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# Bovine ABC transporters: Identification of selected members associated with sterol transfer

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...für meine Oma Anni

**Content**

## Abbreviations

| | |
|---|---|
| AA | amino acid |
| ABC | ATP-binding cassette |
| AP | adaptor-related protein complex |
| ATP | adenosine triphosphate |
| BCRP | breast cancer resistance protein |
| bp | base pair |
| cDNA | complementary DNA |
| CD36 | thrombospondin receptor |
| CP | crossing point |
| CT | cycle threshold |
| D | dry period |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside triphosphate |
| DT | digestive tract |
| FXR | farnesoid receptor |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| HDL | high density lipoprotein |
| HNF3β | hepatocyte nuclear factor 3 beta |
| LT | lactation |
| LRH-1 | liver receptor homolog-1 |
| LXR | liver X receptor |
| MG | mammary gland |
| mRNA | messenger RNA |
| NBD | nucleotide-binding domain |
| NBF | nucleotide-binding fold |
| NF-κB | nuclear factor kappa-B |
| OD | optical density |
| PCR | polymerase chain reaction |
| PEST | sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) |
| PPAR | peroxisome proliferator-activated receptor |
| PXR | pregnane-activated receptor |
| qRT-PCR | quantitative reverse transcription-PCR |
| RG | reference gene |
| RNA | ribonucleic acid |
| RXR | retinoid X receptor |
| SEM | standard error of the mean |
| SOX5 | SRY-box 5 |
| SP1 | specificity protein 1 |
| SR-BI | scavenger receptor class B type 1 |
| SREBP | sterol regulatory element binding protein |
| SRY | sex determing region Y |
| STAT | signal transducer and activator of transcription |
| SXR | steroid-activated receptor |
| TEF | transcription enhancer factor |
| TG | target gene |
| TMD | transmembrane domain |
| ZNF202 | zinc finger protein 202 |

**Abstract**

The family of ATP-binding cassette (ABC) transporters consists of several transmembrane proteins that use the energy of ATP hydrolysis to transport a wide variety of substances through cellular membranes.
ABC transporters play an important role in human physiology and mutations in these genes often result in severe hereditary diseases, like for example Tangier disease (ABCA1), cystic fibrosis (ABCC7), multidrug resistance (ABCB1) or adrenoleucodystrophy (ABCD1).
The ABC transporter A1 (ABCA1) is known to play a significant role in cellular export of phospholipids and cholesterol in humans. Two other members of the family, ABCG5 and ABCG8, are implicated in intestinal absorption and biliary excretion of sterols. ABCA1, ABCG5 and ABCG8 might also play a crucial role in cellular cholesterol homeostasis in the cow and in the transfer of cholesterol into milk, but their presence and tissue distribution in the bovine organism is yet unknown. Therefore the expression of the bovine *ABCA1*, *ABCG5* and *ABCG8* transporter genes was studied using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and their entire coding regions were sequenced. In addition, the proximal promoters were identified and screened for regulatory elements.
Sequence analysis of ABCA1, ABCG5 and ABCG8 presented a high level of length and sequence identity compared to other mammalian species. Expression of bovine *ABCA1* mRNA was found in all tissues tested. Highest expression levels were detected in lung, esophagus, uterus, spleen, and muscle. As anticipated, high expression levels of both *ABCG5* and *ABCG8* were present in liver and digestive tract samples, and interestingly, in the mammary gland. The analysis of the putative *ABCA1* promoter region revealed potential transcription factor binding sites associated with *ABCA1* transcription and lipid metabolism. In the intergenic promoter region of *ABCG5* and *ABCG8*, important factors for lipid regulatory processes were identified.
The physiological role of these and the expression of other ABC transporters in the bovine organism still remains elusive. Based on recent findings in the context of human disorders candidate ABC transporters may be implicated in lipid and cholesterol transport in

the mammary gland, an important organ in conjunction with lipids. Therefore the expression patterns of selected genes associated with sterol transport in lactating and nonlactating mammary glands of dairy cows were investigated. mRNA levels from mammary gland biopsies taken during lactation and in the first and second week of the dry period were analyzed using qRT-PCR. Five genes of the ABC transporter family, namely *ABCA1*, *ABCA7*, *ABCG1*, *ABCG2* and *ABCG5*, and two regulating genes *LXR*$\alpha$, *PPAR*$\gamma$, as well as the milk proteins *lactoferrin* and $\alpha$-*lactalbumin* were assessed.

A significantly enhanced expression in the dry period was observed for *ABCA1* while a significant decrease of expression in this period was detected for *ABCA7*, *ABCG2* and $\alpha$-*lactalbumin*. *ABCG1*, *ABCG5*, *LXR*$\alpha$, *PPAR*$\gamma$ and *lactoferrin* expression was not significantly altered between lactation and dry period. These results indicate that candidate ABC transporters involved in lipid and cholesterol transport show differential expression between lactational stages and the dry period. This may be due to physiological changes in the mammary gland like immigration of macrophages or the accumulation of lipids due to the loss of liquid in the involuting mammary gland.

The mRNA expression analysis of transporters in the bovine organism is the basic requirement to unravel potential novel molecular mechanisms underlying cholesterol and lipid transfer. This work reveals that ABC transporters are a part of the bovine physiology and these findings are fundamental to uncover the physiological importance of the ABC transporters in *Bos taurus*.

## Zusammenfassung

Die Familie der ABC-Transporter ist eine Klasse von Membranproteinen, die als gemeinsames Strukturelement eine ATP-bindende Kassette (englisch: ATP-binding cassette, ABC) besitzen und welche die Energie der ATP-Hydrolyse für den Transport einer Vielzahl von Substanzen über Zellmembranen nutzen.
ABC-Transporter spielen eine wichtige Rolle in der menschlichen Physiologie, und Mutationen in diesen Genen können zu schwerwiegenden Erkrankungen führen, wie zum Beispiel Tangier Krankheit (ABCA1), zystische Fibrose (ABCC7), multiple Arzneimittelresistenz (ABCB1), Adrenoleukodystrophie (ABCD1) und vielen mehr.
Dem ABCA1-Transporter kommt im Menschen eine bedeutende Rolle im zellulären Export von Phospholipiden und Cholesterin zu. Zwei weitere Mitglieder dieser Familie, ABCG5 und ABCG8, sind an der intestinalen Absorption und Exkretion von Sterolen beteiligt. ABCA1, ABCG5 und ABCG8 könnten auch eine entscheidende Rolle in der zellulären Cholesterinhomöostase im Rind und speziell beim Lipidtransport in der Milchdrüse spielen. In dieser Arbeit wurde deshalb die Expression von *ABCA1*, *ABCG5* und *ABCG8* in bovinen Geweben mittels quantitativer Reverse-Transkriptase-Polymerase-Kettenreaktion (qRT-PCR) untersucht und die gesamte kodierende Region der Gene sequenziert. Zusätzlich wurden die proximalen Promotorregionen der Gene inklusive der regulierenden Elemente identifiziert.
Die Analyse von ABCA1, ABCG5 und ABCG8 ergab hohe Längen- und Sequenzhomologien zu analogen Proteinsequenzen anderer Säugetierarten. Die *ABCA1* Genexpression konnte in allen untersuchten Geweben nachgewiesen werden. Höchste Expressionsniveaus wurden in Lunge, Speiseröhre, Gebärmutter, Milz, und Muskel beobachtet. Wie erwartet wurden hohe Expressionen, sowohl von *ABCG5* als auch von *ABCG8*, in Leber- und den Verdauungstrakt-Proben, und interessanterweise auch in der Milchdrüse gefunden. Die Analyse der *ABCA1* Promotorregion offenbarte Bindestellen für Regulatoren des Lipidmetabolismus und für Transkriptionsfaktoren der *ABCA1*

Expression. Im Promotorbereich von *ABCG5* und *ABCG8*, der zwischen den beiden Genen lokalisiert ist, wurden ebenfalls wichtige Faktoren für die Lipidregulation identifiziert.
Die physiologische Rolle dieser und die Expression anderer ABC-Transporter im Rind ist unklar. Ausgehend von Ergebnissen im Zusammenhang mit menschlichen Erkrankungen könnten bestimmte ABC-Transporter auch im Lipid- und Cholesterintransport der bovinen Milchdrüse involviert sein. Aufgrund dieser Hypothese wurde das Expressionsmuster ausgewählter Gene, die mit Steroltransport in Verbindung gebracht werden, in laktierenden und nichtlaktierenden bovinen Milchdrüsen untersucht. Hierzu wurden Milchdrüsenbioptate während der Laktation sowie in der ersten und zweiten Woche der Trockenstellphase entnommen, und die daraus isolierte mRNA mittels qRT-PCR analysiert. Fünf Mitglieder der ABC-Transporter Familie, *ABCA1*, *ABCA7*, *ABCG1*, *ABCG2* und *ABCG5*, und ihre Regulatoren *LXR*$\alpha$, *PPAR*$\gamma$, sowie die Milchproteine *Lactoferrin* und $\alpha$-*Lactalbumin* wurden gemessen. *ABCA1* zeigte einen signifikanten Expressionsanstieg in der Trockenstellphase, während eine signifikante Abnahme der Expression in dieser Zeit für *ABCA7*, *ABCG2* und $\alpha$-*Lactalbumin* beobachtet wurde. Die Expression von *ABCG1*, *ABCG5*, *LXR*$\alpha$, *PPAR*$\gamma$ und *Lactoferrin* zeigte zwischen der Laktations- und Trockenstellphase keine signifikanten Veränderungen.
Die Analysen ergaben deutliche Expressionsunterschiede zwischen Laktation und Trockenstellphase für einige der ausgewählten Transporter. Dies könnte auf die starken physiologischen Veränderungen der Milchdrüse, wie zum Beispiel Immigration von Makrophagen oder die Anhäufung von Lipiden aufgrund von Flüssigkeitsverlust während der Involutionsphase zurückzuführen sein.
Die mRNA Expressionsanalyse von Transportern im bovinen Organismus ist die Grundlage, um mögliche neuartige molekulare Mechanismen aufzudecken, die dem Cholesterin- und Lipidtransfer im Rind unterliegen. Diese Arbeit zeigt, dass ABC-Transporter eine Rolle in der bovinen Physiologie spielen, und diese Ergebnisse bilden die Grundlage um die physiologische Bedeutung der ABC-Transporter im Rind aufzudecken.

## Introduction

*Structure*

The ATP-binding-cassette (ABC) transporters represent the largest family of transmembrane proteins. These proteins hydrolize ATP and use the energy to drive the transport of various substances across the plasma membrane or intracellular membranes of the endoplasmatic reticulum, the peroxisome, and mitochondria. ATP binding cassette transporters are one of the major classes of membrane transporters found in all cell types of all species studied so far. Different ABC transporters translocate different substrates, ranging from small ions, sugars, amino acids, proteins, to large polysaccharides, and they therefore play diverse physiological roles (Childs & Ling 1994, Dean & Allikmets 1995, Higgins 1992).
The ATP-binding-cassette, also known as nucleotide-binding domain (NBD), contains three characteristic motifs. The Walker A and B motif, separated by ~90-120 amino acids (AA), are found in all ATP-binding proteins. The signature C motif, located between the two walker motifs, is characteristic for ABC transporters and is not found in other ATP-binding proteins. The functional protein typically consists of two NBD, and two sets of typically six membrane spanning $\alpha$-helices, referred to as transmembrane domains (TMD).

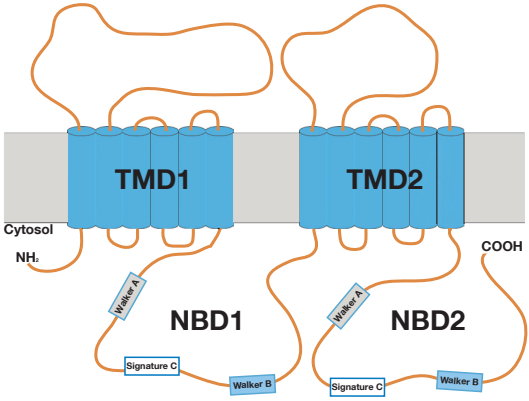


***Figure 1:*** *Typical ABC transporter with two transmembrane (TMD) and two nucleotide-binding domains (NBD).*

The TMD form the binding sites, and provide specificity for the ligand. The two NBD, located in the cytoplasm, bind and hydrolize ATP to drive the translocation of the bound ligand. The NBD, but not the TMD, are homologous throughout the family.
ABC transporters are found, as far as known, in all species. The eukaryotic ABC genes are organized as full transporters containing two sets of TMD and two NBD (Figure 1), or as half transporters, that contain only one NBD and one set of TMD, but these transporters usually form homo- or heterodimers to result in a functional transporter.

*Function*

There is no example of an eukaryotic ABC transporter with a role in uptake into cytoplasm so far – all are exporters. ABC transporters have adapted to serve a wide variety of specialized roles, for example in antigen presentation, transport of drugs (xenotoxins), lipid transport and many others.
Although the number of mammalian ABC proteins is much smaller than found in prokaryotes, several are of major clinical significance; currently, 18 human ABC genes have been associated with genetic diseases (Dean & Annilo 2005), including cystic fibrosis, Stargardt`s macular degeneration, and disturbances in lipid and lipoprotein metabolism.
In recent years, a large group of ABC transporters has been found to be implicated in the translocation of bile acid, phospholipids, and sterols. Therefore, and because ABC genes are prone to be involved in human genetic disorders, ABC transporters are promising target molecules for the treatment of lipid disorders such as cardiovascular disease (Albrecht *et al*. 2004, Soumian *et al*. 2005).

*ABC transporters and lipids*

The human ABC superfamily, which currently consists of 48 known ABC transporters, is divided into seven subfamilies (ABCA to ABCG) by phylogenetic analysis. Some family members, especially of the ABCA and ABCG subfamily, are implicated in the translocation of

lipids. The members of the ABCG family are half-transporters and form homodimers (ABCG1, ABCG2, and ABCG4) or heterodimers (ABCG5 and ABCG8) to become functionally active. Except ABCG2, all members of this family play a significant role in the efflux transport of cholesterol. They facilitate the efflux of excess cholesterol to high-density lipoprotein (HDL), a key player in the reverse cholesterol transport from macrophages to the liver (ABCG1 and ABCG4). ABCG5 and ABCG8 are highly expressed in the intestine and liver cells where they limit the absorption of dietary sterols in the intestine and promote cholesterol elimination from the body through hepatobiliary secretion (Mutch *et al.* 2004, Yu *et al*. 2002).

Unlike other members, ABCG2, also referred to as breast cancer resistance protein (BCRP), accepts a variety of structurally unrelated compounds as substrate and plays important roles in the cancer chemotherapy and drug disposition (Kusuhara & Sugiyama 2007). ABCA1 and ABCA7 are full transporters and members of the ABCA subfamily. The function of ABCA1 is to export excess cellular cholesterol into the HDL pathway (Figure 2) and reduce cholesterol accumulation in macrophages (Oram & Vaughan 2006).

This efflux prevents the accumulation of cellular cholesterol esters and foam cell formation. Cholesterol enters the cell through low-density lipoprotein (LDL) receptor-mediated endocytosis of cholesterol-rich LDL (Figure 2). The ABCA1 transporter facilitates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins, and hence plays a key role in the reverse

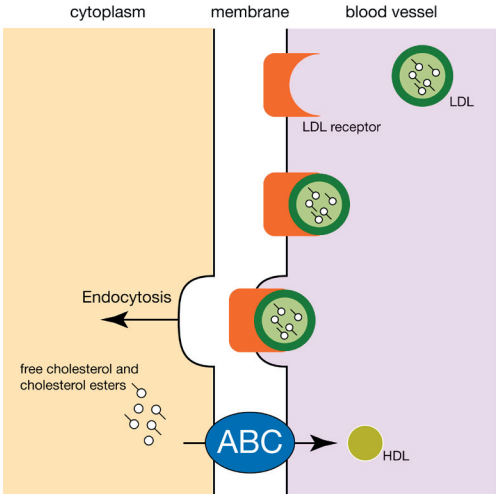


***Figure 2:*** *Receptor mediated endocytosis of cholesterol loaded LDL into the cell and ABCA1 mediated transfer of cholesterol and cholesterol esters into the HDL pathway.*

cholesterol transport. This is a process in which cholesterol is carried in HDL particles back to the liver, where it is converted into bile acids and secreted into bile.
ABCA7 was demonstrated to mediate a similar reaction like ABCA1 to generate HDL in vitro and it may be involved in lipid metabolism in kidney and adipose tissues (Kim *et al.* 2005, Linsel-Nitschke *et al.* 2005, Wang *et al.* 2003b).

*ABC transporter expression and orphan nuclear receptors*

Several mammalian ABC transporters are under tight transcriptional regulation. The orphan nuclear receptors, amongst others, appear to play an important role in this regulation (Fitzgerald *et al*. 2002). Nuclear receptors represent a family of transcription factors that act as heterodimers, which bind to promoter elements and induce gene expression. In general the retinoid X receptor (RXR) is an obligatory partner in the heterodimer; the other partner can be any of the other family members. It is this other partner that determines the specificity for the activating ligand and for the target gene. Quite recently ligands and an increasing number of target genes for these receptors were discovered (Fitzgerald *et al*. 2002, Mitro *et al*. 2007). Nuclear receptors relevant for the expression of ABC transporters are the "liver X receptor" (LXR), the farnesoid receptor (FXR) for which bile salts are important endogenous ligands; and the pregnane- and the steroid-activated receptors (PXR and SXR), which are expressed in rodents and humans, respectively. These two receptors turn out to be important xenobiotics sensing receptors. Finally PPAR$\alpha$ and PPAR$\gamma$, already known as the receptors involved in peroxisome proliferation, are actually key regulators in lipid and carbohydrate metabolism.

## Aim of the Study

Almost nothing is known about ABC transporters in the bovine organism. Therefore the objective of this thesis was to detect and identify ABC transporters, and to describe their expression patterns in different tissues of *Bos taurus*, with special emphasis on ABC transporters involved in the transfer of sterols (Figure 3). Furthermore, the investigation of transporters that may take part in the regulation of lipid translocation in bovine mammary gland was in focus of this work. To elucidate the function of candidate transporters and additionally their regulating genes in mammary gland physiology, expression profiles were analyzed in lactating and non-lactating mammary gland tissues (Figure 3).

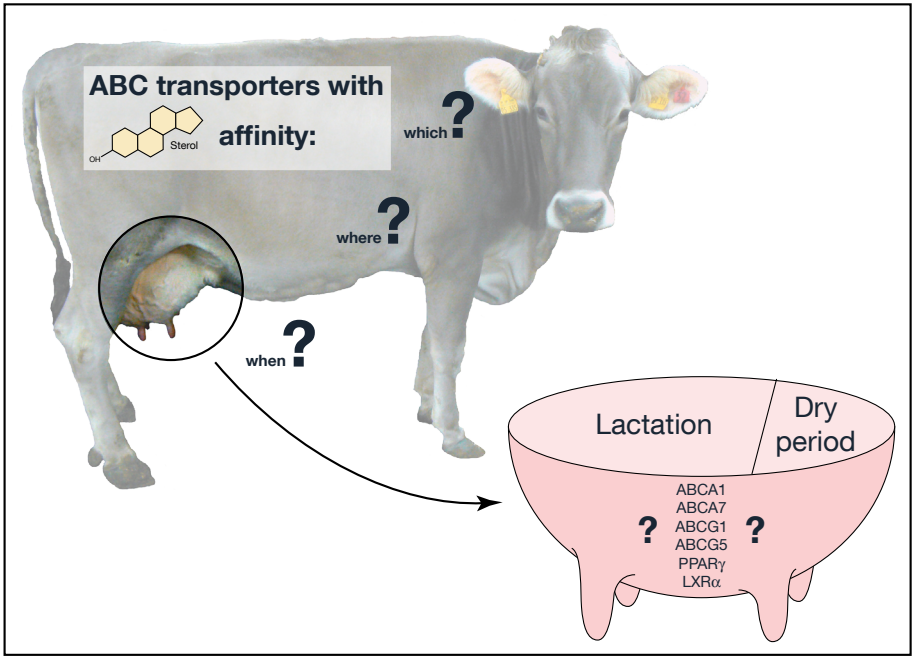


***Figure 3:*** *Schematic presentation of study aims.*

## Material and Methods

*Tissue and biopsy samples*

Bovine tissue samples were collected after slaughtering of lactating Holstein-Friesian cows, without previous history of disease or drug treatment, as described previously (Farke *et al*. 2006, Viturro *et al*. 2006).
Mammary gland biopsies from four healthy dairy cows (German Braunvieh) were carried out as described in Farke *et al*. (2007).

*Total RNA extraction and reverse transcription*

Total RNA was isolated from tissue samples using the RNeasy® Mini and RNeasy® Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) (Farke *et al*. 2006) or peqGOLD TriFast (Peqlab, Erlangen, Germany) (Viturro *et al.* 2006) according to the manufacturers recommendations. To quantify the amount of total RNA, optical density (OD) was measured at 260 nm, obtaining an OD 260/OD280 ratio of 1.7 to 2.0 for all samples. Synthesis of first strand complementary DNA (cDNA) was performed with SuperScript™ III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturers instructions (Farke *et al*. 2006).
Total RNA of mammary biopsy samples was isolated using TriPure (Roche Diagnostics, Mannheim, Germany) according to the manufacturers recommendations. The integrity of the RNA was verified by the OD260/OD280 absorption ratio >1.8. Synthesis of first strand cDNA was performed with SuperScript II (Invitrogen) according to the manufacturers instructions (Farke *et al.* 2007).

*PCR and sequence analysis*

Primer pairs for polymerase chain reaction (PCR) were designed as described previously (Farke *et al*. 2006, Viturro *et al*. 2006). Table 1 lists all primers used for the amplification of the ABCA1, ABCG5 and ABCG8 coding and promoter regions. The PCR reactions and the rapid

amplification of cDNA ends (RACE) were performed as described in detail in Farke *et al*. (2006) and Viturro *et al*. (2006).
PCR products were subjected to gel electrophoresis. The DNA fragments were extracted using Wizard SV Gel and PCR Clean-Up System (Promega) and commercially sequenced (Agowa, Berlin, Germany) from both strands.

***Table 1:*** *PCR primer sequences used for amplification of the ABCA1, ABCG5 and ABCG8 coding and promoter regions.*

| Gene | | Forward primer | | Reverse primer |
|---|---|---|---|---|
| *ABCG5* | | | | |
| | 1.for | 5'-CCGCTGGGAAGTCCTGAG-3' | 1.rev | 5'-AGCTCCCTAAGATGCACATGA-3' |
| | 2.for | 5'-CCTCAAAGATGTCTCCTTGTAC-3' | 2.rev | 5'-GCAGTCATGCAGTCCAG-3' |
| | 3.for | 5'-GTCATGCTGTTTGATGAGCC-3' | 3.rev | 5'-CCAAGTAGCACAAGGGCTTAG-3' |
| | 4.for | 5'-GCGACCAGGAGAGTCAGG-3' | 4.rev | 5'-GACCCGCTTAGTCACAATTTCC-3' |
| *ABCG8* | | | | |
| | 1.for | 5'-GCCTCCAGGACAGCTTGTTC-3' | 1.rev | 5'-GGATTCCTGGGTTCCACAG-3' |
| | 2.for | 5'-CGCGTGGGCAACATCTAC-3' | 2.rev | 5'-ATGATGACGTAGACACAGTGCTCA-3' |
| | 3.for | 5'-CCTGGATGTCATCTCCAAAT-3' | 3.rev | 5'-AATTGTTCAGTTTAGCTTTTGGA-3' |
| *ABCA1* | | | | |
| | 1.for | 5'- GGTTGCTGCTGTGGAAGAAC –3' | 12.rev | 5'- GAATGACATCAGCCCTCAGC –3' |
| | 2.for | 5'- CGGCGGCTTCTCTTGTATAG –3' | 13.rev | 5'- GAAGCCATCTTCCTCTGTGG –3' |
| | 3.for | 5'- TGAGCCTGATGTCTCCTGTG –3' | 14.rev | 5'- GACACACAGGCAGCATCTTC –3' |
| | 4.for | 5'- AAGAGACTGCTGATTGCCAGAC –3' | 15.rev | 5'- ACTGCCAAGACACCTGAACC –3' |
| | 5.for | 5'- TGAAGCTCTCTGCACTAGGATG –3' | 16.rev | 5'- CCTCAGCATCTTGTCCACAG –3' |
| | 6.for | 5'- ACCAGCTTCCGTCTTCACTG –3' | 17.rev | 5'- GTCTGAGAACAGGCGAGACAC –3' |
| | 7.for | 5'- CTGGATGAGAGTCTCTGGAG –3' | 18.rev | 5'- CGGAGATCAGGATCAGGAAG –3' |
| | 8.for | 5'- GCTCTCGACTGTCAAGGCC –3' | 19.rev | 5'- GTCTCATATGGCTCTCGAGTGA –3' |
| | 9.for | 5'- GTCCAGAGGACTGTCCATCTTC –3' | 20.rev | 5'-CCAAGTCGCTCAAGAGACTC –3' |
| | 10.for | 5'- GAAGATGCTGCCTGTGTGTC –3' | 21.rev | 5'- CTATCGGTCAAAGCCTGTTCTC –3' |
| | 11.for | 5'- CACCTGACACTCCAGGTCACAAG –3' | 22.rev | 5'- GAAGATGGACAGTCCTCTGGAC –3' |

## *Real-time PCR*

Primers for the amplification via quantitative reverse transcription-PCR (qRT-PCR) were designed as described in Farke *et al*. (2006),

Farke *et al*. (2007) and Viturro *et al*. (2006). Table 2 lists all primers used for qRT-PCR.
qRT-PCR was carried out using LightCycler® DNA Master SYBR Green technology (Roche Diagnostics). Product-specific PCR conditions are listed in Table 3, App. p. 40, Table 3, App. p. 49 and Table 2, App. p. 76. Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification. Data were analysed using the second derivate maximum calculation described in the LightCycler® Software 3.5. All runs included a negative cDNA control consisting of PCR-grade water, and each sample was measured in duplicate (Farke *et al*. 2006, Farke *et al*. 2007, Viturro *et al*. 2006).

***Table 2:*** *Primers used for quantitative real-time PCR measurements.*

| Gene | Forward primer | Reverse primer | Product size |
|---|---|---|---|
| *ABCA1* | 5'- GGACATGTGCAACTACGTGG –3' | 5'- TGATGGACCACCCATACAGC –3' | 134 bp |
| *ABCA7* | 5'- GCCCAGGTCAACCGAACT -3' | 5'- AGCACGAAGAGCTTCCACTC -3' | 201 bp |
| *ABCG1* | 5'- GACTCGGTCCTCACGCAC -3' | 5'- CGGAGAAACACGCTCATCTC -3' | 203 bp |
| *ABCG2* | 5'- GCTCCTGAAGAGGATGTC -3' | 5'- CAGCGGAAACCTATGGCTC -3' | 174 bp |
| *ABCG5* | 5'-AGCTCAGGCTCAGGGAAAAC-3' | 5'-GTCGCTCTGCAGGACGTAG-3' | 188 bp |
| *ABCG8* | 5'-ATAGGGAGCTCAGGTTGTGG-3' | 5'-TCGTCCACCCTTTTGTCG-3' | 260 bp |
| *GAPDH* | 5'- GTCTTCACTACCATGGAGAAGG –3' | 5'- TCATGGATGACCTTGGCCAG –3' | 197 bp |
| *b-Actin* | 5'- AACTCCATCATGAAGTGTGACG –3' | 5'- GATCCACATCTGCTGGAAGG –3' | 214 bp |
| *Ubiquitin* | 5'- AGATCCAGCATAAGGAAGGCAT –3' | 5'- GCTCCACCTCCAGGGTGAT –3' | 198 bp |
| *18S* | 5'- AAGTCTTTGGGTTCCGGG –3' | 5'- GGACATCTAAGGGCATCACA –3' | 365 bp |
| *Lactoferrin* | 5'- GAACATCCCCATGGGCCTG -3' | 5'- CAGCCAGGCACCTGAAAGC -3' | 203 bp |
| *α-Lactalbumin* | 5'- ACCAGTGGTTATGACACACAAGC -3' | 5'- AGTGCTTTATGGGCCAACCAGT -3' | 233 bp |
| *LXRα* | 5'- CTGCGATTGAGGTGATGCTC -3' | 5'- CGGTCTGCAGAGAAGATGC -3' | 229 bp |
| *PPARγ* | 5'- CTCCAAGAGTACCAAAGTGCAATC -3' | 5'- CCGGAAGAAACCCTTGCATC -3' | 198 bp |

*Data analysis and statistics*

Quantitative real-time PCR data were processed using either the relative quantification ΔΔCT-method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen 2001) as described in Farke *et al*. (2006), or the standard curve method as described in Farke *et al.* (2007).
SigmaPlot software (Systat Software Inc., San Jose, USA) was used for

statistical analysis. The paired t-test with a p-value $<0.05$ was regarded as statistically significant. The linear regression was calculated using the regression wizard included in the SigmaPlot software.

## Results and Discussion

*cDNA and predicted polypeptide structure*

By amplification and sequencing of overlapping PCR fragments, the complete *ABCA1*, *ABCG5* and *ABCG8* coding regions were obtained. The open reading frame of *ABCA1* encodes for a 2,261 AA polypeptide with a predicted molecular weight of 254 kDa (Farke *et al*. 2006). The bovine *ABCG5* and *ABCG8* genes were predicted to encode for 2 proteins of 652 and 674 AA, respectively (Viturro *et al*. 2006). The complete cDNA sequences have been deposited within the GenBank Database under the accession numbers NM_001024693 (ABCA1), NM_001024547 (ABCG5), and NM_001024663 (ABCG8).

The deduced ABCA1 protein is a full ABC transporter with two transmembrane and two nucleotide binding domains, identified by the conserved ATP-binding cassettes including Walker A and Walker B motifs and signature sequences (Farke *et al*. 2006). It has been reported that in some human cells two *ABCA1* gene transcripts due to alternative splicing, one presumably devoid of function, have been observed (Bellincampi *et al*. 2001). The amplification of bovine ABCA1 cDNA with specific primers in this region could not corroborate alternative splicing between exons 3 and 5 for *Bos taurus* in any tissue tested.

Using the software PESTfind (https://emb1.bcc.univie.ac.at), a conserved potential PEST sequence with a PEST score of +16.22 in bovine ABCA1 was identified (Figure 2, App. p. 44). In mouse ABCA1 it has been shown that the PEST sequence enhances the degradation of ABCA1 by calpain protease, and, thus, controls the cell surface concentration and cholesterol efflux activity of ABCA1 (Wang *et al*. 2003a). PEST sequences are found in many proteins undergoing rapid turnover (Rechsteiner & Rogers 1996). According to the very high homology between other mammalian and bovine ABCA1 PEST sequences, it is very likely that they all fulfill similar physiological functions and contribute to the regulation of ABCA1 degradation.

The characteristic signature sequence and Walker A and B motifs were also identified in the half transporters ABCG5 and ABCG8 (Viturro *et al*. 2006).

Homology search with the predicted bovine amino acid sequences revealed very high identity to human, mouse and rat for all three transporters (Table 3).

***Table 3:*** *Homology of the bovine ABCA1, ABCG5 and ABCG8 compared to the human, mouse and rat AA sequences.*

| | AA sequence identity | | |
|---|---|---|---|
| bovine | human | mouse | rat |
| ABCA1 | 94% | 93% | 92% |
| ABCG5 | 80% | 76% | 75% |
| ABCG8 | 77% | 76% | 75% |

Half transporters, like ABCG5 and ABCG8, must form homodimers or heterodimers with other ABC transporter proteins to form a functional transport system. Therefore, simultaneous expression and co-localization of both genes seems to be mandatory for their biological function (Freeman *et al.* 2004). In addition, post-transcriptional processing of both proteins and transport to their final destination is always dependent on the presence of both transcripts (Graf *et al*. 2003).

*Promoter regions*

Analysis of the bovine *ABCA1* promoter identified multiple motifs (Figure 4) that were strongly conserved between human and bovine sequences. Some of these potential transcription factor binding sites are also present in the promoter of receptors involved in lipid metabolism, including the low density *lipoprotein* (*LDL*) *receptor, LDL receptor-related protein, CD36, scavenger receptor class-B type I* (*SR-BI*), and *scavenger receptor A* promoter. These receptor promoters include binding motifs for SP1, activator protein 1 (AP-1), sex determining region Y (SRY/SOX5), and nuclear factor-kappaB (NF-κB) (Armesilla & Vega 1994, Cao *et al*. 1997, Valledor *et al*. 1998). A TATA box, a CAAT box, an E-box and the recognition element for the basic helix-loop-helix leucine zipper containing proteins, such as the sterol

regulatory element binding proteins (SREBP), that are binding sites for sterol regulation (Brown & Goldstein 1997) were identified. Similar E-box motifs have been reported in the promoter for SR-BI (Cao *et al*. 1997, Lopez & McLean 1999), fatty acid synthase (Magana *et al*. 2000), human CD36 (Armesilla & Vega 1994), and the LDL receptor (Brown and Goldstein 1997). These predicted features are consistent with the promoter region of other *ABCA* subfamily members, such as *ABCA2*, *ABCA7*, and *ABCA13* (Kaminski *et al*. 2001, Broccardo *et al*. 2001, Barros *et al*. 2003). The high degree of similarity between motifs in the human and bovine *ABCA1* promoter structure strongly suggests a role for bovine ABCA1 in sterol homeostasis.

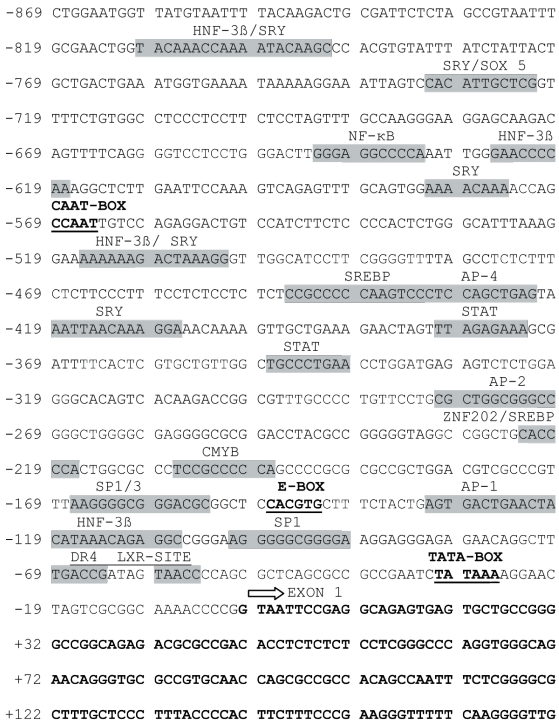


***Figure 4:*** *Putative proximal promoter sequence of the ABCA1 gene with predicted transcription factor binding sites (shaded). TATA box, E-box, and CAAT box motifs are bold and underlined. The putative transcriptional start site, according to the human sequence, is indicated by an arrow and shown in bold (Farke et al. 2006).*

The human *ABCG5* and *ABCG8* 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 always 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 (Viturro *et al*. 2006). In the bovine *ABCG5/8* cluster, the region between the start codons of both genes comprises 430 bp. Similar to the coding regions, analysis of the proximal promoter region revealed a high degree of conservation between the bovine and other mammalian species.

Within the bovine *ABCG5/G8* intergenic region (Figure 2, App. p. 53), response elements for transcription enhancer factor 1 (TEF1), liver receptor homolog-1 (LRH-1), and NF-κB along with 2 GATA boxes were identified. The TEF 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, LRH-1 and NF-κB response elements. The GATA boxes are present in adipocyte precursor cells and control their transition to the mature adipocyte by transcriptional regulation of genes involved (Tong *et al*. 2005). LRH-1 was reported previously (Freeman *et al*. 2004) to stimulate activity of *ABCG5/8* promoter, hypothesizing it to be a key regulator of a number of genes involved in excretion of sterols from liver and intestine. The NF-κB is another widely studied response element because of its crucial role in the regulation of many atherosclerosis-related genes (Israelian-Konaraki & 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 important genes involved in this process.

The bovine promoter sequences have been deposited at the GenBank database under the accession numbers DQ142640, for the *ABCA1* promoter, and DQ086422 for the intergenic spacer between *ABCG5* and *ABCG8*.

*Tissue-specific expression*

In addition to the identification and sequence analysis of the *ABCA1*, *ABCG5* and *ABCG8* genes, their expression was studied in a bovine tissue bank.

The *ABCA1* transcript was detected in all tissues of *Bos taurus* that were analyzed (Figure 5). The highest expression level was observed in lung. This resembles results from Kielar *et al*. (2001) and Langmann *et al*. (2003) in human tissues. The primary function of ABCA1 in human lung might be to modulate lipid pools in alveolary epithelial cells (Agassandian *et al*. 2004). An alternative assumption is that ABCA1 in human lung takes part in cholesterol homeostasis

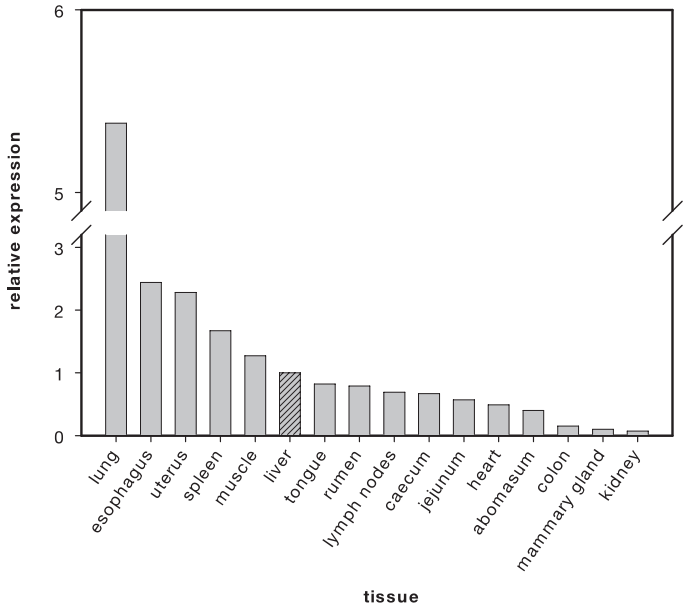


***Figure 5:*** *Quantitative analysis of ABCA1 mRNA in bovine tissues. Bars represent relative quantification calculated as fold-expression compared to liver (crosshatched bar). Values were normalized to the mean of four housekeeping genes (GAPDH, β-actin, ubiquitin, and 18S).*

High expression levels were also found in esophagus, uterus, spleen, and muscle (Figure 5), which is partly in agreement with Langmann *et al*. (2003). Moderate levels of expression were detected for liver, tongue, gastric tissues, cecum, jejunum, heart, and lymph nodes, whereas, congruent with distribution patterns in human tissues, low expression was observed in colon and kidney (Figure 5). The function of intestinal ABCA1 is likely to generate HDL particles that transport dietary cholesterol to the liver. In humans, the resecretion of cholesterol in the intestine is mediated by ABCG5 and ABCG8 (Oram & Heinecke 2005), which could explain the comparatively low distribution of *ABCA1* in these tissues. However, 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 the *ABCA1* transporter under these feeding conditions. It is likely that the function of ABCA1 in kidney may be to maintain normal cholesterol homeostasis and protect against hyperlipidemic renal disease (Wu *et al*. 2004). The detection of *ABCA1* in the mammary gland might indicate a potential role of ABCA1 in the transfer of cholesterol into the milk, a fact that should be verified in further studies.

A matching tissue distribution was observed for *ABCG5* and *ABCG8* (Viturro *et al*. 2006) by means of Block PCR. Unlike *ABCA1*, the expression pattern of *ABCG5* and *ABCG8* was more specific. High intensity bands were present in cDNA samples from liver and colon, however, bands of lesser intensity also appeared in cDNA samples from abomasum, jejunum, lymphatic nodes, mammary gland, leukocytes, and placenta. The results for the remaining tissue bank samples were negative. These results were confirmed by quantitative PCR (Figure 4, App. p. 54) and similar tissue-specific distribution and highly comparable specific expression between *ABCG5* and *ABCG8* were obtained. For both genes, a high level of expression was found in liver and colon samples, with an approximately 10-fold expression compared to other positive tissues. Among these positive tissues are other parts of the digestive system (abomasum and jejunum), the mammary gland, and blood samples. Residual expression was found for lymphatic nodes and placenta.

The expression of *ABCG5* and *ABCG8* in the bovine liver and digestive system is consistent with expression patterns in other species, due to the main role of the ABCG5/8 transport complex in absorption of sterols from the diet and their biliar excretion. It is remarkable that this expression seems to be specifically located along the intestinal tract, as described for mouse (Mutch *et al.* 2004). In the digestive tract of the cow, the highest level of expression of these transporters occurred in the colon, with medium expression levels in jejunum and abomasum, whereas no expression was found in cecum samples. These results are not completely in line 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.
The precise localization of this complex within the udder must be studied in order to define the exact role of ABCG5 and ABCG8 and their potential importance upon regulation 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 three important steps: Intestinal absorption, excretion in bile and excretion in milk.

*ABC transporters in the lactating and nonlactating mammary gland*

It has been reported that milk cholesterol is partially synthesized in the mammary gland but that the major proportion is mainly derived from serum cholesterol (Long *et al.* 1980). But the mechanism of how serum cholesterol is transferred into the milk is still unclear.
The detection of ABC transporters, with sterol affinity, in the bovine mammary gland and the demonstration of their gene levels in the lactating mammary gland may identifiy candidate transporters involved in lipid homeostasis in the lactating mammary gland. Furthermore, expression levels of these transporters in lactating as compared to nonlactating mammary gland tissue may identify a subset of transporters involved in lipid and cholesterol transport into milk.
Mammary gland biopsies from nine consecutive days during lactation and after the first and second week after dry off, respectively, were

analyzed. To determine whether lactation alters transporter gene expression, individual transporter RNA expression levels were compared in lactating and nonlactating bovine mammary glands. For normalization the arithmetic mean of three housekeeping genes (*GAPDH*, β-*actin* and *ubiquitin*) was used.

Concentrations of milk-specific components such as caseins, α-lactalbumin, β-lactoglobulin, and milk fat decline during the first 2-3 weeks of the dry period (Hurley & Rejman 1986). In agreement with these findings we observed a significant decrease in α-*lactalbumin* gene expression (P=0.0113) in the second week of dry period (D2). The expression of *lactoferrin* is regulated differently from that of other milk proteins. An increase of *lactoferrin* gene expression was observed during the first and second week of the dry period, which is in concordance with reports showing that *lactoferrin* is very low in bovine milk during mature lactation and is markedly elevated during mammary involution (Goodman & Schanbacher 1991). Lactoferrin has diverse functions regarding cellular differentiation and growth and plays a role in the immune system where elevated expression levels might help to protect the mammary gland from bacterial infections (Oliver & Sordillo 1989, Schanbacher *et al*. 1993).

Having confirmed that the sampling procedures and RNA measurements were adequate and reliable, the main interest focussed on the expression pattern of selected ABC transporters involved in lipid, phospholipid and cholesterol transport. Therefore gene expression levels of *ABCA1*, *ABCA7*, *ABCG1*, and *ABCG5* in lactating and nonlactating mammary glands were compared.

A significant difference in expression between lactation and dry period was found for *ABCA1*, and *ABCA7* (Table 4). Within the first week after dry off (D1) an increase of *ABCA1* gene expression was observed which reached statistical significance (P=0.0439) in the second week (D2). *ABCA7* expression decreased at the beginning of dry period (D1) and declined significantly (P=0.0323) in the second week (D2). It is possible that the increase of *ABCA1* expression in the nonlactating mammary gland could be associated with the reported immigration of macrophages during involution (Monks *et al*. 2002).

## Results and Discussion

*ABCA1* is highly expressed in tissue macrophages (Lawn *et al*. 2001) and it has been reported that *ABCA1* transcripts are upregulated in macrophages involved in the engulfment and clearance of apoptotic cells (Luciani & Chimini 1996). Interestingly, macrophages from involuting sheep mammary glands have been described as having phagocytic vacuoles containing casein micelles, lipid droplets, and cellular debris (Tatarczuch *et al*. 2000). This suggests that these cells

***Table 4:*** *Expression differences of genes in nonlactating relative to lactating mammary glands. Values indicate n-fold expression (2-ΔΔCT) ± SEM in lactation as compared to the dry period 1 (D1, 1 week after drying off) and 2 (D2, 2 weeks after drying off). A value of 1 indicates no change in relative expression, a value >1 indicates an increase in expression, and a value < 1 indicates a decrease in expression.*

| | **Expression change as compared to lactation phase (LT)** | | | |
|---|---|---|---|---|
| | D1 | P-value | D2 | P-value |
| ABCA1 | 3.12 ± 0.61 | P=0.06 | 2.84 ± 0.37 | P=0.04 |
| ABCA7 | 0.08 ± 1.76 | P=0.08 | 0.04 ± 0.95 | P=0.03 |
| ABCG1 | 1.56 ± 0.69 | P=0.56 | 0.42 ± 2.50 | P=0.65 |
| ABCG2 | 0.16 ± 0.59 | P=0.22 | 0.07 ± 0.42 | P=0.04 |
| ABCG5 | 0.11 ± 0.82 | P=0.18 | 0.13 ± 1.07 | P=0.07 |
| PPARγ | 3.88 ± 2.70 | P=0.32 | 0.93 ± 2.64 | P=0.92 |
| LXRα | 0.66 ± 2.66 | P=0.85 | 3.84 ± 1.45 | P=0.27 |
| Lactoferrin | 2.12 ± 3.57 | P=0.78 | 2.33 ± 2.23 | P=0.67 |
| α-Lactalbumin | 0.03 ± 1.95 | P=0.21 | 0.0004 ± 1.37 | P=0.01 |

play a role in clearance of residual milk and fragmented dead cells. Whether ABCA1 could be implicated in cholesterol and phosholipid transport or intracellular trafficking in the mammary gland is currently unclear. Fong *et al.* (2007) recently identified apolipoprotein (apo) E and apoAI, key acceptors of cholesterol effluxed by ABCA1 in cholesterol loaded macrophages (Oram *et al*. 2000), in bovine milk-fat-globule membranes. These findings indicate, that potential molecular acceptors for ABCA1-meditated cholesterol efflux are present in

bovine milk. However, to shed light on the physiological role of ABCA1 in mammary gland, it is crucial to determine its cellular localisation and to investigate whether *ABCA1* is expressed in milk fat globules or other intracellular compartments.

Surprisingly, the expression data of *ABCA1* and *ABCA7* in the bovine mammary gland showed an opposite trend from lactation to dry period (Figure 6). While *ABCA1* was upregulated *ABCA7* expression decreased during the dry period. Human ABCA1 is induced by

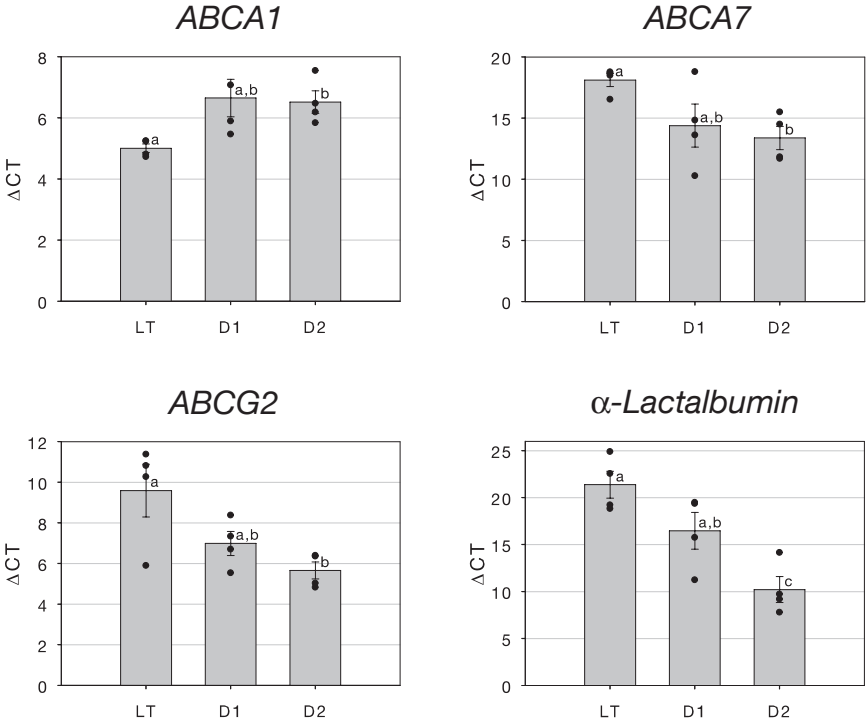


***Figure 6:*** *Significant changes in the mRNA expression of ABCA1, ABCA7, ABCG2 and α-lactalbumin in bovine mammary glands between lactation and dry periods. LT= normalized mean (ΔCT values) for lactation; D1= normalized mean (ΔCT values) for the first week of the dry period; D2= normalized mean (ΔCT values) for the second week of the dry period. Error bars indicate the standard error of the mean (SEM). Means without a common letter are significantly different (P<0.05). Each dot indicates the mean (ΔCT values) of one cow.*

cholesterol through the LXR system (Venkateswaran *et al*. 2000), whereas ABCA7, which is highly homologous to ABCA1, is negatively regulated by cellular cholesterol (Iwamoto *et al*. 2006). Supporting these findings, Wang *et al*. (2003b) demonstrated that, in contrast to *ABCA1*, *ABCA7* shows moderate basal mRNA and protein levels in macrophages but no induction by LXR activation. Their studies show that ABCA7 has the ability to bind apolipoproteins and promote efflux of cellular phospholipids without cholesterol, suggesting a possible role of ABCA7 in cellular phospholipid metabolism in peripheral tissues. This points out that the high homology between ABCA1 and ABCA7 may not be extrapolated to physiological functions. The physiological role of ABCA7 in the mammary gland currently remains elusive. Similar to *ABCA7*, *ABCG5* showed a decreased expression in the dry period (Table 4) which, however, did not reach statistical significance.

Viturro *et al*. (2006) demonstrated *ABCG5* and *ABCG8* expression in the bovine lactating mammary gland for the first time. However, in the present set of samples *ABCG5* expression was significantly lower, with CT values ranging mostly between 30 and 35. Thus, the data gained in these experiments should be interpreted with caution and do currently not allow to postulate an important role for these genes in the mammary gland. It cannot be excluded that ABCG5/8 might be involved in the secretion of sterols in the bovine milk, but further studies are needed to prove a functional role for these half transporters in the mammary gland.

In parallel to the above mentioned lipid transporters, *ABCG2* expression was measured in the mammary gland samples. Jonker *et al*. (2005) demonstrated that the ABCG2 transporter is strongly induced in the mammary gland of mice, cows and humans during lactation and that it is responsible for the active secretion of clinically and toxicologically important substrates into mouse milk. They observed that during involution following cessation of lactation, *ABCG2* expression declined rapidly. In agreement with these data, this study revealed a significant decrease in *ABCG2* expression (P=0.0382) from the lactating to the nonlactating state of the bovine mammary gland (Table 4, Figure 6). Taking into account that contamination of milk with drugs, pesticides

and other xenotoxins can imply a major health risk to the suckling offspring, it is currently unclear why and to which extent ABCG2 is functionally active in the mammary gland. Therefore it is essential to identify physiological ligands for ABCG2, and to investigate which of them may account for the high expression during lactation. In this context van Herwaarden *et al*. (2007) recently demonstrated that ABCG2 not only secretes drugs but also riboflavin (vitamin B2) into milk, implying that vitamin B2 might represent an endogenous ligand for ABCG2 in the mammary gland. Interestingly, a missense mutation in the *ABCG2* gene was recently found to affect milk yield, milk fat and protein concentration in Holstein cattle (Cohen-Zinder *et al*. 2005) suggesting a functional role for ABCG2 in milk secretion.

The expression of several ABC transporters, especially those implicated in lipid homeostasis, is regulated by nuclear receptors. To investigate a potential correlation between the nuclear receptors and their regulatory genes, the expression of *PPARγ* and *LXRα* was analyzed. Assessing the expression of the ABC transporters in relation

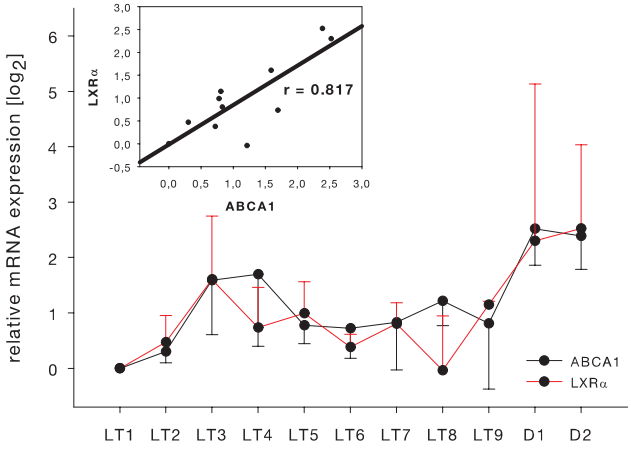


*Figure 7: Relative expression of ABCA1 and LXRα (ΔΔCT values) during 9 consecutive days in lactation (LT1 – LT9) and after dry off (D1, D2) with regression line and correlation coefficient. LT= lactation; D1= first week of the dry period; D2= second week of the dry period; r= correlation coefficient. Error bars indicate the standard error of the mean (SEM).*

to *PPARγ* and *LXRα*, we observed a similar expression pattern for *ABCA1* and *LXRα* with a correlation coefficient (r) of 0.82 (Figure 7, insert), but for none of the other transporters tested. Ligand-bound receptor dimer RXR/LXRα induces the expression of *ABCA1* in mice (Repa *et al*. 2000). They found that this induction was obtained only with specific ligands for LXR and not with ligands for other orphan nuclear receptors. Endogenous ligands for LXR are oxysterols, metabolites of cholesterol. The findings suggest that LXRα is involved in the regulation of *ABCA1* expression in the bovine mammary gland. Indeed *LXRα* was 3.84 ± 1.45 fold increased in the second week of the dry period. However, probably due to low number of animals in this experiment and the high interindividual variation, the differences in *LXRα* expression between lactation and the dry period did not reach statistical significance. All genes tested, except *ABCG1* and *PPARγ* for which no apparent changes in the gene expression during lactation and involution were observed, showed a clear trend towards significance in the second week of dry period (Table 4). The fact that severe changes in the involuting mammary gland take place after the period of approximately one week (Hurley 1989), could strongly support the finding that marked differences in gene expression levels predominantly occurred in the second week of the dry period.

## Conclusions

The identification and characterization of bovine *ABCA1*, *ABCG5* and *ABCG8* and their expression within tissues, including the mammary gland, were in focus of this study (Figure 8). The high degree of similarity to the human analogs in protein sequence, sequence motifs, promoter structure, and expression levels strongly suggests a similar role in sterol homeostasis.

The mammary gland is an important organ in conjunction with lipid and sterol turnover in the cow. By analyzing candidate transporters associated with sterol transfer, insights into gene expression patterns in the lactating and involuting mammary gland were gained. *ABCA1* was up to 3-fold higher expressed in the dry period as compared to lactation, while *ABCA7* and *ABCG5* expression decreased up to 25-fold and 9-fold, respectively, amongst these physiological stages (Figure 8). These findings underscore the need for sensitive, rapid, and accurate methods for the quantification of ABC transporter expression, and a systematic investigation of these molecules in bovine tissues. To unravel the physiological role and underlying molecular mechanisms of sterol transporters in mammary gland, the identification of endogenous ligands by means of transport studies in this organ and immunohistochemical studies, revealing the intracellular localization of the corresponding proteins, are essential.

A better understanding of these transporters and pathways in mammary gland lipid translocation may help to reveal novel molecular mechanisms regulating sterol transfer into milk.

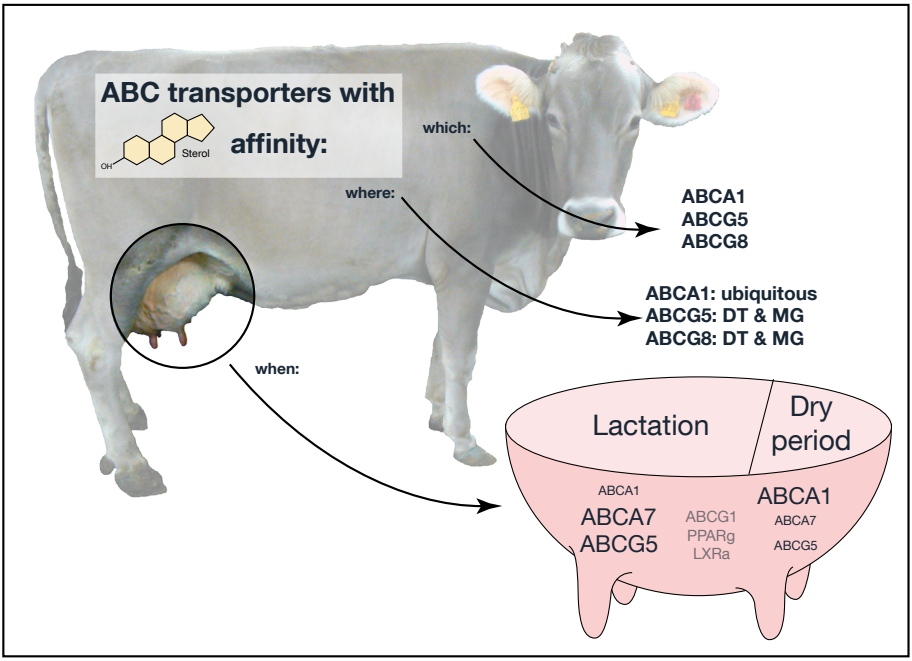


***Figure 8:*** *Schematic presentation of major results obtained in this thesis.*

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## Scientific Communications

*Publications*

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# Identification of the bovine cholesterol efflux regulatory protein ABCA1 and its expression in various tissues[1]

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**ABSTRACT:** The ATP-binding cassette transporter A1 (ABCA1) is known to play a significant role in cellular export of phospholipids and cholesterol in humans. The ABCA1 transporter might also play a crucial role in cellular cholesterol homeostasis in the cow or in the transfer of cholesterol into the milk, but its presence and tissue distribution in the bovine is unknown. Therefore, we studied the expression and distribution of the bovine ABCA1 transporter using quantitative PCR and sequenced the entire *ABCA1* coding region. In addition, the proximal promoter was identified and screened for regulatory elements. Concordant with data from other mammalian species, bovine *ABCA1* mRNA was expressed and detected in all tissues tested. The highest expression levels were detected in lung, esophagus, uterus, spleen, and muscle. Sequence analysis revealed that the open reading frame of this gene consists of 6,786 bases and encodes for a protein of 2,261 AA with a predicted molecular weight of 254 kDa. The deduced bovine ABCA1 protein shows the highest AA sequence homology with human (94%), mouse (93%), rat (92%), and chicken (85%). Analysis of the putative *ABCA1* promoter region revealed potential transcription factor binding sites associated with *ABCA1* transcription and lipid metabolism. This work could open new avenues for elucidating a potential role of ABCA1 in sterol homeostasis in the bovine organism.

**Key words:** ABCA1, ATP-binding cassette transporter, *Bos taurus*, cattle, quantitative PCR, sterol homeostasis



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## INTRODUCTION

The ATP-binding cassette (**ABC**) transporter family represents the largest family of transmembrane proteins. These proteins bind ATP and use the energy to drive the transport of a variety of substrates across cellular membranes (Higgins, 1992; Childs and Ling, 1994; Dean and Allikmets, 1995). Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds within the cell as part of a metabolic process or outside the cell for transport to other organs, or secretion from the body.

Mutations in a number of *ABC* genes are responsible for human inherited diseases. The ABCA1 transporter is involved in disorders concerning cholesterol disposition, such as Tangier disease and familial high-density lipoprotein deficiency (Brooks-Wilson et al., 1999; Albrecht et al., 2004a). With the discovery that mutations in the *ABCA1* gene were causal to Tangier disease, a rare hereditary disease that severely impairs the reverse cholesterol transport (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999), the physiological importance of this protein was recognized. Moreover, ABCA1 has been implicated in atherosclerosis (Albrecht et al., 2004b; Soumian et al., 2005; Oram and Heinecke, 2005) and Scott syndrome, a rare bleeding disorder (Albrecht et al., 2005).

Whereas ABC transporters play a considerable role in hereditary human diseases, only scarce information is available about their expression and function in food-producing animals. Only 5 ABC proteins have been identified in *Bos taurus* (Ambagala et al., 2002; Taguchi et al., 2002; Beharry et al., 2004; Vitarro et al., 2006), and their function remains unknown.

In the current study, the expression of ABCA1 was demonstrated for *Bos taurus,* and its sequence and tissue distribution were characterized in this species. Special interest was placed on characteristics in the proximal promoter and coding region that may indi-

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**Table 1.** Primers used for amplification of the *ABCA1* coding and promoter regions

| | Forward primer | | Reverse primer |
|---|---|---|---|
| 1.for | 5′- GGTTGCTGCTGTGGAAGAAC –3′ | 12.rev | 5′- GAATGACATCAGCCCTCAGC –3′ |
| 2.for | 5′- CGGCGGCTTCTCTTGTATAG –3′ | 13.rev | 5′- GAAGCCATCTTCCTCTGTGG –3′ |
| 3.for | 5′- TGAGCCTGATGTCTCCTGTG –3′ | 14.rev | 5′- GACACACAGGCAGCATCTTC –3′ |
| 4.for | 5′- AAGAGACTGCTGATTGCCAGAC –3′ | 15.rev | 5′- ACTGCCAAGACACCTGAACC –3′ |
| 5.for | 5′- TGAAGCTCTCTGCACTAGGATG –3′ | 16.rev | 5′- CCTCAGCATCTTGTCCACAG –3′ |
| 6.for | 5′- ACCAGCTTCCGTCTTCACTG –3′ | 17.rev | 5′- GTCTGAGAACAGGCGAGACAC –3′ |
| 7.for | 5′- CTGGATGAGAGTCTCTGGAG –3′ | 18.rev | 5′- CGGAGATCAGGATCAGGAAG –3′ |
| 8.for | 5′- GCTCTCGACTGTCAAGGCC –3′ | 19.rev | 5′- GTCTCATATGGCTCTCGAGTGA –3′ |
| 9.for | 5′- GTCCAGAGGACTGTCCATCTTC –3′ | 20.rev | 5′-CCAAGTCGCTCAAGAGACTC –3′ |
| 10.for | 5′- GAAGATGCTGCCTGTGTGTC –3′ | 21.rev | 5′- CTATCGGTCAAAGCCTGTTCTC –3′ |
| 11.for | 5′- CACCTGACACTCCAGGTCACAAG –3′ | 22.rev | 5′- GAAGATGGACAGTCCTCTGGAC –3′ |

cate a potential role of the bovine ABCA1 in lipid homeostasis in bovine cells or tissues.

## MATERIALS AND METHODS

This study was performed according to the requirements of the Bavarian state animal welfare committee.

### *RNA Tissue Bank and Reverse Transcription*

A bovine, noncommercial tissue bank composed of 16 tissues was obtained after slaughter from 1 healthy adult lactating Holstein-Friesian cow without previous history of disease or drug treatment. Total RNA was isolated using the RNeasy Mini Kit or, for mammary gland tissue, the RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany). For fibrous tissues such as heart, tongue, and muscle, a proteinase K step was added after homogenization to increase the RNA yield. The RNA was quantified at 260 nm in a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany), obtaining an OD 260/280 ratio of 1.7 to 2.0 for all samples.

Synthesis of first strand cDNA was performed using 1 μg of total RNA and 200 U of SuperScript III reverse transcription (Invitrogen, Karlsruhe, Germany). The reverse transcription reaction was carried out according to the manufacturer in a 20-μL reaction volume in a PCR thermocycler (Biometra, Goettingen, Germany) and was achieved by successive incubations at 25°C for 5 min and 50°C for 45 min, finishing with enzyme inactivation at 70°C for 15 min.

### *PCR and Sequence Analysis*

The cDNA, stored at –20°C, served as a template for PCR. To screen for evolutionarily conserved sequences within the coding regions, gene sequences from rat, mouse, and human were compared by linear sequence alignment strategies using HUSAR software (DKFZ, Heidelberg, Germany). Primers (Table 1) were designed within the most conserved regions using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Rozen and Skaletsky, 2000) and used in various combinations to amplify overlapping cDNA fragments of 0.3 to 1.0 kb size.

The PCR reactions were performed in a PCR thermocycler (Biometra) and contained 100 ng of cDNA, 10× PCR reaction buffer, 0.4 μ*M* of forward and reverse primers (Metabion, Martinsried, Germany), 200 μ*M* of dNTP (ABgene, Hamburg, Germany), and 1.25 U of the proof-reading enzyme *Pfu* DNA-Polymerase (Promega, Madison, WI), in a final volume of 50 μL. The PCR products were subjected to gel electrophoresis in 1 to 2%-agarose gels containing 1 μg of ethidium bromide/mL. The DNA fragments were extracted using the Wizard SV Gel and PCR Clean-Up System (Promega), and both strands were commercially sequenced (Agowa, Berlin, Germany).

### *Rapid Amplification of cDNA Ends (RACE)*

To determine the 5′ and 3′ end of the *ABCA1* mRNA, RACE was performed using the 5′/3′RACE Kit, second Generation (Roche Diagnostics, Mannheim, Germany) and total RNA from bovine liver as a template. The 5′ RACE fragment was generated using an oligo dT-anchor primer (provided in the kit) and the gene-specific primer 5′-CCT CAG CAT CTT GTC CAC AG-3′. For generating the 3′ RACE fragment, the oligo dT-anchor primer and the gene-specific primer 5′-TGA AGC TCT CTG CAC TAG GAT G-3′ were combined. The amplified products were commercially sequenced (Medigenomix, Martinsried, Germany).

### *Promoter Analysis*

Due to the high degree of identity that has been reported for the human and mouse *ABCA1* promoter regions (Santamarina-Fojo et al., 2000), the human *ABCA1* promoter sequence was compared by linear sequence alignment strategies to Baylor Bovine Data (http://www.hgsc.bcm.tmc.edu/blast/?organism=B taurus) to identify the bovine analogue of the promoter region. According to bovine Contig 222145, specific primers were designed and used with bovine liver genomic DNA. The resulting overlapping fragments were sequenced (Medigenomix) and assembled.

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

| Item | Forward primer | Reverse primer | Product size |
|---|---|---|---|
| ABCA1 | 5′- GGACATGTGCAACTACGTGG –3′ | 5′- TGATGGACCACCCATACAGC –3′ | 134 bp |
| GAPDH[1] | 5′- GTCTTCACTACCATGGAGAAGG –3′ | 5′- TCATGGATGACCTTGGCCAG –3′ | 197 bp |
| β-Actin | 5′- AACTCCATCATGAAGTGTGACG –3′ | 5′- GATCCACATCTGCTGGAAGG –3′ | 214 bp |
| Ubiquitin | 5′- AGATCCAGCATAAGGAAGGCAT –3′ | 5′- GCTCCACCTCCAGGGTGAT –3′ | 198 bp |
| 18S | 5′- AAGTCTTTGGGTTCCGGG –3′ | 5′- GGACATCTAAGGGCATCACA –3′ | 365 bp |

[1]GAPDH = glycerol-3-phosphate dehydrogenase.

The putative *ABCA1* promoter sequence was analyzed for potential transcription factor (**TF**)-binding sites using MatInspector software (http://www.genomatix.de) and MOTIF software (http://motif.genome.jp).

### *Real-Time PCR*

Quantitative reverse-transcription PCR of *ABCA1* mRNA in bovine tissues was carried out using LightCycler DNA Master SYBR Green technology (Roche Diagnostics, Mannheim, Germany). Primer pairs (Table 2) were designed covering 2 exon boundaries to avoid amplification of genomic DNA. The PCR reactions were performed in a final volume of 10 μL, using 1 μL of the LC FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 pmol of each primer, 3 m*M* $MgCl_2$, and 50 ng of cDNA. Before amplification, an initial high-temperature incubation step was performed to activate the DNA polymerase and to ensure complete denaturation of cDNA. All PCR reactions were composed of 40 cycles. Product-specific PCR conditions are listed in Table 3.

Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification. Data were analyzed using the second Derivate Maximum calculation described in the LightCycler Software 3.5. All runs included a negative cDNA control consisting of PCR-grade water, and each sample was measured in duplicate. To minimize any bias related to a potential differential tissue expression of genes used for data normalization, 4 housekeeping genes were included in the analysis [glycerol-3-phosphate dehydrogenase, β-actin, ubiquitin, and 18S, see Tables 2 and 3]. The *ABCA1* mRNA levels were expressed relative to the mean of the 4 housekeeping genes and calculated as fold-expression compared with bovine liver.

**Table 3.** Cycling conditions for quantitative reverse transcription-PCR

| | Denaturation | | Primer annealing | | Elongation | |
|---|---|---|---|---|---|---|
| Gene | T, °C | t, s | T, °C | t, s | T, °C | t, s |
| ABCA1 | 95 | 15 | 55 | 10 | 72 | 20 |
| GAPDH[1] | 95 | 15 | 58 | 10 | 72 | 20 |
| β-Actin | 95 | 15 | 62 | 10 | 72 | 20 |
| Ubiquitin | 95 | 15 | 60 | 10 | 72 | 20 |
| 18S | 95 | 15 | 62 | 10 | 72 | 20 |

[1]GAPDH = glycerol-3-phosphate dehydrogenase.

## RESULTS AND DISCUSSION

### *ABCA1 cDNA and Predicted Polypeptide Structure*

By amplification and sequencing of overlapping PCR fragments, an 8,893-bp cDNA containing the complete coding region of the bovine *ABCA1* gene was obtained. The open reading frame comprises 6,786 bp and encodes for a 2,261 AA polypeptide with a predicted molecular weight of 254 kDa (Figure 1). The complete bovine *ABCA1* cDNA and AA sequence has been deposited within the GenBank Database (Accession No. DQ059505). The deduced protein is a full-size ABC transporter with 2 transmembrane domains and 2 nucleotide binding domains, identified by the conserved ATP-binding cassettes including Walker A and Walker B motifs and signature sequences (Figure 1). Homology search with the predicted bovine ABCA1 AA sequence revealed the greatest identity to human (94%), mouse (93%), rat (92%), and chicken ABCA1 (85%).

It has been reported that in some human cells, such as skin fibroblasts, leukemia T-cells, endothelial and smooth muscle cells, as well as hepatoma cells, 2 *ABCA1* gene transcripts, 1 presumably devoid of function, have been observed (Bellincampi et al., 2001). The PCR amplification of bovine cDNA with specific primers in this region could not confirm alternative splicing between exons 3 and 5 for *Bos taurus* in any tissue tested.

Recently it has been shown that in mouse ABCA1 a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (**PEST** sequence) enhances the degradation of ABCA1 by calpain protease and thus controls the cell surface concentration and cholesterol efflux activity of ABCA1 (Wang et al., 2003). The PEST sequences are found in many proteins undergoing rapid turnover (Rechsteiner and Rogers, 1996). Using the software PESTfind (https://emb1.bcc.univie.ac.at), we identified a conserved potential PEST sequence with a PEST score of +16.22 in bovine ABCA1 (Figure

```
                                                Walker A
bABCA1          IQNLMKVYRDGMKVAVDGLALNFYEGQITSFLGHNGAGKTTTMSILTGLFPPTSGTAYIL  960
hABCA1          IQNLVKVYRDGMKVAVDGLALNFYEGQITSFLGHNGAGKTTTMSILTGLFPPTSGTAYIL

bABCA1          GKDIRSEMSTIRQNLGVCPQHNVLFDMLTVEEHIWFYARLKGLSEKHVKAEMEQMALDVG  102C
hABCA1          GKDIRSEMSTIRQNLGVCPQHNVLFDMLTVEEHIWFYARLKGLSEKHVKAEMEQMALDVG
                              Signature C          Walker B
bABCA1          LPPSKLKSKTSQLSGGMQRKLSVALAFVGGSKVVILDEPTAGVDPYSRRGIWELLLKYRQ  108C
hABCA1          LPSSKLKSKTSQLSGGMQRKLSVALAFVGGSKVVILDEPTAGVDPYSRRGIWELLLKYRQ

bABCA1          GRTIILSTHHMDEADILGDRIAIISHGKLCCVGSSLFLKNQLGTGYYLTLVKKDVESSLS  114C
hABCA1          GRTIILSTHHMDEADVLGDRIAIISHGKLCCVGSSLFLKNQLGTGYYLTLVKKDVESSLS

bABCA1          SCRNSSSTVSYLKKEDSVSQSSSDAGLGSDHESDMLTIDVSVISNLIRKHVAEARLVEDI  120C
hABCA1          SCRNSSSTVSYLKKEDSVSQSSSDAGLGSDHESDTLTIDVSAISNLIRKHVSEARLVEDI

bABCA1          GHELTYVLPYEAAREGAFVELFHEIDDRLSDLGISSYGISETTLEEIFLKVAEESGVDAE  126C
hABCA1          GHELTYVLPYEAAKEGAFVELFHEIDDRLSDLGISSYGISETTLEEIFLKVAEESGVDAE
                                        PEST
bABCA1          TSDGTLPARRNRRVFGDKQSCLRPFTEDDAMDPNDSDIDPESRETDLLSGMDGKGSYQVK  132C
hABCA1          TSDGTLPARRNRRAFGDKQSCLRPFTEDDAADPNDSDIDPESRETDLLSGMDGKGSYQVK

bABCA1          GWKLTQQQFVALLWKRLLIARRSRKGFFAQIVLPAVFVCIALVFSLIVPPFGKYPSLELQ  138C
hABCA1          GWKLTQQQFVALLWKRLLIARRSRKGFFAQIVLPAVFVCIALVFSLIVPPFGKYPSLELQ

bABCA1          PWMYNEQYTFVSNDAPEDVSTQELLNALTGKPGFGTRCMEGNPIPETPCLVGEEKWNTAP  144C
hABCA1          PWMYNEQYTFVSNDAPEDTGTLELLNALTKDPGFGTRCMEGNPIPDTPCQAGEEEWTTAP

bABCA1          VPQTITDLFRNGNWTMENPSPTCQCSSDKIKKMLPVCPLGAGGLPPPQRKQNTADILQNL  150C
hABCA1          VPQTIMDLFQNGNWTMQNPSPACQCSSDKIKKMLPVCPPGAGGLPPPQRKQNTADILQDL

bABCA1          TGRNISDYLVKTYVQIIAKSLKNKIWVNEFRYGGFSLGASNSQSLPPSEEVNDAIKQMKK  156C
hABCA1          TGRNISDYLVKTYVQIIAKSLKNKIWVNEFRYGGFSLGVSNTQALPPSQEVNDAIKQMKK

bABCA1          HLKVVKDSSADRFLSSLGRFMTGLDTKNNVKVWFNNKGWHAISSFLNVINNAILRANLQK  162C
hABCA1          HLKLAKDSSADRFLNSLGRFMTGLDTRNNVKVWFNNKGWHAISSFLNVINNAILRANLQK

bABCA1          GENPSQYGITAFNHPLNLTKQQLSEVALMTTSVDVLVSICVIFAMSFVPASFVVFLIQER  168C
hABCA1          GENPSHYGITAFNHPLNLTKQQLSEVALMTTSVDVLVSICVIFAMSFVPASFVVFLIQER

bABCA1          VSKAKHLQFISGVKPVIYWLSNFVWDMCNYVVPATLVIIIFICFQQKSYVSSTNLPVLAL  174C
hABCA1          VSKAKHLQFISGVKPVIYWLSNFVWDMCNYVVPATLVIIIFICFQQKSYVSSTNLPVLAL

bABCA1          LLLLYGWSITPLMYPASFVFKIPSTAYVVLTSVNLFIGINGSVATFVLELFTNNKLNNIN  180C
hABCA1          LLLLYGWSITPLMYPASFVFKIPSTAYVVLTSVNLFIGINGSVATFVLELFTDNKLNNIN

bABCA1          DILKSVFLIFPHFCLGRGLIDMVKNQAMADALERFGENRFVSPLSWDLVGRNLFAMAVEG  186C
hABCA1          DILKSVFLIFPHFCLGRGLIDMVKNQAMADALERFGENRFVSPLSWDLVGRNLFAMAVEG

bABCA1          VVFFLITVLIQYRFFIRPRPVKAKLPPLNDEDEDVKRERQRILDGGGQNDILEIKELTKV  192C
hABCA1          VVFFLITVLIQYRFFIRPRPVNAKLSPLNDEDEDVRRERQRILDGGGQNDILEIKELTKI
                                        Walker A
bABCA1          YRRKRKPAVDRICVGIPPGECFGLLGVNGAGKSSTFKMLTGDTTVTRGDAFLNKNSILSD  198C
hABCA1          YRRKRKPAVDRICVGIPPGECFGLLGVNGAGKSSTFKMLTGDTTVTRGDAFLNKNSILSN

bABCA1          IHEVHQNMGYCPQFDAITELLTGREHVEFFALLRGVPEKEVGKVGEWAIRKLGLVKYGEK  204C
hABCA1          IHEVHQNMGYCPQFDAITELLTGREHVEFFALLRGVPEKEVGKVGEWAIRKLGLVKYGEK
                     Signature C              Walker B
bABCA1          YAGNYSGGNKRKLSTAMALIGGPPVVFLDEPTTGMDPKARRFLWNCALSIIKEGRSVVLT  210C
hABCA1          YAGNYSGGNKRKLSTAMALIGGPPVVFLDEPTTGMDPKARRFLWNCALSVVKEGRSVVLT

bABCA1          SHSMEECEALCTRMAIMVNGRFRCLGSVQHLKNRFGDGYTIVVRIAGSNPDLKPVQEFFE  216C
hABCA1          SHSMEECEALCTRMAIMVNGRFRCLGSVQHLKNRFGDGYTIVVRIAGSNPDLKPVQDFFG

bABCA1          LAFPGSVLKEKHRNMLQYQLPSSLSSLARIFSSLSQSKKRLHIEDYSVSQTTLDQVFVNF  222C
hABCA1          LAFPGSVLKEKHRNMLQYQLPSSLSSLARIFSILSQSKKRLHIEDYSVSQTTLDQVFVNF

bABCA1          AKDQSDDDHLKDLSLHKNQTVVDVAVLTSFLQDEKVKESYV  2261
hABCA1          AKDQSDDDHLKDLSLHKNQTVVDVAVLTSFLQDEKVKESYV
```

**Figure 1.** Alignment of bovine (bABCA1, Accession No. AAY53813) and human (hABCA1, Accession No. AF285167) ABCA1. Amino acid sequences begin at position 900, close to the first ATP-binding-cassette motif. Walker A; Walker B; signature sequence C motifs; and proline, glutamic acid, serine, and threonine (PEST) sequences are bold and shaded. Differences in the AA sequences are shaded.

| | | | |
|---|---|---|---|
| Cow | 1283 | RPFTEDDAMDPNDSDIDPESRETD | 1306 |
| Human | 1283 | .....A..... | 1306 |
| Chicken | 1283 | .....F..... | 1306 |
| Mouse | 1283 | H.....V..... | 1306 |

**Figure 2.** Alignment of the ABCA1 sequences rich in proline, glutamic acid, serine, and threonine (PEST) across species. Dots indicate identical AA residues compared with bovine ABCA1.

2). According to the very high homology between other mammalian and bovine ABCA1 PEST sequences, it is likely that they all fulfill similar physiological functions and contribute to the regulation of ABCA1 degradation.

### *Promoter Region of the Bovine ABCA1 Gene*

Analysis of the proximal promoter region revealed a high degree of conservation between the bovine and human *ABCA1* genes. The bovine promoter sequence has been deposited at the GenBank database (Accession No. DQ142640).

The genomic region upstream of the transcription initiation site of *ABCA1* (Figure 3) contains several putative elements for transcriptional regulation. Analysis of the bovine *ABCA1* promoter identified multiple motifs that were strongly conserved between human and bovine sequences, pointing to important biological functions. Some of these potential transcription factor binding sites are also present in the promoter of receptors involved in lipid metabolism, including the low density lipoprotein receptor, scavenger receptor A, scavenger receptor class-B type I (SR-BI), and CD36, another member of the class-B scavenger receptor family. These receptors include binding motifs for SP1, activator protein 1 (AP1), sex determining region Y (SRY), and nuclear factor kappa-B (NF-$\kappa$B; Armesilla and Vega, 1994; Cao et al., 1997; Valledor et al., 1998). A TATA box and CAAT box motif were identified at –31 and –569 bp upstream of the transcriptional start site, respectively. In addition, we identified an E-box motif at position –148 and the recognition element for the basic helix-loop-helix leucine zipper containing proteins (position –223), such as the sterol regulatory element binding proteins, which are binding sites for sterol regulation (Brown and Goldstein, 1997). Similar E-box motifs have been reported in the promoter for SR-BI (Cao et al., 1997; Lopez and McLean, 1999), fatty acid synthase (Magana et al., 2000), human CD36 (Armesilla and Vega, 1994), and the low density lipoprotein receptor (Brown and Goldstein, 1997). These predicted features are consistent with the promoter region of other members of the ABCA subfamily, such as *ABCA2, ABCA7,* and *ABCA13* (Broccardo et al., 2001; Kaminski et al., 2001; Barros et al., 2003). The high degree of similarity between motifs in the human and bovine ABCA1 promoter structure strongly suggests a role for ABCA1 in bovine sterol homeostasis.

### *Tissue-Specific Expression of Bovine ABCA1*

The *ABCA1* transcript was detected in all tissues of *Bos taurus* that were analyzed. These tissues are mainly involved in barrier function (lung, intestine), reproductive function (uterus), and metabolic function (liver). The greatest expression level was observed in lung (Figure 4). These results resemble those of Kielar et al. (2001) and Langmann et al. (2003) in human tissues. The primary function of ABCA1 in human lung might be to modulate lipid pools in alveolary epithelial cells (Agassandian et al., 2004). An alternative assumption is that ABCA1 in human lung takes part in cholesterol homeostasis and supports the reverse transport of cholesterol (Santamarina-Fojo et al., 2001).

High expression levels were also found in esophagus, uterus, spleen, and muscle (Figure 4), which is partly in agreement with Langmann et al. (2003). Moderate levels of expression were detected for liver, tongue, gastric tissues, cecum, jejunum, heart, and lymph nodes, whereas, congruent with distribution patterns in human tissues, low expression was observed in colon and kidney (Figure 4). The function of intestinal ABCA1 is likely to generate HDL particles that transport dietary cholesterol to the liver. In humans, the resecretion of cholesterol in the intestine is mediated by 2 other intestinal ABC transporters (ABCG5 and ABCG8; Oram and Heinecke, 2005), which could explain the comparatively low distribution of ABCA1 in these tissues. However, 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 the ABCA1 transporter under these feeding conditions. It is likely that the function of ABCA1 in kidney may be to maintain normal cholesterol homeostasis and protect against hyperlipidemic renal disease (Wu et al., 2004). The detection of ABCA1 in the mammary gland might indicate a potential role of ABCA1 in the transfer of cholesterol into the milk, a hypothesis that should be investigated in further studies. Moreover, studies using in situ hybridization techniques or immunohistochemistry should be performed to determine the cellular and subcellular localization of the bovine ABCA1 transporter in various tissues.

## IMPLICATIONS

We report the identification and characterization of bovine *ABCA1* cDNA, an adenosine triphosphate-binding cassette transporter highly conserved in human, mouse, rat, and chicken. This is supported by 1) the cDNA, which shows 90% sequence homology to the

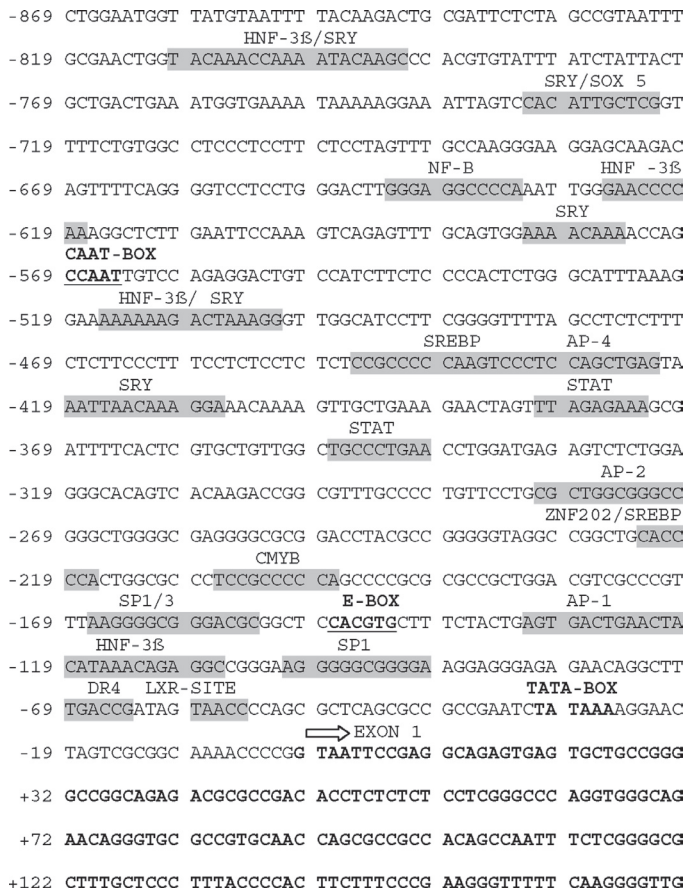


**Figure 3.** Putative proximal promoter sequence of the *ABCA1* gene with predicted transcription factor binding sites (shaded). TATA box, E-box, and CAAT box motifs are bold and underlined. The putative start of exon 1, according to the human sequence (NM_005502), is indicated by an arrow and shown in bold. The translational start site (ATG) is located on exon 2 (not shown in the figure). HNF3$\beta$ = hepatocyte nuclear factor 3 beta; SRY = sex determining region Y; SOX5 = SRY-box 5; NF-$\kappa$B = nuclear factor kappa-B; SREBP = sterol regulatory element binding protein; AP = adaptor-related protein complex; STAT = signal transducer and activator of transcription; ZNF202 = zinc finger protein 202; and LXR = liver X receptor.

human sequence and 2) the deduced ABCA1 protein, which exhibits 94% homology to the human ABCA1 protein. The deduced protein is a full-size adenosine triphosphate-binding cassette transporter with ubiquitous mRNA tissue expression. The high degree of similarity to human ABCA1 in protein sequence, sequence motifs, promoter structure, and expression levels strongly suggests an analogous role of both transporters in sterol homeostasis. Additional studies on substrate specificity and protein localization on the

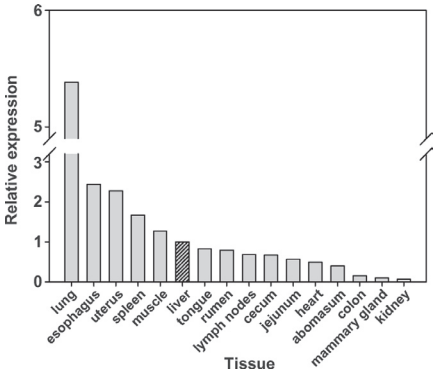


**Figure 4.** Quantitative analysis of *ABCA1* mRNA in bovine tissues. Bars represent relative quantification calculated as fold-expression compared with liver (cross-hatched bar). Values were normalized to the mean of 4 housekeeping genes (glycerol-3-phosphate dehydrogenase, $\beta$-Actin, Ubiquitin, and 18S).

cellular level are needed to further elucidate the physiological role of ABCA1 in *Bos taurus*.

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# 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 cycler (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 m*M* Tris HCl, 100 m*M* KCl, 100 m*M* $(NH_4)_2SO_4$, 20 m*M* $MgSO_4$, 1.0% Triton X-100, and 1 mg/mL nuclease-free BSA), 0.8 μ*M* of both forward and reverse primer (Metabion, Martinsried, Germany), 4.0 m*M* 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′-CCTCAAAGATGTCTCCTTGTAC-3′ | 5′-GCAGTCATGCAGTCCAG-3′ | 463 |
| | 5′-GTCATGCTGTTTGATGAGCC-3′ | 5′-CCAAGTAGCACAAGGGCTTAG-3′ | 905 |
| | 5′-GCGACCAGGAGAGTCAGG-3′ | 5′-GACCCGCTTAGTCACAATTTCC-3′ | 824 |
| ABCG8 | 5′-GCCTCCAGGACAGCTTGTTC-3′ | 5′-GGATTCCTGGGTTCCACAG-3′ | 631 |
| | 5′-CGCGTGGGCAACATCTAC-3′ | 5′-ATGATGACGTAGACACAGTGCTCA-3′ | 896 |
| | 5′-CCTGGATGTCATCTCCAAAT-3′ | 5′-AATTGTTCAGTTTAGCTTTTGGA-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 μL, using 1 μL of the LC FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 pmol of each primer, 3 m*M* $MgCl_2$, and 1 μL 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 nonspecific 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 derivate 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, glyceraldehyde-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′-TCGTCCACCCTTTTGTCG-3′ | 260 |
| GAPDH | 5′-GTCTTCACTACCATGGAGAAGG-3′ | 5′-TCATGGATGACCTTGGCCAG-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 |

[1]ABCG5 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

| | Denaturation | | Primer annealing | | Elongation | | Fluorescence acquisition | |
|---|---|---|---|---|---|---|---|---|
| Gene[1] | °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 |

[1]ABCG5 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-Konaraki 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-

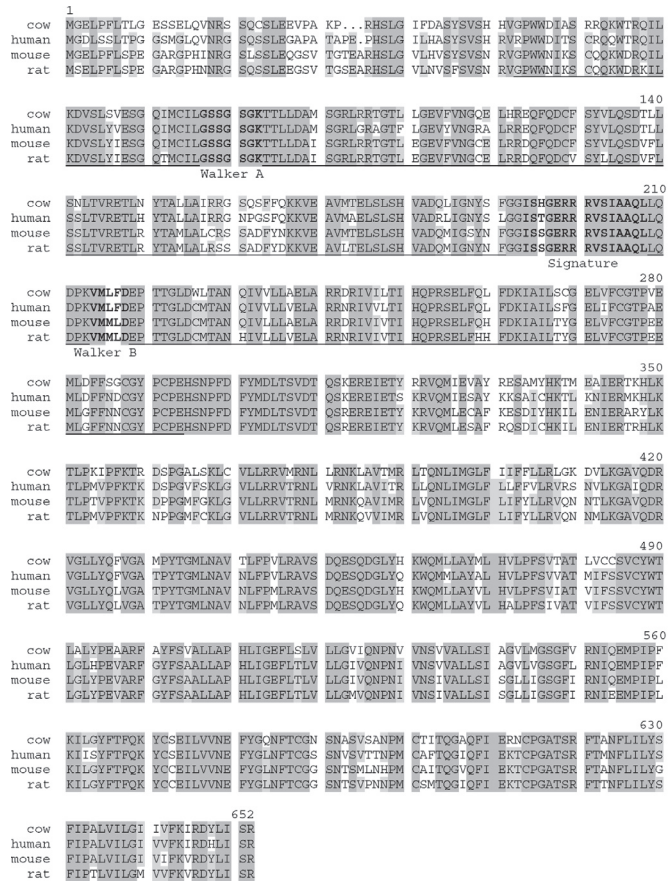


**Figure 1.** Predicted amino acid sequence for the mammalian ATP binding cassette transporter G5. Alignment of the bovine proteins with their homologues in human, mouse, and rat, showing sequence identity (dark and light gray boxes). Nucleotide-binding domain (NBD; underlined) and the conserved Walker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

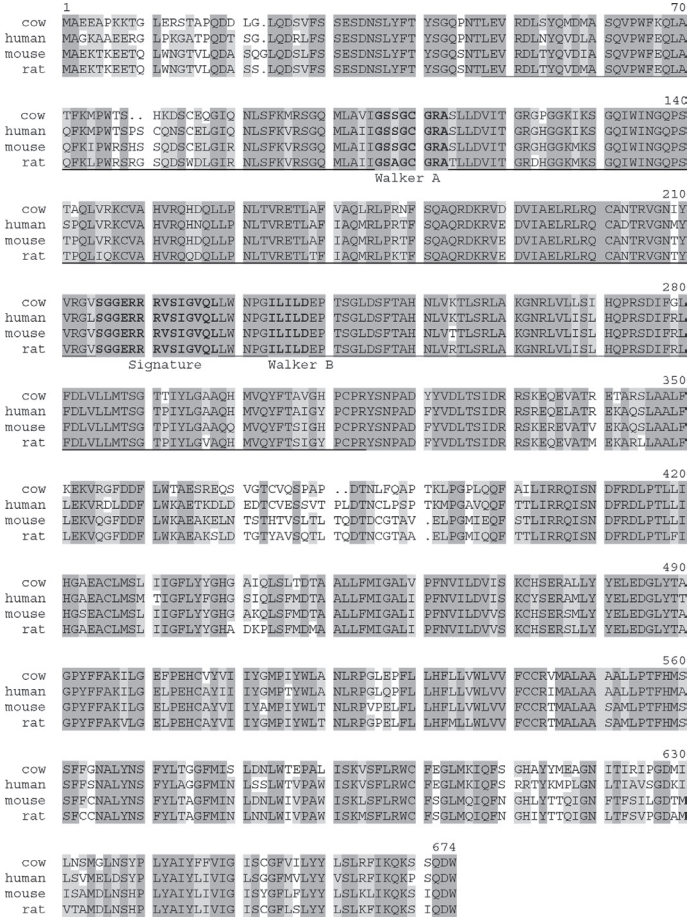


**Figure 2.** Predicted amino acid sequence for the mammalian ATP-binding cassette transporter G8. Alignment of the bovine proteins with their homologues in human, mouse, and rat, showing sequence identity (dark and light gray boxes). Nucleotide-binding domain (NBD; underlined) and the conserved Walker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

species, due to the main role of the ABCG5/8 transport complex in absorption of sterols from the diet and their biliar excretion. It is also remarkable that this expression seems to be specifically located along the intestinal 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-

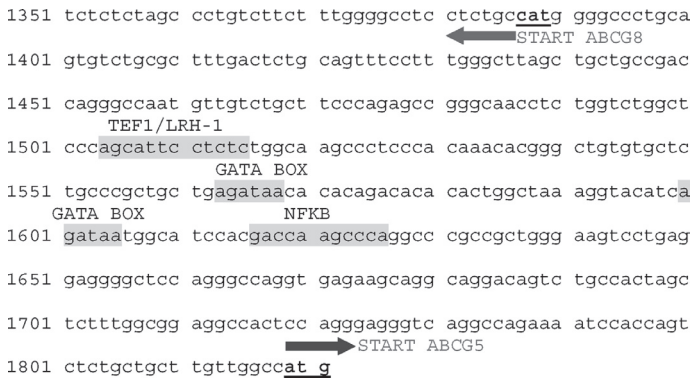


**Figure 3.** Regulatory elements identified in the intergenic promoter region of the bovine ATP-binding cassette transporters G5 and G8. Start codons of both genes are indicated.

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 regulation 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.

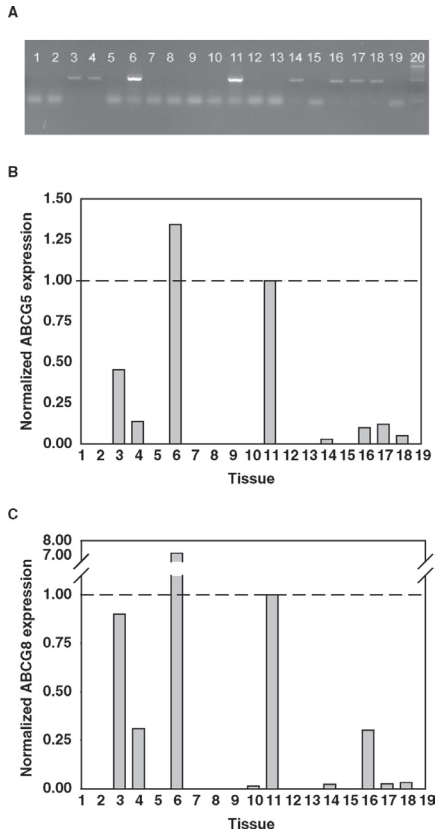


**Figure 4.** Expression of the *ATP-binding cassette transporters G5* and *G8* in bovine tissues (A) Agarose gel electrophoresis for 1 of the overlapping fragments used in *ABCG5* sequencing (amplicon size: 463 bp). Lane 1 = esophagus; 2 = rumen; 3 = abomasum; 4 = jejunum; 5 = cecum; 6 = colon; 7 = 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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# Differential expression of ABC transporters and their regulatory genes during lactation and dry period in dairy cows

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ATP-binding cassette (ABC) transporters play a pivotal role in human physiology, and mutations in these genes often result in severe hereditary diseases. ABC transporters are expressed in the bovine mammary gland but their physiological role in this organ remains elusive. Based on findings in the context of human disorders we speculated that candidate ABC transporters are implicated in lipid and cholesterol transport in the mammary gland. Therefore we investigated the expression pattern of selected genes that are associated with sterol transport in lactating and nonlactating mammary glands of dairy cows. mRNA levels from mammary gland biopsies taken during lactation and in the first and second week of the dry period were analysed using quantitative PCR. Five ABC transporter genes, namely *ABCA1*, *ABCA7*, *ABCG1*, *ABCG2* and *ABCG5*, their regulating genes *LXRα*, *PPARγ*, *SREBP1* and the milk proteins *lactoferrin* and *α-lactalbumin* were assessed. A significantly enhanced expression in the dry period was observed for *ABCA1* while a significant decrease of expression in this period was detected for *ABCA7*, *ABCG2*, *SREBP1* and *α-lactalbumin*. *ABCG1*, *ABCG5*, *LXRα*, *PPARγ* and *lactoferrin* expression was not altered between lactation and dry period. These results indicate that candidate ABC transporters involved in lipid and cholesterol transport show differential mRNA expression between lactation and the dry period. This may be due to physiological changes in the mammary gland such as immigration of macrophages or the accumulation of fat due to the loss of liquid in the involuting mammary gland. The current mRNA expression analysis of transporters in the mammary gland is the prerequisite for elucidating novel molecular mechanisms underlying cholesterol and lipid transfer into milk.

**Keywords:** ABC transporter, dry period, gene expression, lactation, mammary gland, nuclear receptors, SREBP.

The ATP-binding cassette transporter (ABC transporter) superfamily is one of the largest and most ancient families with representatives in all existing phyla from prokaryotes to man. ABC transporters utilize the energy of ATP hydrolysis to transport a wide variety of substrates across cellular membranes, including metabolites, lipids, sterols and drugs (Higgins, 1992; Childs & Ling, 1994; Dean & Allikmets, 1995). The classification as ABC transporter is based on the sequence and organization of the ATP-binding domains, also known as nucleotide-binding folds (NBFs). ABC transporters play a central role in the development of multidrug resistance (MDR). This is caused by several factors, one of which is increased excretion of the drug from the cell by ABC transporters. For example, ABCB1 and ABCG2, also known as P-glycoprotein and breast cancer resistance protein (BCRP), respectively, give resistance to therapeutically relevant drugs such as topotecan, irinotecan and doxorubicin (Allen & Schinkel, 2002; Di Nicolantonio et al. 2005).

More recently ABC transporters were identified that are implicated in the translocation of lipids. They were found either on the basis of homology with known transporters or as causative genes in disease loci. Hence, the function of ABCA1 and ABCG1 is to export excess cellular cholesterol into the HDL pathway and reduce cholesterol accumulation in macrophages (Oram & Vaughan, 2006). ABCA7 was demonstrated to mediate a similar reaction as ABCA1

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to generate HDL in vitro and it may be involved in lipid metabolism in kidneys and adipose tissues (Wang et al. 2003; Linsel-Nitschke et al. 2005; Kim et al. 2005). ABCG5 and ABCG8 are highly expressed in the intestine and liver cells where they form heterodimers that limit the absorption of dietary sterols in the intestine and promote cholesterol elimination from the body through hepatobiliary secretion (Yu et al. 2002; Mutch et al. 2004).

The expression of several ABC transporters is under tight transcriptional regulation and orphan nuclear receptors play an important role in this context. Nuclear receptors comprise a family of transcription factors that act as heterodimers, which bind to promoter elements and induce gene expression. The retinoid X receptor (RXR) is a compulsory partner in the heterodimer. The other partner ascertains the specificity for the activating ligand and for the target gene (Di et al. 1999). Other genes relevant for regulating the expression of ABC transporters involved in lipid and carbohydrate metabolism are the liver X receptors (LXR) $\alpha$ and $\beta$, the peroxisome proliferator-activated receptors (PPAR) $\alpha$ and $\gamma$ and the sterol regulatory element binding proteins (SREBP) 1 and 2.

Whereas ABC transporters play a substantial role in hereditary human diseases, only scarce information is available about their expression and function in food-producing animals. Only few ABC proteins have been identified in *Bos taurus* (Ambagala et al. 2002; Taguchi et al. 2002; Beharry et al. 2004; Cohen-Zinder et al. 2005; Farke et al. 2006; Viturro et al. 2006) and their functions remain mostly speculative.

The aim of the current study was to detect ABC transporters that are known to play a role in lipid transport in the bovine mammary gland. The demonstration of transporter gene levels in the lactating mammary gland may identify candidate transporters involved in lipid homeostasis in the lactating mammary gland. Furthermore, expression levels of these transporters in lactating as compared with nonlactating mammary gland tissue may identify a subset of transporters involved in lipid and cholesterol transport into milk.

## Materials and Methods

### *Cows and their management*

This study was performed according to the requirements of the Bavarian state animal welfare committee (Germany).

Four healthy dairy cows (German Braunvieh), two of them in their first and two in their second lactation, free of clinical udder health problems were used. At the beginning of the experiment, two cows were in early lactation (4–100 d) and two were lactating for more than 300 d. The animals were kept in a loose-housing barn and milked twice daily at 5.00 and 17.00 in a milking parlour. Feeding consisted of maize and grass silage, hay and concentrate according to their individual levels. Water was available ad libitum.

### *Biopsy procedure*

Biopsies were carried out after the morning milking. The experimental quarters were clipped and cleaned. Cows were mildly sedated with an i.v. injection of 0·8 ml xylazine (2%, CP-Pharma, Burgdorf, Germany). Biopsy samples from the rear quarters were taken from a caudal direction and the biopsy site was carefully selected to avoid the cisternal region and larger subcutaneous blood vessels. The area was washed, sterilized with 70% ethanol and then anaesthetized by a s.c. injection of 3·5 ml lidocaine (2%, Chassot, Ravensburg, Germany). Thereafter a puncture incision was made through the skin with a sterile single-use scalpel and one or two biopsies of one single quarter were carried out through this incision using a human Bard®Magnum™ Biopsy Instrument (BARD, Covington, USA) and a Bard®Magnum™ Core Tissue Biopsy Needle (12 g × 10 cm) (BARD). A core of maximum 20 mg mammary tissue was extracted. Until the next morning a swab was fixed with an adhesive Fixomull® stretch plaster (Beiersdorf AG, Hamburg, Germany) to avoid contamination. Mammary biopsies were taken as described above for a total of nine consecutive days as well as one and two weeks after onset of the dry period. Neither during nor after the experiment did the cows need anti-inflammatory or antibiotic therapy. The wound dressing was removed 2 d after taking the last biopsy.

### *RNA isolation and cDNA synthesis*

The tissues were homogenized with an Ultra-Turrax (IKA®-WERKE, Staufen, Germany). Total RNA of mammary gland biopsies was isolated using TriPure (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. To quantify the amount of total RNA extracted, optical density (OD) was determined. The integrity of the RNA was verified by the $OD_{260}/OD_{280}$ absorption ratio >1·8.

Synthesis of first strand complementary DNA (cDNA) was performed with reverse transcriptase (SuperScript II, Invitrogen, Karlsruhe, Germany) and random hexamer primers according to the manufacturer's instructions.

### *Oligonucleotide primers and PCR*

All primer pairs (Table 1) were designed according to bovine or human nucleic acid data bank sequences and covered one or two exon boundaries to avoid amplification of genomic DNA. To test specificity of the primers and to evaluate the optimal annealing temperature, all primer pairs were initially tested by gradient PCR using mammary gland cDNA. PCR reactions were performed in a PCR thermocycler (Biometra, Göttingen, Germany) and contained 50 ng of cDNA, 12·5 µl 2 × PCR Master Mix (Promega, Madison, USA), 0·8 µM of forward and reverse primer (Metabion, Martinsried, Germany) and nuclease-free water in a final volume of 25 µl. PCR products were

# Appendix



**Table 1.** Primer sequences and amplification product sizes of reference and target genes

| Gene | Forward primer | Reverse primer | Product size |
|---|---|---|---|
| GAPDH | 5′-GTCTTCACTACCATGGAGAAGG-3′ | 5′-TCATGGATGACCTTGGCCAG-3′ | 197 bp |
| Ubiquitin | 5′-AGATCCAGCATAAGGAAGGCAT-3′ | 5′-GCTCCACCTCCAGGGTGAT-3′ | 198 bp |
| β-Actin | 5′-AACTCCATCATGAAGTGTGACG-3′ | 5′-GATCCACATCTGCTGGAAGG-3′ | 214 bp |
| Lactoferrin | 5′-GAACATCCCCATGGGCCTG-3′ | 5′-CAGCCAGGCACCTGAAAGC-3′ | 203 bp |
| α-Lactalbumin | 5′-ACCAGTGGTTATGACACACAAGC-3′ | 5′-AGTGCTTTATGGGCCAACCAGT-3′ | 233 bp |
| LXRα | 5′-CTGCGATTGAGGTGATGCTC-3′ | 5′-CGGTCTGCAGAGAAGATGC-3′ | 229 bp |
| PPARγ | 5′-CTCCAAGAGTACCAAAGTGCAATC-3′ | 5′-CCGGAAGAAACCCTTGCATC-3′ | 198 bp |
| SREBP1 | 5′-GACGGCCAGGTGAATCCAGA-3′ | 5′-CAGGACCATCTCTGCCCTCA-3′ | 217 bp |
| ABCA1 | 5′-GGACATGTGCAACTACGTGG-3′ | 5′-TGATGGACCACCCATACAGC-3′ | 134 bp |
| ABCA7 | 5′-GCCCAGGTCAACCGAACT-3′ | 5′-AGCACGAAGAGCTTCCACTC-3′ | 201 bp |
| ABCG1 | 5′-GACTCGGTCCTCACGCAC-3′ | 5′-CGGAGAAACACGCTCATCTC-3′ | 203 bp |
| ABCG2 | 5′-GCTCCTGAAGAGGATGTC-3′ | 5′-CAGCGGAAACCTATGGCTC-3′ | 174 bp |
| ABCG5 | 5′-AGCTCAGGCTCAGGGAAAAC-3′ | 5′-GTCGCTCTGCAGGACGTAG-3′ | 188 bp |

**Table 2.** PCR conditions for quantitative PCR measurements of reference and target genes

| | Denaturation | | Primer annealing | | Elongation | | Fluorescence acquisition | |
|---|---|---|---|---|---|---|---|---|
| | T, °C | T, s | T, °C | T, s | T, °C | T, s | T, °C | T, s |
| GAPDH | 95 | 15 | 58 | 10 | 72 | 20 | † | |
| Ubiquitin | 95 | 15 | 60 | 10 | 72 | 20 | † | |
| β-Actin | 95 | 15 | 62 | 10 | 72 | 20 | † | |
| Lactoferrin | 95 | 15 | 61 | 10 | 72 | 20 | 87 | 3 |
| α-Lactalbumin | 95 | 15 | 59 | 10 | 72 | 20 | † | |
| LXRα | 95 | 15 | 61 | 10 | 72 | 20 | † | |
| PPARγ | 95 | 15 | 63 | 10 | 72 | 20 | 83 | 3 |
| SREBP1 | 95 | 15 | 60 | 10 | 72 | 20 | 87 | 3 |
| ABCA1 | 95 | 15 | 55 | 10 | 72 | 20 | † | |
| ABCA7 | 95 | 15 | 59 | 10 | 72 | 20 | † | |
| ABCG1 | 95 | 15 | 57 | 10 | 72 | 20 | † | |
| ABCG2 | 95 | 15 | 54 | 10 | 72 | 20 | † | |
| ABCG5 | 95 | 15 | 60 | 10 | 72 | 20 | † | |

† Fluorescence acquisition was performed at the end of each elongation cycle

subjected to gel electrophoresis in 2% agarose gels containing 0·05 µl of GelRed™ (Biotium, Hayward, USA) per ml. The DNA fragments were extracted using the Wizard SV Gel and PCR Clean-Up System (Promega), and both strands were commercially sequenced (Agowa, Berlin, Germany).

### *Real-time PCR*

Quantitative real-time PCR (qPCR) in bovine mammary gland tissue was carried out using LightCycler® DNA Master SYBR Green technology (Roche Diagnostics). PCR reactions were performed in a final volume of 10 µl, using 1 µl of the LC FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 pmol of each primer, 3 mM-$MgCl_2$, and 50 ng of cDNA. Before amplification, an initial high-temperature incubation step was performed to activate the DNA polymerase and to ensure complete denaturation of cDNA. Product-specific PCR conditions are listed in Table 2. Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification. Data were analysed using the second Derivate Maximum calculation described in the LightCycler® Software 3.5. All runs included a negative cDNA control consisting of PCR-grade water, and each sample was measured in duplicate.

### *Data analysis and statistics*

Quantitative real time PCR data were processed using the relative quantification ΔΔCT-method ($2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001). Expression changes are shown as relative up- or down-regulation compared with lactation (Table 3). Data were obtained as CT values, which represent the cycle number at which logarithmic plots cross a calculated threshold. For normalization of target gene (TG) expression, the CT values of the reference genes (RGs) *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*),

**Table 3.** Gene expression differences in nonlactating relative to lactating mammary glands. Values indicate n-fold expression ($2^{-\Delta\Delta CT}$) ± SEM in lactation as compared with dry period 1 (D1, 1 week after drying off) and dry period 2 (D2, 2 weeks after drying off). A value of 1 indicates no change in relative expression, values >1 and <1 indicate elevated and decreased mRNA expression levels, respectively

| | Expression change as compared with lactation phase, L | | | |
|---|---|---|---|---|
| | D1 | *P* value | D2 | *P* value |
| ABCA1 | 3·12 ± 0·61 | *P*=0·06 | 2·84 ± 0·37 | *P*=0·04 |
| ABCA7 | 0·08 ± 1·76 | *P*=0·08 | 0·04 ± 0·95 | *P*=0·03 |
| ABCG1 | 1·56 ± 0·69 | *P*=0·56 | 0·42 ± 2·50 | *P*=0·65 |
| ABCG2 | 0·16 ± 0·59 | *P*=0·22 | 0·07 ± 0·42 | *P*=0·04 |
| ABCG5 | 0·11 ± 0·82 | *P*=0·18 | 0·13 ± 1·07 | *P*=0·07 |
| PPARγ | 3·88 ± 2·70 | *P*=0·32 | 0·93 ± 2·64 | *P*=0·92 |
| LXRα | 0·66 ± 2·66 | *P*=0·85 | 3·84 ± 1·45 | *P*=0·27 |
| SREBP1 | 0·13 ± 0·99 | *P*=0·02 | 0·14 ± 0·66 | *P*=0·04 |
| Lactoferrin | 2·12 ± 3·57 | *P*=0·78 | 2·33 ± 2·23 | *P*=0·67 |
| α-Lactalbumin | 0·03 ± 1·95 | *P*=0·21 | 0·0004 ± 1·37 | *P*=0·01 |

*β-actin* and *ubiquitin* were averaged and the mean value served as reference gene index. The following calculations were performed:

$$\Delta CT = CT_{(RG)} - CT_{(TG)}$$

$$\Delta\Delta CT = \Delta CT_{(\text{dry period})} - \Delta CT_{(\text{lactation})}$$

$\Delta CT_{(\text{lactation})}$ = mean ΔCT value of four cows during lactation

$\Delta CT_{(\text{dry period})}$ = mean ΔCT value of four cows during dry period

SigmaPlot software (Systat Software Inc., San Jose CA, USA) was used for statistical analysis.

To analyse differences in the expression levels of the RGs, CT values were compared using a *t* test. For the TGs the expression changes were analysed at the ΔCT levels with a paired *t* test to exclude potential bias because of averaging data that had been transformed through the equation $2^{-\Delta\Delta CT}$. A *P* value <0·05 was regarded as statistically significant. The linear regression was calculated using the regression wizard included in the SigmaPlot software.

## Results

Mammary gland biopsies from nine consecutive days during lactation and after the first and second week after dry off, respectively, were analysed. To determine whether lactation altered transporter gene expression, individual transporter RNA expression levels were compared in lactating and nonlactating bovine mammary glands. For normalization the arithmetic mean of the CT values originating from the three housekeeping genes *GAPDH, β-actin* and *ubiquitin*, was used. The mean of the housekeeping genes showed no significant differences between lactation and dry period (*P*=0·1572, data not shown). However, as *β-actin* represents not an optimal housekeeper in the mammary gland, all expression profiles were also evaluated without *β-actin*, i.e., data were normalized to the mean of the two housekeepers GAPDH and *ubiquitin*. The final outcome, however, remained essentially unchanged (data not shown).

Table 3 summarizes the n-fold differences in target gene RNA expression levels between lactating and nonlactating mammary glands. There was a significant difference in expression between lactation and dry period for *ABCA1, ABCA7, ABCG2, SREBP1* and *α-lactalbumin* (Fig. 1, Table 3). Within the first week after dry off (D1) an increase of *ABCA1* gene expression was observed which reached statistical significance (*P*=0·0439) in the second week (D2). A significant decrease of *SREBP1* gene expression was detected in the first (D1, *P*=0·0204) and second (D2, *P*=0·0435) week of the dry period. *ABCA7* and *ABCG2* expression decreased at the beginning of dry period (D1) and declined significantly (*P*=0·0323 and *P*=0·0382, respectively) in the second week (D2). Likewise, the expression of *α-lactalbumin* showed a declining trend within the first (D1) and a significant decrease (*P*=0·0113) in the second week of dry period (D2).

For *LXRα* and *lactoferrin* an increase in gene expression within the dry period was observed while for *ABCG5* a decreased gene expression from the lactating to nonlactating state was demonstrated. These trends, however, were not statistically significant.

All genes tested, except *ABCG1* and *PPARγ* for which no apparent changes in the gene expression during lactation and involution were observed, showed a clear trend towards significance in the second week of dry period (Table 3).

As LXRα, PPARγ and *SREBP1* are known to be implicated in the regulation of ABC transporters involved in lipid homeostasis, we performed regression analysis with the relative gene expression values of the ABC transporters

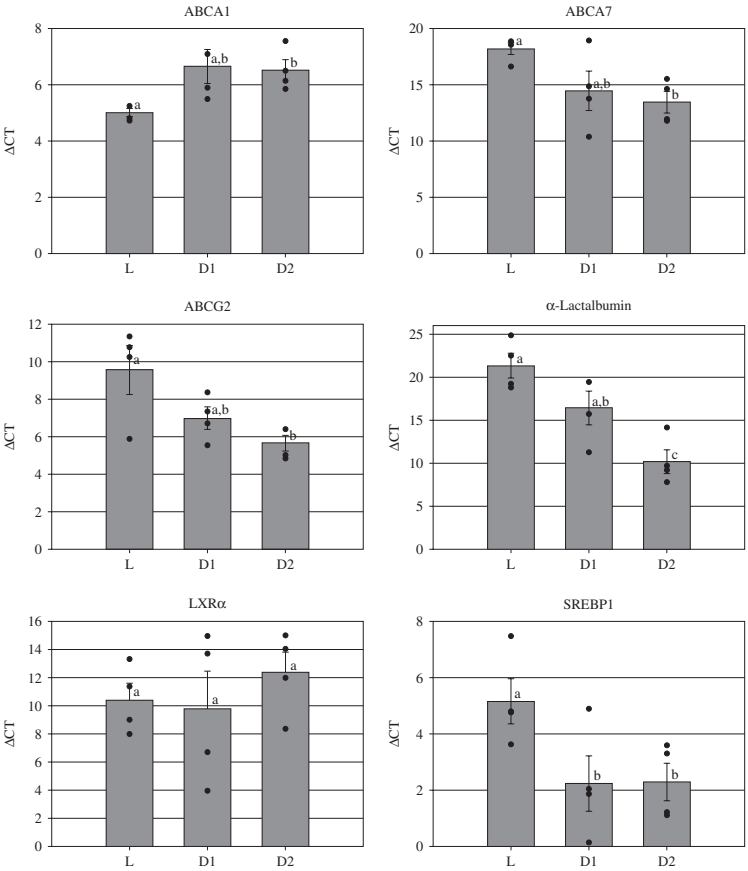


**Fig. 1.** Changes in mRNA expression levels of ABCA1, ABCA7, ABCG2, α-lactalbumin, LXRα and SREBP1 in bovine mammary glands between lactation and dry periods. L = normalized mean (ΔCT values) for lactation; D1 = normalized mean (ΔCT values) for the first week of the dry period; D2 = normalized mean (ΔCT values) for the second week of the dry period. Error bars indicate SEM. Means without a common letter are significantly different ($P < 0{\cdot}05$).

and these factors. We observed a similar trend for *ABCA1* and *LXRα* (Fig. 2A), *ABCA7* and *SREBP1* (Fig. 2B) and *ABCG5* and *SREBP1* (Fig. 2C) gene expression, but for none of the other genes tested (data not shown). The relationship between *ABCA1* and *LXRα, ABCA7* and *SREBP1* and *ABCG5* and *SREBP1* showed a correlation coefficient ($r$) of 0·82 (Fig. 2A, insert), 0·91 (Fig. 2B, insert) and 0·95 (Fig. 2C, insert), respectively.

## Discussion

The active involution process in cows begins with the cessation of regular milk removal, either by drying off or by weaning the calf, and is probably complete by 21–30 d after dry off. It is a transition phase of the mammary gland from the lactating to the nonlactating state; milk continues to accumulate for a couple of days after drying

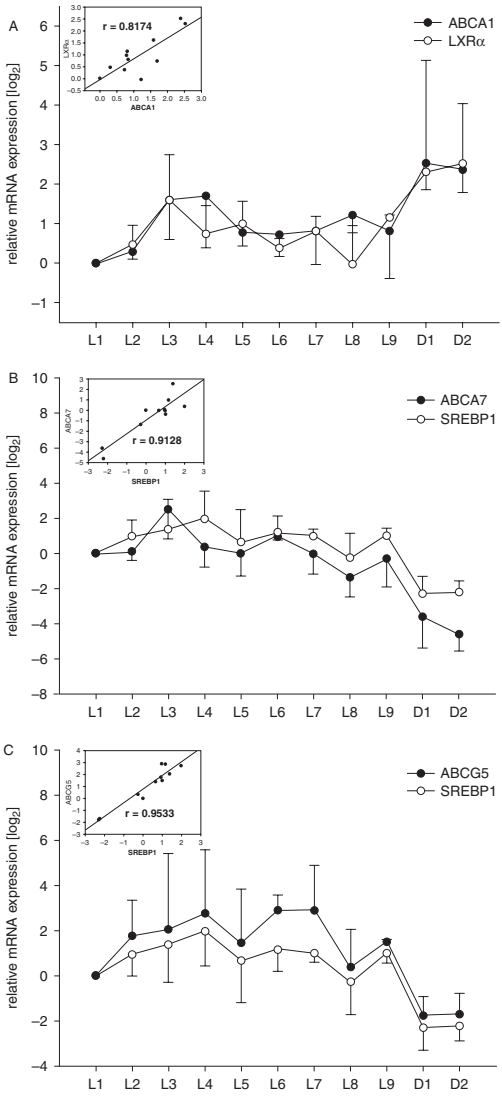


**Fig. 2.** Relative mRNA expression levels (ΔΔCT values) of (A) ABCA1 and LXRα, (B) ABCA7 and SREBP1 and (C) ABCG5 and SREBP1 during nine consecutive days in lactation (L1–L9) and after dry off (D1, D2) with regression lines and correlation coefficients (inserts). L = lactation; D1 = first week of the dry period; D2 = second week of the dry period; *r* = correlation coefficient. Error bars indicate SEM.

# Appendix



off. A significant reduction in fluid volume in the gland occurs between days 3 and 7 of involution (Hurley, 1989). However, it is also known that some cows involute very slowly after non-milking (C Farke, HHD Meyer, RM Bruckmaier and C Albrecht, unpublished observations). These factors may explain our observation of marked differences in gene expression levels predominantly in the second week of the dry period.

Concentrations of milk-specific components such as caseins, α-lactalbumin, β-lactoglobulin, and milk fat decline during the first 2–3 weeks of the dry period (Hurley & Rejman, 1986). In agreement with these findings we observed a significant decrease in *α-lactalbumin* gene expression, confirming adequate biopsy sampling procedures and mRNA measurements and demonstrating that our samples are representative for analysing expression differences between the lactation and dry period (Fig. 1). However, it cannot be completely excluded that, owing to the biopsy sampling technology, varying proportions of peripheral tissues may be included and that the limited numbers of biopsies collected may not completely reflect the majority of the tissue because of local variations. These factors may indicate that very subtle variations in gene expression may not be significantly identified.

The expression of *lactoferrin*, another control gene in this experiment, is regulated differently from that of other milk proteins. An increase of *lactoferrin* gene expression was observed during the first and second week of the dry period, which is in concordance with reports showing that *lactoferrin* is very low in bovine milk during mature lactation and is markedly elevated during mammary involution (Goodman & Schanbacher, 1991). This trend, however, did not reach statistical significance presumably owing to the low number of animals included in this study and a high interindividual variability between the cows. Indeed, when lactoferrin expression profiles were analysed in the context of every single cow, three of the four animals showed the expected increase in *lactoferrin* expression during the dry period while one animal exhibited an opposite trend (data not shown). This could indicate that also for other genes measured in this study a closer association between expression changes might be detected if the results across the genes examined were compared within each animal separately as opposed to using combined averaged data.

Having principally confirmed that our sampling procedures and RNA measurements were adequate and reliable, we focused on the expression pattern of selected ABC transporters involved in lipid, phospholipid and cholesterol transport. We hypothesized that the major structural, morphological and functional differences which occur during and after the onset of the involution period might lead to marked differences in the gene expression levels of candidate ABC transporters implicated in lipid homeostasis in the mammary gland. We therefore compared gene expression levels of ABCA1, ABCA7, ABCG1 and ABCG5 in lactating and nonlactating mammary glands.

It has been reported that milk cholesterol is partially synthesized in the mammary gland but that the major proportion is mainly derived from serum cholesterol (Long et al. 1980). The mechanism by which serum cholesterol is transferred into the milk is still unclear. In human physiology it is well established that ABCA1 represents a cholesterol efflux regulatory gene that plays a major role in the biosynthesis of high density lipoprotein and reverse cholesterol transport (Oram & Vaughan, 2006). ABCA1 is highly expressed in tissue macrophages (Lawn et al. 2001) and it has been reported that *ABCA1* transcripts are up-regulated in macrophages involved in the engulfment and clearance of apoptotic cells (Luciani & Chimini, 1996). We have demonstrated that in bovine tissues ABCA1 is predominantly expressed in lung, oesophagus, uterus, spleen and muscle (Farke et al. 2006). Our present study revealed that ABCA1 gene expression was significantly up-regulated in bovine mammary glands during the dry period. It is possible that this increase of *ABCA1* expression in the nonlactating mammary gland could be associated with the reported immigration of macrophages during involution (Monks et al. 2002). To test this hypothesis we measured mRNA levels of the macrophage-specific *CD14* gene in our mammary gland samples. We observed high interindividual variation in CD14 expression especially in the dry period and found a trend towards an elevated expression in the first week of the dry period (D1; data not shown). This tendency, however, did not reach statistical significance and should be tested not only with more animals but should also be evaluated on protein level. Interestingly, macrophages from involuting sheep mammary glands apparently have phagocytic vacuoles containing casein micelles, lipid droplets and cellular debris (Tatarczuch et al. 2000). This suggests that these cells play a role in clearance of residual milk and fragmented death cells. Whether ABCA1 could be implicated in cholesterol and phospholipid transport or intracellular trafficking in the mammary gland is currently unclear. Fong et al. (2007) recently identified apolipoprotein (apo) E and apoAI, a key acceptor of cholesterol effluxed by ABCA1 (Oram et al. 2000) in bovine milk fat globule membranes. These findings indicate that potential molecular acceptors for ABCA1-meditated cholesterol efflux are present in bovine milk. However, to shed light on the physiological role of ABCA1 in the mammary gland, it is crucial to determine its cellular localization and to investigate whether ABCA1 is expressed in milk fat globules or other intracellular compartments.

Surprisingly, the expression patterns of *ABCA1* and *ABCA7* in the bovine mammary gland showed opposite trends (Fig. 1). While *ABCA1* was up-regulated, *ABCA7* expression decreased during the dry period. ABCA1 is induced by cholesterol through the LXR system (Venkateswaran et al. 2000) whereas ABCA7, which is highly homologous to ABCA1, is negatively regulated by cellular cholesterol (Iwamoto et al. 2006). Wang et al. (2003) demonstrated that, in contrast to *ABCA1*, *ABCA7*

shows moderate basal mRNA and protein levels in macrophages and no induction by LXR. These studies also show that ABCA7 has the ability to bind apolipoproteins and promote efflux of cellular phospholipids without cholesterol, suggesting a possible role of ABCA7 in cellular phospholipid metabolism in peripheral tissues. This indicates that the high homology between ABCA1 and ABCA7 may not be extrapolated to physiological functions. The physiological role of ABCA7 in the mammary gland currently remains elusive.

Similarly to ABCA7, ABCG5 showed a decreased expression in the dry period (Table 3) which, however, did not reach statistical significance. Our laboratories have previously demonstrated high mRNA expression of ABCG5 and ABCG8 in the bovine liver and digestive tract and, interestingly, in the lactating mammary gland (Viturro et al. 2006). However, in the present set of samples ABCG5 expression was significantly lower, with CT values mostly ranging between 30 and 35. Thus the data gained in these experiments should be interpreted with caution and do not currently allow us to postulate an important role for these genes in the mammary gland.

In parallel with the above mentioned lipid transporters, we also measured ABCG2 expression in our mammary gland samples. Jonker et al. (2005) demonstrated that the ABCG2 transporter is strongly induced in the mammary gland of mice, cows and women during lactation and that it is responsible for the active secretion of clinically and toxicologically important substrates into mouse milk. They observed that during involution, *ABCG2* expression declined rapidly. In agreement with these data and other studies (Cohen-Zinder 2005) our investigations revealed a significant decrease of *ABCG2* expression from the lactating to the non-lactating state in the bovine mammary gland (Fig. 1). It is currently unclear why and to what extent ABCG2 is functionally active in the mammary gland. Therefore it is essential to identify physiological ligands for ABCG2 and to investigate which of them may account for the high expression during lactation. In this context, van Herwaarden et al. (2007) recently demonstrated that ABCG2 not only secretes drugs but also riboflavin (vitamin $B_2$) into milk, implying that vitamin $B_2$ might represent an endogenous ligand for ABCG2 in the mammary gland. Interestingly, a missense mutation in the ABCG2 gene was recently found to affect milk yield, milk fat and protein concentration in cattle (Cohen-Zinder, 2005) suggesting a functional role for ABCG2 in milk secretion.

Expression of several ABC transporters, especially those implicated in lipid homeostasis, is regulated by transcription factors such as nuclear receptors and SREBPs. We analysed the expression of *PPARγ*, *LXRα* and *SREBP1* to investigate a potential correlation between the transporters and their regulators. We observed a similar expression pattern for *ABCA1* and *LXRα* (Figs 1 and 2A) suggesting that LXRα is involved in the regulation of ABCA1 expression in the bovine mammary gland. Indeed LXRα was 3·84±1·45 fold increased in the second week of the dry period (Table 3). However, probably owing to the low number of animals in our experiments and the high inter-individual variation, the differences in LXRα expression between lactation and dry period did not reach statistical significance (Fig. 1). Associations between the mRNA expression of transporters and genes involved in their regulation were also observed for *ABCA7* and *SREBP1*, as well as for *ABCG5* and *SREBP1* (Fig. 2B and 2C). These correlations suggest that *SREBP1* is involved in the regulation of ABCA7 and ABCG5 in the bovine mammary gland. These findings are in agreement with previous reports indicating that ABCA7 is regulated by SREBPs but not by LXR (Iwamoto et al. 2006).

In summary, we gained insight into gene expression patterns in the bovine mammary gland during lactation and subsequent involution by analysing candidate genes that are associated with lipid homeostasis. We found significant differences in the expression patterns of a subset of ABC transporters and potential relationships between selected candidate transporters and their regulatory genes. The better understanding of these transporters and the pathways involved in mammary gland lipid transport may help to elucidate novel molecular mechanisms underlying cholesterol and lipid transfer into milk. To unravel the physiological role and underlying regulatory mechanisms of these lipid transporters in the mammary gland, additional cellular localization studies based on immunohistochemistry or in-situ hybridization are essential. Moreover, the identification of endogenous ligands and substrates in the mammary gland are of fundamental importance.

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# Appendix



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