELFQL	FDKIAILSFG	ELIFCGTPAE	
mouse	<u>DPKVMMLDEP</u>	TTGLDCMTAN	QIVVLLAELA	RRDRIVIVT	HQPRSELFQH	FDKIAILTYG	ELVFCGTPBE	
rat	<u>DPKVMMLDEP</u>	TTGLDCMTAN	HIVVLLVELA	RRNRIVIVT	HQPRSELFHH	FDKIAILTYG	ELVFCGTPBE	
		Walker B						350
cow	MLDFFSFCGY	PCPEHSNPF	FYMDLTSVDT	QSKEREIETY	RRVQMIEVAY	RESAMYHKT	EAIERTKHLK	
human	MLDFFNDCGY	PCPEHSNPF	FYMDLTSVDT	QSKEREIETS	KRVQMIESAY	KKSAICHKTL	KNIERMKHLK	
mouse	MLGFFNNGCY	PCPEHSNPF	FYMDLTSVDT	QSREREIETY	KRVQMLECAF	KESDIYHKIL	ENIERARLYK	
rat	MLGFFNNGCY	PCPEHSNPF	FYMDLTSVDT	QSREREIETY	KRVQMLESAF	ROSICHKIL	ENIERTRHLK	
								420
cow	TLPKIPFKTR	DSPGALSCLC	VLLRRVMRNL	LRNKLAVTMR	LTQNLIMGLF	IIFVLLRLGK	DVLKGAVQDR	
human	TLPMPVPKTK	DSPGVFSKLG	VLLRRVTRNL	VRNKLAVITR	LLQNLIMGLF	LLFFVLRVRS	NVLKGAIQDR	
mouse	TLPVVPFKTK	DPPGMFGKLG	VLLRRVTRNL	MRNKQAVIMR	LVQNLIMGLF	LIFYLLRVQN	NVLKGAQDR	
rat	TLPMPVPKTK	NPPGMFCKLG	VLLRRVTRNL	MRNKQVIMR	LVQNLIMGLF	LIFYLLRVQN	NMLKGAQDR	
								490
cow	VGLLYQFVGA	MPYTGMNAV	TLFPVLRAVS	DQESQDGLYH	KWQMLLAYML	HVLPPFSVTAT	LVCCSVCYWT	
human	VGLLYQFVGA	TPYTGMNAV	NLFPVLRAVS	DQESQDGLYQ	KWQMLLAYAL	HVLPPFSVAT	MIFSSVCYWT	
mouse	VGLLYQLVGA	TPYTGMNAV	NLFPMLRAVS	DQESQDGLYH	KWQMLLAYVL	HVLPPFSVIAT	VIFSSVCYWT	
rat	VGLLYQLVGA	TPYTGMNAV	NLFPMLRAVS	DQESQDGLYQ	KWQMLLAYVL	HALPPFSIVAT	VIFSSVCYWT	
								560
cow	LALYPEAARF	AYFSVALLAP	HLIGEFLSLV	LLGVIQNPV	VNSVALLSI	AGVLMGSGFV	RNIQEMPIPF	
human	LGLHPEVARF	GYFSAALLAP	HLIGEFLTLV	LLGIVQNPNI	VNSVALLSI	AGVLVSGGFL	RNIQEMPIPF	
mouse	LGLYPEVARF	GYFSAALLAP	HLIGEFLTLV	LLGIVQNPNI	VNSVALLSI	SGLLIGSGFI	RNIQEMPIPL	
rat	LGLYPEVARF	GYFSAALLAP	HLIGEFLTLV	LLGMVQNPNI	VNSVALLSI	SGLLIGSGFI	RNIQEMPIPL	
								630
cow	KILGYFTFQK	YCSEILVVNE	FYQNFCTCGN	SNASVSANPM	CTITQGAQFI	ERNCPGATSR	FTANFLILYS	
human	KILSYFTFQK	YCSEILVVNE	FYGLNFTCGS	SNVSVTNPM	CAFTQGIQFI	EKTCPGATSR	FTMNFLILYS	
mouse	KILGYFTFQK	YCCEILVVNE	FYGLNFTCGG	SNTSMLNHPM	CAITQGVQFI	EKTCPGATSR	FTANFLILYG	
rat	KILGYFTFQK	YCCEILVVNE	FYGLNFTCGG	SNTSVPNNPM	CSMTQGIQFI	EKTCPGATSR	FTTNFLILYS	
								652
cow	FIPALVILGI	IVFKIRDYLI	SR					
human	FIPALVILGI	VVFKIRDHLI	SR					
mouse	FIPALVILGI	VIFKVRDYLI	SR					
rat	FIPTLVILGM	VVFKVRDYLI	SR					

Figure 1. Predicted amino acid sequence for the mammalian ATP binding cassette transporter G5. Alignment of the bovine proteins with their homologues in human, mouse, and rat, showing sequence identity (dark and light gray boxes). Nucleotide-binding domain (NBD; underlined) and the conserved Walker A, Walker B, and signature motifs (bold) are indicated.

tive tissues are other parts of the digestive system (abomasum and jejunum), mammary gland, and blood samples. Residual expression was found for lymphatic nodes and placenta. Further experiments will be necessary to assess whether these results are of significance, with special interest in placenta, because other mem-

bers of the ABC transporter family were suggested to play an important role in the trafficking of selected substrates in this organ (Christiansen-Weber et al., 2000; Sarkadi et al., 2004).

Expression of *ABCG5* and *ABCG8* in the bovine liver and digestive system is highly consistent with other

Figure 2. Predicted amino acid sequence for the mammalian ATP-binding cassette transporter G8. Alignment of the bovine proteins with their homologues in human, mouse, and rat, showing sequence identity (dark and light gray boxes). Nucleotide-binding domain (NBD; underlined) and the conserved Walker A, Walker B, and signature motifs (bold) are indicated.

tract, as described for the mouse (Mutch et al., 2004). In the bovine digestive tract, the highest mRNA expression levels of these transporters occurred in the colon, with moderate expression levels in jejunum and aboma-


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1351 tctctctagc cctgtcttct ttggggcctc ctctgcatg gggccctgca
                                     ← START ABCG8
1401 gtgtctgcgc tttgactctg cagtttcctt tgggcttagc tgctgccgac

1451 cagggccaat gttgtctgct tcccagagcc gggcaacctc tggtctggct
      TEF1/LRH-1
1501 ccagcattc ctctctggca agccctccca caaacacggg ctgtgtgctc
      GATA BOX
1551 tgcccgctgc tgagataaca cacagacaca cactggctaa aggtacatca
      GATA BOX          NFKB
1601 gataatggca tccacgacca agcccaggcc cgccgctggg aagtccctgag

1651 gaggggctcc agggccaggt gagaagcagg caggacagtc tgccactagc

1701 tctttggcgg agggcactcc agggagggtc aggccagaaa atccaccagt
                                     → START ABCG5
1801 ctctgctgct tgttggccat g

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Figure 3. Regulatory elements identified in the intergenic promoter region of the bovine ATP-binding cassette transporters G5 and G8. Start codons of both genes are indicated.

sum; no expression was found in cecal samples. These results do not completely agree with those presented by Mutch et al. (2004) in which small intestine samples showed slightly higher expression levels than colon samples, a fact that may be related to the special ruminant digestive structures and functions. In future work, the protein expression of ABCG5/8 in the tissues should be confirmed and the cellular localization must be elucidated.

In humans, dietetic supplements containing plant sterols have been widely demonstrated to reduce cholesterol intestinal absorption and subsequent blood concentrations (Ostlund, 2004; Richelle et al., 2004), findings that are related to ABCG5/8 function. Because plant sterols are a major component of the ruminant diet, studies should focus on the functional role of ABCG5/8 in the digestive tract of ruminants. Furthermore, in view of the markedly enhanced plasma concentration of cholesterol in cows fed fat (Blum et al., 1985; Bruckmaier et al., 1998), it would be interesting to study the expression and function of these ABC transporters under these feeding conditions.

Interesting results presented in this work arise from the expression of ABCG5 and ABCG8 mRNA in mammary gland tissue. Importantly, expression of ABCG5 and ABCG8 within the mammary gland, although less than in liver, is highly consistent and confirmed both by conventional and quantitative PCR measurements. Precise protein localization of this complex within the udder must be studied to define the exact role of ABCG5 and ABCG8 and their potential importance upon regu-

lation of milk sterol concentrations. The ABCG5/8 transport complex may be an important intervention point when trying to regulate sterol amounts in the milk, because it may act at 3 important steps: their intestinal absorption, and their excretion in bile and in milk.

In addition, common sequence variants in the ABCG5 and ABCG8 genes within the general human population have been demonstrated to cause interindividual differences in the ability to transport sterols, leading to variation in blood lipid concentrations. Whether the bovine genes present any of these or other significant sequence variations, and whether they affect blood or milk lipid concentrations in cattle, should be addressed in future work.

In summary, the ABCG5 and ABCG8 genes, important factors in sterol homeostasis in mammalian species, have been identified and their sequence and expression characterized in cattle. Their coding sequences, as well as the intergenic promoter sequence, present a high degree of identity with other species, indicating the importance of their function by their evolutionary conservation. Within the promoter region, regulatory elements that are crucial in other lipid homeostatic processes were identified such as GATA boxes and nuclear factor-kappaB response elements. Specificity of tissue distribution was studied in bovine tissue samples, revealing high levels of expression in cDNA samples from liver and digestive tract and relative high expression in the mammary gland.

Figure 4. Expression of the ATP-binding cassette transporters *G5* and *G8* in bovine tissues (A) Agarose gel electrophoresis for 1 of the overlapping fragments used in *ABCG5* sequencing (amplicon size: 463 bp). Lane 1 = esophagus; 2 = rumen; 3 = abomasum; 4 = jejunum; 5 = cecum; 6 = colon; 7 = tongue; 8 = lung; 9 = heart; 10 = kidney; 11 = liver; 12 = spleen; 13 = uterus; 14 = lymphatic node; 15 = muscle; 16 = mammary gland; 17 = blood; 18 = placenta; 19 = negative control; and 20 = 100-bp marker. B) Quantitative reverse transcription-PCR results for *ABCG5* (amplicon size = 161 bp). Bars represent relative quantification values calculated as n-fold expression compared with liver (lane 11, dashed line); lane designations as for panel A. C) Quantitative reverse transcription-PCR results for *ABCG8* (amplicon size = 260 bp).

In conclusion, identification and characterization of bovine *ABCG5* and *ABCG8* genes and their expression within tissues involved in cholesterol control, including the mammary gland, opens a wide range of further investigation, and a future potential intervention point when attempting to control sterol concentrations in milk.

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