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Cellular and Clinical Effects of Genetic Variation in Xenobiotic ABC Transporters

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Cellular and Clinical Effects of Genetic Variation in Xenobiotic ABC Transporters

by

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DISSERTATION

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Cellular and Clinical Effects of Genetic Variation in Xenobiotic ABC Transporters

Jason M. Gow

The xenobiotic ABC transporters are membrane bound proteins that efflux substrates coupled with ATP hydrolysis. These transporters have a broad substrate specificity and tissue distribution in excretory and/or barrier sites, supporting their role in the distribution and elimination of xenobiotics. Many factors regulate the expression and function of xenobiotic ABC transporters, and a wealth of data suggests genetic variation may be a factor. The overall hypothesis investigated in this dissertation is that genetic variation affects clinical phenotypes via modulation of xenobiotic ABC transporter expression and function. Xenobiotic ABC polymorphisms were identified in healthy populations of ethnically diverse individuals. Seven amino acid changing variants and two haplotypes of P-glycoprotein (P-gp), encoded by *ABCB1*, were tested for their *in vitro* effects on P-gp expression and function. The Asn21Asp, Arg669Cys, Ala893Ser, Ala893Thr, Ser1141Thr and Val1251Ile variants, and the Asp21/1236T/Ser893/3435T haplotype showed altered intracellular accumulation of calcein-AM and/or bodipy-FL-paclitaxel. In a substrate-dependent manner, certain P-gp variants showed less sensitivity to cyclosporin A inhibition. *ABCB1* 3'-untranslated region (UTR) variants were investigated for their effects on ABCB1 mRNA stability. Computational methods predicted the impact of 3'-UTR variants on ABCB1 mRNA stability and a cell-based assay measured the mRNA half-life of ABCB1 reference and +89A>T, +146G>A and +193G>A 3'-UTR variants. The mRNA half-life for the 3'-UTR variants was similar to the reference half-life of 9.4 h. Colon cancer patients (n = 33) demonstrated variable

ABCB1 and ABCC1-ABCC3 mRNA expression in matched normal and tumor colon tissue. A preliminary association analysis identified possible trends between specific ABC genotypes and mRNA expression. ABCB1, ABCC1 and ABCC3 showed altered mRNA expression in tumor tissue compared to adjacent healthy tissue, suggesting differential regulation in normal and tumor tissue. The functional relevance of promoter region polymorphisms in *ABCB1*, *ABCC2-ABCC6* and *ABCG2* was predicted using two computational methods. The predictions correctly identified three out of five *ABCC2* promoter variants (−1450A>G, −1193A>G and −920A>G) that were previously shown to have decreased transcriptional activity, illustrating the potential utility of these computational predictions in guiding future studies. In summary, the results of this dissertation research suggest xenobiotic ABC polymorphisms can alter gene expression and transport function.

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Chapter 1

Introduction

1.1 Overview of ABC superfamily

The human ATP-binding cassette (ABC) transporter gene superfamily encodes proteins that transport a diverse set of substrates across membranes. All ABC transporters have three conserved sequence motifs that contain ATP-binding residues, called the nucleotide binding domain (NBD) [1]. The Walker A and B motifs, which are separated by 90-120 amino acids, are found in all proteins that bind ATP, and the ABC superfamily has a signature C motif located just before the Walker B site [2]. ABC transporters are found as both full and half transporters. Full transporters possess two or three transmembrane domains (TMDs), each consisting of 6-11 membrane-spanning α-helices, and two NBDs (Figure 1A). The half-transporters are dimers of single TMD/NBD proteins (Figure 1B). Crystallography data has shown that the two NBDs interact during ATP-binding, suggesting both are needed for energy-dependent function [3-5]. Phylogenetic analysis organizes the superfamily into seven subfamilies designated A through G. The ABCA and ABCC subfamilies solely contain full transporters, while the ABCD and ABCG subfamilies are entirely composed of half transporters. The ABCB subfamily is a mixture of full and half transporters. Interestingly, the proteins encoded by ABCE and ABCF genes do not encode transporters [6, 7]. ABCE and ABCF proteins have two NBDs and no TMDs, but share enough NBD sequence identity to be classified in the ABC superfamily.

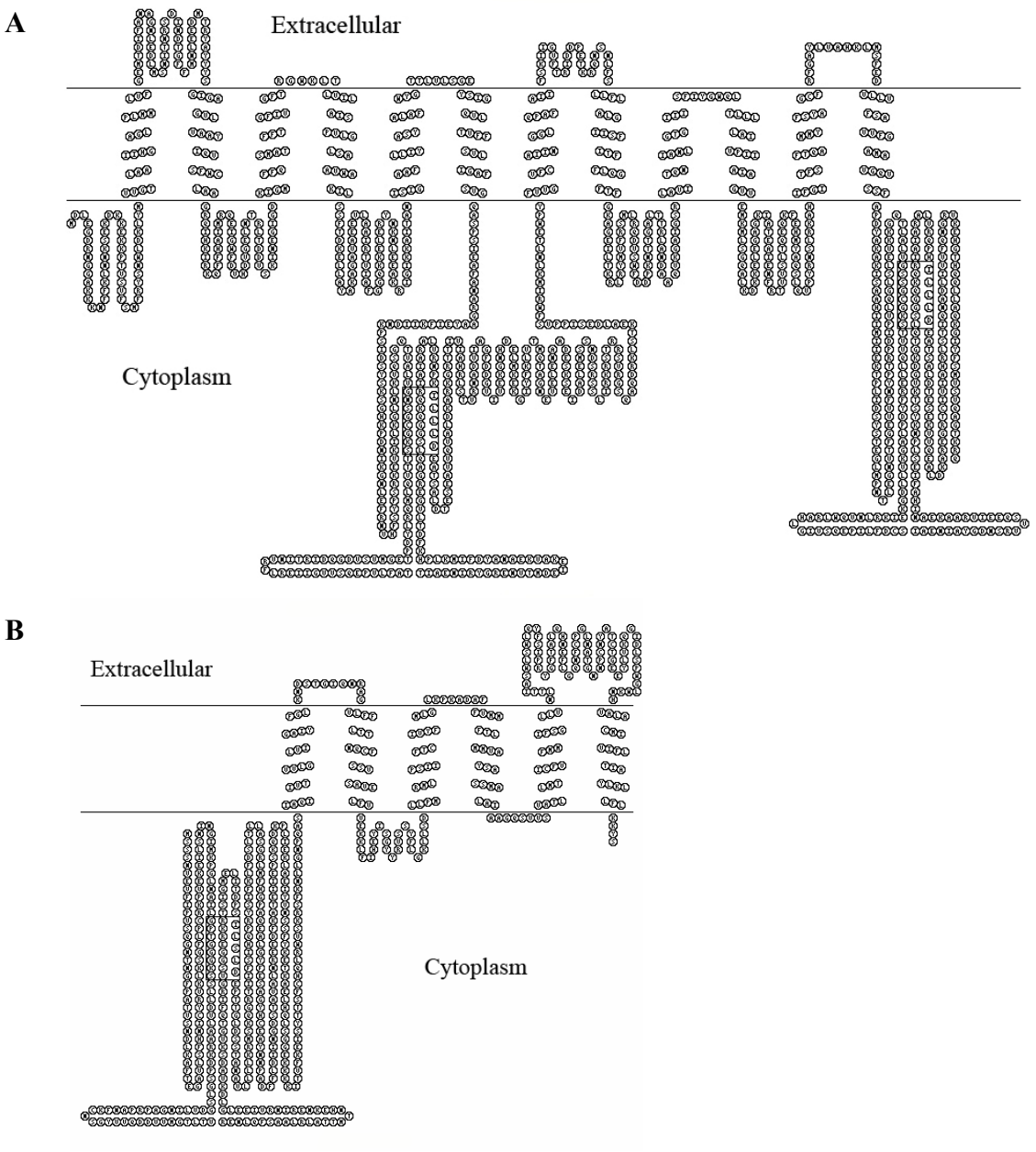

Figure 1.1. Representative full and half ABC transporters. Predicted secondary structure of P-glycoprotein (*ABCB1*) shows two TMDs and two NBDs (A). MXR (*ABCG2*) has one NBD and one TMD (B). The Walker A, Walker B and linker peptide regions comprising the nucleotide binding domains are marked with a box outline. The transmembrane topology schematics were rendered using TOPO (S.J. Johns and R.C. Speth, http://www.sacs.ucsf.edu/TOPO/topo.html, unpublished).

The ABCA family is one of the largest gene clusters with 12 members and many are involved in lipid transport processes (Table 1.1) [8]. Proper functioning of the plasma membrane is dependent on constant maintenance of the asymmetrical distribution of phospholipids and cholesterol. ABCA1 is the prototypical ABCA transporter that primarily effluxes phosphatidylcholine and cholesterol destined for apolipoproteins. Genetic mutations in *ABCA1* cause Tangier Disease, which involves an intracellular accumulation of cholesterol leading to decreased HDL levels [9-11]. Patients often have enlarged organs and/or blood circulation problems due to retained cholesterol [12]. Similar to ABCA1, ABCA2-4, ABCA7, ABCA10 and ABCA12 transport and/or traffic lipids in multiple tissues, and genetic mutations in these transporters, with the exception of *ABCA7* and *ABCA10*, are associated with different diseases [8]. The tissue distribution of ABCA5, ABCA6, ABCA8, ABCA9 and ABCA13 is diverse but their function has yet to be determined [8]. The ABCB proteins have diverse transport functions related to toxin and bile salt secretion, as well as peptide and metal transport [13]. *ABCB1* encodes P-glycoprotein (P-gp) which plays a major role in the removal of xenobiotics from the body. P-gp has a diverse substrate specificity, making it important in xenobiotic toxicity and in the pharmacological effect of drugs and endogenous compounds [14]. Also, P-gp can transport many different drugs, making it important in drug therapy. In the liver, transporters encoded by *ABCB4* and *ABCB11* help regulate normal bile function by secreting phospholipids and bile salts, respectively [15]. Genetic defects in *ABCB4* and *ABCB11* are responsible for heritable cholestasis and liver failure [16, 17]. In contrast, *ABCB2* and *ABCB3* encode the TAP proteins that transport peptide antigens into the endoplasmic reticulum for interactions with class I protein complexes, thus playing a role

Table 1.1
Overview of ABC superfamily

Subfamily	Number of members	Function
ABCA	12	Cholesterol and lipid transport
ABCB	11	Lipid, bile and peptide transport Mitochondrial iron transport Toxin/drug efflux
ABCC	12	Efflux of organinic anions and conjugate Nucleoside transport Chloride ion channel Sulfonylurea receptor Toxin/drug efflux
ABCD	4	Very-long chain fatty acid transport
ABCE	1	Translation initiation?
ABCF	3	Translation initiation?
ABCG	5	Cholesterol and lipid transport Toxin/drug efflux

in immune response [18]. Both transporters are half-sized and heterodimerize with each other. *ABCB6*, *ABCB7* and *ABCB8* also encode half transporters (MTABC3, ABC7, and MABC1, respectively) that are expressed in mitochondria and transport iron for mitochondrial homeostasis [19, 20].

The ABCC subfamily is comprised of 13 genes involved in toxin and ion transport, and signal transduction. A large number of ABCC genes (*ABCC1-ABCC6* and *ABCC10-ABCC13*) encode the multidrug resistance associated proteins (MRPs) that transport a wide range of endogenous substrates, including leukotriene C_4, bile acids and nucleosides [21]. Many MRPs also remove glutathione, glucuronide and sulfate conjugates of toxins and drugs, making them important in xenobiotic effect and toxicity. *ABCC7* encodes cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-

regulated chloride ion channel that influences multiple signaling pathways important for homeostasis [22]. Disruptions in *ABCC7* cause abnormalities in exocrine gland function that lead to cystic fibrosis. *ABCC8* and *ABCC9* encode ATP-dependent potassium channel regulators (SUR1 and SUR2, respectively) that respond to sulfonylureas [23]. Patients with non-insulin-dependent diabetes are treated with sulfonylureas that increase insulin secretion, however, endogenous ligands for SUR1 and SUR2 have yet to be identified.

There are four half transporters in the ABCD subfamily that regulate very-long chain fatty acid (VLCFA) levels via import into the peroxisome. Their role in fatty acid metabolism is supported by evidence for transcriptional regulation by the peroxisome proliferator-activated receptor and retinoid X receptor family of nuclear receptors [24-26]. *ABCD1*, which encodes ALDP, is the most studied ABCD member that is responsible for the X-linked form of adrenoleukodystrophy (ALD), a disorder characterized by late childhood neurodegeneration and adrenal deficiency [27]. Hundreds of *ABCD1* mutations have been identified in ALD patients and yeast homolog studies suggest ALDP transports activated VLCFAs into the peroxisome [28]. Overexpression of ABCD2 (ALDRP) appears to normalize VLFCA levels in *Abcd1* -/- mice [29]. The functions of the two other ABCD proteins are unclear.

The *ABCE* and *ABCF* gene subfamilies encode proteins lacking TMDs but still have NBDs. ABCE and ABCF proteins are cytosolic globular proteins that interact with elongation factors and ribosomal complexes [6, 30]. ABCE1 is the only member of the ABCE subfamily and its yeast homolog (RLI1) is part of the preinitiation complex required for translation. ABCF1 is associated with ribosomes and may be an activator of

eIF-2α kinase, based on *in vitro* studies with the ABCF1 yeast homolog, GCN20. Research is in progress to better understand the physiological roles of ABCE and ABCF proteins.

There are five half transporters in the ABCG subfamily that are involved in toxin and sterol transport. The first gene identified, *ABCG2*, was found in mitoxantrone resistant cells that did not overexpress ABCB1 or ABCC1 [31]. The exact physiological role of ABCG2 is unclear, but its ability to transport natural product drugs as a homodimer suggests it controls toxin exposure. The remaining four ABCG proteins function as sterol transporters in a similar fashion as certain ABCA proteins. Based on the fact that the *ABCG1* and *ABCG4* genes are both induced by cholesterol, they are likely to encode sterol transporters [32, 33]. The current understanding of ABCG5 and ABCG8 transporters is that they are heterodimer partners that efflux plant-derived sterols and cholesterol [34, 35]. Genetic mutations in either *ABCG5* or *ABCG8* cause sisterolemia, which is increased accumulation of phytosterols and cholesterol in the blood and certain tissues.

There are 33 out of 49 members of the ABC superfamily with known function. The proteins expressed on the plasma membrane, such as P-gp and MRP4, generally efflux endogenous and exogenous substrates from the cytoplasm into the extracellular space. Other ABC transporters, for example, ABCB2, ABCB6 and ABCD1, are localized to intracellular organelles, such as ER, mitochondria and peroxisomes, and play a role in cellular homeostasis. Interestingly, four globular ABC proteins (*ABCE* and *ABCF* subfamilies) are most likely involved in translation initiation. Many ABC proteins are associated with specific diseases that highlight the importance and functional

diversity of the ABC gene superfamily. The 12 ABC transporters with unknown function probably serve valuable physiological roles, but also may be important for drug disposition (Table 1.2).

Table 1.2
ABC transporters with unknown function[a]

HUGO gene symbol	Chromosome location	Protein names	Amino acids
ABCA5	17q24.3		1643
ABCA6	17q24.3		1618
ABCA8	17q24.3		1582
ABCA9	17q24.3		1625
ABCA13	7p12.3		5004
ABCB5	7p21.1		813
ABCB8	7q36.1	MABC1	719
ABCB9	12q24.31		767
ABCB10	1q42.13	MTABC2	739
ABCD2	12q11	ALDL1, ALDR	741
ABCD3	1p22.1	PXMP1, PMP70	660
ABCD4	14q24.3	PMP69, P70R	607

[a] Compiled from [8, 13]

1.2 Xenobiotic ABC Transporters

A subset of ABC genes encode xenobiotic efflux transporters that limit toxin exposure and play a significant role in disease treatment by modulating drug disposition and response. Homologous ABC transporters can be found in evolutionarily lower organisms such as yeast, suggesting that certain ABC transporters play a crucial part in

the existence and survival of an organism [36, 37]. Presumably the transport activity of these proteins has a protective effect that has been widely conserved throughout evolution. The best characterized xenobiotic transporters are P-gp, MRP1-MRP5 and MXR. Early studies demonstrated the overexpression of P-gp, MRP1 and MXR in cells showing the multidrug resistance (MDR) phenotype and these transporters are commonly referred to as MDR transporters. A well characterized bile canalicular transporter showed a similar substrate profile as MRP1, but defects in mice and humans were linked to an inherited form of conjugated hyperbilirubinemia known as Dubin-Johnson syndrome. It was later determined that this transporter shared homology to MRP1 and the protein was called MRP2 (encoded by *ABCC2*). ABCC3-5 were identified through genetic approaches and characterized for drug transport. The known xenobiotic transporters have a combined list of substrates that encompass many classes of drugs. Characteristics for individual genes and their encoded proteins are discussed below, and selected properties are listed in Table 1.3.

1.2.1 ABCB1

The majority of multidrug resistance transporter research has focused on P-gp, a xenobiotic efflux transporter that is encoded by *ABCB1*. P-gp was characterized in 1979 as the protein that reduced the permeability of Chinese hamster ovary (CHO) cells, thereby limiting the intracellular accumulation of the anti-inflammatory drug colchicine [38]. In the 1980s, P-gp was found to cause resistance to a wide spectrum of drugs, including anti-cancer agents, and overexpression of the transporter in tumor samples and cell lines was reported [39]. *ABCB1* was cloned and subsequently localized to chromosome 7q21, spanning approximately 200 kb [40]. It has multiple transcriptional

Table 1.3

Characteristics and features of known xenobiotic ABC transporters[a]

HUGO gene symbol	Chromosome location	Protein names	Amino acids
ABCB1	7q21.12	P-gp, MDR1	1280
ABCC1	16p13.12	MRP1	1532
ABCC2	10q24.2	MRP2, cMOAT	1546
ABCC3	17q21.33	MRP3	1528
ABCC4	13q32.1	MRP4	1326
ABCC5	3q27.1	MRP5	1538
ABCC6	16p13.12	MRP6	1504
ABCC10	6p21.1	MRP7	1465
ABCC11	16q12.1	MRP8	1383
ABCC12	16q12.1	MRP9	1360
ABCG2	4q22	MXR, BCRP, ABCP	656

[a] Compiled from [13, 21]

start sites and lacks the traditional TATA box element in the promoter. The 1280 amino acids in P-gp create two TMDs and two NBDs.

Most of the tissues with high P-gp expression are physiological barriers or sites of elimination (Table 1.4). Intestinal localization of P-gp to the apical membrane can hinder drug absorption as demonstrated in Caco-2 cells (cultured enterocytes) expressing high levels of P-gp [41, 42]. The transepithelial flux of numerous P-gp substrates was significantly greater in the basolateral-to-apical direction, suggesting P-gp could reduce intestinal bioavailability [43]. Subsequent animal studies showed *Abcb1a*(-/-) knockout mice had elevated paclitaxel AUC when compared to wildtype controls [44]. The

Table 1.4
Tissue expression and localization of xenobiotic ABC transporters[a]

Gene	Protein	Membrane localization	Tissue
ABCB1	P-gp	Apical	Intestine, liver, blood-brain barrier, kidney, placenta, adrenal cortex
ABCC1	MRP1	Basolateral	Ubiquitous
ABCC2	MRP2	Apical	Liver, kidney, intestine
ABCC3	MRP3	Basolateral	Pancreas, kidney, intestine, liver, adrenal glands
ABCC4	MRP4	Apical and basolateral	Prostate, testis, ovary, intestine, pancreas, lung
ABCC5	MRP5	Basolateral	Ubiquitous
ABCC6	MRP6	Basolateral	Liver, kidney
ABCC10	MRP7	?	Lymphocytes, prostate, lung
ABCC11	MRP8	Apical	Ubiquitous
ABCC12	MRP9	?	Ovary, adipocyte, brain, kidney
ABCG2	MXR	Apical	Breast, placenta, intestine, liver

[a] Compiled from [14, 90, 92]

bioavailability of digoxin is altered in *Abcb1a*(-/-) mice, and induction of intestinal P-gp expression with rifampin caused a greater decrease in bioavailability [45, 46]. Furthermore, the role of P-gp in drug absorption is highlighted by studies demonstrating altered pharmacokinetics when a P-gp inhibitor is coadministered [43].

The blood-brain barrier (BBB) is a layer of endothelial cells that protects the brain from harmful compounds. P-gp is expressed in the BBB and plays an important role in brain exposure to drugs [47]. *Abcb1a*(-/-) mice intravenously administered [^{3}H]-ivermectin (a P-gp substrate) showed modest increases (~3-fold) of drug in the liver,

kidney, small intestine and plasma compared to *Abcb1a*(+/+) mice. The largest differences were in the brain, with an 83-fold increase in [^{3}H]-ivermectin in brain tissue from *Abcb1a*(-/-) mice relative to *Abcb1a* (+/+) mice [48]. Similar increases were seen for [^{3}H]-digoxin and [^{3}H]-cyclosporin levels in the brains of *Abcb1a*(-/-) mice [49]. Loperamide is an opiod antidiarrheal with the potential to induce respiratory depression. It is a P-gp substrate but administration in humans generally results in low systemic exposure and does not cause respiratory depression. When coadminstered with quinidine (P-gp inhibitor), patients develop CNS toxicity most likely related to decreased BBB efflux of loperamide [50]. The available data suggests BBB P-gp expression is a significant component of drug accumulation in the brain, and may limit drug efficacy and toxicity in the CNS [47]. P-gp in other tissues is measurable but more research is needed to understand its effects on drug pharmacokinetics.

P-gp substrates are generally hydrophobic organic cations ranging in molecular weight from 300-2000 Da and include metabolic products, sterols, drugs and other xenobiotics [51]. Table 1.5 shows a partial list of P-gp substrates and demonstrates the pharmacological differences among the drugs transported. With chemotherapy, exposure to one drug can confer resistance to many other anticancer agents due to P-gp overexpression [14, 52]. The exact mechanism of transport is unknown but two pairs of TMDs (5/6 and 11/12) have been shown to modulate substrate recognition [53].

1.2.2 ABCC1

In 1992, the human lung cancer cell line H69 acquired cross-resistance to various unrelated drugs after repeated exposure to the anthracycline doxorubicin. The new H69AR subline did not overexpress P-gp and its MDR was not sensitive to P-gp

Table 1.5
Selected P-glycoprotein substrates[a]

Anticancer	**Antimycotics**	**HIV protease inhibitors**
Actinomycin D	Itraconazole	Indinavir
Daunorubicin	Ketoconazole	Nelfinavir
Doxorubicin		Ritonavir
Etoposide	**Antiepileptics**	Saquinavir
Mitoxantrone	Phenobarbital	
Paclitaxel	Phenytoin	**Antidepressants**
Irinotecan		Fluoxetine
Vinblastine	**Immunosuppressants**	Paroxetine
Vincristine	Cyclosporine	Sertraline
	Sirolimus	St. John's wort
Antihypertensive	Tacrolimus	
Celiprolol	Valspodar	**Antiarrhythmics**
Reserpine		Amiodarone
Talinolol	**Antibiotics**	Digoxin
	Clartihromycin	Quinidine
Cholesterol lowering	Erythromycin	Verapamil
Atorvastatin	Rifampin	
Lovastatin	Tetracycline	***In vitro*** **probes**
		Calcein-AM
Glucocoritcoids	**Antihistamine**	Rhodamine-123
Aldosterone	Fexofenadine	
Cortisol	Terfenadine	
Dexamethasone		

[a] Compiled from [14, 52]

inhibitors. Genetic screening and analysis discovered an overexpressed mRNA encoding a protein with 1531 amino acids, three TMDs and two NBDs homologous to the ABC superfamily [54, 55]. *ABCC1* is located on chromosome 16p13.1 and encodes multidrug resistance associated protein 1 (MRP1). The additional TMD in MRP1 is found at the N-terminus and is necessary for function and localization [56].

MRP1 is ubiquitously expressed and, in contrast to P-gp, is primarily localized to the basolateral membrane (Table 1.4). MRP1 is a key transporter of organic anion conjugates, such as glutathione conjugates (leukotriene C_4 and 2,4-dinitrophenyl-*S*-

glutathione (DNP-SG)) and glucuronides (bilirubin glucuronide), and its basolateral orientation effluxes substrates into the interstitial space rather than excreting them into the bile, urine or gut (Tables 1.4 and 1.6) [21]. Studies with *Abcc1*(-/-) mice have shown it is important for the disposition of various drugs. The testis and kidney normally express high levels of Mrp1, and in *Abcc1*(-/-) mice both tissues showed etoposide-induced toxicity [57, 58]. In mice administered etoposide intravenously, there was ~10-fold increase in drug in the CSF for *Abcc1*(-/-) mice compared to *Abcc1*(+/+) mice [59]. MRP1 is an important xenobiotic transporter given its tissue distribution and capacity for drug transport.

1.2.3 ABCC2

A hepatic transporter in rats was characterized for secretion of biliary glutathione and glucuronate conjugates, and termed canalicular multi-specific organic anion transporter (cMOAT). It was shown to have a similar substrate profile as MRP1 and defects in the protein led to conjugated hyperbilirubinemia (Dubin-Johnson syndrome; Table 1.6) [60]. There are two mutant rat strains with premature stop codons in cMOAT that model DJS. The Groningen Yellow/transporter-deficient strain (TR−) has a single nucleotide deletion and the Eisai hyperbilirubinemic rat (EHBR) strain is caused by a single nucleotide substitution [61, 62]. Sequence analysis determined cMOAT was homologous to MRP1 and was assigned to the ABCC gene family (ABCC2) [63]. *ABCC2* is located on chromosome 10q24 and encodes a 1545 amino acid protein, MRP2, with an extra TMD at the N-terminus relative to P-gp. MRP2 confers cytotoxic resistance to numerous anticancer drugs, and has limited tissue distribution, with highest expression in the liver and moderate levels in small intestine and renal proximal tubules

Table 1.6

Selected substrates for MRP drug and xenobiotic transporters[a]

MRP1	MRP2	MRP3	MRP4	MRP5
Leukotriene C_4	Leukotriene C_4	Leukotriene C_4	**Cyclic nucleotides**	**Cyclic nucleotides**
			cAMP	cAMP
Glutathione conjugates	**Glutathione conjugates**	**Bile acids**	cGMP	cGMP
Aflatoxin B_1 epoxide	Dinitrophenyl	Cholate		
Dinitrophenyl	Prostaglandin A_2	Glycocholate	17β-Estradiol	PMEA
Prostaglandin A_2	Metals (As, Cu, Zn, etc.)	Taurocholate	Prostaglandin E_2	Thioguanine
			Prostaglandin $F_{2\alpha}$	6-mercaptopurine
Glucuronide conjugates	**Glucuronide conjugates**	**Glucuronide conjugates**	Thromboxane	5-fluorouracil
Bilirubin	Bilirubin	Morphine		
17β-Estradiol	17β-Estradiol	Acetaminophen	**Drugs**	
Etoposide	SN-38[b]	17β-Estradiol	Azidothymidine	
	Indomethacin	Etoposide	Gancyclovir	
Sulfate conjugates	Cholate		Methotrexate	
Estrone	*p* -Nitrophenol	**Sulfate conjugates**	*p* -Aminohippurate	
Taurocholate		Taurolithocholate	PMEA[c]	
	Sulfate conjugates	Taurochenodeoxycholate		
Anticancer Drugs	Glycolithocholate			
Daunorubicin	Taurolithocholate	Dinitrophenyl S-glutathione		
Doxorubicin				
Topotecan	**Anticancer Drugs**	**Anticancer Drugs**		
Irinotecan	Same as MRP1	Etoposide		
SN-38	Cisplatin	Methotrexate		
Methotrexate		Vincristine		
Vincristine				
Vinblastine				

[a] Compiled from [21, 51]

[b] 7-Ethyl-10-hydroxycamptothecin

[c] 9-[2-Phosphonomethoxyethyl] adenine

(Table 1.4) [21]. Recently, *Abcc2*(-/-) mice were generated and they demonstrated reduced bile flow, as well as altered methotrexate pharmacokinetics after an intravenous dose [64]. Levels of Mrp3 in the liver and Mrp4 in the kidney were increased, suggesting these transporters may compliment Mrp2 function.

1.2.4 ABCC3

The Human Genome Project allowed novel gene identification via DNA sequence homology with known genes, and circumvented the traditional method of discovering a protein by phenotype. *ABCC3* was identified by data mining of EST databases and is located on chromosome 17q21.33 [65]. The 1528 amino acid protein, MRP3, structurally resembles MRP1 and MRP2, but it cannot transport glutathione (Table 1.6) [66, 67]. It is expressed in many tissues, such as the adrenal glands, pancreas and liver, and is mainly found on the basolateral membrane (Table 1.4). In hepatocytes, MRP3 shows increased expression during cholestasis and possibly compensates for a decrease in MRP2 expression [68]. Cellular cytotoxicity assays demonstrate that MRP3 confers resistance to anticancer agents, such as etoposide, methotrexate and vincristine. Studies with *Abcc3*(-/-) mice have elucidated its physiological role in bile acid and glucuronide conjugate transport, but its impact on drug pharmacokinetics has yet to be investigated [69-72].

1.2.5 ABCC4 and ABCC5

ABCC4 and *ABCC5* encode the "short" MRPs known as MRP4 and MRP5, respectively. They are structurally distinct from MRP1-MRP3 in that they lack the extra TMD at the N-terminus, and thus, are more like P-gp. MRP4 and MRP5 differ from

other xenobiotic ABC transporters in that they transport cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP; Table 1.6) [73]. Nucleoside/nucleotide analogs appear to be MRP4 substrates but the physiological relevance is in question based on the high K_M values [74, 75]. MRP4 can also transport prostaglandins with high affinity, a property not attributed to other MRPs [76]. MRP4 localizes to the basolateral membrane and is highly expressed in the prostate with detectable levels in the lung, testis, ovary, intestine and pancreas (Table 1.4) [77]. In some cell types, MRP4 is targeted to the apical membrane, such as the kidney proximal tubule and the BBB [78, 79]. MRP5 is ubiquitously expressed with high levels in the brain, skeletal muscle, lung and heart (Table 1.4) [65, 80-82]. In polarized epithelial cells, MRP5 is found on the basolateral membrane but it colocalizes with MRP4 on the apical side of capillary endothelial cells. Recently, MRP5 was shown to confer resistance to 5-fluorouracil (5-FU) and transport monophosphorylated metabolites of 5-FU [83].

1.2.6 ABCC6 and ABCC10-ABCC12

The second-generation of ABCC transporters were identified by data mining of EST databases and chromosomal sequences within the last five or six years [84-87]. *ABCC6* is located on chromosome 16p13.12 and encodes a 1504 amino acid protein, MRP6, structurally similar to MRP1-MRP3. The 3' ends of *ABCC1* and *ABCC6* are separated by ~9 kb of genomic DNA. MRP6 transports glutathione conjugated organic anions, such as leukotriene C_4 and DNP-SG, in a similar fashion as MRP1 and MRP2. Nonspecific anion transport inhibitors, including probenecid and indomethacin, attenuate MRP6 function [88, 89]. Dozens of mutations in *ABCC6* have been associated with Pseudoxanthoma elasticum (PXE), which is a rare heritable disorder defined by the

calcification of elastic fibers in the skin, retina and arteries [88]. Many of the mutations change the amino acid sequence in or around the nucleotide-binding domains of MRP6, which most likely produce an aberrant MRP6 transporter. It is possible that MRP6 expression in the kidney and liver is necessary for metabolite transport related to PXE.

Basic features of MRP7 (*ABCC10*), MRP8 (*ABCC11*) and MRP9 (*ABCC12*) are listed in Tables 1.3 and 1.4, but very limited data exists for these transporters. MRP7 and MRP8 transport leukotriene C_4 and estradiol 17β-glucuronide, but with lower affinity than MRP1 and MRP2. Traditional anticancer agents, such as docetaxel, vinblastine and vincristine, are less cytotoxic in cells overexpressing MRP7 [90-92]. Also, MRP8 transports cyclic nucleotides and confers resistance to 5-fluorouracil and poly-methoxyethylacrylate (PMEA) [93]. MRP8 and MRP9 contain two TMDs and two NBDs, similar to MRP4 and MRP5. MRP7 resembles MRP1-MRP3 in that it has an additional TMD at the N-terminus [92].

1.2.7 ABCG2

ABCG2 was independently cloned from three different sources and given distinct names reflecting the tissue or cell source, or the cytotoxicity profile. A breast cancer cell line surviving drug selection showed overexpression of an mRNA transcript encoding a 655 amino acid protein that was named breast cancer resistance protein (BCRP) [94]. An *ABCG2* encoded transporter isolated from cells exposed to mitoxantrone was called mitoxantrone resistance protein (MXR) [31], and a placental cDNA library showed enrichment of a putative protein termed placenta-specific ABC transporter (ABCP) [95]. For the sake of simplicity, the protein encoded by *ABCG2* will be referred to as MXR. It was later determined that MXR isolated from the drug-resistant cell lines contained an

amino acid mutation at residue 482 that arose from the drug selection process [52]. MXR is a half transporter with the NBD near the N-terminal domain that homodimerizes and is apically expressed on the plasma membrane [96, 97]. It is found in many tissues with highest expression in the liver, intestine, BBB, placenta, breast and various stem cells (Table 1.4) [97]. MXR protects the body from harmful endogenous toxins as evident from investigations with *Abcg2*(-/-) mice [96, 98]. Interstingly, under hypoxic conditions MXR prevents the cellular accumulation of heme and/or porphyrins [99]. Many MXR substrates are anticancer drugs but it also can transport glutamated forms of methotrexate and folates (Table 1.7) [100, 101]. The MXR mutant at residue 482 has a slightly different substrate profile than wildtype, with daunorubicin, doxorubicin and rhodamine 123 [52, 102].

Table 1.7
Selected MXR substrates[a]

Anticancer drugs	**Conjugates**	**Plant derived compounds**
Mitoxantrone	Estrone-3-sulfate	Flavonoids
Bisantrene	Estradiol-17β-glucuronide	Porphyrins
Methotrexate	Dinitrophenyl-*S* -glutathione	
Topotecan	Dehydroepiandrosterone sulfate	**Nucleoside analogs**
Irinotecan		Azidothymidine
SN-38[b]	**Fluoroquinolone antibiotics**	Lamivudine
Etoposide	Ciprofloxacin	
Daunorubicin[c]	Ofloxacin	***In vitro*** **probes**
Doxorubicin[c]	Norfloxacin	Rhodamine 123[c]

[a] Compiled from [51, 52]

[b] 7-Ethyl-10-Hydroxycamptothecin

[c] Only with Arg482Gly mutant

1.3 Genetic Variation in ABC Transporters

Systematic examination of genetic variation has been carried out for a number of ABC transporter genes, including members of the *ABCB*, *ABCC* and *ABCG* families. Most of these SNPs can be found in several publicly available databases, including the Pharmacogenetics Knowledge Base (PharmGKB) that catalogs genotypic and phenotypic pharmacogenetic data [103]. In most cases, only the coding region and surrounding intron-exon boundaries were examined based on the hypothesis that nonsynonymous variation leading to an amino acid change would be most likely to influence transport function [104-109]. Furthermore, polymorphisms in untranslated regions may influence mRNA stability and translation, while promoter region variants may influence transcription and gene/protein expression. In light of the relative paucity of common coding region variants that affect transport function (see section below), there is a growing interest to determine whether genetic variation in these noncoding regions may have a greater impact on transporter function. Many studies have focused on a single ethnic population and have examined a sufficient number of representative DNA samples to provide reasonable estimates of minor allele frequencies for common variants (>5% minor allele frequency) in that population [105, 109-122]. Others have carried out deep resequencing in multiple ethnic groups, including Caucasians, African Americans, Mexican Americans and Asian Americans [107, 108, 123-135]. The latter approach is important since genetic variation will differ across ethnic groups and population-specific information is necessary for application to the clinical setting.

A recent analysis of 19 solute carrier (SLC) and five ABC membrane transporter genes that were screened for polymorphisms in exonic and flanking intronic regions

revealed some interesting trends in genetic variation [136]. A total of 680 single nucleotide polymorphisms (SNPs) were identified in 96 kb of sequence that was screened in DNA samples from 247 individuals, including 100 Caucasians, 100 African Americans and 30 Asians. Interestingly, the number of synonymous (silent) and nonsynonymous (resulting in an amino acid change) SNPs identified in the 24 membrane transporter genes was also similar (175 and 155, respectively) [136]. However, statistical genetic analysis of the observed variation that takes into account the frequency of the SNP and the number of alleles that were screened (denoted as the average heterozygosity, π) revealed that variation was about three- to four-fold more common at synonymous positions than at non-synonymous positions. This suggests that there is some selective pressure on membrane transporter genes to suppress dramatic changes in transporter function. Amino acid diversity was also much lower in the transmembrane regions of the ABC transporters compared to the loops, with calculated π values for non-synonymous sites varying more than 13-fold between loops and transmembrane domains. In the loop regions, non-synonymous SNPs were much more common at evolutionarily unconserved sites compared to conserved sites. Of the five ABC transporters analyzed in this study, average heterozygosity in the transmembrane regions was extremely low at all sites, irrespective of sequence conservation across species [136]. A number of algorithms were evaluated that parsed non-synonymous SNPs into various categories based on chemical similarities and evolutionary relatedness. Assuming that deleterious amino acid changes would be selected against and thus found at low frequency, consideration of conservation at the variant site across orthologous species is predicted to be the best indicator of a detrimental effect on transporter function [130, 136].

Haplotypes define the combination of genotypes across a given gene or a multigenic region and are expected to more accurately predict functional consequences of genetic variation than consideration of single SNPs. The near completion of the HapMap project to define haplotype blocks across all human genes provides a wealth of information regarding genetic variation in individuals of African, European and Asian descent and this information is being increasingly used in the design of genetic association studies [137]. Haplotype structure has been determined for several membrane transporter genes based on the variants identified during population screening [107, 108, 111, 124, 131, 132, 134, 138]. Numerous haplotypes have been estimated for *ABCB1*, although relatively few contain non-synonymous variants that would result in altered protein sequence [107, 108]. Of these, *ABCB1**13 is the most common haplotype in Caucasians and Asian Americans and contains three intronic variants, two synonymous changes (1236C>T and 3435C>T) and a single non-synonymous site (2677G>T/A, Ala893Ser) [108].

1.4 Functional Implications of Xenobiotic ABC Polymorphisms

Efforts into understanding the functional consequences of genetic variation in the ABC genes lag behind the genetic analysis of these genes. Some nonsynonymous changes have been tested for their effect on transport function. Even less data are available regarding genetic variation in the untranslated and promoter regions of membrane transporter genes. These non-coding polymorphisms may influence transcription, mRNA stability, translation, and gene/protein expression. Functional and/or clinical pharmacogenomic data on xenobiotic ABC genes is available for *ABCB1*,

ABCC1-ABCC3 and *ABCG2*, and are discussed below. To date, there are no clinical or *in vitro* pharmacogenetic studies with *ABCC5* or *ABCC10-ABCC12*.

1.4.1 ABCB1

The results of functional studies of variants in *ABCB1* are summarized in Table 1.8. Early research screened for *ABCB1* mutations to explain P-gp overexpression in tissue and drug-resistant cell lines. These phenotype-to-genotype studies identified *ABCB1* mutations that appeared in diseased patients or after drug selection [139-141]. One mutation at residue 185 (Gly>Val) was found during colchicine selection and it demonstrated altered *in vitro* transport of colchicine and vinblastine, as well as different resistance patterns to these drugs [142]. The results provided an association between P-gp expression and genetic changes, but further work is needed to understand the consequences of the functional changes.

More recently, *in vitro* genotype-to-phenotype investigations have been the primary route of study for the functional effects of *ABCB1* nonsynonymous variants. There are dozens of polymorphic sites in *ABCB1* that have been identified through multiple SNP discovery efforts [107, 108, 121]. Functional analyses of all of the nonsynonymous variants are slowly accumulating but the most frequently studied SNP is the triallelic variant at amino acid position 893 that is located in the sixth intracellular loop near the C-terminus. The reference Ala can change into a common Ser or a lower frequency Thr [107, 108]. The Ala to Ser variant was first seen in the drug-resistant cell line AdR MCF-7, which is a breast cancer cell line that overexpresses P-gp [140]. ABCB1 was cloned from AdR MCF-7 and transfected into different drug-sensitive cell

Table 1.8
Functional consequences of polymorphisms in *ABCB1*

Variant or haplotype	Reference	Function[d]	Substrates	Expression method
Promoter region [a]				
-1517T>C/-1017T>C/ -41A>G/-129T>C[b]	Takane H 2004	↑	Luciferase	Transient, HepG2 cells
-274A>G	Wang B 2006	↑	β-Galactosidase	Transient, HEK293 cells
-146C>T	Wang B 2006	↑	β-Galactosidase	Transient, HEK293 cells
-41A>G/-145C>G/ -129T>C[b]	Wang B 2006	↓	β-Galactosidase	Transient, HeLa cells
-129T>C	Wang B 2006	↑	β-Galactosidase	Transient, HEK293 cells
Coding region				
Asn21Asp	Kimchi-Sarfaty 2002	↔	Bisantrene, calcein-AM, daunorubicin, bodipy-FL forskolin, bodipy-FL prazosin, bodipy-FL verapamil, bodipy-FL vinblastine	Transient, HeLa cells
Phe103Leu	Kimchi-Sarfaty 2002	↔	Bisantrene, calcein-AM, daunorubicin, bodipy-FL forskolin, bodipy-FL prazosin, bodipy-FL verapamil, bodipy-FL vinblastine	Transient, HeLa cells

Ser400Asn	Kimchi-Sarfaty 2002	↔	Bisantrene, calcein-AM, daunorubicin, bodipy-FL forskolin, bodipy-FL prazosin, bodipy-FL verapamil, bodipy-FL vinblastine	Transient, HeLa cells
	Woodahl 2004	↓	Rhodamine 123	Stable, LLC-PK1 cells
		↑	Vinblastine, vincristine	Stable, LLC-PK1 cells
		↔	Doxorubicin	Stable, LLC-PK1 cells
	Woodahl 2005	↑	Amprenavir, indinavir, lopinavir, ritonavir and saquinavir	Stable, LLC-PK1 cells
	Crouthamel 2006	↑	Doxorubicin, paclitaxel, vinblastine, vincristine	Stable, HEK293 cells
Ser400Ile[c]	Crouthamel 2006	↓	Doxorubicin, paclitaxel, vinblastine, vincristine	Stable, HEK293 cells
Ala893Ser	Kim 2001	↑	Digoxin	Stable, NIH3T3-GP+E86 cells
	Kimchi-Sarfaty 2002	↔	Bisantrene, calcein-AM, daunorubicin, bodipy-FL forskolin, bodipy-FL prazosin, bodipy-FL verapamil, bodipy-FL vinblastine	Transient, HeLa cells
	Kroetz 2003	↔	Calcein-AM	Transient, HEK293T cells

	Morita 2003	↔	Cyclosporin A, digoxin, verapamil, vinblastine	Stable, LLC-PK1 cells
	Oselin K, Gerloff 2003	↔	Rhodamine 123	Lymphocytes (*ex-vivo*)
	Salama NN 2006	↓	Rhodamine 123, vincristine, vinblastine	Stable, LLC-PK1 cells
	Schaefer M 2006	↑	Vincristine	Transient, HighFive insect vesicles
Ala893Thr	Schaefer M 2006	↑	Vincristine	Transient, HighFive insect vesicles
Ala998Thr	Kimchi-Sarfaty 2002	↔	Bisantrene, calcein-AM, daunorubicin, bodipy-FL forskolin, bodipy-FL prazosin, bodipy-FL verapamil, bodipy-FL vinblastine	Transient, HeLa cells
3435C>T	Hitzl M 2001	↓	Rhodamine 123	Lymphocytes (*ex-vivo*)
	Oselin K, Gerloff 2003	↔	Rhodamine 123	Lymphocytes (*ex-vivo*)

[a] Positions relative to the start of exon 1a

[b] -145C>G and -129T>C are relative to the ATG start codon

[c] Only found in leukemia patients

[d] ↑, ↓ and ↔ represent increased, decreased and no change, respectively, in variant function compared to reference

types. The cells acquired the drug resistance phenotype but no comparison was made with the reference Ala at codon 893.

The functional effects of the Ala893Ser polymorphism were first examined in a specialized mouse fibroblast cell line (NIH3T3-GP+E86)[107]. In this study the Ser893 P-gp showed enhanced efflux of the model substrate digoxin. In contrast, analysis of the function of five nonsynonymous variants, including Ala893Ser, in HeLa cells (human cervical cancer cell line) using seven different substrates showed no differences in transport compared to the reference P-gp [143]. Two other studies looking at Ala893Ser also concluded that there was no *in vitro* functional difference using established P-gp substrates, such as calcein-AM and verapamil [108, 144]. There is one report of decreased function in stable LLCPK1 cells expressing 893Ser as measured by rhodamine-123 accumulation [145], but this conflicts with kinetic findings from Sf9 insect cell membrane vesicles overexpressing Ala893Ser/Thr, in which the 893Ser and 893Thr variants had increased V_{max} and transport rates of ^{3}H-vincristine compared to reference [146]. This is supported by studies examining the ATPase activity of ten nonsynonymous variants [147]. It is possible that the varying results for the Ala893Ser/Thr variant reflect differences between the heterologous expression systems used in these analyses or result from substrate-dependent effects of this polymorphism.

Other P-gp variants have been characterized *in vitro*, including Asn21Asp, Phe103Leu, Ser400Asn and Ala998Thr [143, 148, 149]. In most cases there were no differences in function between the reference and variant P-gps; however, Ser400Asn shows increased resistance to vinblastine and vincristine, and decreased transepithelial flux of rhodamine-123 [149, 150]. The Ser400Asn variant also has altered affinity to

several protease inhibitors [148]. Interestingly, a third variant at residue 400 encoding an isoleucine was identified in leukemia patients with an allele frequency of 2.3%. It functions differently than the Asn400 variant, showing decreased resistance to vinblastine and vincristine [150]. Ile400 is probably a disease related polymorphism because numerous *ABCB1* genotyping and SNP discovery studies have not identified Ile400 in large populations (>100) [107-109].

ABCB1 promoter region polymorphisms may alter P-gp expression because of their possible location in transcriptional regulatory regions. The exact size of the *ABCB1* promoter is unknown, but it is generally accepted that the first 300 bases are the core promoter and the subsequent 500-1500 bases are for enhancer or inducer elements [151]. The region upstream of the *ABCB1* transcriptional start site (TSS) has been screened for polymorphisms in Caucasians, African Americans, Japanese, Chinese, Malaysians and Indians [152, 153]. The highest allele frequency is about 10% and many SNPs are ethnic specific. One haplotype containing three promoter variants (-1517T>C, -1017T>C and -41A>G) and a 5'-UTR variant (-129T>C) shows a possible trend for increased mRNA expression in liver and placental tissue from Japanese patients [152]. In addition, this haplotype demonstrated ~50% greater luciferase activity than reference ABCB1 in a reporter gene assay, as well as altered binding of nuclear proteins. A subsequent study concluded that other promoter haplotypes changed β-galactosidase activity but the effects were not always seen in the three different cell lines tested [153]. Similar to amino acid variants having substrate-dependent effects, promoter SNPs may alter transcription only in certain tissues.

ABCB1 mRNA and protein expression in barrier and excretory tissues is important clinically because they indirectly affect P-gp function. Studies associating *ABCB1* polymorphisms with mRNA and P-gp levels are summarized in Table 1.9. The catalyst for many clinical *ABCB1* pharmacogenetic studies was the report that the variant T allele at 3435 was associated with decreased P-gp levels in the duodenum and corresponding increases in serum levels of digoxin [121]. The T allele is also associated with decreased P-gp in the placenta but not in the kidney or liver [154-156]. Examination of the association between *ABCB1* 2677G>T and P-gp expression indicates decreased expression with the variant T allele in placenta but not in the duodenum, heart, kidney or liver [121, 154, 156-159]. It is difficult to say how the *ABCB1* 2677 and 3435 polymorphisms influence tissue expression of P-gp because there are many confounding variables in the studies to date, such as population ethnicity and *ABCB1* haplotype structure. Limited data exists on SNPs with lower allele frequencies than 2677G>T and 3435C>T because study populations are of limited size. The 5'-UTR variant -129T>C was shown to correlate with decreased P-gp levels in placenta [159] while two promoter variants, -2352G>A and -692T>C, demonstrated no effects on placental P-gp expression [154].

ABCB1 genotype associations with mRNA expression suggest that the 2677 and 3435 polymorphisms do not have a global effect or are tissue specific. Most studies do not find associations between mRNA levels and the 2677 and 3435 genotypes for the duodenum, heart, kidney, liver and lymphocytes [154-158, 160] (Table 1.9). There is conflicting evidence demonstrating either increased or decreased mRNA expression

Table 1.9
ABCB1* genotype associations with ABCB1 mRNA and P-glycoprotein levels *in vivo

Variant	Outcome		Tissue	Reference
	mRNA	Protein		
-2352G>A (promoter)	No change	No change	Kidney	Haenisch S 2006
-692T>C (promoter)	No change	No change	Kidney	Haenisch S 2006
-129T>C (5'-UTR)	n.d.[a]	50% decrease	Placenta	Tanabe M 2001
2677G>T	No change	No change	Duodenum	Moriya Y 2002, Hoffmeyer S 2000, Siegmund W 2002
	Increased	No change	Heart	Meissner K 2004
	No change	No change	Kidney	Haenisch S 2006, Uwai Y 2004
	No change	No change	Liver	Owen A 2005
	50% decrease	n.d.	Liver	Song P 2006
	No change	n.d.	Lymphocyte	Hitzl M 2001; Oselin K, Nowakowski 2003
	n.d.	50% decrease	Placenta	Tanabe M 2001
3435C>T	Increased	n.d.	Duodenum	Moriya Y 2002, Nakamura 2002
	No change	No change	Duodenum	Siegmund W 2002
	n.d.	Decreased	Duodenum	Hoffmeyer S 2000
	No change	n.d.	Heart	Meissner K 2004
	No change	No change	Kidney	Haenisch S 2006, Uwai Y 2004
	No change	No change	Liver	Owen A 2005
	50% decrease	n.d.	Liver	Song P 2006
	No change	n.d.	Lymphocyte	Hitzl M 2001; Oselin K, Nowakowski 2003
	n.d.	50% decrease	Placenta	Hitzl M 2004
	n.d.	No change	Placenta	Tanabe M 2001

[a] n.d. = no data

associated with 2677G>T or 3435C>T, but these results represent a fraction of the available data.

Recent mechanism-based *in vitro* studies suggest the synonymous 3435 variant may alter mRNA expression and P-gp tertiary structure. Transient expression of ABCB1 3435T in CHO cells showed lower mRNA expression than 3435C as determined by allelic imbalance. This result was attributed to decreased ABCB1 mRNA stability with 3435T, possibly due to changes in mRNA secondary structure [161]. In a transient expression system, the P-gp inhibitors cyclosporin A and verapamil were less effective on Rhodamine 123, bodipy-FL-paclitaxel, bodipy-FL-verapamil, daunorubicin, bodipy-FL-vinblastine and calcein-AM transport for the 3435T variant compared to reference [162]. The 3435T allele uses an infrequent codon compared to 3435C that may alter the timing of cotranslational folding. Consequently, these different P-gp tertiary structures may explain the inhibitor-specific effects of the 3435 variant on P-gp function.

1.4.2 ABCC1

Limited *in vitro* functional data exists for polymorphisms of *ABCC1* encoding MRP1 and these results are summarized in Table 1.10. In a single study the function of ten nonsynonymous variants were examined using three different MRP1 substrates. There appeared to be minor substrate-dependent changes for some of the variants, however, only the Ala989Thr was shown to have a significant functional effect. Specifically, the 989Thr MRP1 showed a 50% decrease in function when transporting estradiol 17β-glucuronide but had normal transport function with leukotriene C_4 and glutathione [163]. Amino acid 989 is at the membrane interface of the seventh extracellular loop and may play an important role in substrate interactions. The

Table 1.10
Functional consequences of polymorphisms in *ABCC1*

Variant	Reference	Function[b]	Substrates	Expression Method
Promoter region[a]				
-260G>C	Wang Z 2005	↑	β-Galactosidase	Transient, KB3-1, MCF-7, Hep3B and HepG2 cells
-118$(GCC)_7$>$(GCC)_{14}$	Nicolis E 2006	↔	Luciferase	Transient, 16HBE14o-AS3 cells
Coding region				
Cys43Ser	Leslie E 2003	↓	Sodium arsenite, vincristine	Stable, HeLa cells
		↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Stable, HeLa vesicles
Thr73Ile	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Ser92Phe	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Thr117Met	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Arg230Lys	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles

Arg433Ser	Conrad 2002	↓	Leukotriene C_4, oestrone sulfate	Transient, HEK293T vesicles; Stable, HeLa vesicles
		↔	Estradiol 17β-glucuronide	Transient, HEK293T vesicles; Stable, HeLa vesicles
		↑	Doxorubicin	Stable, HeLa cells
Arg633Gln	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Gly671Val	Conrad 2001	↔	Leukotriene C_4, estradiol 17β-glucuronide, estrone sulfate	Transient, HEK293 vesicles
Arg723Gln	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Ala989Thr	Letourneau 2005	↓	Estradiol 17β-glucuronide	Transient, HEK293 vesicles
		↔	Leukotriene C_4, glutathione	Transient, HEK293 vesicles
Cys1047Ser	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Arg1058Lys	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles
Ser1512Leu	Letourneau 2005	↔	Leukotriene C_4, estradiol 17β-glucuronide, glutathione	Transient, HEK293 vesicles

[a] Positions relative to transcriptional start site

[b] ↑, ↓ and ↔ represent increased, decreased and no change, respectively, in variant function compared to reference

functional effects of a variant at amino acid 433, located at the interface between the cytoplasm and the plasma membrane, have also been investigated [164]. The Arg433Ser polymorphism occurs in the 4th cytoplasmic loop close to the membrane interface of transmembrane domain 8 and has a minor allele frequency of ~1% [106]. MRP1 was transiently expressed in HEK293 and HeLa cells and membrane vesicles were used to measure transport of leukotriene C_4, estradiol 17β-glucuronide and oestrone sulfate. The Ser433 MRP1 showed a 50% decrease in transport of leukotriene C_4 and oestrone sulfate as well as a decrease in V_{max} for both substrates relative to the reference transporter [164]. It is possible that the conversion from a positively charged amino acid (Arg) to a neutral one (Ser) disrupted substrate affinity [165].

There is one study that investigated *ABCC1* promoter polymorphisms and it focused on a G-to-C transversion 260 base pairs upstream (-260G>C) of the transcriptional start site. Based on an inter-population genome analysis, it was predicted that -260G>C underwent recent positive selection and as a result this promoter variant may be functionally relevant [166]. The transcriptional activity of the reference and –260G>C variant of *ABCC1* was studied in four different mammalian cell lines using reporter constructs and in each case the variant C allele had at least a 2-fold greater level of promoter activity. Bioinformatic analysis indicates that this SNP resides in a putative c-ETS-1 transcription factor binding site [166]. The variant C-allele disrupts the c-ETS-1 binding sequence and may negate the repressive properties c-ETS-1 has on transcription. Another *ABCC1* polymorphism located in the 5'-UTR was tested for transcriptional activity. It is a polymorphic GCC repeat at −118 that has seven to 14 repeats and cystic

fibrosis airway epithelial cells expressing either $(GCC)_7$ or $(GCC)_{14}$ showed no difference in reporter gene expression [167].

To our knowledge there are only two studies investigating the effects *ABCC1* polymorphisms have on tissue expression and the results are shown in Table 1.11. Duodenal mRNA levels of ABCC1 were measured and compared to four synonymous SNPs: 128G>C, 218C>T, 2168G>A and 2137G>A. In 13 Japanese patients no differences in mRNA were found between the reference and variants [160]. Healthy German volunteers were genotyped for 816G>A, 825T>C, 1684T>C and 4002G>A synonymous variants and there were no correlations with mRNA expression in lymphocytes [168].

Table 1.11
ABCC1* genotype associations with ABCC1 mRNA levels *in vivo

Variant	Outcome	Tissue	Reference
128G>C	No change	Duodenum	Moriya Y 2002
218C>T	No change	Duodenum	Moriya Y 2002
816G>A	No change	Lymphocytes	Oselin K 2003
825T>C	No change	Lymphocytes	Oselin K 2003
1684T>C	No change	Lymphocytes	Oselin K 2003
2168G>A	No change	Duodenum	Moriya Y 2002
3137G>A	No change	Duodenum	Moriya Y 2002
4002G>A	No change	Lymphocytes	Oselin K 2003

1.4.3 ABCC2

The functional consequences of selected *ABCC2* polymorphisms have been studied and the results are summarized in Table 1.12. A deficiency in MRP2 function can lead to altered transport of conjugated bilirubin in the liver resulting in the hyperbilirubinemic disease known as Dubin Johnson Syndrome (DJS) [169, 170]. Certain mutations in *ABCC2* are thought to form an inactive protein product and are regarded as the molecular basis of DJS [171-173]. In some cases, including the Arg768Trp and I1173Phe variants, loss of MRP2 transport function is a result of defects in protein maturation and sorting to the apical membrane [172, 174]. In contrast, the Gln1382Arg and Arg1150His polymorphisms have no effect on localization but disrupt the nucleotide binding domain and ATP-dependent transport of MRP2 substrates [172, 173]. A rare mutation resulting in the Arg412Gly variant of MRP2 has recently been associated with loss of methotrexate function in vitro and higher plasma methotrexate levels in vivo [175]. The most common non-synonymous variant of MRP2 described to date is a Val417Ile change found at a frequency of 12-17% in major ethnic populations [103]. A comparison of reference and Ile174 MRP2 transport of estradiol 17β-glucuronide, leukotriene C_4, and 2,4-dinitrophenol-S-glutathione showed no significant changes in function [176].

There is ongoing research as to how ABCC2 mRNA and MRP2 protein levels associate with *ABCC2* polymorphisms, and the available data is summarized in Table 1.13. The 1446C>G, 3563T>A (Val1188Glu) and 4544G>A (Cys1515Tyr) polymorphisms are associated with increased mRNA expression in the liver [177, 178], but no differences in expression were found between carriers of the 1249G>A

Table 1.12
Functional consequences of polymorphisms in *ABCC2*

Variant	Reference	Function[a]	Substrates	Expression Method
Arg412Gly	Hulot 2005	↓	Methotrexate, glutathione-methylfluorescein	Transient, CHO cells
Val417Ile	Hirouchi M 2004	↔	2,4-Dinitrophenyl-S-glutathione, leukotriene C_4, estradiol 17β-glucuronide	Transient, LLC-PK1 vesicles
Arg768Trp	Hulot 2005	↓	Methotrexate, glutathione-methylfluorescein	Transient, CHO cells
	Hashimoto 2002	↓↓	Leukotriene C_4, glutathione-monochlorobimane	Stable, HEK293 cells and vesicles
Ser789Phe	Hirouchi M 2004	↑	2,4-Dinitrophenyl-S-glutathione, leukotriene C_4, estradiol 17β-glucuronide	Transient, LLC-PK1 vesicles

Arg1150His	Mor-Cohen 2001	↓	Carboxyfluorescein	Transient, HEK293 cells
Ile1173Phe	Keitel 2003	↓↓↓	Leukotriene C_4, estradiol 17β-glucuronide	Stable, HEK293 vesicles
	Mor-Cohen 2001	↓	Carboxyfluorescein	Transient, HEK293 cells
Glu1382Arg	Hashimoto 2002	↓↓	Leukotriene C_4, glutathione-monochlorobimane	Stable, HEK293 cells and vesicles
Ala1450Thr	Hirouchi M 2004	↔	2,4-Dinitrophenyl-S-glutathione, leukotriene C_4, estradiol 17β-glucuronide	Transient, LLC-PK1 vesicles

[a] ↑, ↓ and ↔ represent increased, decreased and no change, respectively, in variant function compared to reference. The number of arrows represent the magnitude of the functional change.

Table 1.13
ABCC2* genotype associations with ABCC2 mRNA and MRP2 levels *in vivo

Variant	Outcome		Tissue	Reference
	mRNA	Protein		
-24C>T (5'-UTR)	No change	n.d.[a]	Duodenum	Moriya Y 2002
	Decrease	No change	Kidney	Haenisch S 2006
	No change	No change	Placenta	Meyer zu Schwabedissen HE 2005
1249G>A	No change	No change	Kidney	Haenisch S 2006
	Decrease	Decrease (n.s.)[b]	Placenta	Meyer zu Schwabedissen HE 2005
1286G>A (Val417Ile)	No change	n.d.	Liver	Meier Y 2006
1446C>G	Increase	n.d.	Liver	Niemi M 2006
3600T>A (Val1188Glu)	Increase	n.d.	Liver	Meier Y 2006
3972C>T	No change	No change	Kidney	Haenisch S 2006
	No change	No change	Placenta	Meyer zu Schwabedissen HE 2005
4581G>A (Cys1515Tyr)	Increase	n.d.	Liver	Meier Y 2006

[a] n.d. = no data

[b] n.s. = not statistically significant

(Val417Ile) and 3972C>T variants and those with wildtype genotypes in either the kidney or liver [154, 177]. In 82 kidney cancer patients, -24C>T (5'-UTR) was found to have lower mRNA levels in normal kidney tissue but no changes in protein expression [154]. In matched tumor samples, there were no differences in expression between carriers of the -24CC, -24CT and -24TT genotypes. Also, ABCC2 mRNA and protein in normal and tumor kidney tissue were not associated with the 1249G>A and 3972C>T genotypes. In one study, the variant 1249A allele was associated with decreased preterm placental ABCC2 mRNA levels and possibly decreased MRP2 expression, although these results need to be confirmed with a larger sample size [179].

1.4.4 ABCC3

Data for *ABCC3* genetic variation is growing and one study identified a nonsynonymous variant at 3890 (G>A) with an allele frequency of 8% [180]. It causes an Arg-to-His substitution at residue 1287 and was predicted to be functionally important due to its evolutionary conservation and proximity to the Walker A in NBD2. When stably expressed in MDCKII cells, MRP3 1287His levels were equivalent to the levels of MRP3 1287Arg. Additionally, transport of leukotriene C_4, estradiol 17β-glucuronide and dehydroepiandrosterone sulfate (DHEAS) in membrane vesicles was not affected by the Arg1287His polymorphism [180]. As discussed previously, promoter region variants may alter transcriptional activity, and an *ABCC3* promoter polymorphism 211 base pairs upstream of the transcriptional start site (-211C>T) results in a 50% decrease in hepatic mRNA levels [122]. Interestingly, a recent report determined -211C>T is not an indicator of mRNA levels in lymphoblasts [181].

ABCC3 clinical pharmacogenetic research has investigated the effects of genetic variation in liver and lymphoblasts (Table 1.14). The *ABCC3* variant alleles 1552A>C, 3039C>T, 3890G>A (Arg1297His), 3942C>T and 4509A>G are not associated with mRNA or protein levels in the liver [122].

1.4.5 ABCC4

HIV-infected patients receiving the antiretroviral drugs indinavir, lamivudine-triphosphate and zidovudine-triphosphate were tested for possible pharmacokinetic associations with *ABCC4* genotypes. The +38T>G (3'-UTR) *ABCC4* variant was associated with a 20% increase in lamivudine-triphosphate plasma levels while the 3609G>A variant showed a trend for elevated zidovudine-triphosphate levels in plasma. The *ABCC4* variants 1497C>T and 3348G>A did not have any associations with the antiretroviral drugs [182]. A similar study investigated the effects *ABCC4* polymorphisms have on tenofovir disoproxil fumarate-induced renal proximal tubulopathy. In HIV patients no significant associations with toxicity were observed for the 559G>T, 669C>T, 912G>T, 951G>A, 969G>A, 1497C>T, 3310T>C, 3348A>G and 3609G>A *ABCC4* variants [183].

1.4.6 ABCG2

Functional analysis of MXR variants is summarized in Table 1.15. Much of the functional data on *ABCG2* polymorphisms focuses on the Gln141Lys variant in the cytoplasmic domain of the protein. This polymorphism is found at a frequency of approximately 9% in Caucasians and less than 2% in African Americans [103, 184]. Initial studies showed that Lys141 MXR had lower *in vitro* protein expression as well as

Table 1.14
ABCC3* genotype associations with ABCC3 mRNA and MRP3 levels *in vivo

Variant	Outcome		Tissue	Reference
	mRNA	Protein		
-211C>T (promoter)	50% decrease	Decrease (n.s.)[a]	Liver	Lang T 2004
	No change	n.d.[b]	Lymphocytes	Doerfel C 2006
1552A>C	No change	No change	Liver	Lang T 2004
3039C>T	No change	No change	Liver	Lang T 2004
3890G>A (Arg1297His)	No change	No change	Liver	Lang T 2004
3942C>T	No change	No change	Liver	Lang T 2004
4509A>G	No change	No change	Liver	Lang T 2004

[a] n.s. = not statistically significant

[b] n.d. = no data

Table 1.15
Functional consequences of polymorphisms in *ABCG2*

Variant	Reference	Function[a]	Substrates[b]	Expression method
Val12Met	Mizuarai 2004	↓	Topoisomerase I inhibitor, mitoxantrone, topotecan, doxorubicin	Stable, LLC-PK1 cells
	Imai 2002	↔	SN-38, topotecan, mitoxantrone	Transient, PA317 murine fibroblast cells
	Kondo 2004	↔	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles
	Morisaki K 2005	↔	Mitoxantrone, topotecan and SN-38	Stable HEK293 cells
	Yanase K 2006	↔	Mitoxantrone, topotecan and SN-38	Transient, PA317 murine fibroblast cells
Gln141Lys	Mizuarai 2004	↓	Topoisomerase I inhibitor, mitoxantrone, topotecan, doxorubicin	Stable, LLC-PK1 cells
	Imai 2002	↓	SN-38, topotecan, mitoxantrone	Transient, PA317 murine fibroblast cells
	Kondo 2004	↔	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles
	Morisaki K 2005	↓	Mitoxantrone, topotecan and SN-38 ATPase activity	Stable HEK293 cells
	Yanase K 2006	↓	Mitoxantrone, topotecan and SN-38	Transient, PA317 murine fibroblast cells
Ala149Pro	Kondo 2004	↔	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles

Arg163Lys	Kondo 2004	↔	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles
Gln166Glu	Kondo 2004	↔	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles
Ile206Leu	Vethanayagam 2005	↑	Bodipy FL-prazosin, mitoxantrone, pheophorbide a	Stable, HEK293 cells
Pro269Ser	Kondo 2004	↔	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles
Ser441Asn	Kondo 2004	↓	Estrone 3-sulfate, DHEAS, PAH, methotrexate	Transient, HEK293 vesicles
Arg482Gly (mutation)	Shafran 2005	↑	GW1843, methotrexate, tomudex	Stable, HEK293 cells
Asn590Tyr	Vethanayagam 2005	↓	Bodipy FL-prazosin, mitoxantrone, pheophorbide a	Stable, HEK293 cells
Asp620Asn	Vethanayagam 2005	↓	Bodipy FL-prazosin, mitoxantrone, pheophorbide a	Stable, HEK293 cells

[a] ↑, ↓ and ↔ represent increased, decreased and no change, respectively, in variant function compared to reference

[b] DHEAS = dehydroepiandrosterone-3-sulphate, PAH = *p* -aminohippurate, SN-38 = 7-ethyl-10-hydroxycamptothecin

decreased *in vitro* drug resistance to mitoxantrone, topotecan and SN-38 [116]. Also, the 141Lys variant can alter MXR activity by limiting membrane expression [185]. More recently, the Gln141Lys variant has been linked with a defect in ATPase activity associated with an increased K_m for ATP [184, 185]. This is somewhat surprising since the polymorphism of note is not located in the nucleotide binding domain. Changes in ATPase activity were also associated with increased sensitivity to mitoxantrone, doxorubicin, and topotecan. The evidence for the *in vitro* effects of Gln141Lys is convincing since the results have been replicated by different investigators using unrelated cell lines [116, 184-186]. However, 141Lys MXR may cause substrate-specific effects because it transports esterone-3-sulfate, DHEAS, methotrexate and *p*-aminohippurate (PAH) similarly to 141Gln MXR [187].

Substantial research has focused on the *in vitro* effects of Val12Met MXR. This variant is found in many ethnic groups at varying allele frequencies (2-20%) and is located in the first intracellular domain [104, 188]. It appears the 12Met MXR variant does not modulate cellular resistance to mitoxantrone, topotecan and SN-38 in LLCPK1, HEK293 or PA317 cell lines overexpressing ABCG2 [116, 185, 186]. There is one report of the Val12Met impairing proper membrane localization in LLCPK1 cells, which resulted in greater topoisomerase I inhibitor sensitivity [187].

Eight other *ABCG2* nonsynonymous variants have been studied *in vitro* and demonstrate varying functional effects (Table 1.15). In transport assays, the Ser441Asn, Asn590Tyr and Asp620Asn variants decrease efflux of model substrates, such as methotrexate and mitoxantrone [187, 189]. The Ala149Pro, Arg163Lys, Gln166Glu and Pro269Ser MXR variants do not influence transport of estrone-3-sulfate and methotrexate

in HEK293 vesicles [187]. Conversely, Ile206Leu MXR showed increased transport of mitoxantrone and bodipy-labeled prazosin [189].

The tissue expression of ABCG2 mRNA and MXR protein does not seem affected by several SNPs (Table 1.16). The nonsynonymous 34G>A (Val12Met) variant is not associated with changes in mRNA or protein in the placenta, heart or intestine [188, 190, 191]. This correlates with the previously discussed *in vitro* data showing the 12Met MXR variant does not alter membrane expression. There is one report of a 50% decrease in MXR protein associated with the 421C>A (Gln141Lys) *ABCC2* polymorphism in placenta from Japanese patients but no associated decrease in mRNA [190]. The mRNA levels were not determined during the *in vitro* functional assays of the 421C>A *ABCG2* polymorphism but protein expression was lower at the plasma membrane. The 34G>A and 421C>A *ABCG2* SNPs did not associate with altered mRNA or protein levels in the heart and intestine [188, 191].

Table 1.16
ABCG2* genotype associations with ABCG2 mRNA and MXR levels *in vivo

Variant	Outcome		Tissue	Reference
	mRNA	Protein		
34G>A (Val12Met)	No change	No change	Placenta	Kobayashi D 2004
	No change	No change	Intestine	Zamber CP 2003
		No change	Heart	Meissner K 2006
376C>T (Gln126stop)	No change	No change	Placenta	Kobayashi D 2004
421C>A (Gln141Lys)	No change	50% decrease	Placenta	Kobayashi D 2004
	No change	n.d.[a]	Heart	Meissner K 2006
	No change	No change	Intestine	Zamber CP 2003

[a] n.d. = no data

1.5 Focus of Dissertation

Xenobiotic ABC transporters influence drug absorption, distribution and elimination. The wealth of polymorphic data suggests genetic variation in xenobiotic ABC genes may play a role in drug response. The overall goal of these studies is two-fold. The first focus is to determine how genetic variation modulates the cellular processes controlling P-gp expression and function. The second focus is to investigate multiple ABC transporter variants for associations with mRNA expression in colon cancer, and their potential effects on transcription. P-glycoprotein is the main transporter investigated in this dissertation research because of its importance in the efflux of countless xenobiotics. The objectives of this dissertation are as follows:

1. Determine whether nonsynonymous P-gp variants change protein expression and function (Chapter 2). Transient expression of reference and variant P-gps in mammalian cells was used to measure surface P-gp expression and accumulation of P-gp substrates.
2. Investigate the effects of *ABCB1* 3'-UTR polymorphisms on ABCB1 mRNA stability (Chapter 3). Computational methods were used to predict the effects of *ABCB1* 3'-UTR variants on mRNA stability. Stable cell lines were created in order to measure the mRNA half-life of ABCB1 reference and selected 3'-UTR variants.
3. Identify associations between *ABCB1* and *ABCC1-ABCC3* variants and their respective mRNA expression in colon cancer (Chapter 4). Genotypes for *ABCB1* and *ABCC1-ABCC3* were determined in colon cancer patients and compared to mRNA levels in matched normal and tumor colon tissue.

4. Predict and determine the functional relevance of promoter region polymorphisms in xenobiotic ABC genes (Chapter 5). Possible transcription factor binding sites were identified in promoter sequences of selected ABC genes by comparisons with other species and analysis with predictive algorithms. Promoter region polymorphisms located in transcription factor binding sites could alter transcription and gene/protein expression.

In summary, these studies provide a better understanding as to how polymorphisms in ABC transporters mechanistically alter drug efflux.

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Chapter 2

The Effects of *ABCB1* Coding Region Polymorphisms on P-glycoprotein Activity

2.1 Introduction

P-glycoprotein (P-gp) is an important efflux transporter that can influence the pharmacokinetics and pharmacodynamics of many drugs. Important barrier or excretory tissues express P-gp in an orientation that protects the body from harmful compounds and also limits access of drugs to target tissues. The blood-brain barrier expresses P-gp and brain concentrations of P-gp substrates, such as digoxin and loperamide, are increased in *mdr1a* -/- mice [192, 193]. P-gp activity in the intestines is a major determinant of oral bioavailability for countless therapeutic compounds [194] and modulators of P-gp, such as rifampicin, can change absorption [195]. Once a drug is at the site of action it is possible for P-gp to limit intracellular levels. The mulidrug resistance (MDR) phenotype seen in the treatment of cancer is often caused by an overexpression of P-gp in solid tumors [196, 197].

Variability in drug response is widely observed for P-gp substrates in the treatment of many diseases. One potential mechanism for this variability is interindividual differences in P-gp activity. The number of P-gp transporters on the cell membrane and the level of P-gp transport function are the two most important factors that control the apparent activity of P-gp. Genetic variation in *ABCB1*, which encodes P-gp, is thought to be one of the factors that influence P-gp expression and function. The statistical analysis of *ABCB1* genetic variation data indicates that there is considerable

nucleotide diversity in this gene [108, 198]. A polymorphism that affects P-gp activity at the protein level most likely will be an amino acid changing nonsynonymous variant. It is possible that synonymous or promoter region variants can influence expression level of P-gp, thereby affecting P-gp transport function. Amino acid changes may alter key domains necessary for substrate binding, ATP-hydrolysis, or protein folding. The majority of pharmacogenetic studies for P-gp have focused on two polymorphisms, a synonymous change at 3435 (C>T) and a nonsynonymous change at 2677 (G>T, Ser893Ala) [199-202]. Unfortunately, there is no clear consensus as to how these polymorphisms affect P-gp at the *in vivo* or cellular level. Single nucleotide polymorphism (SNP) identification studies have discovered numerous other nonsynonymous and synonymous SNPs [103]. The SNPs at positions 2677 and 3435 may simply be markers for other functionally relevant sites that have yet to be investigated. The question still remains as to whether nonsynonymous P-gp variants can alter activity.

In these studies we investigated how seven nonsynonymous variants and two common haplotypes alter P-gp function in a newly developed *in vitro* assay that utilizes transient expression of P-gp in HEK293T cells. Substrates were screened to determine how well they are transported by P-gp in these cells and calcein-AM and bodipy-FL-paclitaxel were selected to test the function of the P-gp variants. The data from these studies suggest that certain P-gp variants have altered function that is substrate specific. In addition, some variants have different sensitivities to the P-gp inhibitor cyclosporin A that is also substrate dependent.

2.2 Materials and Methods

2.2.1 Materials

Fetal bovine serum (FBS) and Lipofectamine 2000 used in cell culture were purchased from Invitrogen (Carlsbad, CA). Calcein-acetoxymethylester (calcein-AM), bodipy-FL-paclitaxel, bodipy-FL-prazosin, and bodipy-FL-vinblastine were purchased from Invitrogen and stored diluted in DMSO at -20°C in a desiccated container. Cyclosporin A, daunorubicin and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO) and stored in DMSO at 4°C. Murine IgG2a MRK16 antibody was obtained from Kamiya Biomedical Co. (Seattle, WA, USA) and goat anti-mouse IgG allophycocyanin (APC) from Invitrogen. Eagle's minimum essential medium (EMEM) with Earle's BSS and L-glutamine, non-essential amino acids, sodium pyruvate and antibiotics were prepared by the University of California, San Francisco Cell Culture Facility. The pcDNA5/FRT mammalian expression vector was obtained from Invitrogen and the pCIneo mammalian expression vector was obtained from Promega (Madison, WI). Round-bottom 5 mL tubes (12x75 mm) were obtained from BD Falcon (Bedford, MA).

2.2.2 Cell Culture

HEK293T cells were obtained from ATCC and maintained according to their instructions. Briefly, EMEM medium containing 10% FBS, 1% nonessential amino acids, 0.11 μg/mL sodium pyruvate, 100 μg/mL streptomycin and 100 units/mL penicillin was used to propagate the cells in 5% CO_2 at 37°C. Cells were maintained in T75 flasks and passaged every 2-3 days.

2.2.3 ABCB1 Plasmids and Transfection

ABCB1 nonsynonymous polymorphisms were identified previously [108]. A full-length ABCB1 cDNA in pCIneo was used as a template to create variant plasmids. Selected variants of *ABCB1* while in pCIneo were created with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Primer sequences are listed in Table 2.1. Plasmid constructs for 2677T (893Ser), the 1236T/2677T(893Ser)/3435T haplotype, and a nucleotide binding domain mutant (NBD) were made previously [108]. Each amino acid change was introduced by altering a single nucleotide and the listed nucleotide positions are determined from the first base of the ATG start codon. The following variants were created from the reference *ABCB1* plasmid in pCIneo: Asn21Asp (61A>G), Ser400Thr (1199>A), Arg669Cys (2005C>T), Ala893Thr (2677G>A), Ser1141Thr (3421T>A) and Val1251Ile (3751G>A). The 61G(Asp21)/1236T/2677T(Ser893)/3435T construct was made from 1236T/2677T(Ser893)/3435T plasmid using the 61A>G primers. All variants and the reference were verified by direct sequencing. Reference ABCB1 was subcloned into the pcDNA5/FRT vector.

HEK293T cells were seeded in T25 flasks at $1.2x10^6$ cells/flask in EMEM supplemented with 10% FBS, 1% nonessential amino acids and 0.11 μg/mL sodium pyruvate, and allowed to grow for 24 hours. Cells were transfected at a confluency of 70-85% with 9.2 μg pcDNA5/FRT, 8.7 μg ABCB1 reference plasmid DNA or 9.2 μg variant plasmid DNA with 25 μL Lipofectamine-2000 in a final flask volume of 5 mL. Fresh medium was replaced ~5 hours after the addition of transfection reagents.

Table 2.1
Site-directed mutagenesis primers for *ABCB1* nonsynonymous variants

Base change	Mutagenesis primers[a]	
61A>G	Forward	5'-TTTAAACTGAACGATAAAAGTGAAAAAG
	Reverse	5'-CTTTTTCACTTTTATCGTTCAGTTTAAA
1199G>A	Forward	5'-GAAATGTTCACTTCAATTACCCATCTCGAA
	Reverse	5'-TTCGAGATGGGTAATTGAAGTGAACATTTC
2005C>T	Forward	5'-GAAAAAGATCAACTTGTAGGAGTGTCCGTG
	Reverse	5'-CACGGACACTCCTACAAGTTGATCTTTTTC
2677G>A	Forward	5'-GAAAGAACTAGAAGGTACTGGGAAGATCGC
	Reverse	5'-GCGATCTTCCCAGTACCTTCTAGTTCTTTC
3421T>A	Forward	5'-CAGCCGGGTGGTGACACAGGAAGAGATTG
	Reverse	5'-CAATCTCTTCCTGTGTCACCACCCGGCTG
3751G>A	Forward	5'-GTTTCAGAATGGCAGAATCAAGGAGCATGG
	Reverse	5'-CCATGCTCCTTGATTCTGCCATTCTGAAAC

[a] In each primer the SNP position is underlined.

2.2.4 Substrate Accumulation Assays

All substrates were diluted to the appropriate concentrations in PBS with or without 10 μM cyclosporin A for the substrate screening assays, which were performed once. The accumulation assays were repeated a total of three times for 1.5 μM calcein-AM and 100 nM bodipy-FL-paclitaxel in the absence or presence of 10 μM cyclosporin A. Triplicate samples were used for both +/- cyclosporin A conditions for each P-gp variant. After 24 hours transfected cells were removed from the flask with 0.05% trypsin and counted in order to achieve a concentration of $5x10^5$ cells/sample in PBS. Cells in PBS were seeded in 96-well plates at $5x10^5$ cells/well in 100 μL and centrifuged at 150*g* for 3 minutes. The PBS was aspirated and 100 μL substrate with or without 10 μM

cyclosporin A in PBS was added to resuspend the cells. Samples were allowed to incubate for 45 minutes in the dark at 37°C with a brief agitation period 25 minutes after substrate addition. All subsequent manipulations were done on ice with cold reagents or at 4°C for the centrifugation steps. Accumulation was stopped by centrifuging the cells at 150*g* for 3 minutes, followed by a PBS wash. The cells were resuspended in 75 µL of PBS containing 6.67 µg/mL MRK16 primary antibody and incubated on ice in the dark for 30 minutes. After two wash steps, 60 µL of PBS containing 2.5 µg/mL of secondary antibody with APC was used to resuspend the cells and the mixture was incubated on ice in the dark for 25 minutes. Cells were washed twice, resuspended and transferred into 5 mL tubes in 250-300 µL PBS.

The substrate accumulation assay was used to measure the IC_{50} of cyclosporin A for P-gp reference and the V1251I variant using bodipy-FL-paclitaxel as the substrate. Increasing concentrations of cyclosporin A (3.2 nM to 100 µM) were tested with 100 nM bodipy-FL-paclitaxel using the same methods as described above and an IC_{50} was estimated using Prism (GraphPad, San Diego).

2.2.5 Flow Cytometry

Cell samples were run on a dual-laser FACScalibur machine (Becton-Dickinson, San Jose, CA) with excitation wavelengths at 488 and 635 nM controlled by CellQuest software (Becton-Dickinson, San Jose, CA). Emission filters at 530 nM (FL1) detected calcein-AM and bodipy-FL compounds, 661 nM (FL3) detected daunorubicin and doxorubicin, and >670 nM (FL4) detected APC fluorescence. A total of 15,000 events were counted and FlowJo software (Treestar, Ashland, OR) was used to analyze the flow cytometry data. Forward- and side-scatter analysis established the R1 gate for the

healthy, single-cell population. Substrate (FL1 or FL3) and APC (FL4) fluorescence for the empty vector samples was determined from cells in the R1 gate. The P-gp positive gate was established in the FL4 channel of R1 and was set so that <1% of the empty vector population resided in the gate. Substrate and APC measurements for the P-gp transfected samples were determined from the R1/P-gp positive gate.

2.2.6 Statistical Analyses

Transfection efficiency was calculated in the FL4 channel (APC fluorescence) from the percentage of cells in the R1 gate that also resided in the P-gp positive gate. The P-gp expression level was determined from the median APC fluorescence of the cells in the R1/P-gp positive gate. Median calcein and bodipy-FL-paclitaxel fluorescence values from the R1/P-gp positive cells were averaged from the 3 separate experiments for P-gp reference, variants and a NBD mutant. The averaged fluorescence for the NBD mutant and each variant was converted to a percent of reference, with reference set as 100, and this represented the substrate accumulation in the absence of cyclosporin A. Inhibitor sensitivity was determined from the median calcein and bodipy-FL-paclitaxel fluorescence values in the presence of cyclosporin A for P-gp reference, variants and NBD mutant. The percent difference in fluorescence with and without inhibitor for P-gp reference, variants and the NBD mutant was calculated from the following equation:

$$(+CsA_{fluorescence} - Avg(-CsA_{fluorescence})) / Avg(-CsA_{fluorescence})$$

Percent difference values for the variants and NBD mutant were normalized to reference. Student's *t*-test with Bonferroni's correction ($p<0.006$) was used to calculate the statistical significance of the mean values calculated for substrate accumulation and percent difference of inhibitor.

2.3 Results

2.3.1 P-gp Variants

The coding regions and flanking intronic regions of *ABCB1* were screened for SNPs in 247 ethnically diverse subjects from the Coriell Institute [108]. Thirteen nonsynonymous sites were identified that span the entire region of the *ABCB1* gene (Table 2.2). The allele frequencies in Caucasians and African Americans vary from 0.5 to 46.4% and some of the SNPs are ethnic specific. Amino acid sequence alignments were performed using P-gp amino acid sequences from six other mammalian species. The degree of conservation is often linked to the allele frequency; that is, the reference amino acid for low frequency (<1%) nonsynonymous SNPs tend to be more conserved across the different species. One exception is the 2547A>G variant which encodes Ile849Met. In this case four out of six species had the Ile and the remaining two had an amino acid at this position that is distinct from the reference or variant. For certain residue positions, such as 21 and 89, neither the reference nor variant amino acid for human are found in the other mammalian species. Grantham values were determined for the 13 nonsynonymous SNPs to gauge how drastic the amino acid change is in terms of chemical properties [203]. All of the amino acid changing variants are located in the loop domains of P-gp, except for Ile849Met which resides in the 9th transmembrane domain (Figure 2.1). There are no nonsynonymous SNPs in the functional ATP-binding domains, including the Walker A, Walker B and linker regions. The amino acid altering variants that were studied were chosen based on an allele frequency greater than 2% in any one ethnic group or having a Grantham value >150 (Table 2.2). There are six polymorphic residues that meet this criteria: N21D, S400N, R669C, A893S/T, S1141T

Table 2.2
Allele frequencies, evolutionay conservation and Grantham values for *ABCB1* nonsynonymous polymorphisms and selected haplotypes

Variant or haplotype		Allele frequencies (%)[b]		Amino acid conservation[d]		Grantham
Nucleotide change[a]	Amino acid change	AA	CA	Reference	Variant	value[e]
61A>G	Asn21Asp	2.5	8	-	-	23
266T>C	Met89Thr	0	0.5	-	-	81
781A>G	Ile261Val	1.5	0	d, ha, mk, ms, r, s	-	29
1199G>A	Ser400Thr	1	2.5	d, ha, mk	ms, r	46
1985T>G	Leu662Arg	0	0.5	d, ha, mk, ms, r, s	-	102
2005C>T	Arg669Cys	1	0	d, ha, mk, r, s	-	180
2547A>G	Ile849Met	0	0.5	d, ha, mk, s	-	10
2677G>T	Ala893Ser	10	46.4	ha, ms, r	d, mk, s	99
2677G>A	Ala893Thr	0.5	3.6	ha, ms, r	-	58
3151C>G	Pro1051Ala	0.5	0	d, ha, mk, ms, r, s	-	27
3322T>C	Trp1256Lys	0.5	0	d, ha, mk, ms, r, s	-	101
3421T>A	Ser1141Thr	11.1	0	d, ha, mk, ms, r, s	-	58
3751G>A	Val1251Ile	0[c]	0	d, ha, mk, ms, r	s	29
3767C>A	Thr1256Lys	0	0.5	d, ha, mk, ms, r, s	-	78
1236C>T/2677G>T/ 3435C>T	Ala893Ser	6	34	N/A	N/A	N/A
61A>G/1236C>T/ 2677G>T/3435C>T	Asn21Asp/ Ala893Ser	2.5	8	N/A	N/A	N/A

[a] The variants and/or haplotypes chosen for study are shown in bold.

[b] Minor allele frequencies for African American (AA) and Caucasian (CA) populations. Haplotype frequencies represent the percentage of chromosomes that have the respective SNPs [7].

[c] The 3751G>A variant has an allele frequency of 5% in Mexican Americans.

[d] Determined from sequence alignment with dog (d), hamster (ha), monkey (mk), mouse (ms), rat (r) and sheep (s).

[e] Grantham values were taken from Grantham [14].

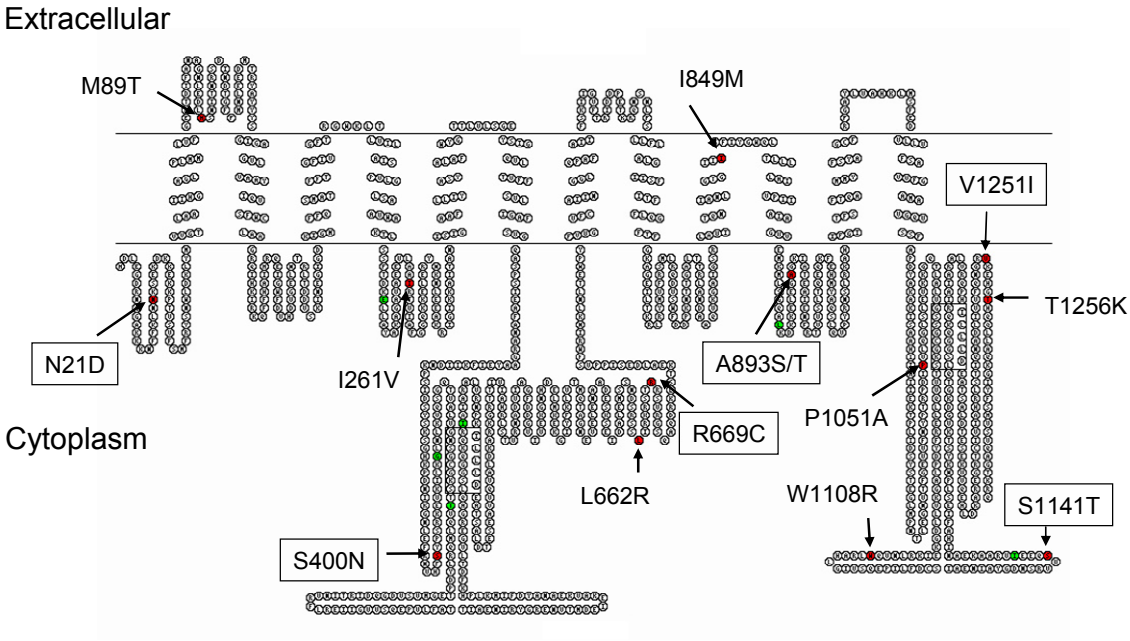


Figure 2.1. Secondary structure of P-gp and its nonsynonymous variants. The transmembrane topology schematic was rendered using TOPO (S.J. Johns and R.C. Speth, transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO/topo.html, unpublished). The position of amino acid changes in P-gp resulting from nonsynonymous SNPs is indicated. The Walker A, Walker B and linker peptide regions comprising the nucleotide binding domains are marked with a box outline. Variants chosen for study are marked with a box outline.

and V1251I. Residue 893 is interesting because it is triallelic and occurs naturally in haplotypes with the two common synonymous SNPs 1236C>T and 3435C>T as well as N21D [108] (Figure 2.1). As a result, two different haplotype constructs were also studied that contain Ser893, 1236T and 3435T with either Asn21 or Asp21.

2.3.2 P-gp Substrate Validation

Various compounds were screened in our assay to see how well they are transported by P-gp in our cell-based system before testing them with all of the P-gp

variant constructs. Six different compounds were selected—calcein-AM, bodipy-FL-paclitaxel, daunorubicin, doxorubicin, bodipy-FL-prazosin and bodipy-FL-vinblastine—based on previous data showing they are P-gp substrates [143, 204]. HEK293T cells transiently transfected with either empty vector, reference P-gp or a non-functional P-gp mutant (NBD P-gp) were exposed to different concentrations of each compound. The P-gp inhibitor, cyclosporin A, was used to verify that substrate transport is in fact specific to P-gp. The accumulation of substrate in the cells was measured using flow cytometry and only healthy, single-cell events were gated to measure substrate fluorescence in the empty vector samples. Substrate fluorescence in the reference P-gp and NBD P-gp samples were determined only from cells that were positive for P-gp expression in order to eliminate untransfected cells. The reference P-gp should transport any substrate, which will result in lower accumulation levels. The empty vector and NBD P-gp samples cannot transport substrate and will represent the upper limit of accumulation. Cyclosporin A inhibits P-gp activity and results in increased accumulation of substrate that should be comparable to empty vector and NBD mutant.

Calcein-AM passively and rapidly enters the cell due to its high lipid solubility. High-capacity esterases cleave the acetoxymethylester group enabling the calcein metabolite to fluoresce [205]. There was a dramatic increase in calcein fluorescence in the vector transfected cells compared to the blank, and calcein levels in the reference transfected cells were many times less than in the NBD-transfected cells (Figure 2.2A). Reference P-gp consistently showed a 20-fold less accumulation of calcein than the NBD mutant and empty vector controls at 0.5-3 μM calcein-AM (Figure 2.2A). Bodipy-FL-paclitaxel had a significantly large dynamic range between reference P-gp and the two

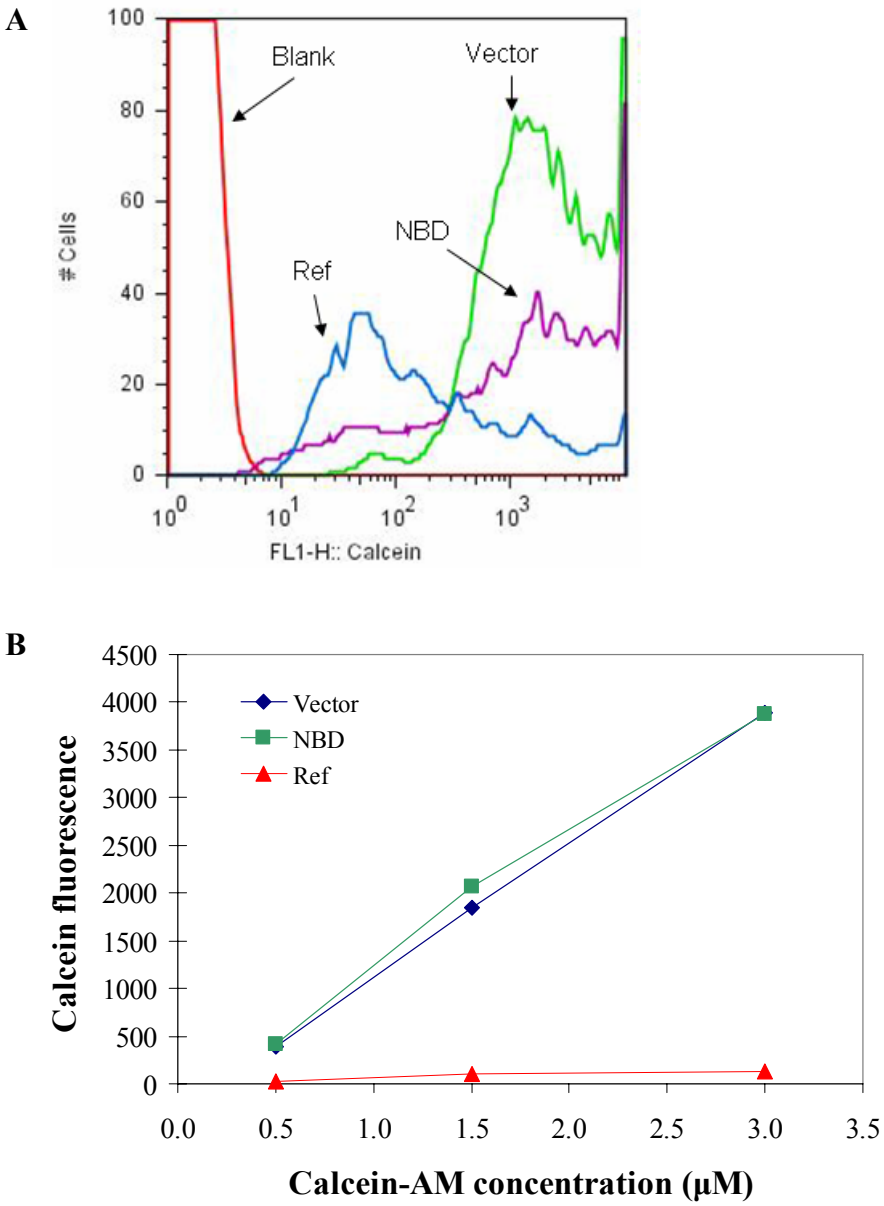


Figure 2.2. Calcein-AM is transported by P-gp in transfected HEK293T cells. The accumulation of 1.5 µM calcein-AM was determined for empty vector, NBD mutant and reference transfected HEK293T cells using flow cytometry. The blank sample shows the background fluorescence of HEK293T cells in the FL1 channel (A). Calcein-AM fluorescence for the three different transfected samples was directly correlated with substrate concentration between 0.5 and 3 µM (B). Mean values are shown (n = 2).

negative controls, empty vector and NBD P-gp (Figure 2.3A and B). Concentrations of 50, 150 and 450 nM bodipy-FL-paclitaxel were analyzed in the absence or presence of 10 μM cyclosporin A. At all three concentrations there was at least a 15-fold difference between reference P-gp and the negative controls, including reference P-gp with cyclosporin A. The tested concentrations did not appear to be saturating (Figure 2.3B).

Daunorubicn and doxorubicin did not show significant transport over background in this assay. Reference P-gp had similar intracellular levels of daunorubicin and doxorubicin as empty vector and NBD P-gp. Furthermore, cyclosporin A did not cause an increase in accumulation as was expected for a P-gp substrate (Figures 2.4A and B and Figure 2.5). At 900 nM bodipy-FL-prazosin, there was a ~6-fold increase in fluorescence for reference P-gp with cyclosporin A when compared to no cyclosporin A (Figure 2.6A). However, the high concentration of 900 nM caused a small secondary peak in the absence of cyclosporin A that was not seen at the two lower concentrations (Figure 2.6A). Three concentrations of bodipy-FL-prazosin (100, 300 and 900 nM) consistently showed lower accumulation for reference P-gp than empty vector and NBD P-gp (Figure 2.6B). Bodipy-FL-vinblastine consistently showed modest transport and had a ~2-fold difference between reference P-gp and the negative controls at concentrations between 0.2-1.2 μM concentrations (Figures 2.7A and B). The addition of cyclosporin A increased accumulation in reference-transfected cells by 2-fold. Based on these screens, calcein-AM and bodipy-FL-paclitaxel were chosen for study of P-gp variant transport function.

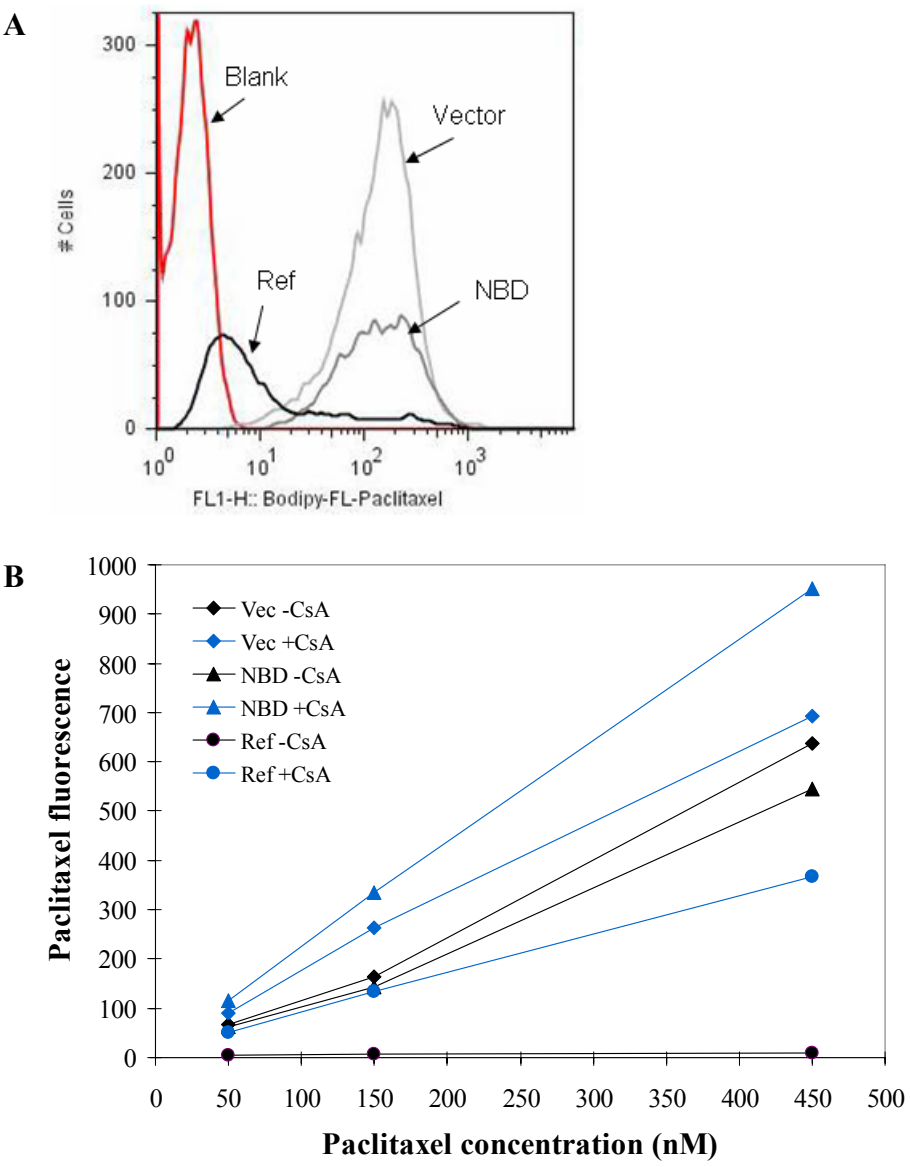


Figure 2.3. Bodipy-FL-paclitaxel is transported by P-gp in transfected HEK293T cells. The accumulation of 150 nM bodipy-FL-paclitaxel was determined for empty vector, NBD mutant and reference transfected HEK293T cells using flow cytometry (A). Bodipy-FL-paclitaxel fluorescence for the three different constructs was determined at 50, 150 and 450 nM with or without 10 μM cyclosporin A (B). Each point is the mean of duplicate samples.

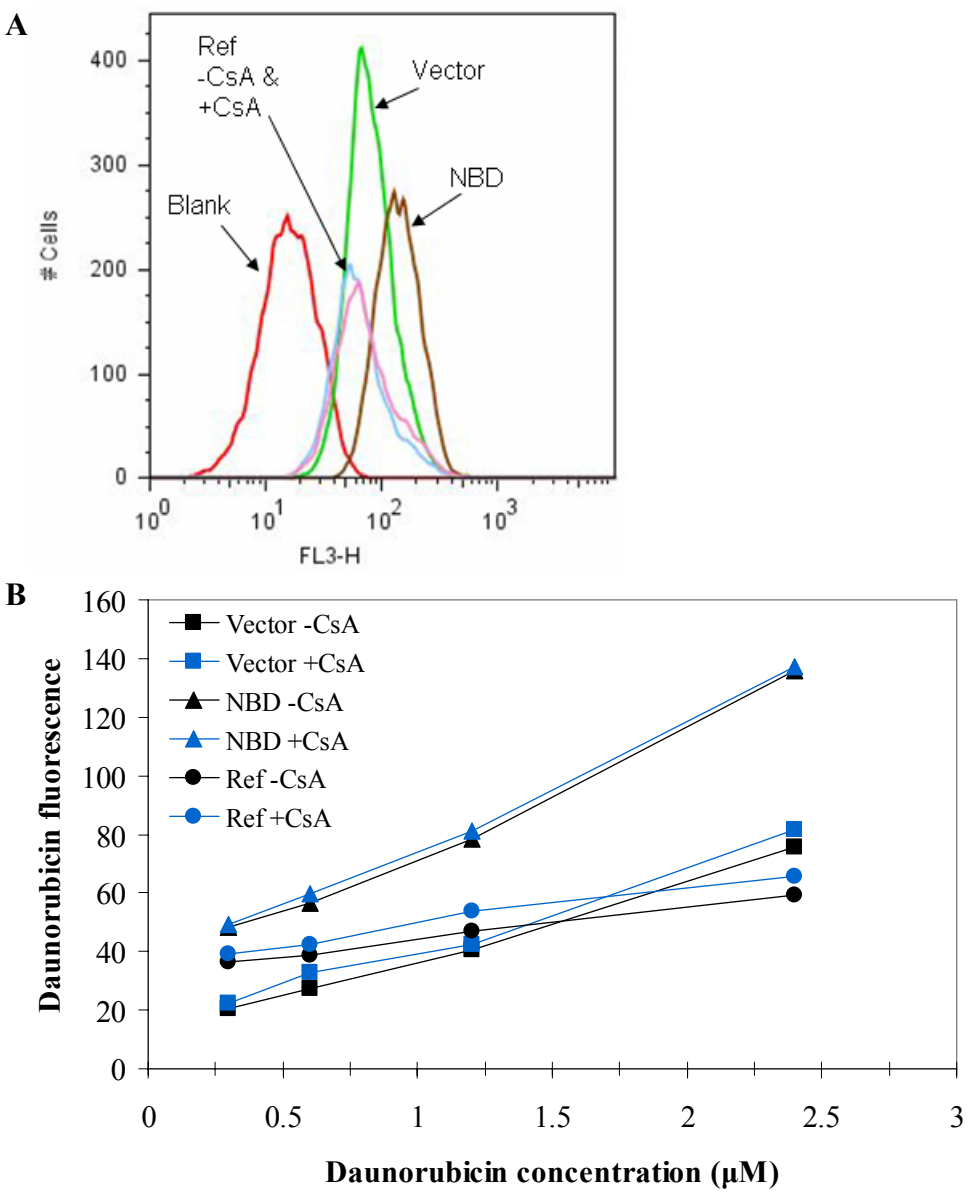


Figure 2.4. Bodipy-FL-daunorubicin is not significantly transported in P-gp transfected HEK293T cells. The accumulation of 2.4 µM daunorubicin was determined for empty vector, NBD mutant and reference transfected HEK293T cells using flow cytometry (A). Four concentrations of daunorubicin were tested with the various P-gp constructs and transport was insensitive to 10 µM cyclosporin A at all concentrations (B). Each point represents the mean of duplicate samples.

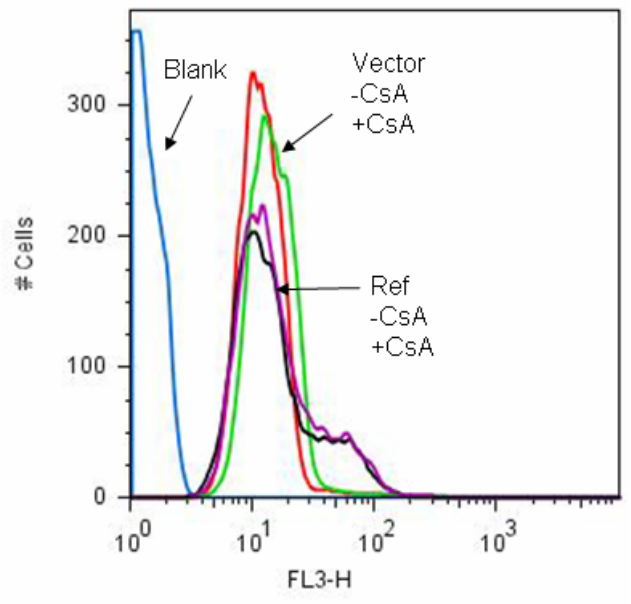


Figure 2.5. Doxorubicin is not significantly transported in P-gp transfected HEK293T cells. The accumulation of 3 μM doxorubicin was determined for empty vector, NBD mutant and reference transfected HEK293T cells using flow cytometry. In some cases, 10 μM cyclosporin A was included in the incubation.

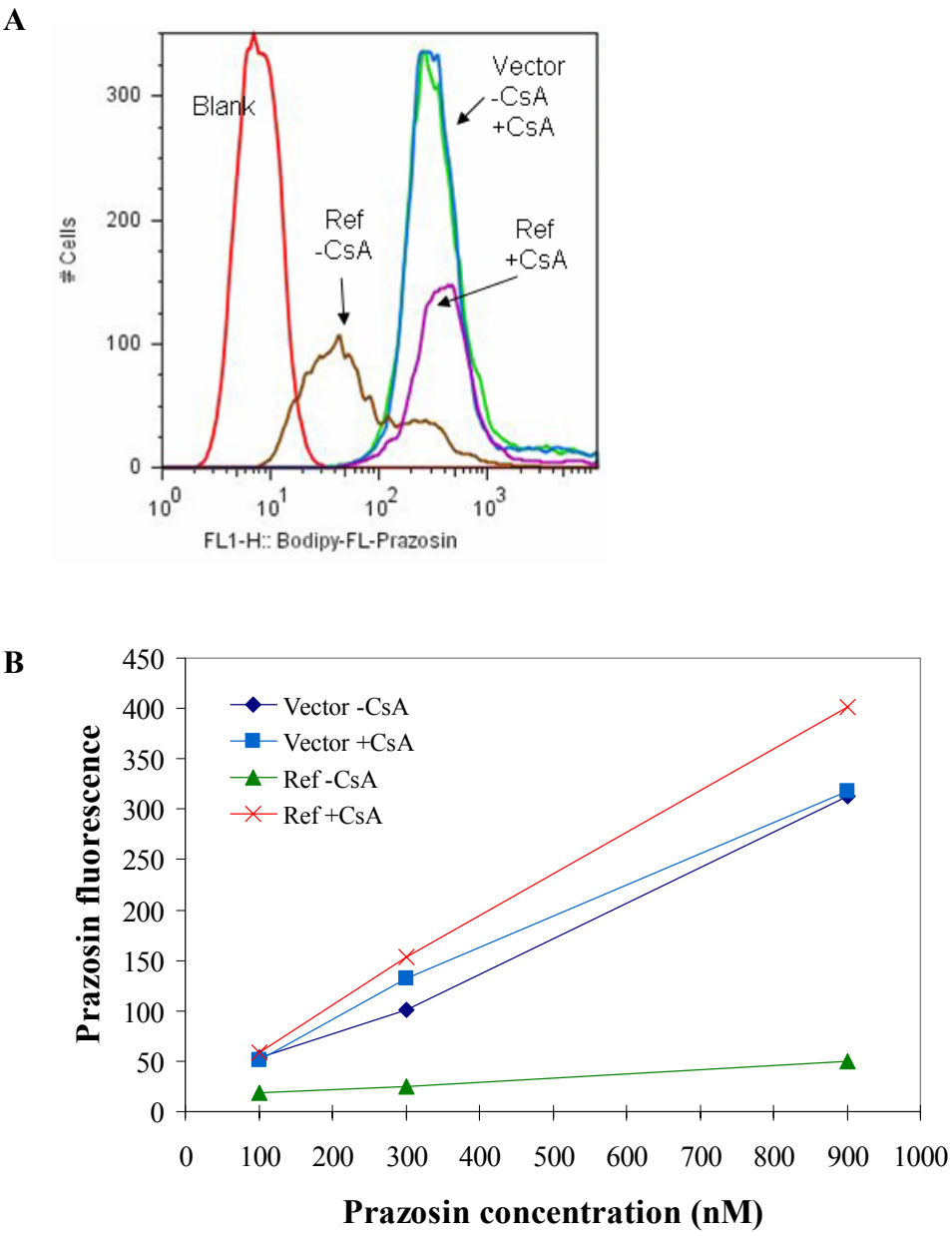


Figure 2.6. Bodipy-FL-prazosin is transported by P-gp in transfected HEK293T cells. The accumulation of 900 nM bodipy-FL-prazosin was determined for the NBD mutant and reference P-gp transfected HEK293T cells using flow cytometry (A). Three concentrations of bodipy-FL-prazosin were tested with the vector and reference constructs and only the reference P-gp was sensitive to cyclosporin A (B). Each point represents the mean of duplicate samples.

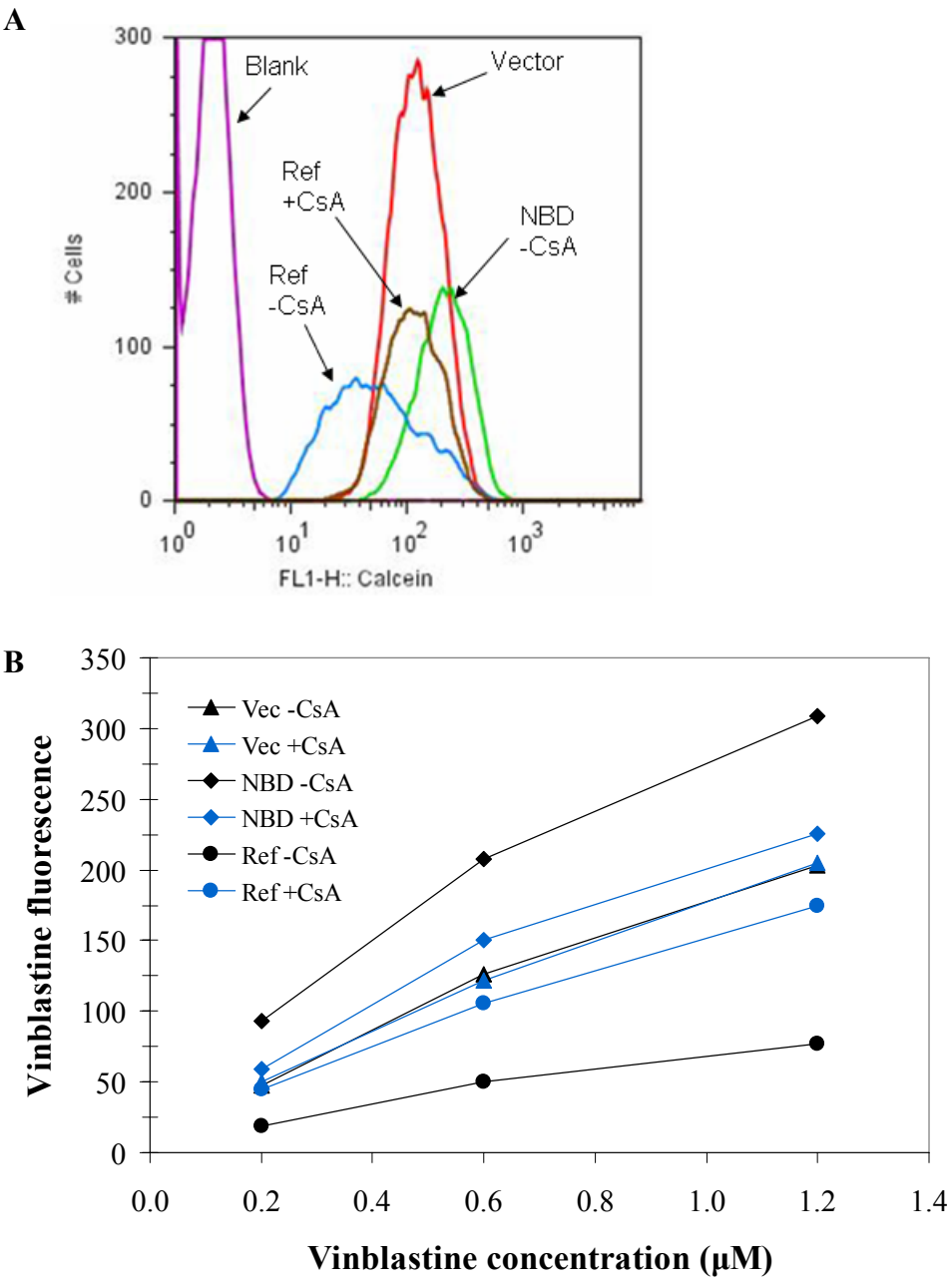


Figure 2.7. Bodipy-FL-vinblastine shows modest transport in P-gp transfected HEK293T cells. The accumulation of 0.6 µM bodipy-FL-vinblastine was measured for empty vector, NBD mutant and reference transfected HEK293T cells using flow cytometry (A). Three concentrations of bodipy-FL-vinblastine (0.2-1.2 µM) were tested with the vector, reference and NBD constructs and the reference P-gp transfected cells were sensitive to cyclosporin A (B). Mean values are shown (n = 2).

2.3.3 Calcein-AM Transport by P-gp Variants

Fluorescence measurements of substrate accumulation in the P-gp reference and variant transfected HEK293T cells were only based upon the subpopulation of cells staining positively for surface P-gp expression. Surface epitopes are recognized by the P-gp antibody, MRK16, which in turn was recognized by a secondary antibody conjugated with the fluorescent compound, allophycocyanin (APC). Figure 2.8 shows APC staining for empty vector, reference, NBD and variant P-gp expressing cells with a "P-gp positive" gate superimposed to mark the population of cells overexpressing P-gp. The P-gp positive gate is set so that less than 1% of empty vector sample cells reside in the region. Two important expression measurements are determined from the APC staining histogram: transfection efficiency and P-gp expression levels. Transfection efficiency is the percentage of healthy cells located in the P-gp positive gate. The second measurement, P-gp expression level, is the median APC fluorescence of the cells that are P-gp positive. The empty vector has increased APC staining over the blank sample due to endogenous P-gp levels in the HEK293T cells (Figure 2.8). Reference, NBD and variant P-gp samples have similar levels of transfection efficiency and protein expression within an experiment and, for the most part, across experiments (Figures 2.8, 2.9 and 2.13).

Transfection efficiency for the calcein accumulation experiments ranged from 48-56% of cells expressing P-gp on the cell surface (Figure 2.9A). All of the variants, including NBD, were within 5% of reference for transfection efficiency. P-gp expression levels for all of the variants were within 20% of reference across three separate experiments (Figure 2.9B). Accumulation of 1.5 μM calcein-AM in the P-gp positive cells for reference and the variants was dramatically less than for the NBD mutant

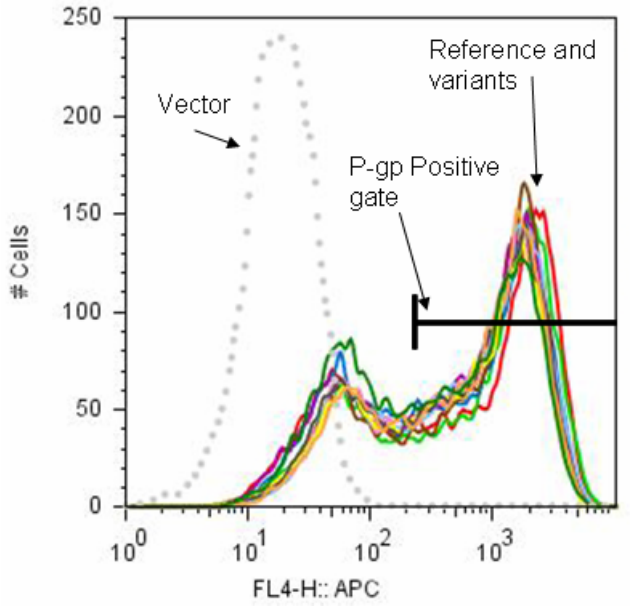
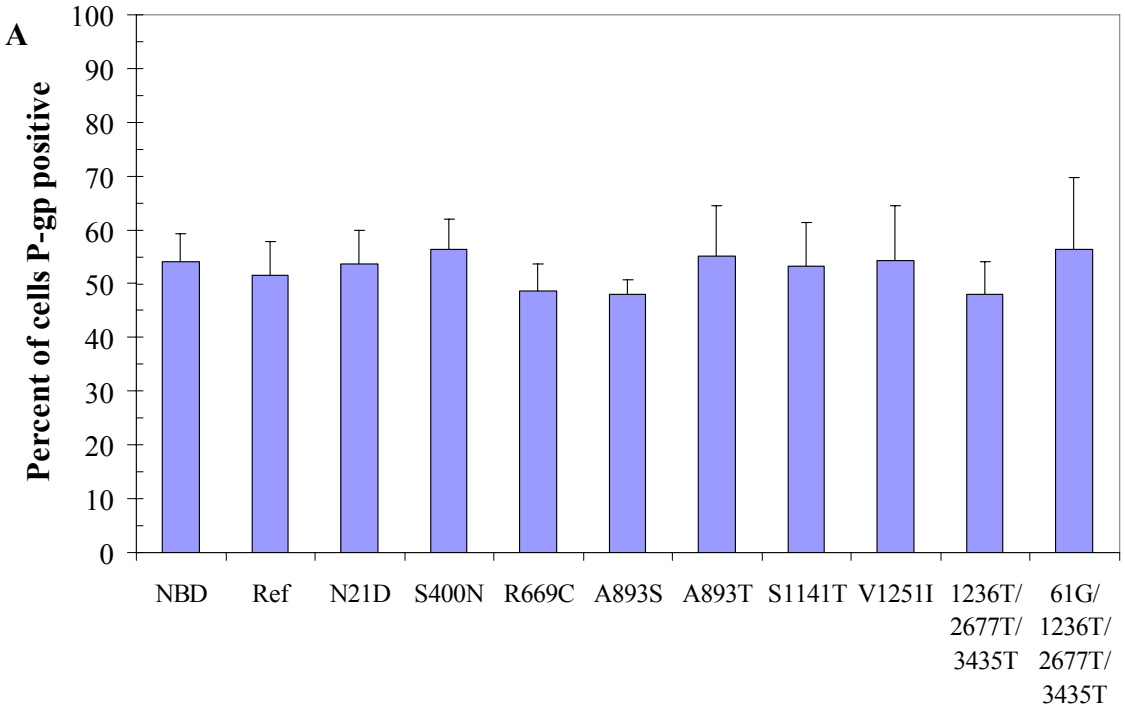


Figure 2.8. Transient P-gp expression in HEK293T cells for reference and variants. After the accumulation assay, HEK293T cells were stained for P-gp expression using a primary MRK16 antibody and a secondary APC antibody. The representative histogram of APC fluorescence for empty vector, NBD mutant, reference and variant transfected cells shows P-gp expression based on the intensity of APC fluorescence, as read on the x-axis. The P-gp positive gate was determined as stated in the Materials and Methods section and represents the threshold for cells that overexpress P-gp.


A
Percent of cells P-gp positive
100
90
80
70
60
50
40
30
20
10
0
NBD
Ref
N21D
S400N
R669C
A893S
A893T
S1141T
V1251I
1236T/ 2677T/ 3435T
61G/ 1236T/ 2677T/ 3435T

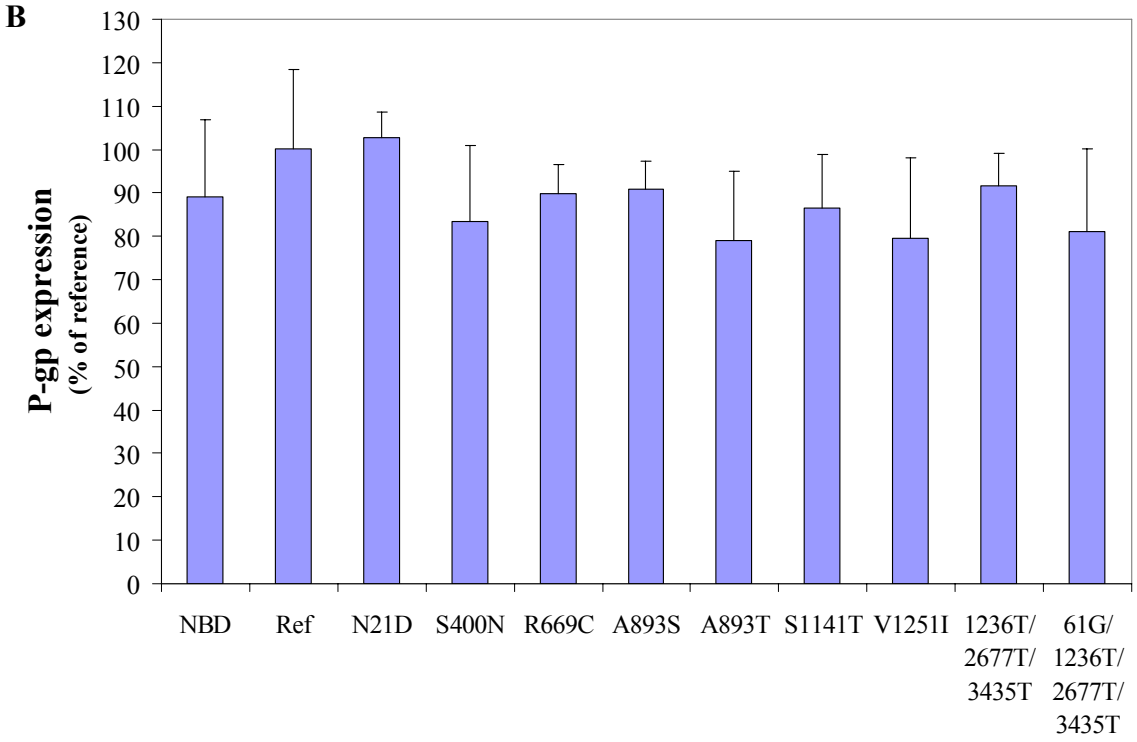

B
P-gp expression
(% of reference)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
NBD
Ref
N21D
S400N
R669C
A893S
A893T
S1141T
V1251I
1236T/ 2677T/ 3435T
61G/ 1236T/ 2677T/ 3435T

Figure 2.9. Transfection efficiency and P-gp expression in the calcein-AM accumulation experiments. The reproducibility of transient expression for P-gp in HEK293T cells was monitored during the calcein-AM accumulation assasys (N=3). Transfection efficiency shows the percentage of cells overexpressing P-gp as determined by the P-gp positive gate. The maximum value possible is 100% and the data represents the absolute transfection efficiency for reference and all variants (A). P-gp expression levels for reference and all variants were calculated from the median APC fluorescence and then expressed as a percentage of reference APC fluorescence (B). In both panels, the values represent the mean ± SD from three replications.

(Figure 2.10A). Median calcein fluorescence values for all variant constructs were normalized to the reference sample, which was arbitrarily set at 100. The median calcein fluorescence of almost all variants was less than reference, with a range of 69-97% (Figure 2.11). The decreased accumulation of calcein by 893Thr and 1251Ile was statistically significant ($p<0.006$), with respective accumulation values of 69% and 74% of reference.

Calcein accumulation was also investigated in the presence of 10 μM cyclosporin A (Figure 2.12) and the data was analyzed to show how sensitive each variant is to inhibition. The percent difference in intracellular fluorescence between control and cyclosporin A-treated cells was calculated individually for each variant and then normalized to reference which was arbitrarily set at 100. The NBD mutant has very high levels of calcein accumulation both in the absence and presence of cyclosporin A. As a result, the percent difference for the NBD mutant is very low (2.1%) and this corresponds to negligible sensitivity to inhibition. In contrast, the reference and variant P-gp constructs have low calcein levels without cyclosporin A but high calcein levels with

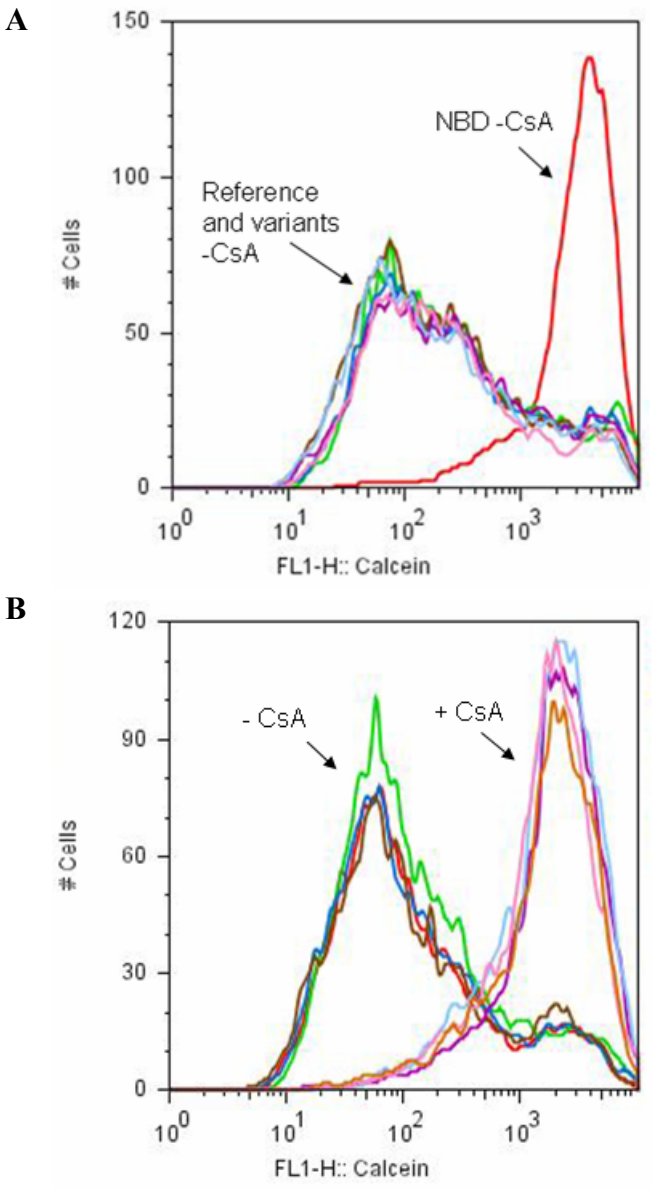


Figure 2.10. Calcein-AM accumulation in P-gp reference and variant transfected HEK293T cells. Fluorescence of calcein as measured in the FL1 channel was determined for P-gp reference, variants and NBD mutant. The data collected in the absence of cyclosporin A for P-gp reference, NBD mutant, S400N, A893T, S1141T, V1251I and haplotype N21D/A893S/1236C>T/3534C>T is shown in a representative fluorescence histogram (A). The effects of cyclosporin A on calcein-AM accumulation are displayed in a representative histogram for P-gp reference, N21D, R669C and A893S (B).

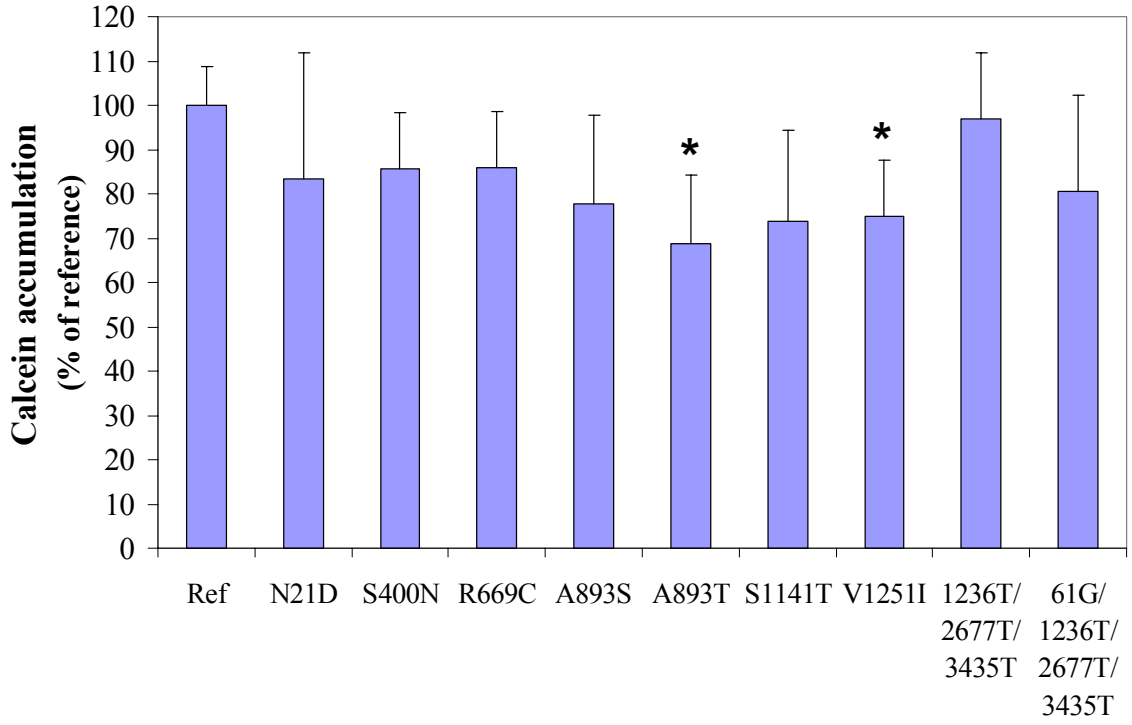


Figure 2.11. Relative calcein-AM accumulation for P-gp reference and variant constructs. Calcein fluorescence data from three replicate experiments were averaged and then normalized to reference which was set at 100. The NBD mutant demonstrated ~1700% calcein accumulation compared to reference (data not shown). Student's t-Test with Bonferroni correction was used to calculate the statistical significance of the mean accumulation values for all nine variant constructs relative to reference. Significant differences ($p<0.006$) are marked (*). Each value represents the mean ± SD of three replicates.

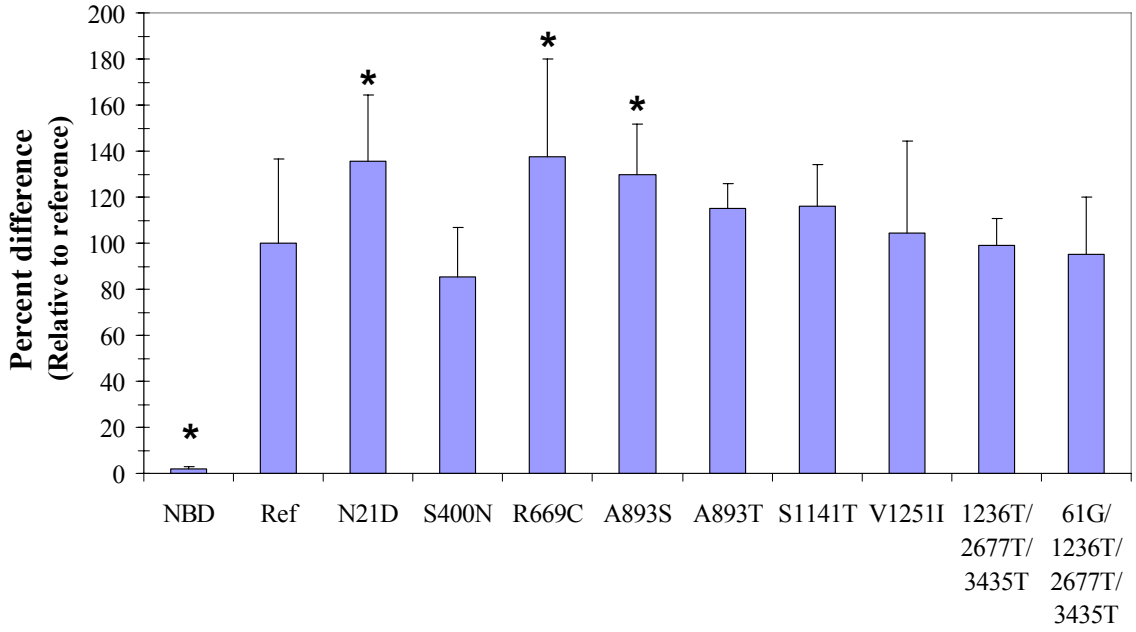


Figure 2.12. Inhibition of calcein accumulation by cyclosporin A in P-gp reference and variant transfected HEK293T cells. The level of cyclosporin A inhibition was determined individually for P-gp reference, NBD mutant and the nine variant constructs. The percent difference in intracellular fluorescence with inhibitor compared to no inhibitor was calculated as described in the Materials and Methods section. All values are means ± SD and are expressed relative to reference. Three variants showed increased sensitivity to cyclosporin A ($p<0.04$) and are marked (*).

cyclosporin A (Figure 2.10B). Interestingly, the N21D, R669C and A893S variants are 30-40% more sensitivite to cyclosporin A ($p<0.04$) compared to reference (Figure 2.12).

2.3.4 Bodipy-FL-paclitaxel Accumulation

The three replicate experiments for bodipy-FL-paclitaxel accumulation had similar levels of P-gp transfection efficiency for reference and all nine variant constructs (56-66%; Figure 2.13A). P-gp expression levels for the variants were within 30% of the reference (Figure 2.13B). Of the cells that were P-gp positive, those expressing reference

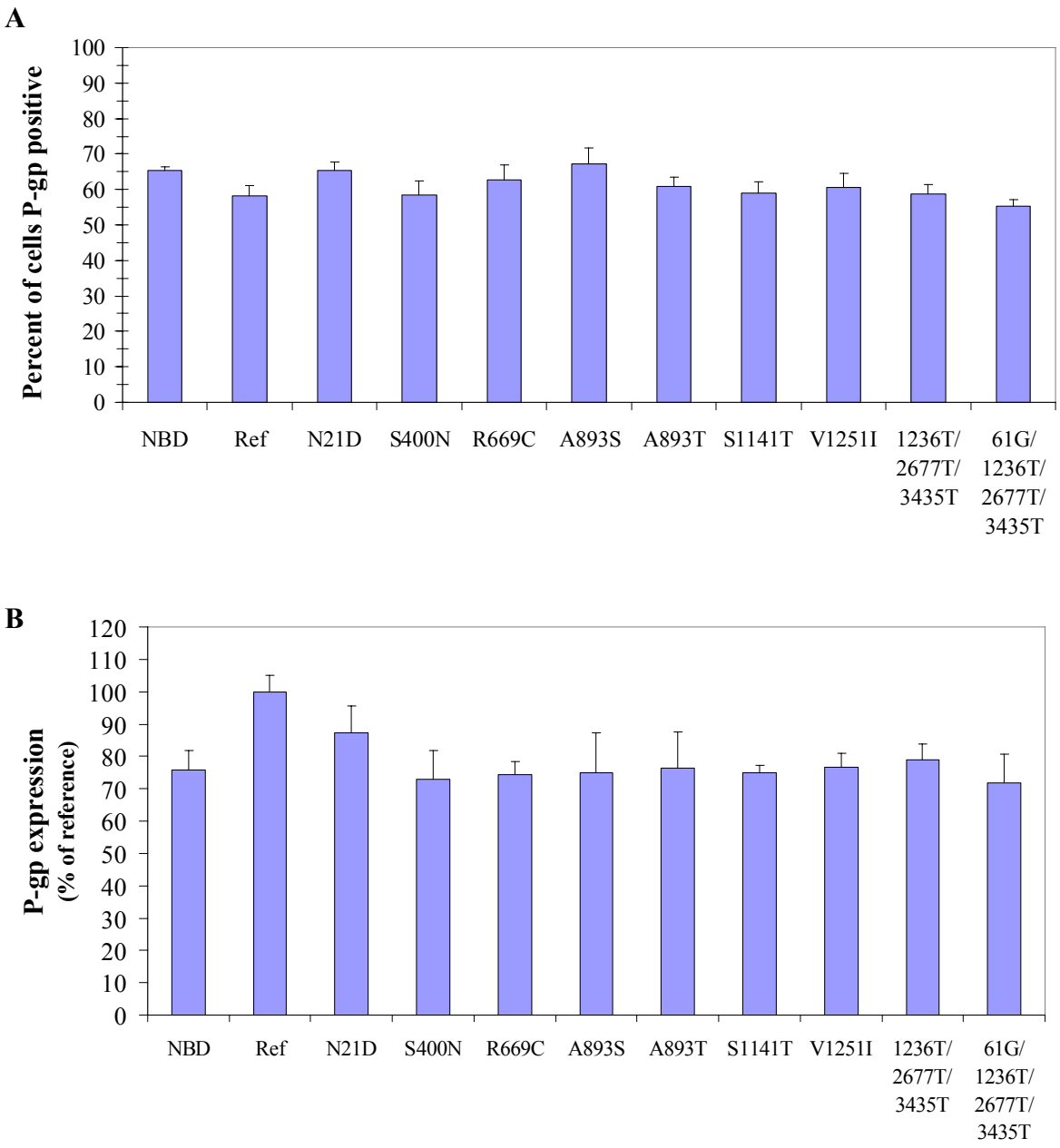


Figure 2.13. Transfection efficiency and P-gp expression in the bodipy-FL-paclitaxel experiments. The reproducibility of transient expression for P-gp in HEK293T cells was monitored during the bodipy-FL-paclitaxel accumulation assasys (N=3). Transfection efficiency shows the percentage of cells overexpressing P-gp as determined by the P-gp positive gate. The maximum value is 100% and the data represents the absolute transfection efficiency for reference and all variants (A). P-gp expression levels for reference and all variants were calculated from the median APC

fluorescence and then expressed as a percentage of reference APC fluorescence (B). In both panels, values are expressed as mean ± SD from three experiments.

or any variant plasmid DNA had significantly less accumulation of 100 nM bodipy-FL-paclitaxel than NBD or empty vector (Figure 2.14A and B). In addition, the presence of cyclosporin A dramatically increased bodipy-FL-paclitaxel accumulation. Median bodipy-FL-paclitaxel fluorescence values for all variant samples were normalized to reference, which was arbitrarily set at 100. The N21D, S400N, R669C, A893T variants and the A893S 1236C>T 3435C>T haplotype were within 5% of the reference (Figure 2.15). The A893S and V1251I P-gp variants and the N21D/A893S/1236C>T/3435C>T haplotype accumulated paclitaxel at 114, 118 and 124% of reference, respectively ($p<0.006$), indicating decreased P-gp function.

Accumulation of 100 nM bodipy-FL-paclitaxel was also measured in the presence of 10 μM cyclosporin A. Again, the percent difference in intracellular fluorescence in the absence and presence of cyclosporin A was calculated in order to determine how effective cyclosporin A inhibits each variant (Figure 2.16). Three single-variant constructs—A893S, A893T and S1141T—were 27-30% less sensitive than reference while V1251I was 65% less sensitive than reference ($p<0.006$). The haplotype containing N21D/A893S/1236C>T/3435C>T had a percent difference that was 53% of reference ($p<0.006$). As a follow-up, the IC_{50} of cyclosporin A with bodipy-FL-paclitaxel was determined for reference and the V1251I variant. No significant differences were seen in accumulation at any concentration of cyclosporin A (N=2; Figure 2.17). Nevertheless, the IC_{50} is estimated to be less than 10 μM for cyclosporin A.

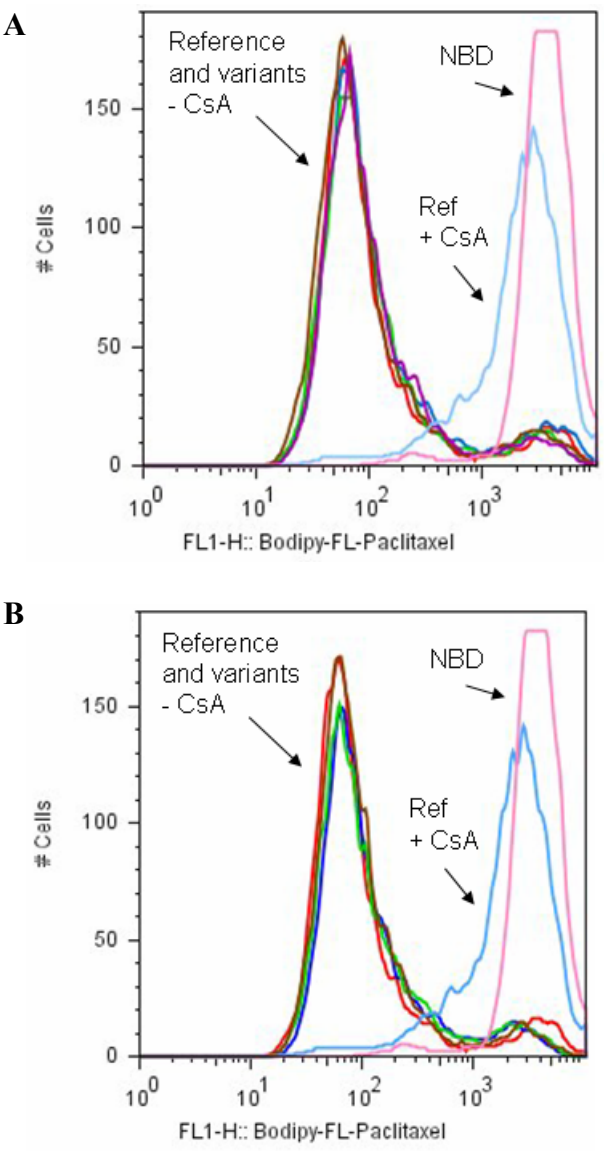


Figure 2.14. Bodipy-FL-paclitaxel accumulation in P-gp reference and variant HEK293T cells. Fluorescence of bodipy-FL-paclitaxel as measured in the FL1 channel was determined for P-gp reference, variants and NBD mutant. The data collected in the absence of cyclosporin A for N21D, S400N, R669C and S1141T (A) and A893S, A893T and V1251I (B) are shown in representative fluorescence histograms. Bodipy-FL-paclitaxel fluorescence measurements for reference ± cyclosporin A and NBD are shown in both histograms.

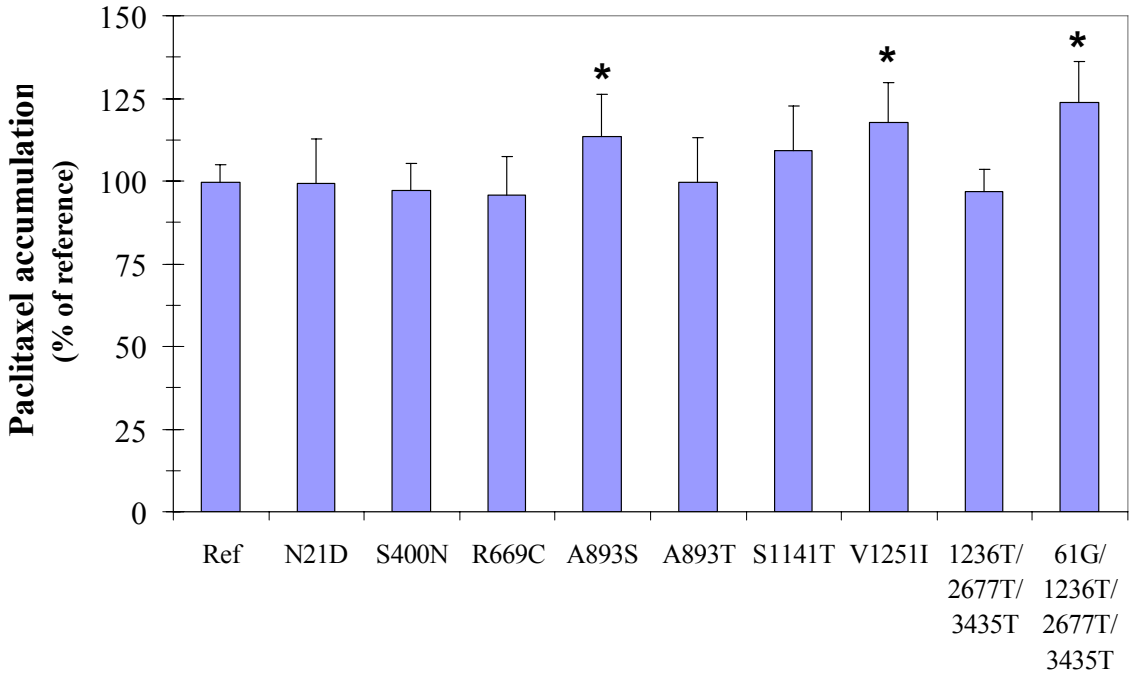


Figure 2.15. Relative bodipy-FL-paclitaxel accumulation for P-gp reference and variant constructs. Bodipy-FL-paclitaxel fluorescence data from three replicate experiments were averaged and then normalized to reference which was set at 100. Student's *t*-test with Bonferroni correction was used to calculate the statistical significance of the mean accumulation values for all nine variant constructs. Significant differences are noted (*). Values shown are the mean ± SD from three experiments.

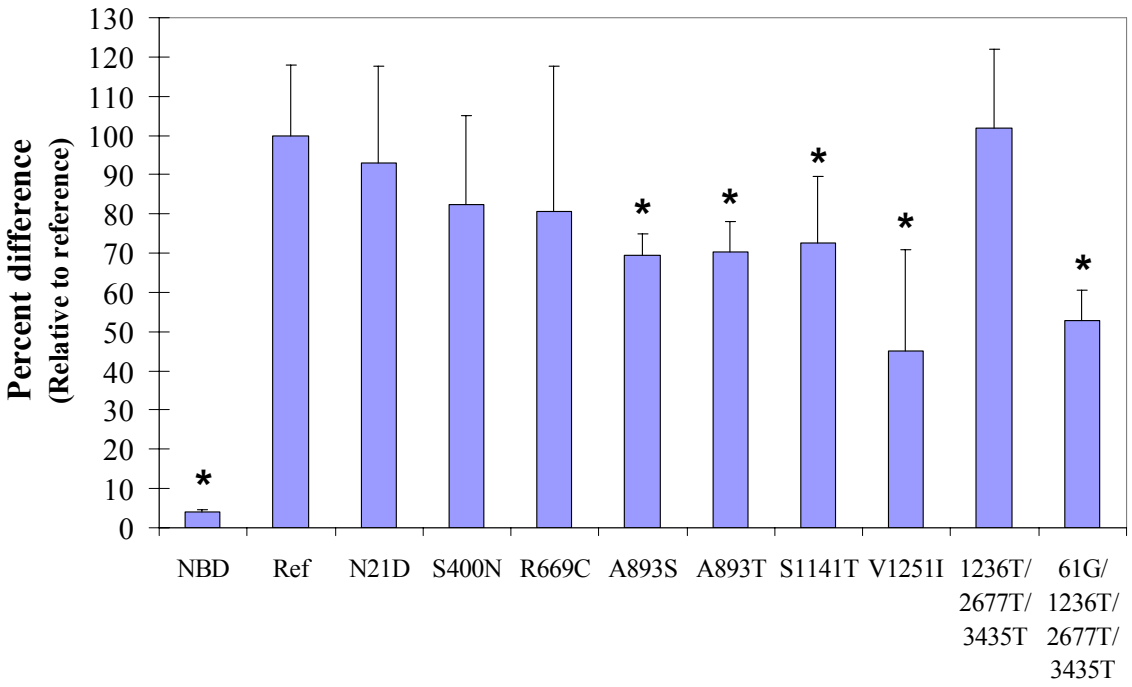


Figure 2.16. Inhibition of bodipy-FL-paclitaxel accumulation by cyclosporin A in P-gp reference and variant transfected HEK293T cells. The level of cyclosporin A inhibition was determined individually for P-gp reference, NBD mutant and the nine variant constructs. The percent difference in intracellular fluorescence with inhibitor compared to no inhibitor was calculated as described in the Materials and Methods section. All values are expressed relative to reference and are the mean ± SD from three determinations. Five variants showed decreased sensitivity to cyclosporin A and are marked (*).

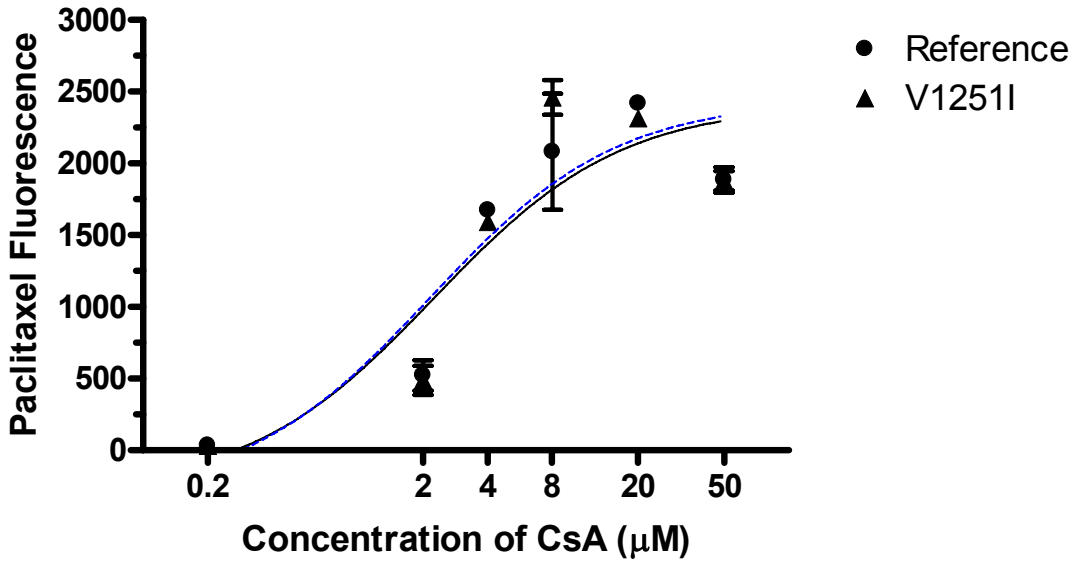


Figure 2.17. IC_{50} estimation for cyclosporin A inhibition of bodipy-FL-paclitaxel accumulation. P-gp reference and the V1251I variant were used to investigate the IC_{50} for cyclosporin A inhibition. Data from one of the two experiments shows increasing concentrations of cyclosporin A cause an increase in bodipy-FL-paclitaxel fluorescence. The IC_{50} is estimated to be less than 10 μM.

2.4 Discussion

ABCB1 polymorphism discovery efforts have been carried out using DNA samples from subjects in multiple ethnic groups. Subsequent studies that genotyped for the previously identified SNPs have helped to verify the actual allele frequencies for many of the known SNPs. There seems to be a consensus, at least within ethnic groups, regarding the common nonsynonymous variants, but rare nonsynonymous SNPs tend to be population-specific [107, 108, 206, 207]. Kroetz *et al*. identified 13 nonsynonymous variants in a group of 247 ethnically diverse patients, which is arguably the most

extensive *ABCB1* investigation to date [108]. Current research is focused on understanding how common P-gp variants can influence clinical phenotypes because the interpatient variability associated with P-gp substrates most likely is not caused by individual or rare *ABCB1* SNPs. Based on this reasoning, five out of 13 nonsynonymous variant sites were chosen for study based on having an allele frequency >2%: N21D, S400N, A893S/T, S1141T and V1251I (Table 2.2). Even though R669C has an allele frequency of only 1% in African Americans it was chosen because the variant amino acid causes a drastic chemical change as calculated by the Grantham value (D=180). Further studies involving the seven low frequency variants are warranted, but the results may not be as applicable at the clinical level. Pharmacogenetic research involving haplotypes is also important because many polymorphisms do not exist individually. Two haplotypes commonly found in African Americans and Caucasians [108] were investigated in this study. These haplotype constructs contained A893S, 1236C>T and 3435C>T either with or without N21D. In the end, a total of nine *ABCB1* variant constructs were studied. While the synonymous 1236C>T and 3435C>T changes are not expected to directly affect transport function, they could indirectly influence mRNA secondary structure and influence function.

The *in vitro* assay used in this study to measure P-gp function utilizes lipid-based transient transfection of *ABCB1* plasmid DNA in HEK293T cells in order to overexpress P-gp. Many *in vitro* functional studies used viral systems [107, 143] or stable cell lines that overexpress P-gp [144, 208]. The substrates screened have been shown to work effectively in these other expression systems, however, their utility will be dependent on the sensitivity of a given assay and background transport by endogenous proteins.

Calcein-AM, bodipy-FL-paclitaxel, daunorubicin, doxorubicin, bodipy-FL-prazosin and bodipy-FL-vinblastine were tested at various concentrations in the absence or presence of cyclosporin A. Comparing the function of P-gp variants to reference requires a large dynamic range in which to detect differences. Calcein and bodipy-FL-paclitaxel have ~15- and ~20-fold differences in accumulation between reference P-gp and the negative controls, respectively (Figures 2.2 and 2.3). Such a large difference can be attributed to their high affinity/capacity for P-gp transport. Calcein-AM is a commonly used substrate for many P-gp function assays but bodipy-FL-paclitaxel has only been used in a few studies [143, 209]. Bodipy-FL-prazosin showed at best a 6-fold difference between the upper and lower limits of accumulation (Figure 2.6). While its dynamic range is somewhat less than that of calcein-AM, future work should examine this substrate. At all three concentrations tested for bodipy-FL-vinblastine, the biggest range observed was 2-fold (Figure 2.7), and was deemed not sensitive enough to detect differences between reference and variant P-gps. Daunorubicin and doxorubicin were not transported by HEK293T cells overexpressing P-gp (Figures 2.4 and 2.5) and were not considered further. It should be noted that these two compounds are effluxed by other ABC transporters [197, 210] and HEK293T cells express various ABC transporters at appreciable levels (Gow, unpublished). This may explain the lack of significant accumulation of daunorubicin and doxorubicin.

Multiple types of expression systems have been used to measure the effects of P-gp variants on transport function. The studies that employ stable cell lines have a homogenous population of cells that constantly overexpress P-gp [107, 144, 208]. An obvious drawback is that creating stable cell lines can be very time consuming and in the

end many cell lines will have P-gp transport function that is similar to the reference transporter. The transient expression system we developed, in contrast, has consistent transfection efficiency and P-gp expression levels that allow for a reliable way to quickly screen P-gp variants against multiple substrates.

P-gp function is characterized by the efflux of intracellular substrates. Direct measure of efflux is generally performed with transcellular transport assays that utilize polarized cells, such as LLCPK1, to measure the movement of substrate across the basolateral and apical membranes [211]. The assay used in the present studies uses a suspension of HEK293T cells and substrate accumulation is detected as an indirect measure of P-gp efflux. It is assumed that uptake is constant in the transfected HEK293T cells but efflux may vary depending on which P-gp variant is expressed. After the accumulation period, the final measurement of intracellular substrate levels corresponds to P-gp mediated efflux. Calcein-AM is a well-documented substrate with a high affinity for P-gp [205]. The A893T and V1251I variants had lower intracellular levels of calcein, which suggests increased efflux by P-gp (Table 2.3). To our knowledge, the 1251Ile variant has not been studied previously, but recent data shows 893Thr has increased transport of ^{3}H-vincristine [146]. Previous work consistent with the current findings has shown N21D, S400N and A893S do not change calcein-AM transport [108, 143]. The transport of digoxin by the A893S variant was originally reported to be increased [107], although a subsequent study found similar digoxin transport by the 893Ser and reference P-gp [144]. Using rhodamine-123, vinblastine and vincristine, Salama et al. demonstrated decreased function for 893Ser in accumulation and transepithelial flux

Table 2.3
Substrate- and inhibitor-dependent effects of P-gp variants on transport function

Variant or haplotype	Calcein-AM		Bodipy-FL-paclitaxel	
	−CsA[a]	+CsA[b]	−CsA	+CsA
Asn21Asp		↑		
Ser400Thr				
Arg669Cys		↑		
Ala893Ser		↑	↓	↓
Ala893Thr	↑			↓
Ser1141Thr				↓
Val1251Ile	↑		↓	↓
1236C>T/Ala893Ser/ 3435C>T				
Asn21Asp/1236C>T/ Ala893Ser/3435C>T			↓	↓

[a] Arrows indicate statistically significant changes in P-gp function relative to reference in the absence of cyclosporin A (CsA)

[b] Arrows indicate statistically significant changes in sensitivity to CsA inhibition relative to reference P-gp

assays [145]. A893S has been the focus of many *in vivo* and *in vitro* pharmacogenetic studies but it remains to be seen if this variant is a causative SNP or merely a marker. Woodahl *et al*. created an LLC-PK1 stable cell line expressing 400Asn P-gp. This variant showed a decrease in function for rhodamine 123 but increased function for HIV protease inhibitors, such as saquinavir and ritonavir [148, 208]. One possible explanation is that the S400N variation alters P-gp function in a substrate specific manner.

Paclitaxel is an antimicrotubule agent used in the treatment of many types of cancers, including ovarian and breast (nlm.nih.gov/medlineplus). It disrupts mitosis by stabilizing microtubule formation [212] and *in vitro* studies use paclitaxel to test P-gp

transport and cytotoxicity [211]. The S400N variant has been shown to confer higher drug resistance to paclitaxel but the stable cell line overexpressing P-gp was selected for G418 resistance [213]. It is unknown what other background changes occur during the stable cell creation process that could affect total cellular resistance. Cellular efflux studies require paclitaxel to be radiolabeled but a recent alternative is to use the bodipy-labeled form that allows for fluorescence measurements. The bodipy modification may alter interactions with P-gp but studies, including ours, show the compound is transported to a high degree [143, 209].

Limited data exists on the effects of *ABCB1* genetic variation on bodipy-FL-paclitaxel transport. Kimchi-Sarfaty et al investigated N21D, F103L, S400N, A893S, A998T and three double mutants (N21D/S400N, N21D/A893S and S400N/A893S) using a baculovirus expression system with bodipy-FL-paclitaxel and showed no differences in function. Our results are similar for N21D and S400N, but we found that A893S and N21D/A893S have decreased transport of bodipy-FL-paclitaxel (Table 2.3). The percent change in function compared to reference for these hypofunctional variants is at most 24% but the statistical significance indicates the data has relevance at the *in vitro* level. It should be noted that V1251I P-gp displays decreased function with bodipy-FL-paclitaxel (Figure 2.15) but increased function with calcein-AM (Figure 2.11 and Table 2.3). The 1251 residue is evolutionarily conserved across five other mammalian species (Table 2.2) and is only found in the Mexican American population [108]. There is no other data on the function of the V1251I variant so testing other classes of substrates may elucidate the importance of this amino acid change.

Limited research is available on how an inhibitor mechanistically alters the interactions between P-gp and a substrate. Various investigations have shown substrates can bind to different P-gp domains [211, 214] but it is difficult to say *a priori* what domains are important for each class of compounds. In addition, predictive 3D modeling of how an amino acid change will affect transport function is still in its infancy. We tested the hypothesis that an amino acid changing variant can alter sensitivity to an inhibitor and our results suggest this is possible. Furthermore, the variants that function differently in the presence of cyclosporin A do so in a substrate dependent manner. The N21D, R669C and A893S P-gp variants show increased inhibition of calcein transport by cyclosporin A. In contrast, the A893S, A893T, S1141T and N21D/A893S/1236C>T/3435C>T variants are less sensitive to cyclosporin A inhibition of bodipy-FL-paclitaxel transport (Table 2.3). Current clinical research is interested in the coadministration of paclitaxel and cyclosporin A for the treatment of various cancers [215, 216]. The data from our investigation may improve the planning of such cancer studies as well as predicting the outcomes. The simultaneous interaction of substrate and inhibitor with P-gp is a complex interplay that is not fully understood but knowing how drug-drug interactions are influenced by P-gp variants can help improve drug therapy.

In summary, P-gp variants and haplotypes were investigated for possible functional effects on P-gp expression and function. Intracellular accumulation of calcein-AM and/or bodipy-FL-paclitaxel was altered by Ala893Ser, Ala893Thr, Val1251Ile and Asn21Asp/1236C>T/Ala893Ser/3435C>T. In addition, certain variants and haplotypes showed different sensitivities to cyclosporin A inhibition (Table 2.3). Substrate-specific differences illustrate how naturally occurring variants do not affect P-gp function in a

deleterious manner. To date, no study has discovered a naturally occurring P-gp variant that drastically changes function [217]. *In vitro* studies generate reproducible data regarding how a variant influences function but the consequences may not be as dramatic at the *in vivo* level. Unless a variant is characterized against multiple substrates it is difficult to predict the extent of its clinical significance. However, *in vitro* data continues to contribute to a better understanding of how P-gp operates at the molecular level, and may influence drug design and discovery.

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Chapter 3

THE EFFECTS OF *ABCB1* 3'-UTR VARIANTS ON mRNA STABILITY

3.1 Introduction

P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) transporter superfamily and its physiological role is to remove intracellular compounds via energy-dependent efflux. The distribution of P-gp in various barrier and excretory tissues can hinder drug therapy because many different types of drugs are P-gp substrates [197, 210]. Furthermore, variability in drug response is widely observed for P-gp substrates, suggesting that there are interindividual differences in P-gp expression and function. The kinetics of P-gp function are dependent upon transport activity and the abundance of P-gp molecules. It is generally accepted that the structure of P-gp, encoded by the *ABCB1* gene, regulates P-gp transport function. However, the mechanisms that control membrane P-gp expression levels are expected to be distinct from those that control transport activity.

The level of ABCB1 mRNA expression is an important determinant of P-gp expression levels. The interindividual differences in ABCB1 mRNA expression levels observed in the liver [218] small intestine [160, 219, 220] and kidney [221] may contribute to the variable drug response of P-gp substrates. Genetic variation in *ABCB1* is one possible mechanism influencing ABCB1 mRNA levels. Several studies have investigated the effects of coding region *ABCB1* polymorphisms, such as 2677G>T and 3435C>T, on mRNA expression. The 3435C>T synonymous variant is the most

commonly studied with respect to mRNA levels. It was initially linked with increased mRNA expression levels [160], however, data from subsequent studies are not in agreement [154, 156, 158, 221]. An *in vitro* study measured different mRNA stabilities between the C and T alleles of 3435 [161], so it is possible that this variant causes changes at the cellular level that may extend to clinical phenotypes.

Mechanisms that alter mRNA levels can change P-gp expression and potentially P-gp transport activity. Steady-state ABCB1 mRNA levels reflect the balance between gene transcription (*i.e.* synthesis) and mRNA stability (*i.e.* decay). Recent evidence has demonstrated that the 3'-untranslated region (UTR) of mRNA is an important regulatory site controlling interactions with mRNA degradation machinery (Figure 3.1) [222-225]. 3'-UTR RNA-binding proteins that recognize specific mRNA sequence elements and secondary structure dictate the fate of mRNA transcripts. Polymorphisms in the 3'-UTR of *ABCB1* could disrupt native RNA-protein interactions, resulting in altered mRNA stability.

The current studies identified nine *ABCB1* 3'-UTR polymorphisms and used a series of computational analyses to predict the functional importance of each variant. Transient and stable expression systems in multiple cell types were screened to find a reliable model to measure the mRNA half-life. An mRNA half-life assay was finally developed in stable cells to test the effects of three *ABCB1* variants on mRNA stability.

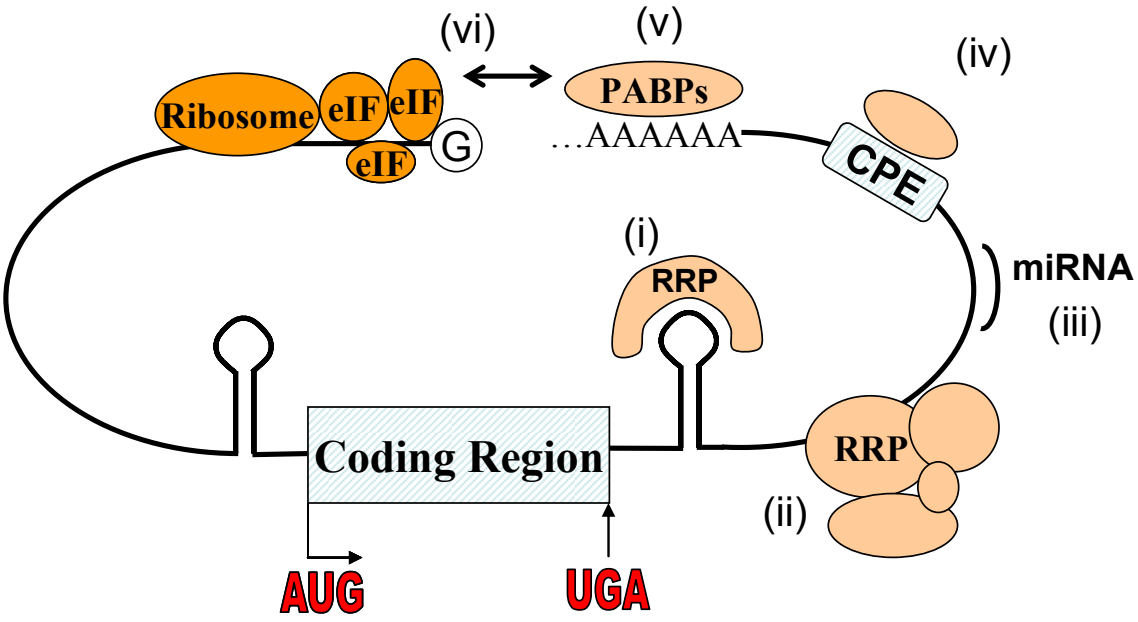


Figure 3.1. Regulatory elements in the 3'-untranslated region. (i) RNA-recognition proteins (RRPs) bind sequence motifs and mRNA secondary structure elements (*e.g.* stem-loop) for the purpose of controlling mRNA degradation. (ii) Multiple proteins often form complexes that interact with degradation machinery. (iii) Short 20-30-nucleotide antisense microRNAs (miRNAs) naturally transcribed in the genome can alter RRP binding sites. (iv) Cytoplasmic polyadenylation elements bind regulatory proteins that influence the length of the poly-A tail. (v) The poly-A tail contributes to nuclear and cytoplasmic processes controlling mRNA function. The poly-A binding proteins (PABPs) enable the "closed loop" structure of mRNA that influences mRNA stability and translation. PABPs can interact with (vi) translation initiation factors located at the 5' cap.

3.2 Materials and Methods

3.2.1 Materials

Trizol, lipofectamine 2000, zeocin, hygromycin, the pFRT/lacZeo, pcDNA5/FRT and pOG44 plasmids and HEK293 Flp-In (Flp293) cells were obtained from Invitrogen

(Carlsbad, CA). Calcein-AM (Invitrogen) was dissolved in 100% DMSO and stored in a desiccated container at -20°C. Cyclosporin A and actinomycin D (Sigma-Aldrich, St. Louis, MO) were dissolved in 100% DMSO and stored at 4°C and -20°C, respectively. LLCPK1 and HeLa cells were purchased from ATCC. HEK293T cells were obtained from the Gladstone Institute of Virology and Immunology (San Francisco, CA). Murine IgG2a MRK16 antibody was obtained from Kamiya Biomedical Co. (Seattle, WA, USA) and goat anti-mouse IgG allophycocyanin (APC) from Invitrogen. Cell culture media and reagents were purchased from the UCSF cell culture facility (San Francisco, CA).

3.2.2 Cell Culture

LLCPK1 cells were maintained in M-199 medium supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293T and HeLa cells required EMEM containing 10% FBS, 1% nonessential amino acids, 0.11 μg/mL sodium pyruvate, and 1% penicillin/streptomycin. Growth media for HEK293 Flp-In cells contained DME-21 supplemented with 10% FBS, 1% penicillin/streptomycin and 100 μg/mL zeocin. All cell lines were passaged every 2-4 days in T75 flasks that were kept in 5% CO_2 at 37° C.

3.2.3 Identification and Haplotype Analysis of ABCB1 3'-UTR Variants

Genomic DNA from 247 human samples from the Coriell Institute were used to identify 3'-UTR polymorphisms in *ABCB1*. Direct sequencing of the 3'-UTR was performed with forward and reverse primers (5'-GGTGTTTCAGAATGGCAGAGTC-3' and 5'-CTGCTTAACCATTCCCACAAAA-3', respectively) based on previously reported methods [108, 198]. Haplotypes were determined using a Bayesian inference

algorithm as described previously [226] and included known *ABCB1* coding and intronic variants from the same samples [108].

3.2.4 ABCB1 plasmids

Full-length ABCB1 cDNA was subcloned into pcDNA5/FRT and used as a template to create 3'-UTR variant plasmids. The +193A>G variant was already present and was changed back to reference. Mutagenesis primers for +89A>T, +146G>A and reversion of +193A>G are listed in Table 3.1. The QuikChange Site-directed Mutagenesis Kit (Stratagene) with *Pfu* turbo polymerase was used (according to the manufacturer's protocol) to introduce the desired variants. Direct sequencing verified the base changes.

Table 3.1
Site-directed mutagenesis primers for *ABCB1* reference and 3'-UTR variants

Base change	Mutagenesis primers[a]	
+89A>T	Forward	5'-AACACTTACAGAATTTTGAAGAGGTATCTGT
	Reverse	5'-ACAGATACCCTCTTCAAAATTCTGTAAGTGTT
+146G>A	Forward	5'-GTCTTCAGAGACTTCATAATTAAAGGAACAG
	Reverse	5'-CTGTTCCTTTAATTATGAAGTCTCTGAAGAC
+193G>A	Forward	5'-AAGTGGAGAGAAATCATAGTTTAAACTGCAT
	Reverse	5'-ATGCAGTTTAAACTATGATTTCTCTCCACTT

[a] In each primer the SNP position is underlined

3.2.5 Sequence Alignment of Mammalian 3'-UTRs

The 3'-UTR sequence immediately after the TGA stop codon was taken from the following GenBank accession numbers: NM_000927.3 (human), NM_011076.1 (mouse), NM_133401.1 (rat) and AY582534.1 (rhesus monkey). The UCSC genome browser

(genome.ucsc.edu) helped identify the correct 3' end point for each 3'-UTR. Clustal W (version 1.83) was used to align the 3'-UTRs of human, rhesus monkey, mouse and rat.

3.2.6 Identification of 3'-UTR Sequence Motifs

The internet-based UTRscan program (www.ba.itb.cnr.it/UTR) searches for 5'- and 3'-UTR degenerative sequence elements deposited in the UTRdb database [227]. The 380 bp region of the human *ABCB1* 3'-UTR was queried with UTRscan to identify possible sequence motifs known to bind regulatory proteins.

3.2.7 Predicted mRNA Secondary Structures

A segment of approximately 130 bp encompassing each 3'-UTR variant was analyzed by MFOLD (bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). The sequence between the variants in each haplotype pair (+21/+169, +77/+146 and +89/+193), plus 30-40 base pairs 5' and 3' of the region were used for MFOLD analysis. A qualitative analysis of the predicted structures determined whether or not a valid comparison could be made between the reference and variant alleles.

3.2.8 ABCB1 mRNA Time Course in Transiently Transfected HEK293T and HeLa Cells

Time course experiments in HEK293T (n = 1) and HeLa cells (n = 1) were performed in triplicate for each time point. Cells were seeded at 1.8 x 10^5 cells/well (HEK293T) or 1.2 x 10^5 cells/well (HeLa) in 12-well plates and allowed to grow for 24 hours. Cells were transfected at a confluency of 70-85% with 1.35 μg *ABCB1* reference plasmid DNA and 3.4 μL Lipofectamine 2000 in a final well volume of 1 mL. At time points after transfection, cells were harvested over a 72-hour period for HEK293T cells or a 48-hour period for HeLa cells in order to isolate total RNA. RNA was quantitated

using UV spectrophotometry (NanoDrop Technologies, Wilmington, DE) for each sample and equal amounts of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. TaqMan primers and probe for *ABCB1* were designed using Primer Express (Applied Biosystems, Foster City, CA) with help from Dr. David Ginzinger (UCSF Cancer Center). Reactions were run on an ABI Prism 7700 and cycling conditions were: 12 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The threshold limit was set so that it intersected all samples during the log-linear phase of amplification and the corresponding C_T values for each set of triplicates were averaged. The averaged C_T values were then normalized to the t = 5 h (HEK293T cells) time point using the equation for percent mRNA remaining:

% remaining = $(2^{(C_{T,\ t=5} - C_{T,\ t=x})}) * 100$, where x equals times after t = 5 in hours.

A similar equation was used for HeLa cells where percent mRNA remaining was expressed relative to 4 hours.

3.2.9 ABCB1 mRNA Half-life in Transiently Transfected HEK293T Cells

Cells were seeded and transfected with ABCB1 reference plasmid as described above with triplicate samples for each time point. Growth medium containing 15 μg/mL actinomycin D was added either 12 or 24 hours after transfection to begin the mRNA half-life experiment. Total RNA was isolated 0-24 hours after actinomycin D exposure using Trizol and quantitated with UV spectrophotometry (Nanodrop Technologies). Reverse transcription and TaqMan PCR were performed as above; the triplicate C_T values were averaged for each time point and then normalized to the t = 0 time point using the equation for percent mRNA remaining. The resulting percent of mRNA

remaining was plotted versus time and a decay slope (*k*) was determined using logarithmic data fitting (Excel). The half-life was calculated from the equation:

$$t_{1/2} = \ln 2 / k.$$

3.2.10 LLCPK1 FRT Stable Cell Lines

LLCPK1 cells were seeded at 3 x 10^5 cells/well in 6-well plates in medium without antibiotics. One day later cells were transfected with 4 μg pFRT/LacZeo plasmid and 6 μL Lipofectamine 2000 in a total well volume of 2 mL. Fresh medium was added ~6 hours after transfection. The following day cells from each well were split into six new wells and 2-3 hours later medium containing 200 μg/mL hygromycin was added. Drug selection lasted 10-14 days and fresh hygromycin media was added every 2-3 days. Surviving cell colonies were seeded in 96-well plates at a concentration of 1 cell/well in 100 μL hygromycin media in order to establish FRT clones from individual cells. Propagation of cells continued with increasing well sizes until there were enough cells to assay for stable integration of the pFRT/LacZeo plasmid.

Genomic DNA was isolated from parental LLCPK1 and LLCPK1 FRT colonies using a QIAamp DNA mini kit (Qiagen) following the manufacturer's protocol. PCR was performed on the genomic DNA using forward (5'-ACACTACGTCTGAACGTCGAA-3') and reverse (5'-ATGATGCTCGTGACGGTTAA-3') primers specific for the LacZ gene. The 150 bp PCR amplicon was visualized by gel electrophoresis and ethidum bromide staining. Southern blot analysis of LLCPK1 FRT genomic DNA was performed as described previously [228]. Briefly, DNA was digested with either *Xba*I or *Hind*III restriction enzymes and separated on an agarose gel by electrophoresis. The DNA gel was

transferred to a nitrocellulose membrane and then hybridized with a ^{32}P-labeled probe made from the above LacZ PCR amplicon. A phosphor screen collected the radioactive emissions and was imaged using a Storm Molecular Imager (GMI, Ramsey, MN).

LLCPK1 FRT clones that were positive for LacZ were assayed for β-galactosidase activity according to the manufacturer's instructions (Promega). Briefly, cells were grown to confluency in 24-well plates, washed twice with PBS and 150 µL of 1X reporter lysis buffer was added. Cells were lysed by pipetting up and down and then transferred to a 1.5 mL tube on ice. After 10-15 seconds of vortexing, the lysate was centrifuged at 16000x*g* for 2 minutes at 4°C. The supernatant was transferred to a fresh tube and stored at -80°C. All lysate samples were thawed and 30 µL were added to a 96-well plate containing 20 µL 1X reporter lysis buffer, followed by 50 µL Assay 2X buffer. The mixture was incubated at 37°C for 1 hour and then 150 µL of 1 M sodium carbonate was added to stop the reaction. The absorbance at 420 nm was read for each sample using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

3.2.11 ABCB1 *Flp293 Stable Cell Lines*

HEK293 Flp-In cells (Flp293) were seeded at 5 x 10^5 cells/well in 6-well plates in medium without antibiotics. One day later cells were transfected with 3.6 µg p0G44, 0.4 µg ABCB1/pcDNA5/FRT and 6 µL lipofectamine 2000 in a total well volume of 2 mL. Fresh medium was added ~5 hours after transfection. The following day cells were split into six new wells and 2-3 hours later medium containing 75 µg/mL hygromycin was added. Drug selection lasted 10-14 days and fresh hygromycin media was added every 2-3 days. Surviving cell colonies were propagated and screened for surface P-gp expression by flow cytometry (described below). P-gp positive clones were further

screened for ABCB1 mRNA expression using TaqMan quantitative real-time PCR as described for the half-life assays.

3.2.12 Detection of P-gp Surface Expression

Flow cytometry was used to measure the P-gp surface expression of *ABCB1* LLCPK1 and Flp293 stable clones and cell lines. Samples were assayed using a suspension of 5 x 10^5 cells initially incubated with 75 µL of PBS containing 6.67 µg/mL MRK16 primary antibody on ice for 30 minutes. After two wash steps with cold PBS, 60 µL of PBS containing 2.5 µg/mL of APC-labeled secondary antibody was used to resuspend the cells. Following incubation on ice in the dark for 25 minutes the cells were washed twice with cold PBS, resuspended in 250-300 µL PBS and transferred into 5 mL tubes on ice.

Cell samples were run on a dual-laser FACScalibur machine (Becton-Dickinson, San Jose, CA) with excitation wavelengths at 488 and 635 nM controlled by CellQuest software (Becton-Dickinson, San Jose, CA). Emission filters at 530 nM (FL1) detected calcein and >670 nM (FL4) detected APC fluorescence. A total of 15,000 events were counted and FlowJo software (Treestar, Ashland, OR) was used to analyze the flow cytometry data. Forward- and side-scatter analysis established the R1 gate for the healthy, single-cell population. Calcein (FL1) and/or APC (FL4) fluorescence was determined from cells in the R1 gate.

3.2.13 Calcein-AM Accumulation in ABCB1 *LLCPK1 and Flp293 Stable Cell Lines*

ABCB1 LLCPK1 +193 and *ABCB1* Flp293 stable cell lines were seeded in 96-well plates in suspension at a concentration of 5 x 10^5 cells/well in 100 µL PBS and

centrifuged at 150x*g* for 3 minutes. For *ABCB1* LLCPK1 +193, the PBS was aspirated and 100 µL 0.25-2 µM calcein-AM ± 2.5 µM cyclosporin A or ± 1 µM GF120918 in PBS was added to resuspend the cells; for *ABCB1* Flp293 stable cell lines, the PBS was aspirated and 100 µL 1 µM calcein-AM ± 10 µM cyclosporin A was added to resuspend the cells. Samples were allowed to incubate for 45 minutes in the dark at 37° C with a brief agitation period 25 minutes after substrate addition. Cells were washed twice in cold PBS and then transferred into 5 mL tubes in 250-300 µL PBS for flow cytometry analysis.

3.2.14 ABCB1 mRNA half-life in Flp293 Stable Cell Lines

ABCB1 reference, +89A>T, +146G>A and +193A>G Flp293 cell lines were seeded in 12-well plates at 6 x 10^5 cells/well and allowed to grow for 24 hours. For each cell line, triplicate wells were set up for each time point. Medium containing 7.5 µg/mL actinomycin D was added to the wells and cells were harvested over a 24 hour period. Total RNA isolation, reverse transcription and TaqMan PCR were carried out as described above. ABCB1 mRNA levels were averaged for each time point and normalized to t = 0 using the above equation to calculate percent mRNA remaining. The decay slope (*k*) for mRNA versus time was determined using logarithmic data fitting (Excel). The half-life was calculated from the equation $t_{1/2} = \ln 2 / k$. The experiment was carried out in triplicate.

3.2.15 Statistical Analysis

Half-life values from three experiments for *ABCB1* reference, +89A>T, +146G>A and +193A>G Flp293 cell lines were averaged and the standard deviation was calculated.

Student's *t*-test was used to determine if the mean half-lives for the variants were different than reference with an α value of 0.05.

3.3 Results

3.3.1 Identification of ABCB1 3'-UTR Variants and Associated Haplotypes

The 3'-UTR of *ABCB1* was sequenced in 247 DNA samples from ethnically diverse populations from the Coriell Institute. There were nine variant sites identified in this 380 bp region with allele frequencies ranging from 0.5 to 14% (Table 3.2). All of the variants were found in the African American population except for +355T>C, and only three variant positions (+89, +169 and +193) were found in the Caucasian population. There is a four base pair deletion starting at +77 (ACTT>-) and there is an eight base pair insertion starting at +169 (G>GACAGAGA) with allele frequencies of 7% and 9.5% in African Americans, respectively. Only +193A>G was also found in the smaller DNA sample collections from Asian American and Mexican American populations, making it a cosmopolitan SNP.

Polymorphisms in the entire *ABCB1* gene were constructed into haplotypes using PHASE analysis and Clustal W. There were 28 distinct haplotypes containing 3'-UTR variants distributed among 60, 28, 10 and 1 chromosome(s) for African Americans, Caucasian Americans, Asian Americans and Mexican Americans, respectively (Figure 3.2). Some of the 3'-UTR polymorphisms are almost always found in pairs, such as +21 and +169, and +89 and +193. The deletion variant at +77 is always found with SNP +146 and the nonsynonymous variant in exon 26 (3241T>A, Ser1141Thr). Only one haplotype was inferred for +316G>A and +355T>C, but three out of four haplotypes for

Table 3.2
Alleles, frequencies and sequence similarities of *ABCB1* 3'-UTR polymorphisms

Position[a]	Alleles	Allele frequency (%)				# of species aligned[c]
		AA[b] n=100	CA n=100	AS n=30	ME n=10	
+21	T>C	8	0	0	0	3/3
+77	ACTT>-	7	0	0	5	N/A[d]
+89	A>T	11	6.5	0	0	3/3
+146	G>A	9	0	0	5	3/3
+169	G>GACAGAGA	9.5	0.5	0	0	1/3
+193	A>G	13	14	15	5	3/3
+252	A>C	4.5	0	0	0	3/3
+316	G>A	3	0	0	0	2/3
+355	T>C	0	0	1.7	0	3/3

[a] SNP location determined from first base after TGA stop codon
[b] AA = African Americans, CA = Caucasian Americans, AS = Asian Americans and ME = Mexican Americans
[c] Refers to the number of sequences from monkey, rat and mouse that have the same base as human at each SNP position.
[d] See sequence alignment (Figure 3.3).

+252A>C were in linkage disequilibrium with the previously mentioned African American specific nonsynonymous SNP (3241T>A, Ser1141Thr).

3.3.2 Genetic Analysis of the ABCB1 3'-UTR

Little research has been done on the 3'-UTR of *ABCB1* so predictive measures may be helpful to identify which SNPs may be important for mRNA stability. One such approach is to carry out sequence alignments with other mammalian species. Clustal W was used to align the 3'-UTR of human *ABCB1* with corresponding sequences from mouse, rat and rhesus monkey (Figure 3.3). The sequences ranged in length from 350 to 401 base pairs and 175 base positions were 100% identical when compared to the human sequence. Using the human sequence as a reference, the sequence similarity of these four

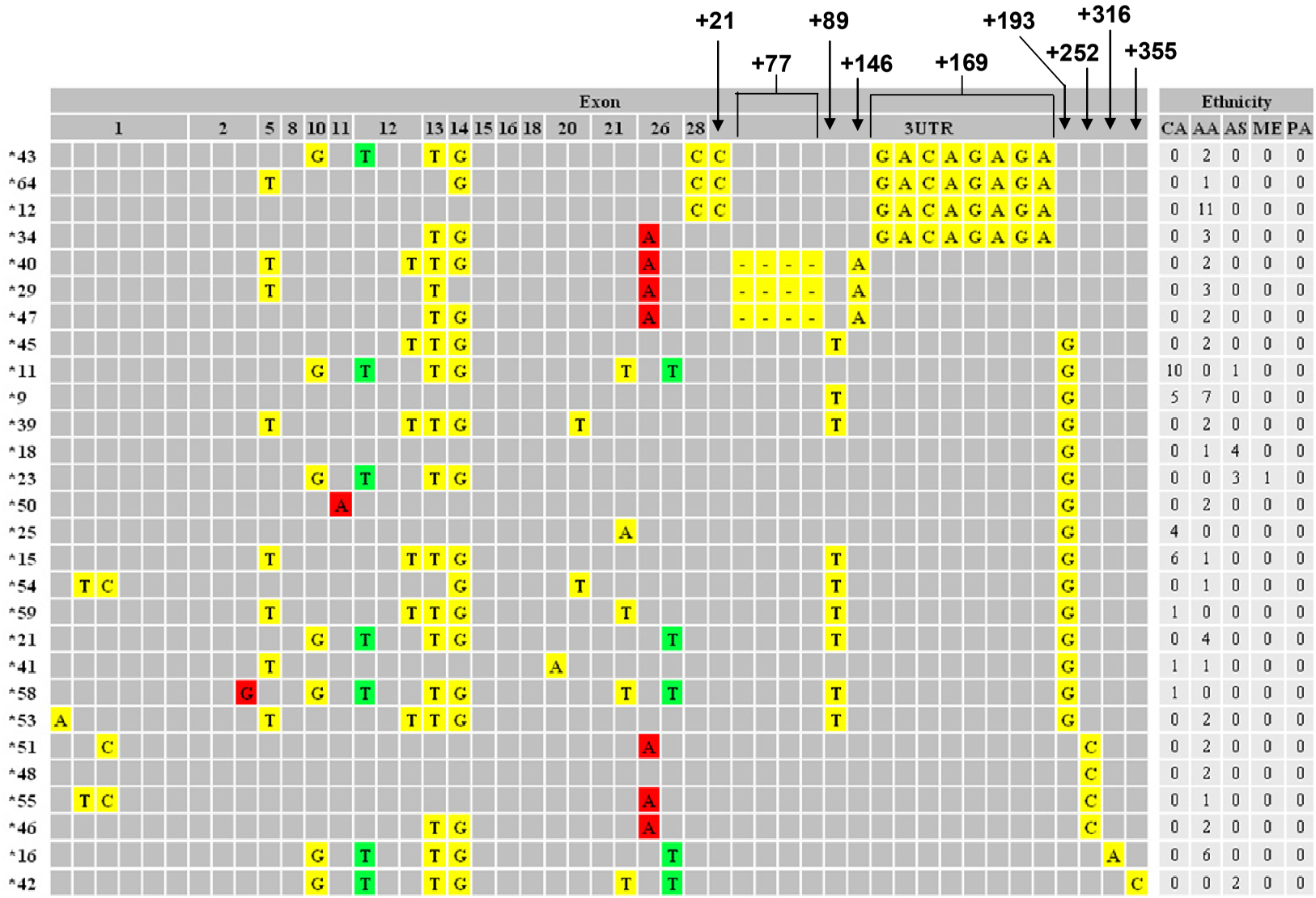

+21
+89
+193
+316
+77
+146
+169
+252
+355
Exon
1
2
5
8
10
11
12
13
14
15
16
18
20
21
26
28
3UTR
Ethnicity
CA AA AS ME PA
*43 0 2 0 0 0
*64 0 1 0 0 0
*12 0 11 0 0 0
*34 0 3 0 0 0
*40 0 2 0 0 0
*29 0 3 0 0 0
*47 0 2 0 0 0
*45 0 2 0 0 0
*11 10 0 1 0 0
*9 5 7 0 0 0
*39 0 2 0 0 0
*18 0 1 4 0 0
*23 0 0 3 1 0
*50 0 2 0 0 0
*25 4 0 0 0 0
*15 6 1 0 0 0
*54 0 1 0 0 0
*59 1 0 0 0 0
*21 0 4 0 0 0
*41 1 1 0 0 0
*58 1 0 0 0 0
*53 0 2 0 0 0
*51 0 2 0 0 0
*48 0 2 0 0 0
*55 0 1 0 0 0
*46 0 2 0 0 0
*16 0 6 0 0 0
*42 0 0 2 0 0

Figure 3.2. *ABCB1* haplotypes that contain 3'-UTR variants. Inferred haplotypes for *ABCB1* coding region, 5' and 3' UTRs variants are organized based on the presence of 3'-UTR variants and arbitrarily numbered. Nonsynonymous (dark squares) and synonymous (light squares) SNPs are organized by exon position and represented by the nucleotide base of the variant allele. The number of chromosomes each haplotype is found on is listed according to ethnicity.

species is 46% and it increases to 90% if only human and rhesus monkey are compared. There are six 3'-UTR variant sites that are 100% identical in the three other species: +21, +89, +146, +193, +252 and +355. The other three variant sites are only conserved in rhesus monkey. The multiple species alignment also revealed contiguous sections of 100% conservation that could indicate functional importance, so the *ABCB1* 3'-UTR sequence was analyzed against a database of known 5' and 3'-UTR sequence elements [227]. Surprisingly, an IRES element, which enables translation initiation independent of the 5' cap, was found between +282 and +380. However, IRES elements generally are located in the 5'-UTR [229]. No other potential RNA binding sites were identified using UTRscan.

3.3.3 Predicted mRNA Secondary Structures of ABCB1 3'-UTR Variants

We used an internet-based program called MFOLD to predict how *ABCB1* 3'-UTR variants affected mRNA secondary structure. The MFOLD program determines mRNA secondary structure based on multiple parameters, such as lowest free energy and stem loop formation [230]. In general, the number of bases and the number of different structures predicted are directly proportional. Approximately 130 base pair regions

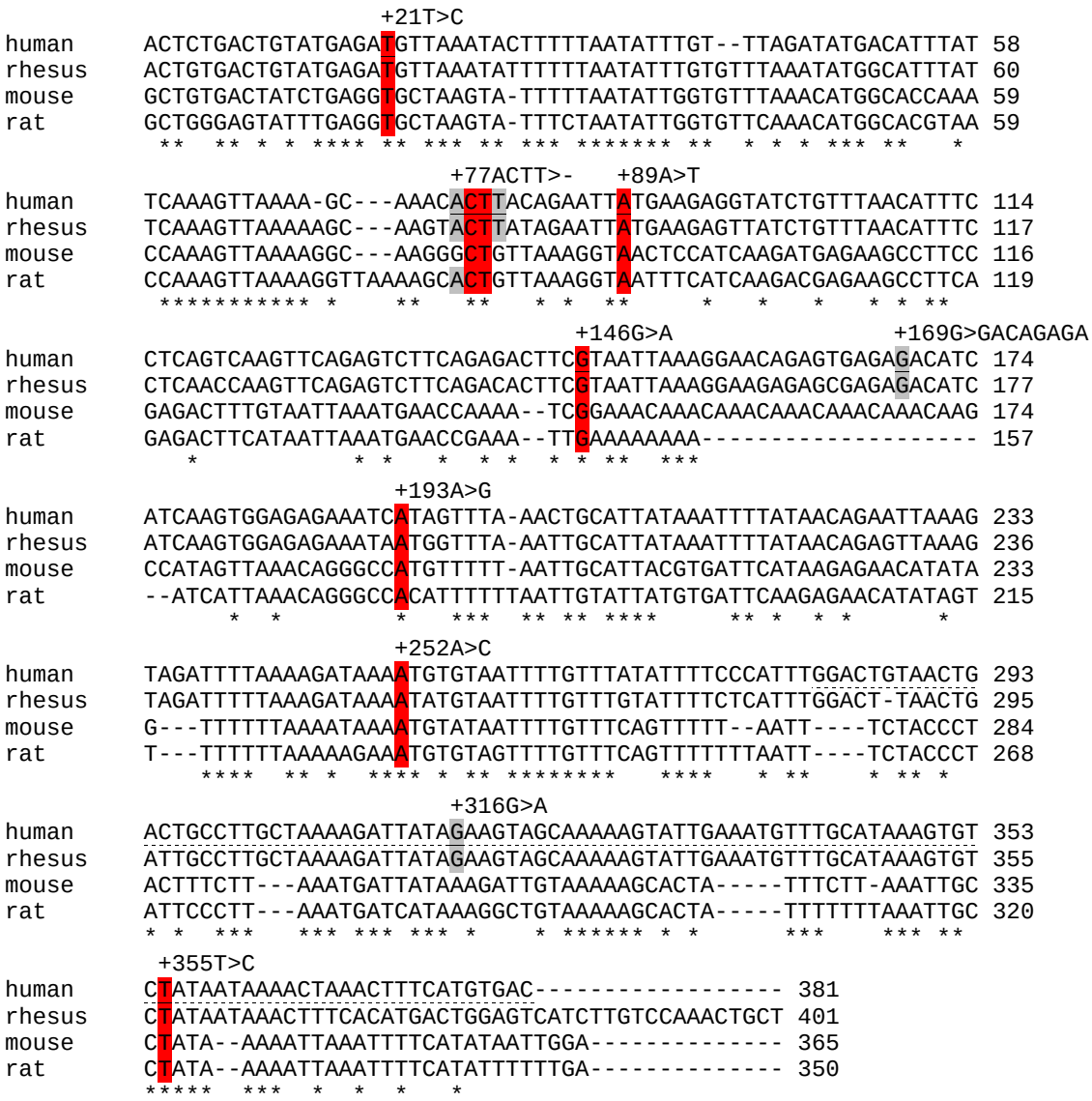
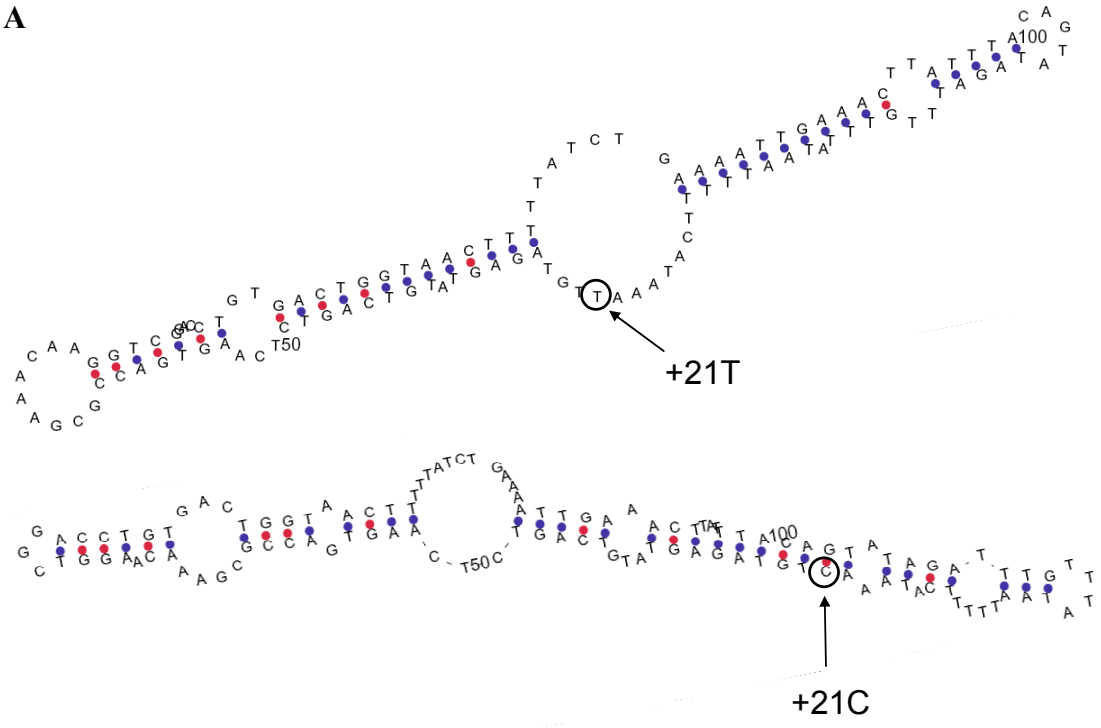


Figure 3.3. *ABCB1* 3'-UTR sequence alignment with selected mammalian species. Clustal W was used to align the *ABCB1* 3'-UTRs of human, rhesus monkey, rat and mouse. Variant positions are designated in the human sequence (underlined) and reference allele homology for all (dark highlight) or some (light highlight) species is shown. Complete homology for each base position is marked (*). UTRscan predicted an IRES element from +282 to +381 and it is shown in the human sequence with a dashed underline.

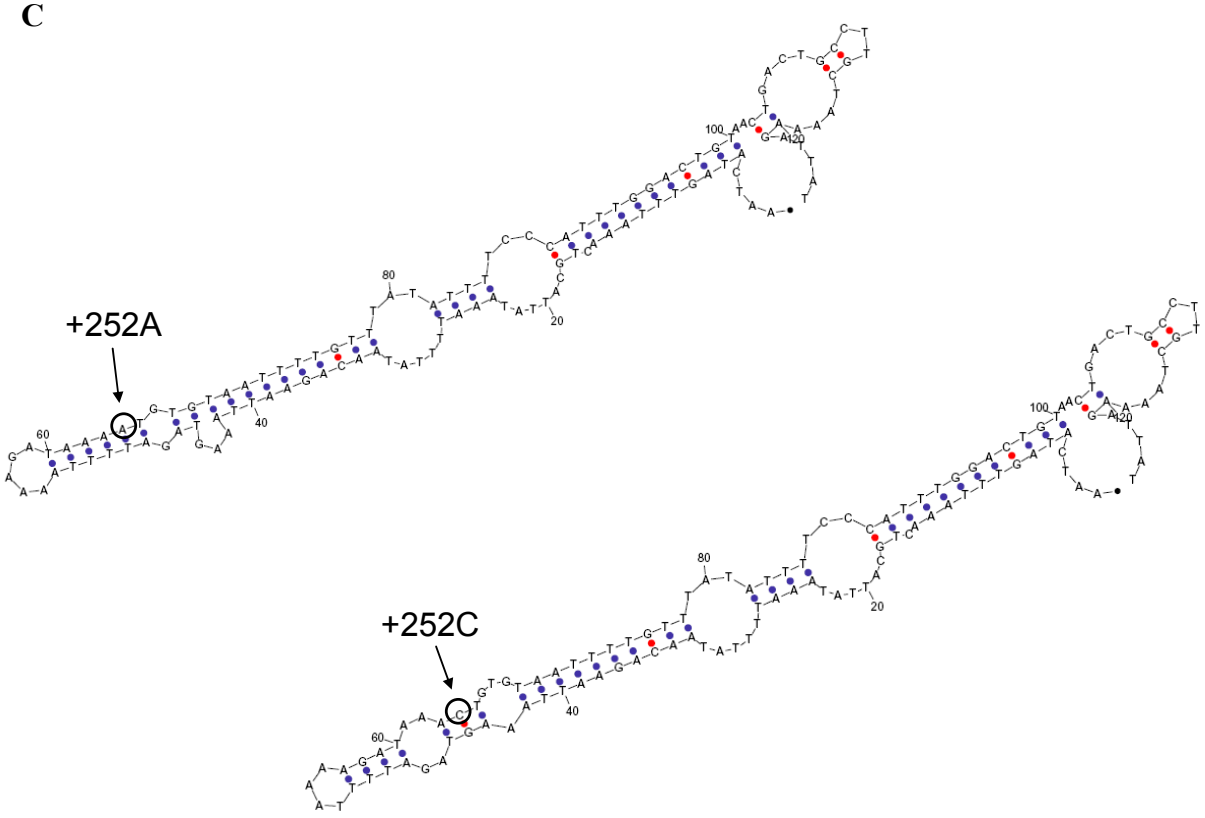
centered on each polymorphism were used since this minimized the number of predicted structures while maximizing the most probable nucleotide-nucleotide interactions. The predictions for the 3'-UTR variants were considered viable based on a qualitative analysis of the reference and variant structures. If there were not enough reference structures that looked similar then any changes seen in the variant structures could not be attributed to the polymorphism. The variant alleles for *ABCB1* +21T>C, +169G>GACAGAGA and +252A>C altered localized stem-loop or hairpin formation but no other regions were changed (Figures 3.4A, B and C). The +77ACTT>-/+146G>A haplotype caused a bigger change that affected almost half of the predicted structure (Figure 3.4D). *ABCB1* 3'-UTR variants that did not alter mRNA secondary structure are summarized in Table 3.3 and +89A>T is shown as a representative example (Figure 3.4E). Inconclusive results for +316G>A, +355T>C and the +21T>C/+169G>GACAGAGA and +89A>T/+193A>G haplotypes were due to conflicting and incomparable structures for the reference and variant alleles (Table 3.3).

3.3.4 Assessment of ABCB1 Transient Expression for mRNA Stability Experiments

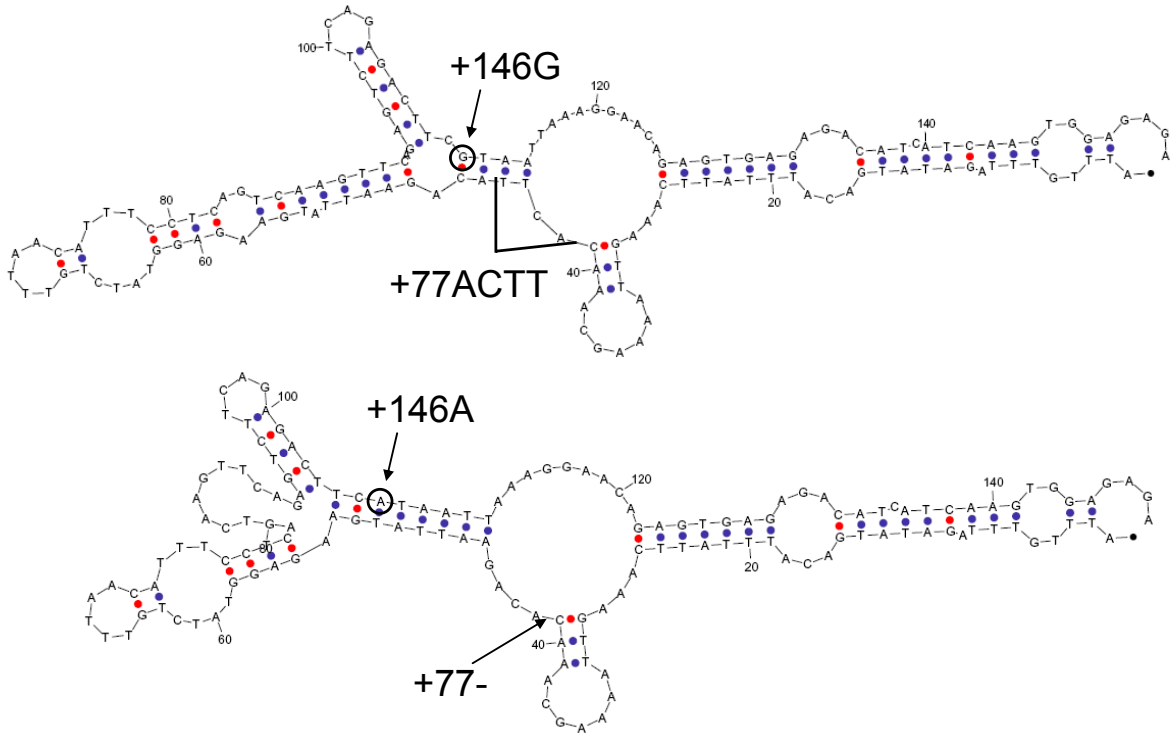
ABCB1 reference and 3'-UTR variants had to be expressed in a chosen cell line that would allow for accurate and reproducible mRNA measurements. The first method tried was transient expression of the reference and variant plasmids in HEK293T and HeLa cells. Both of these cell lines survive well during transfection and have relatively high transfection efficiency. Measurement of mRNA half-life requires that mRNA levels are at a steady-state level before transcription is stopped, otherwise one cannot determine the true rate of decay. Transient transfection of ABCB1 reference plasmid was tested in HEK293T and HeLa cells, and ABCB1 mRNA levels were measured over 48-72 hours.


A
+21T
+21C
B
+169G
+169GACAGAGA

C



D



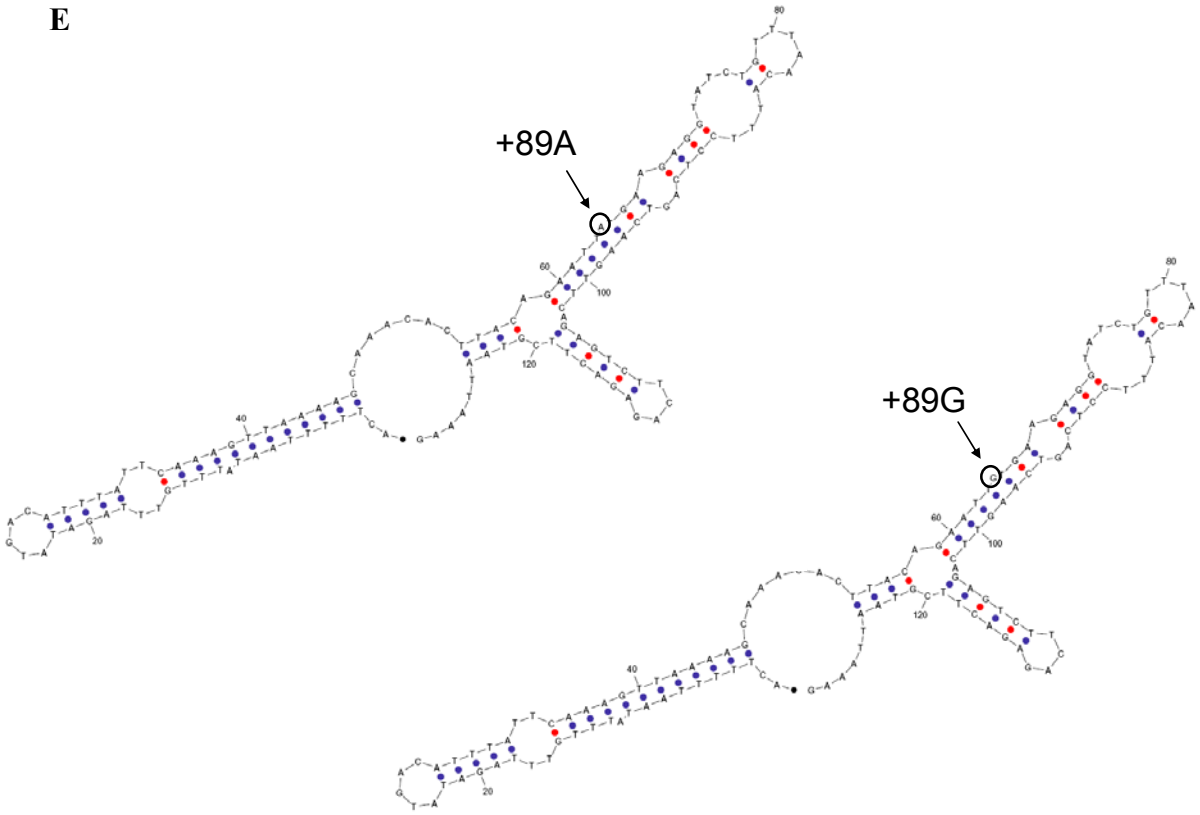


Figure 3.4. *ABCB1* 3'-UTR polymorphisms that change mRNA secondary structure. MFOLD predictions for +21T>C (A), +169G>GACAGAGA (B), +252A>C (C) and the +77ACTT>-/+146G>A haplotype (D) suggest these polymorphisms alter mRNA secondary structure. The +89A>T variant is shown as a representative example with no changes in secondary structure (E). The reference (top) and variant (bottom) sequences are shown.

Table 3.3
Effects of *ABCB1* 3'-UTR polymorphisms on predicted mRNA structure

Variant or haplotype	Secondary structure change?
+21T>C	yes
+89A>T	no
+146G>A	no
+169G>GACAGAGA	yes
+193A>G	no
+252A>C	yes
+316G>A	inconclusive
+355T>C	inconclusive
+21T>C and +169G>GACAGAGA	inconclusive
+77ACTT>- and +146G>A	yes
+89A>T and +193A>G	inconclusive

Inconclusive results were due to conflicting and incomparable structures for the reference and variant alleles

A 72-hour time course was chosen for the HEK293T cells and at t = 0 the mRNA levels represent the endogenous expression of *ABCB1* (Figure 3.5A). At five hours post-transfection there is ~1000-fold increase in ABCB1 mRNA levels. ABCB1 mRNA expression peaks at 24 hours post-transfection and then decreases through the 72-hour time point. A similar pattern is seen in HeLa cells transfected with the ABCB1 plasmid (Figure 3.5B). There is a dramatic rise in mRNA levels only 4 hours after transfection with the peak expression occurring at 16 hours. The subsequent decrease in mRNA continues until the final time point at 48 hours post-transfection in HeLa cells. The rate of decay was linear after the peak expression time point with a calculated half-life of 12-15 hours for both transiently transfected HEK293T and HeLa cells.

The time course experiments suggested that in the absence of actinomycin D, mRNA degradation was dominant at any point 16-24 hours post-transfection. In

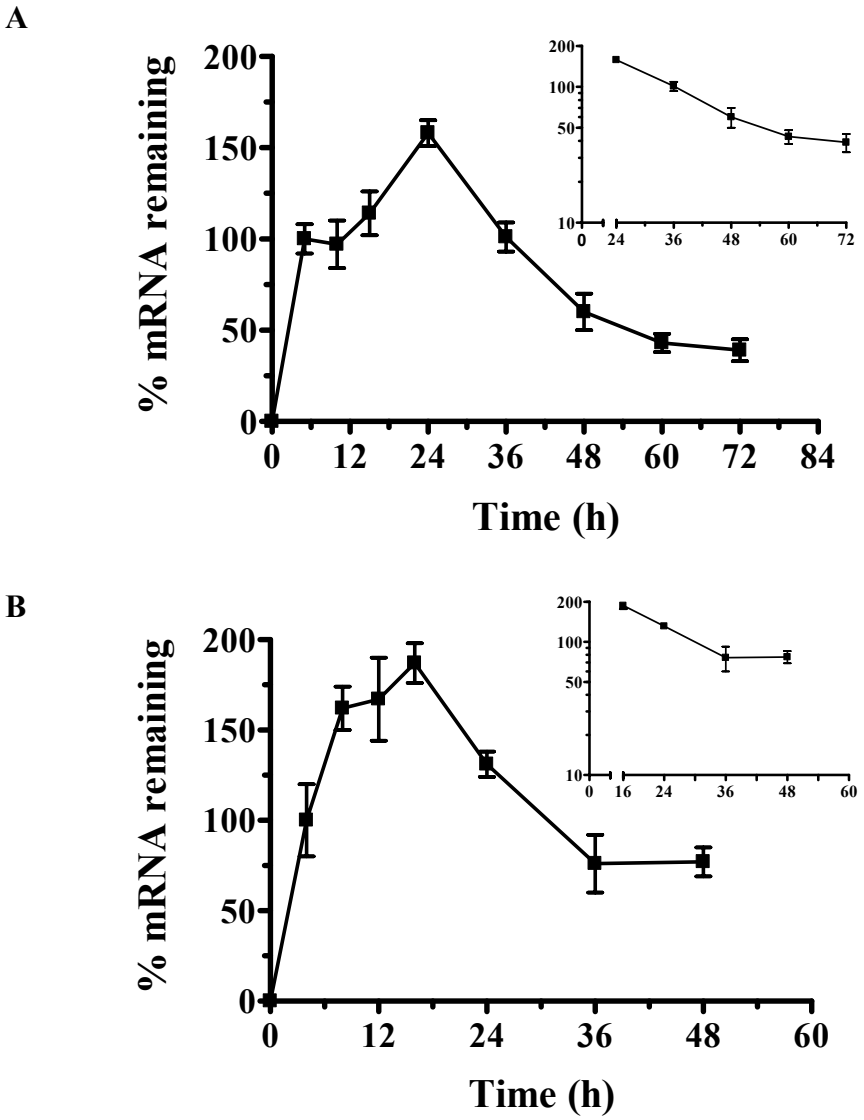


Figure 3.5. Time course of ABCB1 mRNA levels after transient transfection in HEK293T and HeLa cells. ABCB1 reference plasmid was transiently expressed in HEK293T (A) and HeLa (B) cells and mRNA levels were recorded over a 48-72 hour period. Expression levels are normalized to the first time point after t = 0 and represent ~1000-fold increase over background. Each point is the mean ± standard deviation from triplicate determinations. Figure insets are log-linear plots of the mRNA decay period.

accordance, half-life experiments in HEK293T cells conducted 24 hours after transfection showed that the half-life in the presence of actinomycin D was similar to that in its absence (Figure 3.6A). Without further information it is unclear whether this half-life reflects the true rate of degradation or a cellular response to overexpression. An alternative approach was taken to stop transcription at 12 hours post-transfection, which is before the peak in mRNA expression. The half-life data obtained from these experiments was not reproducible nor was there an obvious linear decay of ABCB1 mRNA (Figure 3.6B). These data do not support the use of a transient expression system for determining ABCB1 mRNA half-life.

3.3.5 Development of ABCB1 LLCPK1 Stable Cells for mRNA Stability Experiments

Stable expression of genes in mammalian cells provides a system where expression of mRNA and protein is constant. ABCB1 LLCPK1 stable cell lines were created using the Flp-In system, which is designed to express a single copy of a gene at the same transcriptional level, allowing us to eliminate transcription as a variable contributing to mRNA levels. Flp recombinase integrates exogenous DNA into a host genome assuming both contain an FRT (flp recombinase target) site. Mammalian cells do not have FRT sites so LLCPK1 cells possessing a single copy of the FRT site were created. This was accomplished using the pFRT/LacZeo plasmid that contains a zeocin drug-selection marker, the FRT site and the LacZ gene. Colonies that passed drug selection were screened by PCR for the presence of the LacZ gene, which also is not found in mammalian cells (Figure 3.7A). Genomic DNA from nine different LLCPK1 clones was analyzed by Southern Blot to determine how many FRT sites were present.

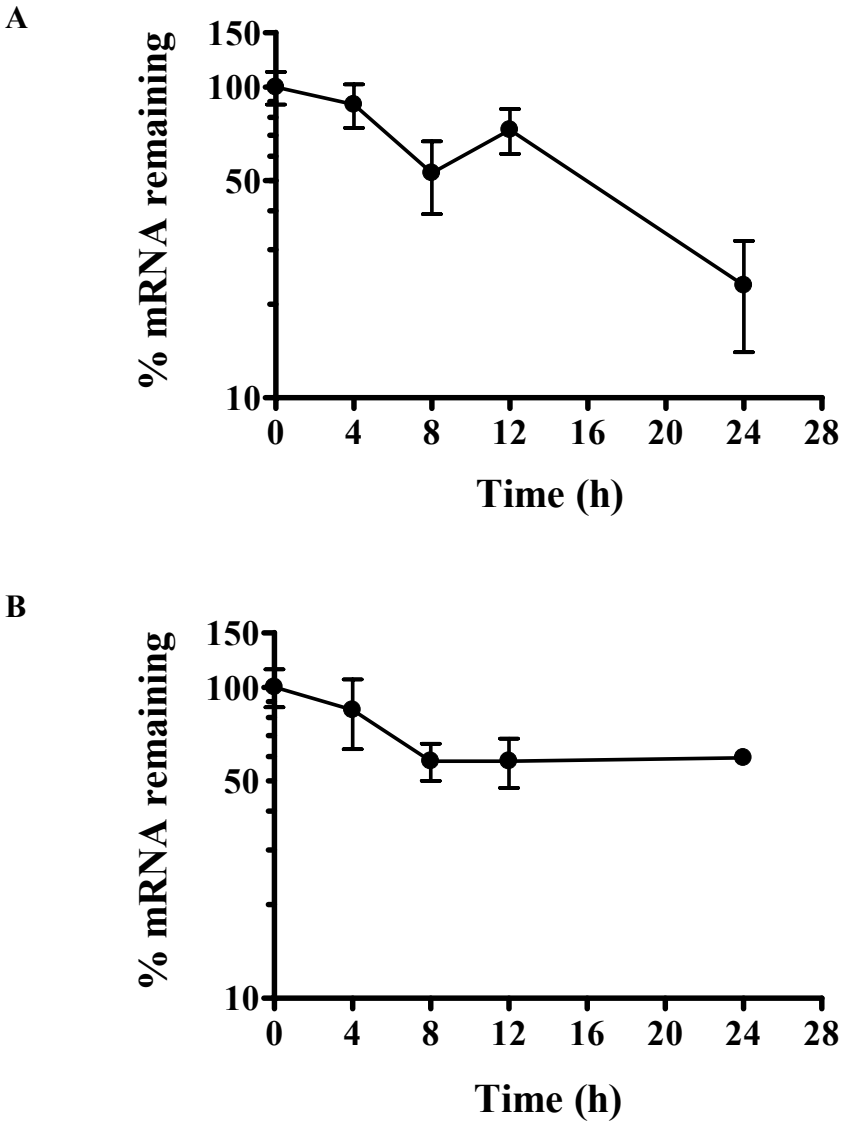


Figure 3.6. ABCB1 mRNA half-life in transiently transfected HEK293T cells. ABCB1 reference plasmid was transiently expressed in HEK293T cells and mRNA levels after actinomycin D exposure were determined 12 (A) and 24 (B) hours post-transfection. ABCB1 mRNA expression after transcription was stopped was normalized to the t = 0 time point. Each value represents the mean ± standard deviation from triplicate determinations.

Using either *Hind*III or *Xba*I to digest the genomic DNA, seven LLCPK1 FRT clones had a single hybridization band when probed with a radiolabeled LacZ oligonucleotide. Figure 3.7B shows five of the seven LLCPK1 FRT clones with single FRT sites. The seven clones were tested for β-galactosidase activity to indicate the transcriptional activity of the transfected gene. Clone I had one of the highest levels of β-galactosidase activity (Figure 3.7C), and its cellular morphology and growth characteristics best resembled LLCPK1 cells. As a result, clone I was chosen to be the parental cell line (LLCPK1 FRT) for the ABCB1 3'-UTR constructs.

LLCPK1 FRT cells were used to make ABCB1 stable cell lines expressing reference, +89A>T, +146G>A and +193A>G. These three 3'-UTR variants were chosen based upon their high allele frequencies and 100% evolutionary conservation (Table 3.2 and Figure 3.3). Each ABCB1/FRT plasmid was cotransfected with the pOG44 vector that would express the flp recombinase protein and effectively integrate ABCB1 into the FRT site. Stable integration of the ABCB1/FRT plasmid would destroy the zeocin resistance originally inferred by the pFRT/LacZeo vector and add resistance to hygromycin. Colonies surviving hygromycin exposure were tested for surface P-gp expression to screen for positive ABCB1 clones. P-gp surface expression measurements showed that only +193A>G had increased P-gp levels while reference, +89A>T and +146G>A were the same as LLCPK1 FRT (Figure 3.8). All attempts in making ABCB1 reference, +89A>T and +146G>A stable LLCPK1 cell lines were unsuccessful so a different cell line was tested.

The LLCPK1 ABCB1 +193 stable cell line was characterized for use in future experiments. TaqMan gene expression analysis did not detect anything in the LLCPK1

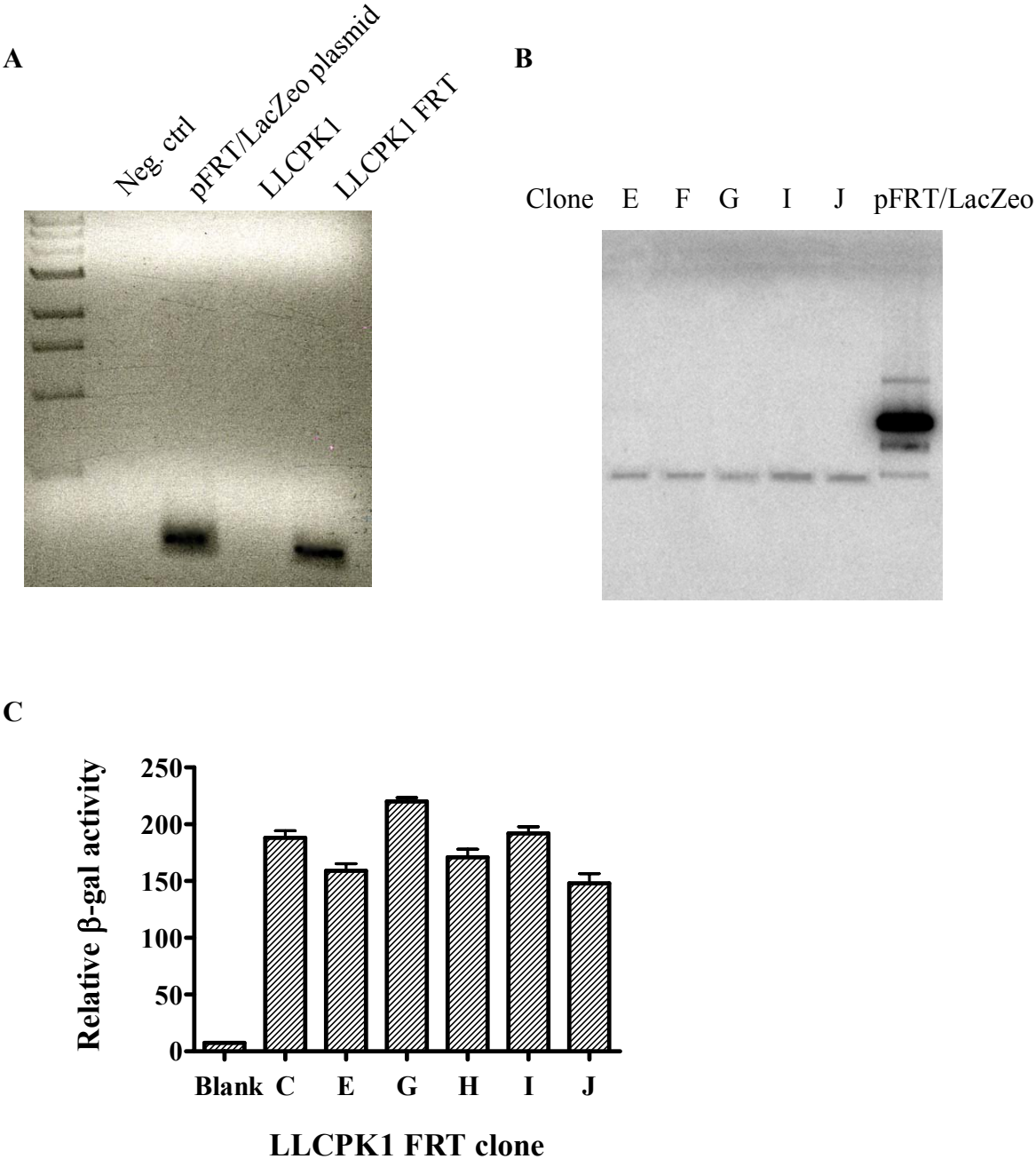


Figure 3.7. Verification of pFRT/LacZeo plasmid insertion in LLCPK1 FRT clones. Stable integration of the pFRT/LacZeo plasmid into the LLCPK1 genome was validated by detection of the LacZ gene and β-galactosidase activity. A LacZ amplicon detected via PCR was seen in the LLCPK1 FRT clones but not in LLCPK1 host cells (A). Southern blot analysis of five representative LLCPK1 FRT clones shows a single copy of the LacZ gene. The pFRT/LacZ plasmid was a positive control and the minor bands are

probably from different physical forms of the plasmid (B). Transcriptional activity of the inserted pFRT/LacZeo plasmid was determined using a β-galactosidase assay of cell lysates from six representative clones (C). Each bar represents the mean ± standard deviation from triplicate samples.

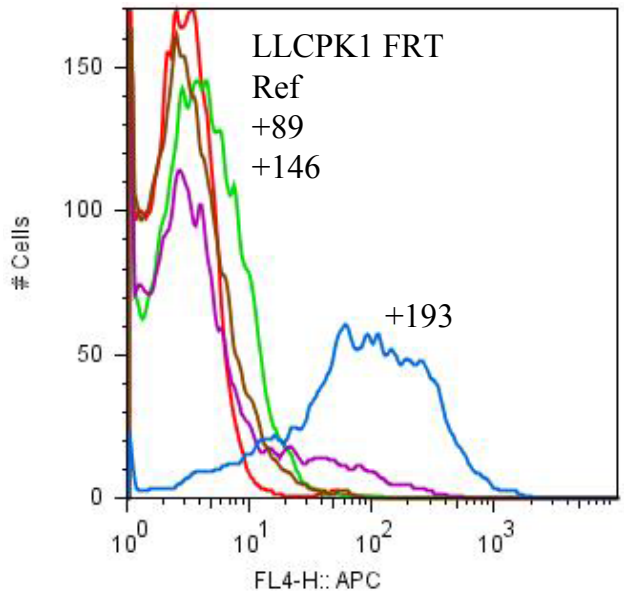


Figure 3.8. P-gp surface expression in *ABCB1* reference and variant LLCPK1 stable clones. *ABCB1* LLCPK1 stable clones were stained for P-gp expression using a primary MRK16 antibody and a secondary APC antibody. The representative histogram for the LLCPK1 FRT cell line, reference, +89A>T, +146G>A and +193A>G stable clones shows P-gp expression based on the intensity of APC fluorescence as read on the x-axis. Only the +193A>G clone showed increased P-gp expression relative to the LLCPK1 FRT cells.

FRT host cell line but the LLCPK1 ABCB1 +193 cell line had high levels of ABCB1 mRNA comparable to transiently transfected HEK293T cells (data not shown). The TaqMan assay is designed for human ABCB1 and apparently there was no cross reactivity with the porcine ABCB1 in LLCPK1 cells.

P-gp function was tested in the stable +193 cell line using the calcein-AM accumulation assay. Host LLCPK1 FRT cells displayed higher levels of calcein accumulation (0.25-2 μM) compared to LLCPK1 ABCB1 +193 cells, indicating the overexpression of functional human P-gp (Figure 3.9). In addition, the P-gp inhibitors cyclosporin A and GF120918 increased calcein levels in the +193 stable cell line.

3.3.6 Development and Characterization of ABCB1 Flp293 Stable Cells for mRNA Stability Experiments

The third attempt at developing a reliable ABCB1 mRNA expression system used the Flp-In system with commercially available host HEK293 FRT (Flp293) cells. ABCB1 reference and +146G>A stable cell lines were made by polyclonal selection of multiple colonies showing hygromycin resistance. All of the polyclones had two populations of surface P-gp expression that were either similar to host Flp293 cells or showed overexpression of P-gp. Selected polyclones for reference and +146G>A were sorted using flow cytometry to remove the non-P-gp expressing cells and the sorted cells had one population that overexpressed P-gp. The final cell lines selected for ABCB1 reference and +146G>A most closely resembled the cellular morphology and growth characteristics of the host Flp293 cell lines, and their ABCB1 mRNA and P-gp expression levels were dramatically increased 70- and 25-fold, respectively, over Flp293 cells (Figures 3.10A and B).

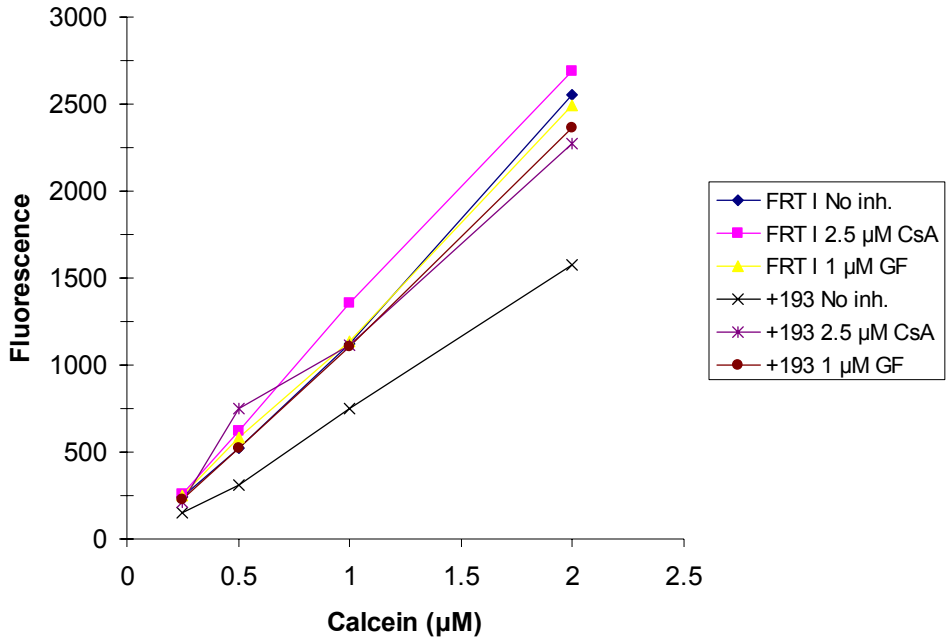


Figure 3.9. Calcein-AM accumulation in *ABCB1* LLCPK1 +193 stable cell line. P-gp function in the parental LLCPK1 FRT and the *ABCB1* LLCPK1 +193 stable cells line was determined with 0.25-2 µM calcein-AM in the absence or presence of two P-gp inhibitors (2.5 µM cyclosporin A and 1 µM GF120918). Flow cytometry was used to measure intracellular calcein in the samples. Each point represents the mean of duplicate samples.

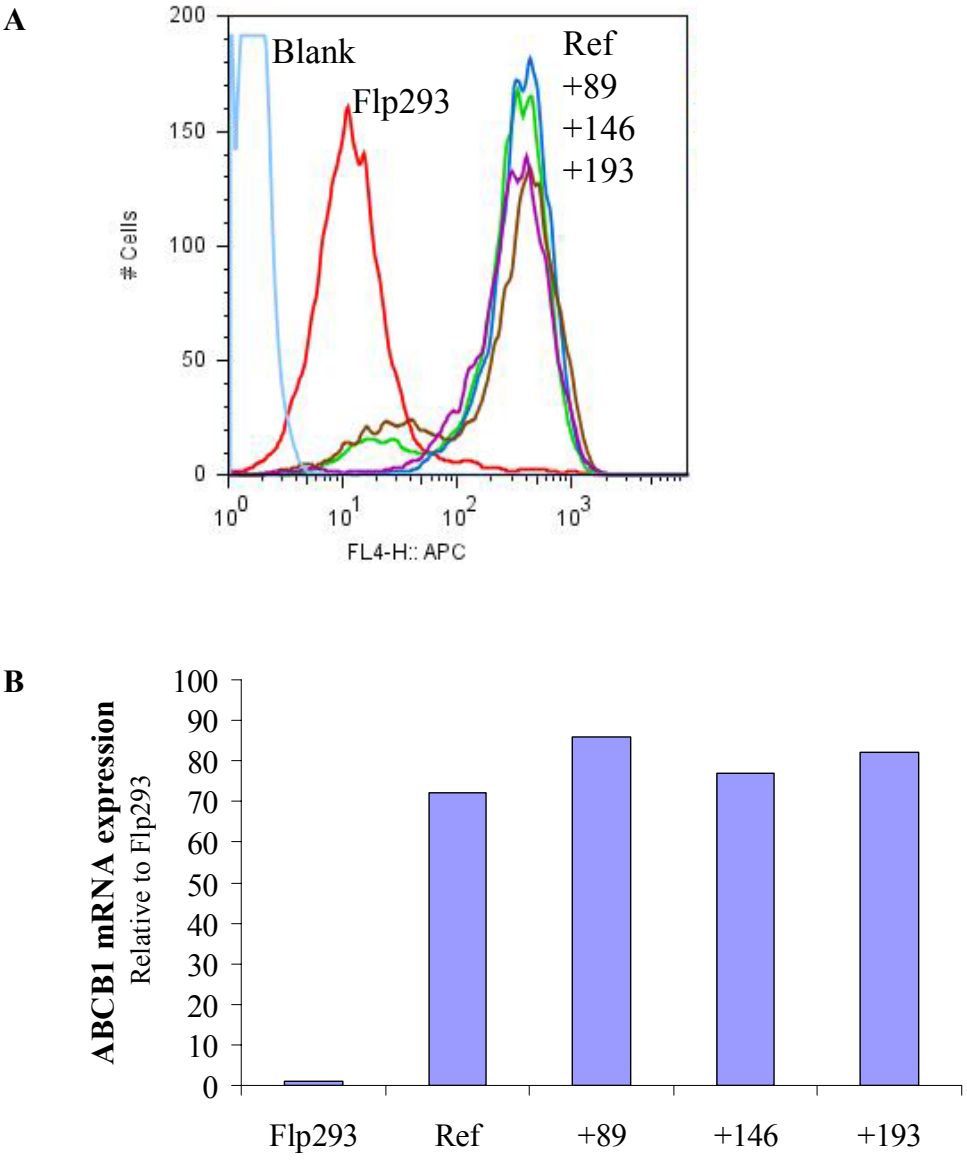


Figure 3.10. Characterization of *ABCB1* reference and variant Flp293 stable cell lines. Stable integration of *ABCB1* reference and variant plasmids in Flp293 cells was confirmed with surface P-gp expression and mRNA levels. *ABCB1* Flp293 stable cell lines were stained for P-gp expression using a primary MRK16 antibody and a secondary APC antibody as measured by flow cytometry (A). TaqMan quantitative PCR was used to compare ABCB1 mRNA levels of reference and variants to the parental Flp293 cell line. Each bar represents the fold change in ABCB1 mRNA compared to Flp293 cells (N=1; B).

Single colonies surviving hygromycin selection for ABCB1 +89A>T and +193A>G were individually propagated in order to minimize false positives. Clones were initially screened for P-gp surface expression and those that displayed 100% overexpression were tested for ABCB1 mRNA levels. The final cell lines for ABCB1 +89A>T and +193A>G that showed high mRNA and P-gp expression were chosen for their similarities to the host Flp293 cells. The four ABCB1 stable cell lines (reference, +89A>T, +146G>A and +193A>G) that were created had a ~25-fold increase in P-gp expression (Figure 3.10A) and at least a 70-fold increase in mRNA expression (Figure 3.10B).

The newly made ABCB1 stable cell lines were tested for P-gp function to insure that the cells were viable for future experiments. The accumulation of 1 μM calcein-AM in Flp293 cells was ~4-fold higher than accumulation for ABCB1 reference, +89A>T, +146G>A and +193A>G, indicating that the P-gp overexpressing cells have increased efflux (Figure 3.11A). Cyclosporin A increased calcein levels in the four ABCB1 stable cell lines to that of Flp293 cells (Figure 3.11B).

3.3.7 mRNA Half-life of ABCB1 3'UTR Variants in Flp293 Stable Cells

The mRNA stability of transcripts is commonly determined by measuring mRNA half-life. ABCB1 Flp293 reference, +89A>T, +146G>A and +193A>G stable cell lines were exposed to actinomycin D to stop transcription and the mRNA levels over a 24 hour period were normalized to the t = 0 point to convert the data into percent mRNA remaining for each cell line. The calculated half-life for ABCB1 reference was 9.4 ± 1.3 h (N=3) and was similar to that estimated for the 3'-UTR variants (N=3, Figure 3.12 and Table 3.4). As a negative control, the ABCB1 reference cell line was not exposed to

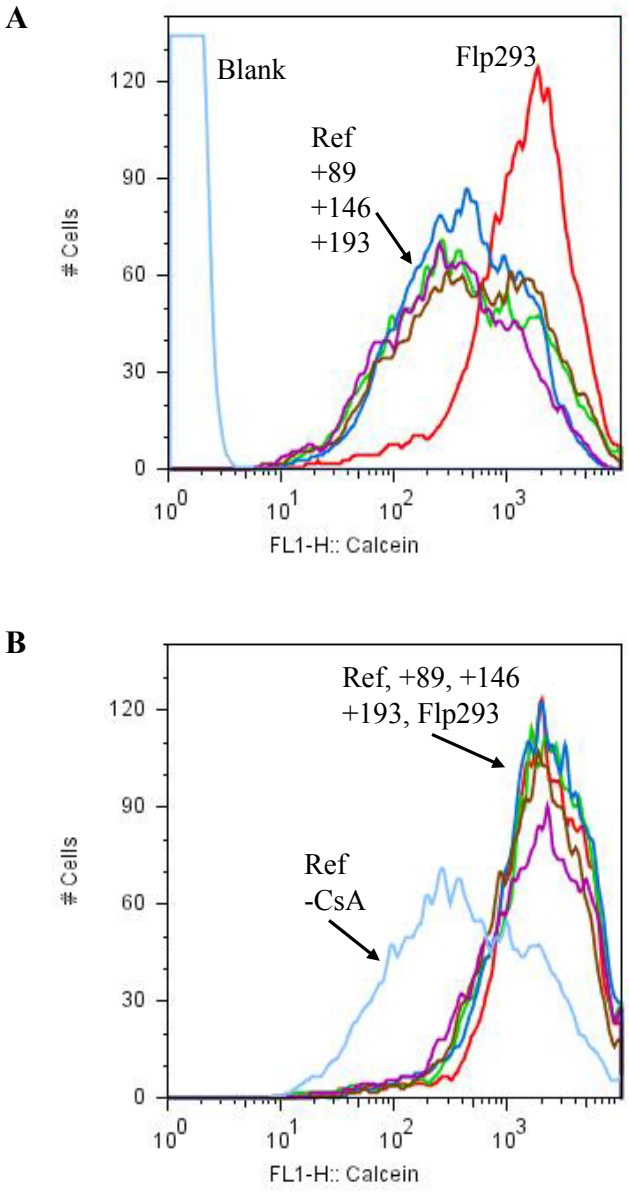


Figure 3.11. Calcein-AM accumulation in *ABCB1* reference and variant Flp293 stable cell lines. P-gp function in *ABCB1* reference and variant cell lines was determined by measuring the intracellular accumulation of 1 μM calcein. Parental Flp293 cells showed higher levels of calcein fluorescence (A). The addition of 10 μM cyclosporin A increased calcein fluorescence in the reference and variant cell lines to a similar level as Flp293 cells (B).

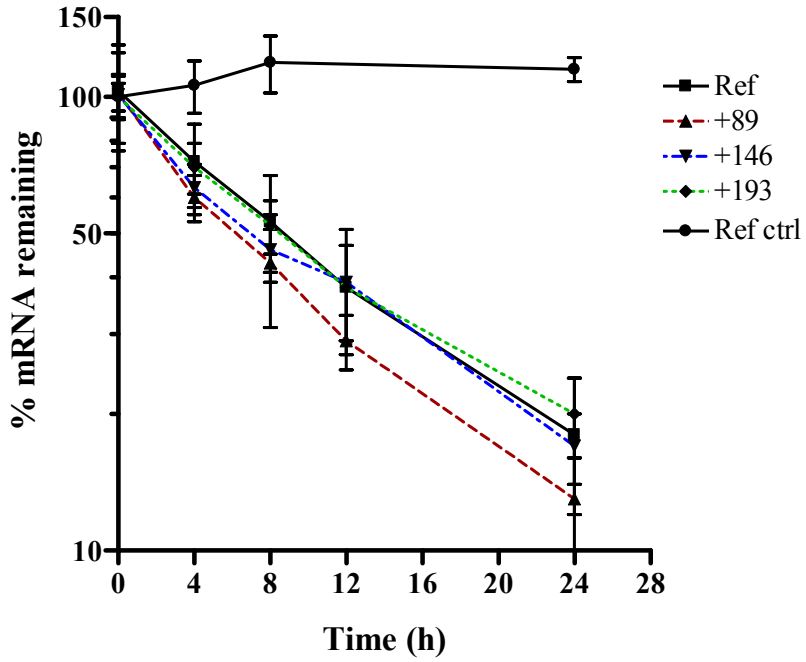


Figure 3.12. mRNA half-life of ABCB1 reference and 3'-UTR variants in Flp293 stable cells. The mRNA decay of ABCB1 reference and 3'-UTR variants was measured over 24 hours in the presence of 7.5 μg/mL actinomycin D. The reference cell line without actinomycin D served as a negative control. The percent mRNA remaining was calculated according to the Materials and Methods section. Each point is the mean ± S.D. of three experiments.

Table 3.4
Calculated mRNA half-lives of ABCB1 reference and 3'-UTR variants

3'-UTR variant	mRNA half-life mean ± S.D. (h)	*t* -test p-value
Reference	9.4 ± 1.3	
+89A>T	8.3 ± 1.4	0.35
+146G>A	9.6 ± 0.8	0.88
+193A>G	10.3 ± 1.2	0.44

The rate of mRNA decay for ABCB1 reference and 3'-UTR variants was used to calculate mRNA half-life as described in the Materials and Methods section. Statistical significance of mean half-life values for +89A>T, +146G>A and +193A>G were compared to reference using a Student's *t* -test.

actinomycin D to verify constant ABCB1 mRNA expression during the 24 hour period (Figure 3.12).

3.4 Discussion

ABCB1 mRNA degradation is an important cellular mechanism controlling P-gp expression and ultimately impacts apparent P-gp activity. The 3'-UTR of mRNA transcripts is considered the key regulatory region that interacts with mRNA degradation machinery. Protein binding sites and secondary structure in the 3'-UTR will either promote or repress mRNA degradation, resulting in changes to steady-state mRNA levels. Our studies were designed to test the hypothesis that *ABCB1* 3'-UTR variants alter mRNA stability. The functional importance of each of nine 3'-UTR variants was estimated with predictive measures and three variants were tested in an mRNA half-life assay.

There are many large population studies that identified *ABCB1* genetic variants but few of them adequately covered the 3'-UTR [107, 108, 206, 207]. The +21T>C, +77ACTT>-, +89A>T, +146G>A, +193A>G, +252A>C and +316G>A variants were previously deposited in dbSNP (www.ncbi.nih.gov/SNP) or Ensembl (www.ensembl.org). Using an ethnically diverse population of 247 DNA samples, our studies identified the seven known variants plus two novel 3'-UTR variants (+169G>GACAGAGA and +355T>C; Table 3.1). In the 5' UTR, coding regions and flanking intronic regions of *ABCB1* there is a polymorphic site every 150 base pairs, on average [108]. Given that the 3'-UTR is 380 bp there should be 2-3 polymorphisms, however, the observed number is three times greater suggesting genetic variation is

tolerated more in the 3'-UTR than in coding regions of *ABCB1*. There were distinct ethnic patterns of variation with the majority of polymorphisms found in the African American population and less so in Caucasian, Asian and Mexican Americans. Also, the inferred *ABCB1* haplotypes indicate many of these 3'-UTR polymorphisms are in significant linkage disequilibrium with each other (Figure 3.2).

Regulatory proteins bind to specific elements in the 3'-UTR and interact with RNA degradation machinery by promoting or repressing degradation [224]. A common sequence motif containing AU repeats is thought to confer rapid degradation and one study hypothesized the AU-rich regions of the c-myc and ABCB1 3'-UTRs could destabilize β-globin mRNA. In HepG2 cells, an immortalized liver cancer cell line, c-myc, ABCB1 and β-globin mRNA have half-lives of 30 minutes, 8 hours and >24 hours, respectively [231]. Chimeras containing the β-globin coding region with either the ABCB1 3'-UTR or the c-myc 3'-UTR showed that β-globin half-life was only decreased by c-myc [231]. It should be noted that our measured half-life for reference ABCB1 in HEK293 cells (~9.4 h, Figure 3.12) is very similar to that seen in HepG2 cells.

The mRNA stability of ABCB1 is a regulated process dependent on multiple factors, such as stress and cell type. The human leukemia cell line K562 is a disease-state model for studying mechanisms controlling P-gp overexpression due to drug exposure. ABCB1 mRNA half-life in K562 cells is 1 h but short-term incubation with drugs, such as vinblastine and doxorubicin, increased the half-life to 10 h with no changes in transcriptional activity [232]. Studies in rats have discovered that carcinogenesis in the liver can modulate Pgp1-3 (human P-gp orthologs) mRNA stability by dramatically increasing their normally short half-life (2 vs. 12 h) [233]. Subsequent research

determined that the increased mRNA stability in rat liver tumors was associated with a decrease in Pgp mRNA fragments, indicating there was less mRNA degradation [234]. Even in healthy organs free of stress, rat Pgp mRNA has tissue-specific half-lives that range from 2-12 h [235].

There are no literature reports discussing possible regulatory sites in the 3'-UTR of *ABCB1*. Our sequence analysis indicates that there may be functionally important segments based on high conservation with other mammalian species (Figure 3.3), however, the UTRscan database did not find any probable elements. The 3'-UTR field is rapidly growing and has yet to reach the same knowledge base as promoter research, which has identified thousands of transcription factors and sequence elements [236]. In contrast, UTRscan currently searches for 31 known degenerative elements so it is possible there are many more yet to be identified.

The stability of mRNA may be altered by 3'-UTR polymorphisms if recognition of specific mRNA sequence and secondary structure by regulatory proteins is disrupted [222, 224]. A polymorphism in the 3'-UTR of human TNFα changes binding affinity for a multi-protein complex that contains the HuR regulatory protein [237]. HuR binds AU-rich elements in the 3'-UTR of certain genes [238] and has been shown to stabilize mRNA containing TNFα 3'-UTR sequence motifs [239]. There is one report that found the 3435C>T synonymous variant decreases mRNA stability [161], but to our knowledge no pharmacogenetic research of this type has been conducted for *ABCB1* 3'-UTR variants. Thus, our mRNA half-life data represent novel findings as to the effects +89A>T, +146G>A and +193A>G have on ABCB1 mRNA stability. Future mRNA stability experiments should investigate the other *ABCB1* 3'-UTR variants, especially the

haplotype pairs of +21/+169, +77/+146 and +89/+193. The most interesting data may come from the +77ACTT>-/+146G>A haplotype because these two variants are always linked (Figure 3.2) and they dramatically change secondary structure (Figure 3.4D). Furthermore, the 5'-UTR may play a role in mRNA stability based on the closed loop mRNA structure (Figure 3.1). The possible interactions between both UTRs in the cytosol suggest 5'-UTR polymorphisms could modify regulatory protein interactions that propagate to the 3'UTR and impact mRNA stability.

3.5 References

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Chapter 4

The Effects of Genetic Variation on ABC Gene Expression in Colon Cancer

4.1 Introduction

Colon cancer is one of the most prevalent malignant diseases in the U.S. with over 120,000 people diagnosed every year [1]. The 5-year survival rates are about 70% if detected early enough, however, more advanced stages of the disease are less responsive to treatment and have a lower survival rate (30-60%) [2, 3]. Surgical resection of the tumor is performed first and then adjuvant therapy, such as chemotherapy and radiation therapy, is given based on the extent of the cancerous region. Often a stage IV or V colon tumor will also metastasize to the liver, which requires surgery on both organs and an aggressive adjuvant regimen. Unfortunately, the median survival for metastatic cases is 18-20 months regardless of treatment type [4].

Development of multidrug resistance (MDR) is one explanation for ineffective chemotherapy of colon cancer because many patients lose sensitivity to drugs. One cause of this phenotype is reduced intracellular drug levels in the tumor due to overexpression of ATP-binding cassette (ABC) efflux transporters, such as P-glycoprotein (P-gp) and the multidrug resistance-associated proteins (MRPs). These xenobiotic transporters are a focus of MDR research because drugs administered to colon cancer patients, such as 5-fluorouracil (5-FU), doxorubicin and irinotecan, are substrates for P-gp and the MRPs [5, 6]. In healthy duodenal tissue, P-gp, MRP1 and MRP3 are found at appreciable levels

[7]. Patient response to chemotherapy has been associated with P-gp and MRP expression [8-10] and studies with cancer cell lines have found high levels of multidrug resistance transporter mRNA and protein [6]. It is not fully understood what regulates expression of these transporters during carcinogenesis and drug exposure; however, the observed variability in treatment suggests genetic variation is one possibility.

P-gp is encoded by *ABCB1*, which has more than 70 single nucleotide polymorphisms (SNPs) with an allele frequency greater than 5% [11-13]. There is evidence for associations between clinical phenotypes and *ABCB1*/P-gp variants in healthy and disease populations, such as cancer and HIV [14, 15]. Less clinical data are available for MRP1 (*ABCC1*), MRP2 (*ABCC2*) and MRP3 (*ABCC3*), possibly due to the overlapping substrate specificity for these transporters [16]. The abundance of SNPs for these four genes is consistent with the hypothesis that genetic variation contributes to expression variability.

The goal of the present study was to investigate mRNA expression levels of *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* in colon cancer. Tissue samples from matched normal and cancerous regions were used to measure mRNA levels and observed variability in gene expression prompted a preliminary association analysis between genotype and mRNA levels. Inferred haplotypes for each gene were constructed and included in the association analysis.

4.2 Materials and Methods

4.2.1 Materials

Trizol was purchased from Invitrogen (Carlsbad, CA) and stored at 4°C. All other reagents were molecular biology grade and were purchased from Fisher Scientific.

4.2.2 Tissue Collection and Storage

Drug-naïve colon cancer patients underwent surgery at the UCSF Moffit-Long Hospital for tumor resection. Noncancerous (normal) and cancerous (tumor) colon tissue was removed, and separately snap-frozen in liquid nitrogen. Matched tissue samples were given a numerical identifier and stored at -80°C. The noncancerous samples were considered normal after a pathological evaluation.

4.2.3 DNA and RNA Isolation

Genomic DNA was isolated from ~50 mg normal colon tissue using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The DNA was quantitated with UV spectrophotometry (NanoDrop Technologies, Wilmington, DE) and stored at 4°C. Approximately 100 mg of normal and tumor colon tissue was used to isolate total RNA. The tissue was added to 1 mL of Trizol while still frozen and total RNA was extracted according to the manufacturer's protocol. Quantitation of total RNA was determined with UV spectrophotometry and the samples were stored at -80°C.

4.2.4 Validation of TaqMan Assays

Serial dilutions of total RNA (0.125-1 μg) from human liver were used to determine the reverse transcription (RT) linearity for ABCB1, ABCC1-3 and hGus

TaqMan assays. Total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. TaqMan primers and probes for ABCB1, ABCC1, ABCC2, ABCC3 and hGus were designed using Primer Express (Applied Biosystems, Foster City, CA) with help from Dr. David Ginzinger (UCSF Cancer Center; Table 4.1). Reactions were run in triplicate on an ABI Prism 7700 and cycling conditions were: 12 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The threshold limit was set so that it intersected all samples during the log-linear phase of amplification and the corresponding PCR cycle number at threshold (C_T) values for each set of triplicates were averaged. RT linearity was determined from the slope of C_T vs. arbitrary RNA units (Excel).

PCR efficiency was determined from 10-fold serial dilutions of cDNA (reverse transcribed from liver total RNA). TaqMan reactions were run in triplicate as described above and the C_T values were averaged for each cDNA concentration. The slope of C_T vs. cDNA dilution was used to calculate PCR efficiency: $(10^{(-1 / \text{slope})} - 1) * 100$.

4.2.5 Relative Gene Expression

Total RNA for normal and tumor tissue was reverse transcribed and triplicate reactions for each sample were used in *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3* and *hGus* TaqMan assays. The threshold limit was set so that it intersected all samples during the log-linear phase of amplification and the corresponding C_T values for each set of triplicates were averaged. Relative gene expression was determined using the ΔC_T method which compares target gene expression to a control gene. The equation used to calculate ΔC_T in normal and tumor tissue is:

$$\Delta C_T = C_{T,\ \text{ABC gene}} - C_{T,\ \text{hGus}}.$$

Table 4.1
TaqMan primers and probes for ABC genes and hGus

Gene	Forward and reverse primers	TaqMan probe[a]
ABCB1	5'-TGATCATTGAAAAAACCCCTTTG 5'-TCCAGGCTCAGTCCCTGAAG	5'-(6-FAM)-ACGGAAGGCCTAATGCCGAACACATT-(BHQ1)
ABCC1	5'-GACCATGAATGTGCAGAAGGC 5'-CTGGATGAGGTCGTCCGTTT	5'-(6-FAM)-AGAACCTCAGTGTCGGGCAGCGC-(BHQ1)
ABCC2	5'-CCTGCGCTTTCCCCTGA 5'-TGGCAGATGTGTCCAAGTCATC	5'-(6-FAM)-CTCCAGGCCAGTGTTTCCACAGAGC-(BHQ1)
ABCC3	5'-CTTGCTGATTCCACTCAACGG 5'-TGATGCGCGAGTCCTTCA	5'-(6-FAM)-CGCGCCTTCCAGGTAAAGCAAATG-(BHQ1)
hGus	5'-CTCATTTGGAATTTTGCCGATT 5'-CCGAGTGAAGATCCCCTTTTTA	5'-(6-FAM)-TGAACAGTCACCGACGAGAGTGCTGG-(BHQ1)

[a] 5' modification = 6-carboxyfluorescein (6-FAM); 3' modification = black hole quencher 1 (BHQ1).

The triplicate C_T values for each gene in each sample were averaged and used in the ΔC_T equation. Relative ABCB1, ABCC1, ABCC2 and ABCC3 mRNA levels were calculated in normal and tumor tissue using the following equation:

$$\text{Rel. expression} = 2\text{^}(-(\Delta C_T)).$$

4.2.6 ABC Transporter Genotypes and Haplotypes

Single nucleotide polymorphisms (SNPs) of *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* were genotyped in colon cancer patients using genomic DNA. Direct sequencing was carried out with previously designed primers available at www.pharmacogenetics.ucsf.edu. Chromatograms were analyzed using Sequencher (Gene Codes Corp., Ann Arbor, MI) to classify the variants. Haplotypes were inferred using PHASE and aligned using Clustal W [17].

4.2.7 Statistical Analysis

Mean expression of ABC mRNA in normal and tumor colon tissue with ΔC_T values was compared using a paired Student's *t* test. Cross-gene analysis of mean mRNA levels in normal tissue was determined by a Student's *t* test using ΔC_T values. Associations between genotype and mRNA expression were determined for reference and variant allele carriers. The mean ΔC_T values for each group(s) were converted to relative expression by the above equation. The Mann-Whitney *U* test compared mean ΔC_T values of two genotype groups and the Kruskal-Wallis test compared mean ΔC_T values for three genotype groups. Relative expression ratios for genotypes and haplotypes with notable p-values (<0.15) were calculated as follows: rel. $\text{expression}_{\text{variant}}$ / rel. $\text{expression}_{\text{reference}}$.

4.3 Results

4.3.1 Validation of TaqMan Assays

Real-time quantitative PCR is a highly sensitive assay that requires optimized conditions for accurate and reproducible results. TaqMan assays for ABCB1, ABCC1-3 and hGus were custom designed, and two optimization steps were performed. The reverse transcription (RT) linearity measures the consistency of the RT reaction over a concentration gradient and the PCR efficiency measures the reproducibility of the PCR reaction over a range of cDNA concentrations. The RT linerarity was performed with 2-fold serial dilutions of total RNA from 1-0.125 μg total RNA. In theory, two samples with a 2-fold difference in RNA concentration will differ by one C_T ($2^1 = 2$), thus a linear-log plot of C_T vs. RNA concentration should have a slope of one. The RNA concentration was substituted for arbitrary units so that a simple linear regression could be used to determine the slope (Figure 4.1A). The slopes for ABCB1, ABCC1-3 and hGus were linear and within the acceptable range of 1 ± 0.15 (Table 4.2). PCR efficiency was determined from a 1000-fold concentration

range of cDNA in 10-fold serial dilutions. The difference in C_T between each dilution should be 3.33 based on the exponential nature of PCR, *i.e.* $2^{3.33} = 10$ (a 4-fold concentration difference equates to a 2 C_T difference, $2^2 = 4$). The relationship between C_T and cDNA dilution provides a slope which reflects the actual efficiency of the PCR reaction. A 100% efficient reaction will have a slope of -3.33 as determined from a linear regression analysis and the acceptable efficiency range is 80-110%. ABCB1, ABCC1-3 and hGus showed linear PCR amplification with efficiencies close to 100% (Figure 4.1B and Table 4.2).

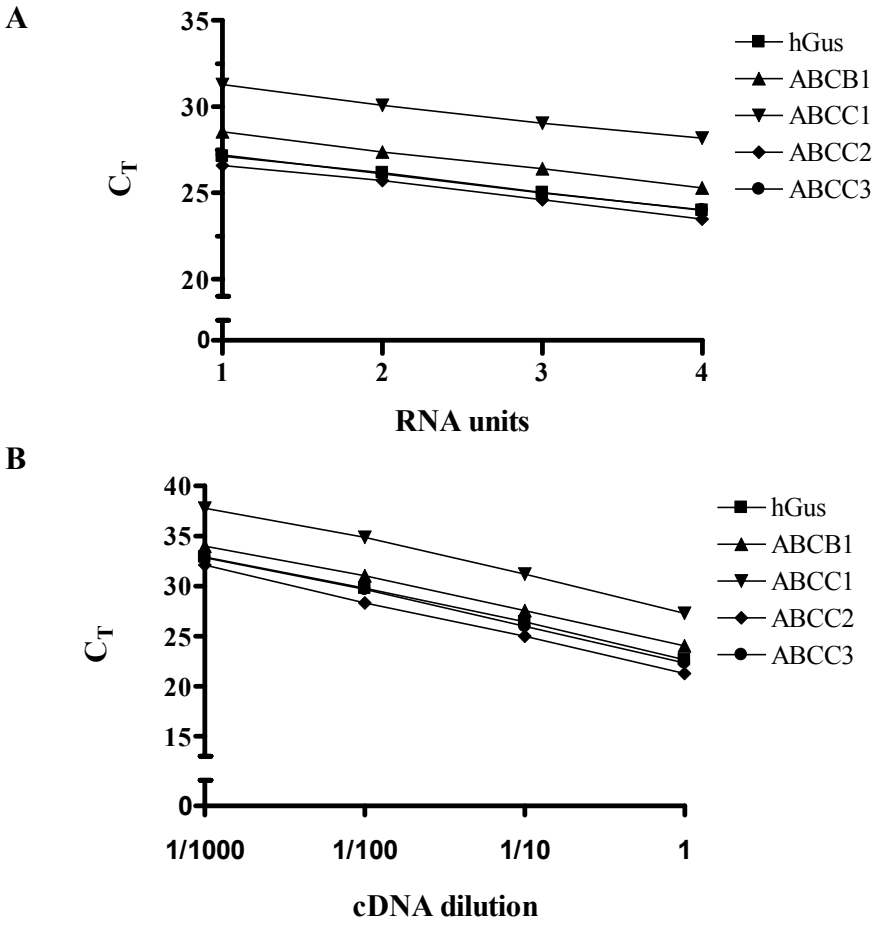


Figure 4.1. Validation of hGus, ABCB1, ABCC1, ABCC2 and ABCC3 TaqMan assays. The RT linearity examines the consistency of the RT reaction from 0.125-1 μg of RNA. RNA units represent two-fold increases in RNA concentration. C_T values are the mean of duplicate determinations for 2-fold serial dilutions of RNA (A). The PCR efficiency determines how well the PCR reaction amplifies template over a 1000-fold concentration range. cDNA samples were made from a single RT reaction and serially diluted 10-fold. C_T values are the mean of duplicate determinations (B).

Table 4.2
Validation parameters for TaqMan assays

TaqMan Assay	RT linearity	PCR efficiency (%)
hGus	1.15	96
ABCB1	1.08	99
ABCC1	0.94	93
ABCC2	0.92	90
ABCC3	0.97	92

Ideal values for RT linearity and PCR efficiency are 1 and 100%, respectively.

4.3.2 ABC Gene Expression in Colon Cancer Patients

Pathologically normal colon tissue with matched tumor was surgically removed from 32 drug-naïve colon cancer patients and frozen for storage. Total RNA was isolated and the mRNA levels for ABCB1, ABCC1, ABCC2 and ABCC3 were normalized to the control gene, *hGus* (β-glucuronidase). TaqMan real-time quantitative PCR measurements showed that ABCC3 had the highest expression in healthy and tumor tissue. ABCB1 and ABCC1 were approximately 20% and 10%, respectively, less than ABCC3 ($p<0.0001$), and ABCC2 had the lowest expression (<2% of ABCC3, $p<0.0001$; Figure 4.2). There was greater mRNA expression variability in the tumor compared to normal tissue for ABCB1 (~5-fold) and ABCC2 (~10-fold), however, variability in ABCC1 levels decreased ~50% in the tumor (Table 4.3). Even though the range of ABCC3 mRNA levels was similar for both tissue types, there was significant variability (~60-fold). It should be noted that there were mRNA expression outliers for ABCC1-ABCC3 in normal tissue that altered the calculated variability (Table 4.3)

ABC gene expression levels in the tumor were compared to normal tissue in order to determine if any significant changes occurred during carcinogenesis. Relative mRNA

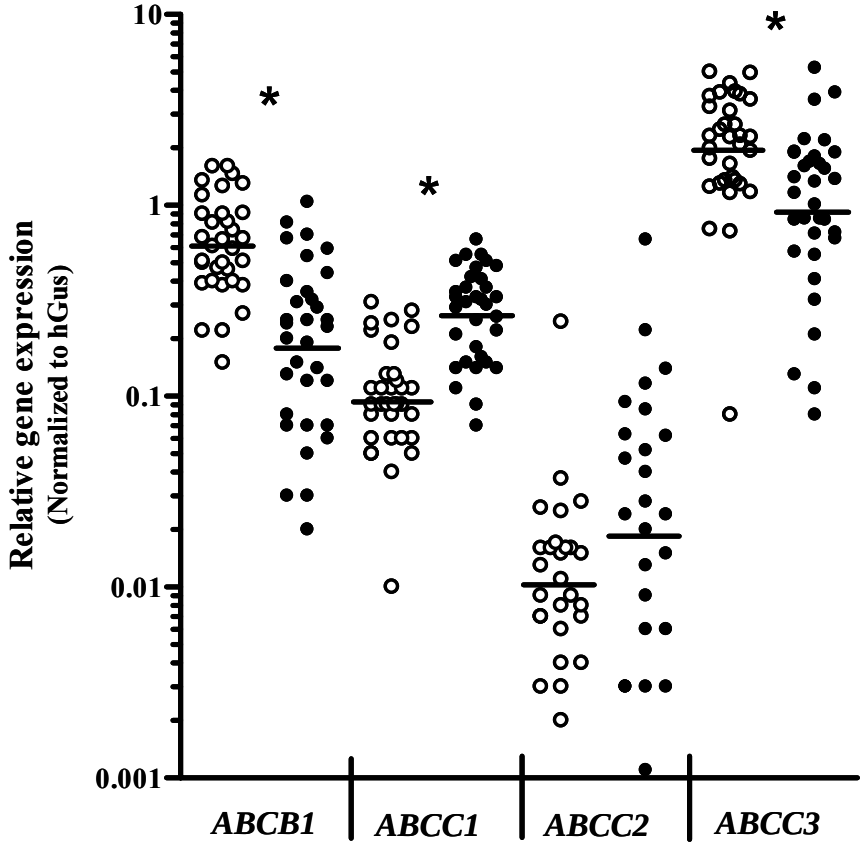


Figure 4.2. ABC gene expression in normal and tumor colon. ABCB1, ABCC1, ABCC2 and ABCC3 mRNA expression in normal (○) and tumor (●) colon tissue was determined using TaqMan real-time PCR. Measurements were normalized to hGus and represent relative mRNA expression levels with mean values noted. A statistically significant difference ($p<0.0001$) between normal and tumor is marked (*).

Table 4.3
ABC gene expression variability in normal and tumor colon tissue

	Expression variability[a]		Tumor-to-normal ratio[b]
	Normal	Tumor	
ABCB1	10	48	0.31*
ABCC1	24 (10)	10	2.81*
ABCC2	129 (20)	1078	1.45
ABCC3	61 (10)	66	0.45*

[a] Fold-difference between the lowest and highest values. Values in parentheses excluded outliers.

[b] Data calculated from Figure 4.1. A value of "1" means no change between tumor and normal.

* $p<0.0001$

expression of ABCB1 and ABCC3 in tumor decreased by 70% and 55%, respectively, but ABCC1 increased 2.8-fold ($p<0.0001$; Table 4.3). There was a modest increase in ABCC2 expression in the tumor (1.45-fold), but it was not statistically significant.

4.3.3 ABC Genetic Variation in Colon Cancer Patients

Genetic differences between colon cancer patients may contribute to the variable and altered ABC gene expression. Thus, genomic DNA from normal tissue was genotyped for known *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* single nucleotide polymorphisms (SNPs; pharmacogenetics.ucsf.edu). Power calculations suggested a 50% difference in mRNA expression between reference and variant groups could be detected for SNPs with a 10% minor allele frequency. The majority of the patients were Caucasian, so only SNPs with a minor allele frequency >10% in this ethnic group were considered. There were ten polymorphic sites genotyped for *ABCB1*, seven for *ABCC1*, four for *ABCC2*, and five for *ABCC3* (Table 4.4). *ABCB1* +89A>T ($q_{\text{Caucasion}} = 6.5\%$) was genotyped because it was part of the same sequencing amplicon as +193A>G. All SNPs displayed a normal distribution of alleles based on Hardy-Weinberg equilibrium, except for *ABCC2* Intron 26 (-34)T>C (data not shown).

Polymorphisms for each gene were used to estimate haplotypes using PHASE. Haplotypes were aligned using Clustal W and were numbered based on the recommendations of Nebert *et al*. [18] (Figure 4.3). The most common haplotype for *ABCB1* (4) contained three exonic and three intronic SNPs (Figure 4.3A), and was reported by Kroetz *et al*. as *ABCB1**13 [12]. Haplotype 1 represented the reference sequence for *ABCB1* and seven out of ten haplotypes consisted of at least two SNPs. *ABCC1* reference haplotype was not estimated (Figure 4.3B). The two highest frequency

Table 4.4
Genotyped polymorphisms for *ABCB1, ABCC1, ABCC2* and *ABCC3*

Gene	NCBI SNP ID[a]	Genomic region	cDNA position[b]	Alleles	Amino acid change	CA allele frequency (%)[c]	Patient allele frequency
ABCB1	rs2235015	Intron 4	(-25)	G>T		15.8	17.2
	rs10276036	Intron 9	(-44)	A>G		45.0	53.1
	rs1128503	Exon 12	1236	C>T	Synonymous	45.9	53.1
	rs2235033	Intron 13	(+24)	C>T		52.1	60.9
	rs2235013	Intron 14	(+38)	A>G		50.5	60.9
	rs2235040	Intron 20	(+24)	G>A		12.1	9.4
	rs2032582	Exon 21	2677	G>T	Ala893Ser	46.4	45.3
	rs2032582	Exon 21	2677	G>A	Ala893Thr	3.6	1.6
	rs1045642	Exon 26	3435	C>T	Synonymous	56.1	56.3
	rs17064	3'-UTR	+89	A>T		6.5	4.7
	rs3842	3'-UTR	+193	A>G		14.0	18.8
ABCC1	rs35587	Exon 9	1062	C>T	Synonymous	31.0	34.4
	rs35588	Intron 9	(+8)	A>G		31	34.4
	rs3765129	Intron 11	(-48)	C>T		16.2	21.9
	rs35605	Exon 13	1684	C>T	Synonymous	19.5	14.1
	rs2074087	Intron 18	(-30)	G>C		18.8	12.5
	rs2239330	Exon 28	4002	G>A	Synonymous	26.8	21.9
	rs35939983	Intron 30	(+18)	G>A		20.4	12.5
ABCC2	rs717620	5'-UTR	-24	C>T		19.5	18.8
	rs2273697	Exon 10	1249	G>A	Val417Ile	17.0	21.9
	rs17216177	Intron 26	(-34)	T>C		17.0	3.1
	rs3740066	Exon 28	3972	C>T	Synonymous	38.3	29.7
ABCC3	rs739923	Intron 5	(-22)	G>A		25.6	34.4
	rs4148415	Intron 19	(-123)	C>T		38.8	32.8
	rs2072365	Intron 20	(+29)	C>T		38.8	32.8
	rs2072365	Intron 20	(+53)	A>G		38.1	35.9
	rs2277624	Exon 27	3942	C>T	Synonymous	25.6	25.0

[a] Reference SNP ID number from NCBI dbSNP (build 126; www.ncbi.nih.gov/SNP)

[b] Nucleotide position relative to ATG start codon. Intronic positions are relative to the beginning of the intron (*i.e.* Intron 13(+24)) or the start of the following exon (*i.e.* Intron 4(-25)).

[c] Caucasian allele frequence based on Kroetz *et al.* [12].

A

Haplotype	Exon 2 61 A>G	Intron 4 (-25) G>T	Intron 9 (-44) A>G	Exon 12 1236 C>T	Intron 12 (+44) C>T	Intron 13 (+24) C>T	Intron 14 (+38) A>G	Intron 20 (+24) G>A	Exon 21 2677 G>T/A	Exon 26 3435 C>T	**Number and percent of chromosomes**
1											11 (16.7)
4			**G**	**T**		**T**	**G**		**T**	**T**	23 (34.8)
4a	**G**		**G**	**T**		**T**	**G**		**T**	**T**	8 (12.1)
7a		**T**						**A**			6 (9.1)
2										**T**	6 (9.1)
5			**G**	**T**		**T**	**G**				5 (7.6)
6a		**T**			**T**	**T**	**G**				4 (6.1)
7		**T**									1 (1.5)
6					**T**	**T**	**G**				1 (1.5)
3									**A**	**T**	1 (1.5)

B

Haplotype	Exon 9 1062 C>T	Intron 9 (+8) A>G	Intron 11 (-48) C>T	Exon 13 1684 C>T	Intron 18 (-30) G>C	Exon 28 4002 G>A	Intron 30 (+18) G>A	**Number and percent of chromosomes**
2	**T**							24 (36.3)
1		**G**						10 (15.2)
2a	**T**						**A**	6 (9.1)
2b	**T**					**A**		5 (7.6)
6		**G**	**T**			**A**		4 (6.1)
4	**T**		**T**					2 (3.0)
3	**T**			**T**	**C**			2 (3.0)
3a	**T**			**T**	**C**	**A**		2 (3.0)
7		**G**	**T**	**T**				2 (3.0)
8		**G**	**T**	**T**	**C**	**A**		2 (3.0)
5		**G**	**T**					2 (3.0)
10		**G**					**A**	2 (3.0)
12	**T**		**T**	**T**	**C**			1 (1.5)
11		**G**	**T**				**A**	1 (1.5)
9		**G**				**A**		1 (1.5)

C

Haplotype	5'-UTR -24 C>T	Exon 10 1249 G>A	Intron 26 (-34) T>C	Exon 28 3972 C>T	**Number and percent of chromosomes**	**% of chromosomes**
1					29 (43.9)	43.9
2		**A**			14 (21.6)	21.6
3a	**T**			**T**	11 (16.7)	16.7
3				**T**	10 (15.2)	15.2
4	**T**				2 (3.0)	3.0

D

Haplotype	Intron 5 (-22) G>A	Intron 19 (-123) C>T	Intron 20 (+29) C>T	Intron 20 (+53) A>G	Exon 27 3942 C>T	**Number and percent of chromosomes**
1						17 (25.7)
3a	**A**	**T**	**T**			18 (27.3)
2a				**G**	**T**	17 (25.7)
2				**G**		6 (9.0)
3	**A**					5 (7.6)
4		**T**	**T**			3 (4.5)

Figure 4.3. ABC gene haplotypes found in colon cancer patients. Polymorphisms for *ABCB1* (A), *ABCC1* (B), *ABCC2* (C), and *ABCC3* (D) were grouped into haplotypes based on PHASE inference and Clustal W alignments. Haplotype numbers were assigned based on evolutionary considerations and variant base changes are shown. Dark shading and light shading denote nonsynonymous and synonymous variants, respectively. Haplotypes tested for mRNA expression association are in bold.

haplotypes for *ABCC1* contained only one SNP, and together they represented almost 50% of the chromosomes. Four polymorphisms for *ABCC2* were investigated; hence this gene had the fewest number of inferred haplotypes (Figure 4.3C). Only ABCC2 haplotype 3a contained two SNPs. but there was a reference haplotype. Three out of six *ABCC3* haplotypes accounted for 80% of the chromosomes (Figure 4.3D).

4.3.4 ABC Genetic Variation and mRNA Expression

The mRNA levels for ABCB1, ABCC1, ABCC2 and ABCC3 in normal and tumor colon tissue were grouped according to each patient's genotype and haplotype for an association analysis. Mean mRNA expression between reference and variant alleles was analyzed separately for normal and tumor tissue to determine whether ABC genetic variation influences expression (Figure 4.2). SNP associations were evaluated using two different statistical methods based on genotype distribution. For example, the Kruskal-Wallis one-way analysis of variance was used for *ABCB1* 1236C>T because there were seven samples homozygous for C/C, 14 heterozygous for C/T and 11 homozygous for T/T. In contrast, only two samples were homozygous G/G for *ABCB1* +193A>G, so these were grouped with the heterozygotes and compared to homozygous reference A/A using the Mann-Whitney *U* test. Both statistical methods rank-order the data and do not assume a normal distribution (*i.e.* non-parametric). Gene expression and haplotype associations used the Mann-Whitney *U* test because only two groups were compared. Samples with one or two copies of the haplotype were combined and compared to those with no copies. Only haplotypes that were seen on at least six chromosomes (9%) were considered. The p-values reported were not adjusted for multiple comparisons because we wanted to identify possible associations to support the collection of additional

samples (Tables 4.5 and 4.6). There is an increased chance for a false positive without multiple comparisons correction; however, this investigation was designed as an exploratory study. Possible associations between expression and genotype were considered interesting with a $p<0.15$.

There were two cases for *ABCB1* that indicated a possible trend in mRNA expression differences due to genotype. In normal tissue, the 3'-UTR SNP at +193 was associated with a 40% decrease in expression relative to +193AA genotype ($p=0.09$; Figure 4.4A). The power calculations used to set the >10% allele frequency threshold considered a 50% difference in means as viable, so the difference with +193A>G is near the limit of detection. As mentioned earlier, +89A>T was genotyped because it was part of the same sequencing reaction as +193A>G, and there were only three samples harboring the variant T allele. Regardless, there may be a trend for higher expression with +89T than +89A ($p=0.12$; Figure 4.4B). *ABCB1* haplotypes did not associate with mRNA expression in normal or tumor colon tissue (Table 4.6).

ABCC1 showed three polymorphisms in normal tissue that may associate with differential mRNA expression. The synonymous 1684C>T has a 1.5-fold increase in mRNA levels for heterozygous C/T samples ($p=0.13$, Figure 4.5A). A similar trend was observed for intron 18 (-30)G>C in which the variant allele showed a 1.5-fold increase ($p=0.13$; Figure 4.5B). These two SNPs were found together in four haplotypes and differ by one patient in the heterozygous group, indicating that they are in tight linkage disequilibrium. In normal tissue, the variant allele for Intron 30 (-18)G>A and haplotype 2 are potentially associated with a 40% decrease in mRNA expression ($p=0.15$ and $p=0.09$; Figures 4.5C and D). Haplotype 2 contains Intron 30 (-18)G>A and the

Table 4.5
Statistical analysis of mRNA expression differences for ABC SNPs in normal and tumor colon tissue

Gene	NCBI SNP ID	p-value[a]		Gene	NCBI SNP ID	p-value[a]	
		Normal	Tumor			Normal	Tumor
ABCB1	rs2235015	0.28	0.75	*ABCC2*	rs717620	0.21	0.60
	rs10276036	0.27	0.58		rs2273697	0.57	0.29
	rs1128503	0.52	0.58		rs17216177	NA	NA
	rs2235033	0.99	0.56		rs3740066	0.30	0.92
	rs2235013	0.99	0.56				
	rs2235040	0.66	0.87	*ABCC3*	rs739923	0.27	0.90
	rs2032582	0.63	0.27		rs4148415	0.30	0.30
	rs2032582	NA	NA		rs2072365	0.30	0.30
	rs1045642	0.51	0.99		rs2072365	0.57	0.25
	rs17064	0.92	**0.12**		rs2277624	0.62	0.29
	rs3842	**0.09**	0.62				
ABCC1	rs35587	0.63	0.18				
	rs35588	0.63	0.18				
	rs3765129	0.72	0.58				
	rs35605	**0.13**	0.77				
	rs2074087	**0.13**	0.97				
	rs2239330	0.52	0.37				
	rs35939983	**0.15**	0.99				

[a] Nonparametric analyses were used to calculate p-values as described in the Materials and Methods section. Numbers in bold represent possible trends.

Table 4.6
Statistical analysis of mRNA expression differences for ABC haplotypes in normal and tumor colon tissue

Gene	Haplotype[a]	p-value[b]	
		Normal	Tumor
ABCB1	1°	0.51	0.19
	4	0.47	0.92
	4a	0.41	0.93
	7a	0.70	0.87
ABCC1	2	**0.09**	0.58
ABCC2	1°	0.52	0.54
	3a	**0.10**	**0.10**
ABCC3	1°	0.70	0.17
	2a	0.74	0.24
	3a	0.78	0.77

[a] Patients that carried at least one allele of the haplotype were grouped together and compared to samples that lacked the haplotype.

[b] Mean mRNA expression values were compared using the Mann-Whitney *U* test and only haplotypes that contained at least two SNPs were analyzed, except for reference (°).

Numbers in bold suggest a possible trend.

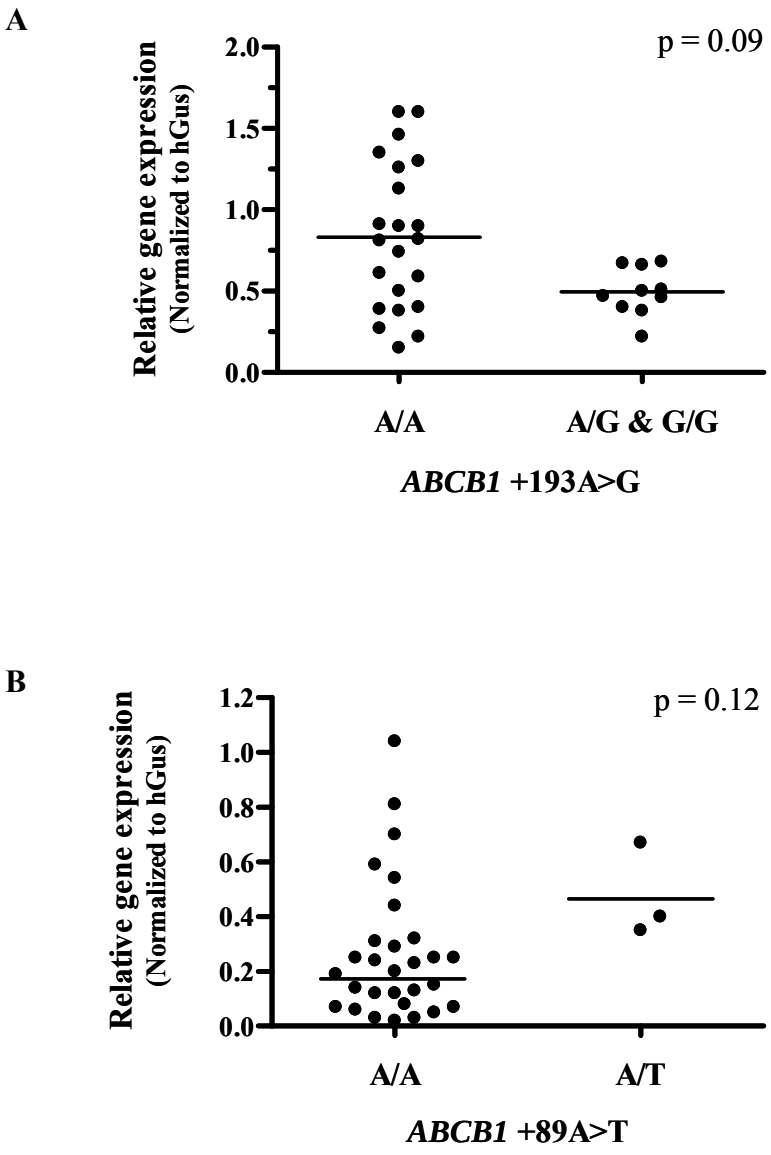
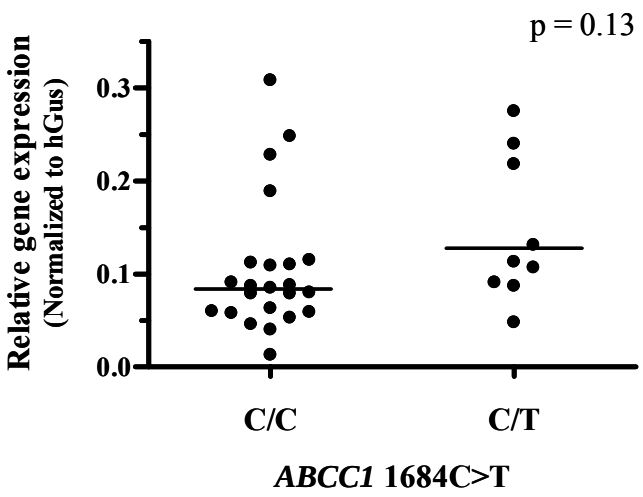


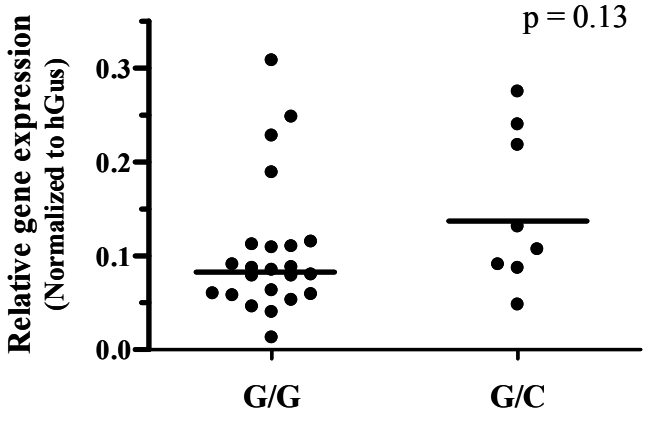
Figure 4.4. Possible *ABCB1* genotype and expression associations. ABCB1 mRNA expression was normalized to hGus in normal and tumor colon tissue for each patient. Each data point represents patient expression and genotype for *ABCB1* +193A>G in normal (A) and +89A>T in tumor (B). Mean expression for each genotype is shown. The Mann-Whitney *U* test was used to calculate statistical significance between mean expression values.

A



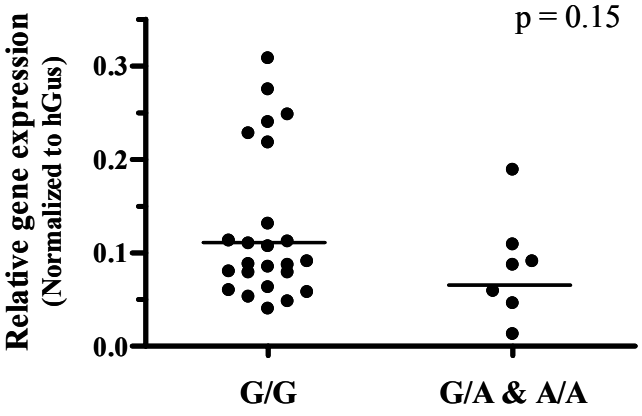
ABCC1 1684C>T

B



ABCC1 Intron 18(-30)G>C

C



ABCC1 Intron 30(+18)G>A

D

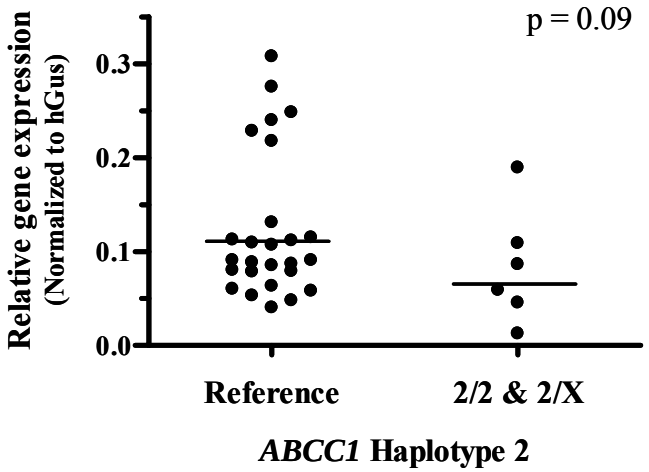


Figure 4.5. Possible *ABCC1* genotype and expression associations. ABCC1 mRNA expression was normalized to hGus in normal colon tissue for each patient. Each data point represents genotype and patient expression in healthy for *ABCC1* 1684C>T (A), Intron 18 (-30)G>C (B), Intron 30 (+18)G>A (C), and haplotype 2 (D). Mean expression for each genotype is shown. The Mann-Whitney *U* test was used to calculate statistical significance between mean expression values.

synonymous variant 1062C>T, and it was the only *ABCC1* haplotype containing two polymorphisms that appeared in at least 6 chromosomes. There were no associations found in tumor tissue for *ABCC1* genotype/haplotype and mRNA levels.

ABCC2 haplotype 3a was marginally associated with ABCC2 mRNA levels in healthy colon and colon tumor. *ABCC2* haplotype 3a contains the 5'-UTR -24C>T and the synonymous 3972C>T variants. In healthy colon, there was a 1.8-fold increase in mRNA in normal tissue from patients with the *ABCC2* haplotype 3a compared to the rest of the population (p=0.10; Figure 4.6A). In addition, ABCC2 expression in the tumor for haplotype 3a carriers was 3.4-fold higher than for patients carrying all other haplotypes (p=0.10; Figure 4.6B). This is the only indication for an association between ABC

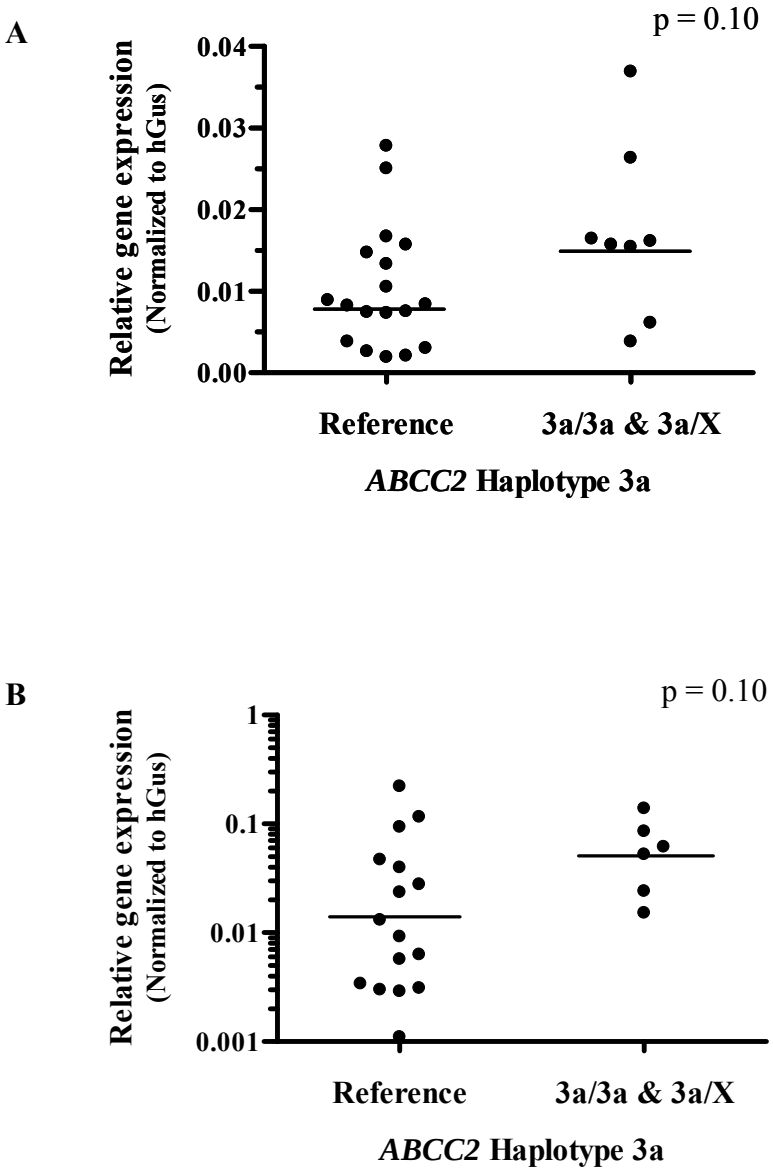


Figure 4.6. Possible *ABCC2* genotype and expression associations. ABCC2 mRNA expression was normalized to hGus in normal and tumor colon tissue for each patient. Each data point represents genotype and patient expression for haplotype 3a in healthy (A) and tumor (B). Mean expression for each genotype is shown. The Mann-Whitney *U* test was used to calculate statistical significance between mean expression values.

expression differences in normal and tumor colon tissue and ABC genotype. There were no trends observed for *ABCC3*.

4.4 Discussion

Gene expression of xenobiotic ABC transporters may influence colon cancer treatment. Our studies examined mRNA levels of ABCB1, ABCC1, ABCC2 and ABCC3 in 32 drug-naïve colon cancer patients to investigate genetic causes for expression variability. Common SNPs in the four genes were genotyped and formed into haplotypes for a comprehensive association analysis with mRNA expression. In addition, we identified altered gene expression in tumor tissue when compared to healthy.

The four ABC genes tested showed at least a 10-fold range in expression for normal and tumor colon tissue but the exact magnitudes were gene-dependent and tissue-dependent (Figure 4.2 and Table 4.2). Data from bladder and kidney cancer studies found similar trends in ABCB1, ABCC1, ABCC2 and ABCC3 mRNA variability [9, 19]. We also found mRNA expression in the tumor to be different than normal tissue for ABCB1 (70% decrease), ABCC1 (2.8-fold increase) and ABCC3 (55% decrease). The data implies patients may initially be more sensitive to drugs that are ABCB1 and ABCC3 substrates, but less sensitive to those transported by ABCC1. Hinoshita *et al*. reported comparable decreases for ABCB1 and ABCC3, but showed no change with ABCC1 in 45 Japanese colon cancer patients [20]. In addition, the study reported significantly greater ABCC2 mRNA expression in colon tumor. The differences between our results may be due to ethnicity, because the majority of the samples were from Caucasians in our study (Robert Warren, personal communication). A recent study demonstrated that P-gp knockout mice from a cancer-model strain developed smaller

tumors than their wildtype controls [21]. It is possible that ABC gene expression is linked to carcinogenesis but the mechanisms have yet to be understood.

The goal of clinical pharmacogenetic research is to examine how naturally occurring polymorphisms influence phenotypes. The population sample size dictates how many variant allele carriers will be present for a given polymorphism. Our study contained a relatively small population of 32 Caucasian patients so it was important to choose common SNPs based on Caucasian allele frequency. Less stringent statistical thresholds were used because the study was designed to identify possible associations as a premise for collecting more samples. Also, we investigated inferred haplotypes for *ABCB1*, *ABCC1*, *ABCC2* and *ABCC3* because one SNP may merely be a marker for a functional variant and/or the presence of multiple SNPs may have synergistic effects. Genomic DNA from tumor was not analyzed but it would be interesting to see if somatic mutations exist and can alter ABC gene expression and drug exposure in colon cancer.

Our data identified possible ABC genotypes associated with mRNA expression in normal and tumor colon tissue. The 3'-UTR SNPs in *ABCB1* (+89A>T and +193A>G) were associated with altered mRNA expression (Figure 4.4), which could mean these polymorphisms are involved in regulation of mRNA stability. Expression of ABCB1 in a human kidney cell line did not find differences in mRNA half-life for +89A>T and +193A>G (Chapter 3), but regulation of mRNA stability can be tissue dependent [22, 23]. There are a limited number of reports investigating how *ABCB1* genotypes associate with gene expression in the colon. One study showed that diplotypes of polymorphisms 4kb upstream of the transcriptional start site decreased ABCB1 mRNA, and showed decreased promoter activity in a luciferase transactivation assay [24]. Also, transcription

factor binding assays showed some of the upstream variants altered protein binding. In other tissues, such as the duodenum, liver and kidney, 2677G>T/A and 3435C>T have been the primary focus of *ABCB1* pharmacogenetic investigations. There are examples of altered mRNA expression associated with these two variants, but the data are not in agreement [7, 19, 25-28]. Ideally, the mechanisms influencing *ABCB1* genotype associated expression should be examined.

It is unclear how the three *ABCC1* variants and haplotype 2 altered mRNA expression but these preliminary results need to be confirmed in additional samples. The synonymous 1684C>T and intron 18 (-30)G>C variants were associated with a 40% lower mean mRNA expression in healthy tissue than homozygous reference patients. It is possible these SNPs are linked to the same unidentified functional variants because they show the same trend and are almost always in linkage. In contrast, intron 30(-18)G>C appears to be an important marker associated with increased mRNA alone and in the context of haplotype 2. The SNP at position 1062 is part of haplotype 2 and it did not show any trends with expression. To our knowledge, the only other study investigating *ABCC1* SNPs did not find an association between mRNA expression and four other synonymous polymorphisms in healthy duodenum [7].

Haplotype 3a was the only *ABCC2* genotype or haplotype to show possible mRNA expression trends in both normal and tumor tissue. It is arguable that polymorphic effects are dependent on disease state, as evident in our results from specific genetic associations found in normal or tumor colon tissue, but not in both. The two variants found in haplotype 3a are either in the 5'-UTR (-24C>T) or near the 3'-UTR (3972C>T), and they may alter mRNA secondary structure resulting in changes to

mRNA stability. One theory suggests the 5'- and 3'-UTRs interact in the cytosol to control mRNA stability and translation [29]. Perhaps further investigation of haplotype 3a will provide new insights into its role in mRNA levels in the colon. Individually, -24C>T is associated with decreased mRNA in kidney but not in the duodenum or placenta, and 3972C>T has no effect on mRNA in kidney and placenta [30, 31]. The combination of previous work and our results reinforces the hypothesis that genotype/expression associations may be haplotype and tissue dependent. With the discovery of numerous polymorphisms in *ABCB1* and *ABCC1-3* (www.ncbi.nih.gov/SNP), deciding which polymorphisms to analyze is a challenge. Tagging SNPs (tSNPs) that have common allele frequencies and exist in distinct haplotypes could be used as initial variants to investigate phenotype-associated differences and enable subsequent research on lower frequency variants linked to the tSNPs.

Cancer pharmacogenetic research has shown common polymorphisms impact clinical phenotypes, such as pharmacokinetics. Irinotecan is an FDA approved drug in the treatment of colon cancer and *ABCB1* 1236C>T demonstrated increased AUC of irinotecan and its metabolite, SN-38 [5]. There is further evidence that the 1236T/2677T/3435T haplotype of *ABCB1* is associated with decreased irinotecan renal clearance, which translates into increased AUC [32]. Both of these studies improve our understanding of the effects genetic variants have on pharmacokinetic parameters, which may eventually lead to improved treatment with irinotecan. It is also critical to understand how genetic variants influencing expression of MDR transporters modulate

drug exposure and response. Continued collection of healthy and tumor tissue will be required to critically analyze genotype-expression associations.

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Chapter 5

Functional Prediction of Xenobiotic ABC Promoter Region Polymorphisms

5.1 Introduction

Pharmacogenetic studies of xenobiotic ABC transporters have mainly focused on the functional effects of coding region polymorphisms [1-3]. The noncoding regions, such as the promoter and untranslated regions, also play an important role in transporter expression by controlling various cellular processes involved in gene and protein production.

The interaction of numerous transcriptional elements regulates gene expression. Structural modifications in the genome, such as DNA/histone methylation and chromatin condensation, can have global effects on transcriptional activity [4-7]. At the gene level, the core promoter region contains commonly shared transcription factor binding sites that are critical for RNA polymerase II binding and activity. Classic examples are the TATA and CCAAT box motifs located within 35 and 100 bp of the transcriptional start site (TSS), respectively [8-11]. Gene-specific elements, such as enhancers and repressors, recruit protein complexes that further modify RNA polymerase II binding.

The promoter regions of certain human xenobiotic ABC transporters have been analyzed for transcription factor binding sites (TFBS). Interestingly, some do not have a TATA box, but instead rely on an initiator element located near the TSS. *ABCB1* and *ABCG2* have multiple initiator elements that produce mRNAs with different 5'-untranslated region lengths in a disease- and drug-dependent manner [12, 13] Many

unrelated factors regulate ABCB1 expression, such as heat shock proteins, p53, and pregnane X receptor (PXR) [14]. The *ABCG2* promoter has several SP1 and AP1 sites, as well as an estrogen receptor element that may influence its expression in breast cancer [15, 16]. Putative TFBSs in *ABCC1-ABCC3* and *ABCC6* have been identified but their role in the regulation of these genes is not understood [17-21].

The goal of these studies was to predict the functional significance of promoter variants of *ABCB1*, *ABCC2-ABCC6* and *ABCG2*. Polymorphisms were identified in an ethnically diverse population of healthy volunteers totaling more than 300 individuals. Computational methods were used to identify possible regulatory regions and TFBSs within the resequenced region of the promoters. The results provided in these studies will allow for a prioritized list as to which polymorphisms should be investigated in *in vitro* functional assays. Previous work from this lab has functionally characterized *ABCC2* promoter variants and the predictions for these variants can be compared to the functional data [22].

5.2 Materials and Methods

5.2.1 SNP Identification in ABC Promoters

The promoter region of *ABCB1* and *ABCC2* was screened previously for DNA sequence variation in 247 ethnically-diverse samples from the Coriell Institute [22, 23]. The polymorphisms were renumbered in relation to the TSS, which is 140 bp upstream of the ATG start codon in the mRNA sequence for ABCB1 (NM_000927) and 99 bp upstream of the ATG start codon in the mRNA sequence for ABCC2 (NM_000392). The SOPIE cohort, consisting of African Americans (n = 78), Caucasians (n = 78), Asian Americans (n = 78) and Mexican Americans (n = 78), was screened for promoter variants

in *ABCC3-ABCC6* and *ABCG2*. Sequencing primers and methods are available on-line (http://pharmacogenetics.ucsf.edu).

5.2.2 Population Genetic Parameters

The population mutation parameter (θ) was calculated for promoter region variants using the following equation [24]:

$$(\#\text{ of variants} / n) / a_1,$$

$$\text{where } a_1 = \sum_{i=1}^{n-1} \frac{1}{i}$$

For a_1, n is the number of chromosomes in the sample.

The average heterozygosity parameter (π) was calculated for promoter region variants using the following equation [24]:

$$(k / (1 - 1/n)) / \#\text{ of nucleotides screened},$$

$$\text{Where } k = \sum_{j=1}^{S} 2 p_j (1 - p_j)$$

For k, S is the number of variants and p_j is the allele frequency of the jth variant. Synonymous and nonsynonymous values for θ and π were previously calculated by the Pharmacogenetics of Membrane Transporters bioinformatics core and are available at http://pharmacogenetics.ucsf.edu.

5.2.3 Mammalian ABC Promoter Sequences

Human *ABCB1*, *ABCC1-ABCC6* and *ABCG2* gene locations were identified according to their RefSeq from the University of California, Santa Cruz (UCSC) genome browser website (http://genome.ucsc.edu). The transcriptional start site (TSS) was assumed to be the first base of the RefSeq, thus, the region upstream of the TSS was

considered the promoter. DNA sequence from −1000 bp of the TSS to +100 bp of the TSS was obtained from the March 2006 build. Promoter sequence for mouse and rat homologs were determined in a similar manner using genome browser versions February 2006 and November 2004, respectively. The human, mouse and rat TSSs for *ABCB1*, *ABCC1-ABCC6* and *ABCG2* are listed in Table 5.1.

Table 5.1
Transcription start sites for selected mammalian ABC genes

Gene	Chromosomal location (strand)[a]		
	Human	Mouse	Rat
ABCB1	chr7:87068119 (-)	chr5:8804192 (+)[b]	chr4:21829489 (+)
ABCC1	chr16:15950935 (+)	chr16:14275138 (+)	chr10:575700 (-)
ABCC2	chr10:101532563 (+)	chr19:43834682 (+)	N/A[c]
ABCC3	chr17:46067227 (+)	chr11:94209066 (-)	chr10:83030799 (-)
ABCC4	chr13:94751684 (-)	chr14:117589897 (-)	chr15:103611238 (-)
ABCC5	chr3:185218421 (-)	chr16:20339937 (-)	chr11:82714334 (+)
ABCC6	chr16:16224815 (-)	chr7:45898324 (-)	chr1:96524655 (-)
ABCG2	chr4:89299035 (-)	chr6:58526250 (+)	chr4:87502584 (+)

[a] Positions based on UCSC genome browser (http://genome.ucsc.edu). Build versions for human, mouse and rat are March 2006, February 2006 and November 2004, respectively.
[b] *Abcb1b*
[c] Promoter sequence obtained from AF261713.1. Rat *Abcc2* has a provisional status on the UCSC genome browser.

5.2.4 ABC Promoter Sequence Alignments

The promoter regions for human, mouse and rat *ABCB1*, *ABCC1-ABCC6* and *ABCG2* were aligned using Clustal W (ver. 1.83) and edited in Jalview (ver. 2.2) [25]. Sequence identities for ~1000 bp regions between human, mouse and rat were

determined from pairwise comparisons using Clustal W. Conservation near promoter variants was

determined from an arbitrary threshold of 70% sequence identity and was based on at least seven out of ten bp encompassing a SNP matching the mouse and/or rat sequence.

5.2.5 Transcription Factor Binding Site Prediction

Promoter sequences for human *ABCB1*, *ABCC1*, *ABCC3-ABCC6* and *ABCG2* were submitted to Patch (based on the TransFac database, http://www.gene-regulation.com) and ConSite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite) for prediction of transcription factor binding sites [26]. A similar approach was used previously to predict transcription factor binding sites for *ABCC2* [22].

5.3 Results

5.3.1 Genetic Variation in Xenobiotic ABC Promoters

Promoter region variants for *ABCB1* and *ABCC2* have been reported previously [22, 23]. An ethnically diverse cohort of healthy volunteers was used to identify polymorphisms in the promoter regions of *ABCC3-ABCC6* and *ABCG2*. There were 78 individuals each from four different ethnic groups: African Americans, Caucasians, Asian Americans and Mexican Americans. Approximately 500-800 bp upstream of the TSS containing the core promoter, enhancer elements and 100 bp downstream of the TSS were sequenced [27]. A total of 56 polymorphisms were identified in the promoters of *ABCB1*, *ABCC2-ABCC6* and *ABCG2*, and the results are summarized in Table 5.2. Work is ongoing to screen *ABCC1* for promoter SNPs. The majority of the ABC promoter

Table 5.2
Promoter SNPs identified in *ABCB1* , *ABCC2* -*ABCC6* and *ABCG2*

Gene	Chromosome (strand)	Golden path[a]	NCBI SNP ID[b]	Position[c]	Alleles	Allele frequency (%)[d]			
						AA	CA	AS	ME
ABCB1	chr7 (-)	87068604	rs35462624	-464	G>A	1.6	0	0	0
		87068553	rs34762047	-413	C>T	0	0.5	0	0
		87068476	rs28381799	-336	T>C	0.5	0	0	0
		87068390	rs28381800	-250	A>T	1	0	0	0
		87068371	rs2188524	-231	A>G	0	0	1.7	0
		87068241	rs35265821	-101	G>A	0	0	1.9	0
		87068145	rs34976462	-5	C>G	0	0	1.8	0
ABCC2	chr10 (+)	101531029	rs17222653	-1464	G>A	0.5	0	0	0
		101531043	rs1885301	-1450	A>G	52	57	85	80
		101531300	rs17222667	-1193	A>G	1.5	0	0	0
		101531353	rs17222646	-1140	G>A	3	0	0	5
		101531527	rs17216128	-966	C>A	0.5	0	0	5
		101531533	rs17216121	-960	C>CG	0.5	0	0	0
		101531569	rs7910642	-924	G>A	14	10.5	26.7	30
		101531573	rs2804402	-920	A>G	37	43	16.7	20
		101531794	rs17222533	-699	C>A	0.5	0	0	0
		101531859	rs17216135	-634	G>A	0	0	1.7	0
ABCC3	chr17 (+)	46067037	rs9895420	-190	T>A	24	10	3.1	8.9
		46067086	rs4793665	-140	C>T	54	56	92.9	53.6
ABCC4	chr13 (-)	94751858		-174	C>G	0	0.7	0	0.8
		94751797		-113	A>G	0	0	0.8	0
		94751732		-48	C>T	0	0	0.8	0

ABCC5 chr3 (-)	185218915		-494	G>A	2.2	0	0	1.5
	185218874		-453	G>A	0	0	3	0
	185218788		-367	G>C	2.2	0	0	1.5
	185218784		-363	C>G	0.7	0	0	0
	185218676		-255	C>T	0	0.7	0	0
	185218603		-182	C>T	0	0.7	0	1.5
	185218584		-163	G>A	0	0	0.8	0
	185218548		-127	C>T	6	0	0	1.5
	185218545		-124	G>C	1.5	0	0	0
	185218531		-110	A>G	0.7	0	0	0
	185218486		-65	A>C	0	0	2.3	0
	185218430		-9	T>C	0	0	0.8	0
ABCC6 chr16 (-)	16225444	rs4780624	-629	C>T	0.6	1.3	0.9	0
	16225329		-514	G>A	0.6	0	0	0
	16225324		-509	C>T	0	0.6	0	0
	16225286	rs4781770	-471	C>T	0	0	0	1
	16225260		-445	C>T	3.7	0	0	0
	16225246		-431	C>G	0	0	0	1
	16225243	rs3743530	-428	T>C	21	6.3	27.6	8
	16225011		-196	A>C	18	3.8	0.8	2
	16224931		-116	C>T	0	1.9	0	2
	16224924		-109	C>T	9.4	2.6	1.7	1
	16224919		-104	C>T	1.9	14.7	0.8	20
	16224898		-83	C>A	0.6	0	0	0
	16224877		-62	T>C	0	0	0.8	1
	16224857		-42	C>G	1.3	0	0	0

ABCG2 chr4 (-)	89299519		-484	T>A	4.2	0	0	0
	89299518		-483	C>T	0	0	0.8	0
	89299459		-424	G>A	0	0	0	0.9
	89299425		-390	C>A	7.5	0	0	1.7
	89299375	rs2231134	-340	G>C	2.5	6.5	0	6
	89299301		-266	AGTGTTT>-	3.8	4.2	0	4.7
	89299186		-251	G>C	0	0	0	0.8
	89299119		-184	C>T	0	0	0.8	0

[a] UCSC genome browser (http://genome.ucsc.edu), March 2006 build

[b] Reference SNP ID number from NCBI dbSNP (build 126; www.ncbi.nih.gov/SNP)

[c] Relative to transcriptional start site

[d] For *ABCC3* -*ABCC5* and *ABCG2* , n = 136 for African Americans (AA), Caucasians (CA), Asian Americans (AS) and Mexican Americans (ME). For *ABCB1* and *ABCC2* , n = 200 for AA and CA, 60 for AS and 20 for ME. For *ABCC6* , n = 160 for AA, 158 for CA, 116 for AS and 100 for ME.

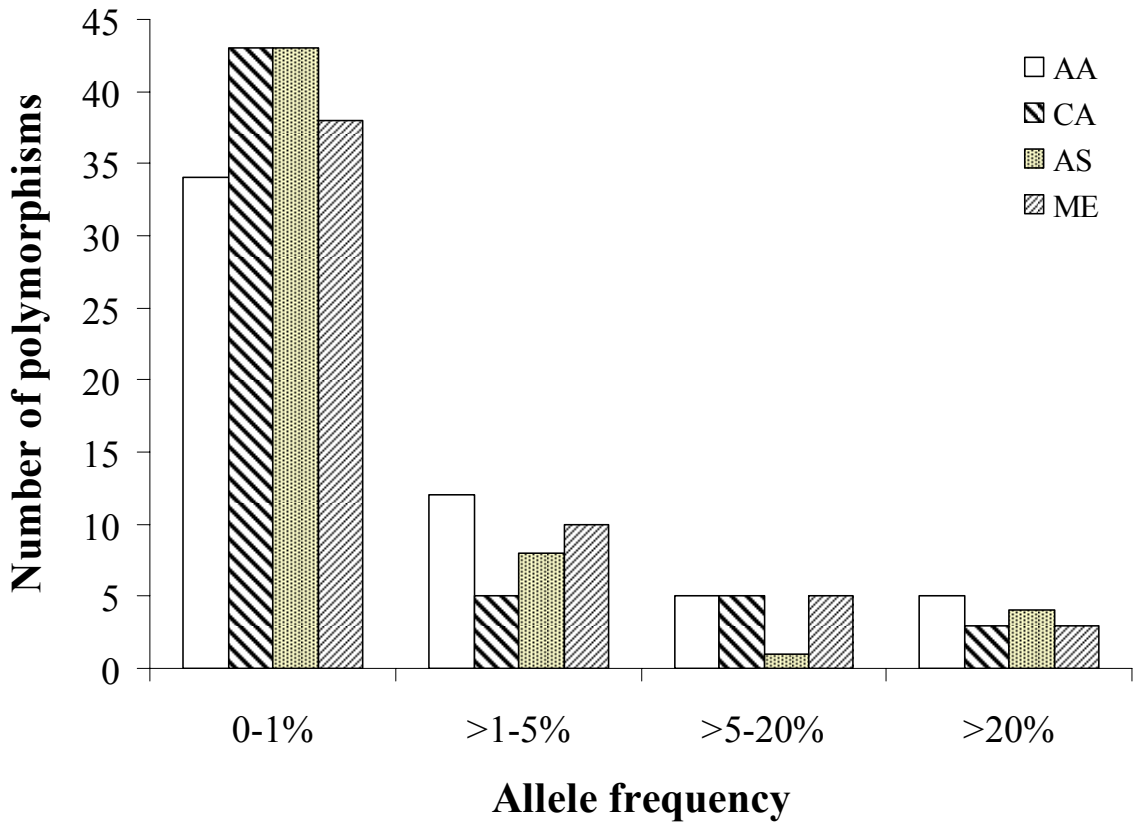


Figure 5.1. Frequency distribution of 56 promoter SNPs in seven ABC genes. Ethnic-specific allele frequencies for African Americans (AA), Caucasians (CA), Asian Americans (AS) and Mexican Americans were grouped into four bins. Each bar represents the number of SNPs found in each allele frequency bin.

polymorphisms have an allele frequency <1% (Figure 5.1) and are specific to one ethnic group. In general, the variants were located within 500 bp of the TSS, with the exception of *ABCC2* which has no variants in the 500 bp region upstream of the TSS. *ABCC5* and *ABCC6* had the largest number of polymorphisms. Only two high frequency, cosmopolitan SNPs for *ABCC3* were discovered and only three *ABCC4* singleton variants were identified. Interestingly, there was a seven base pair deletion in *ABCG2* found in

African Americans, Caucasians and Mexican Americans with an allele frequency between 4-5%.

5.3.2 Nucleotide Diversity of Xenobiotic ABC Promoter Variants

The population genetic parameter, θ, measures the degree of mutation for a gene based on the number of nucleotides and individuals screened, as well as the number of variants identified. This parameter can be calculated for any characterized region of a gene and/or for different types of polymorphisms, such as coding and noncoding. The first 300 bp (−1 to −300) of the promoter are considered the core regulatory region necessary for transcription while the upstream flanking region contains enhancer or repressor elements [27]. Accordingly, θ was calculated for the two promoter regions, −1 to −300 bp and upstream of −300 bp, in order to determine if there was similar genetic variation in the core promoter and upstream regions. *ABCB1* and *ABCG2* had similar levels of mutation in the core promoter and flanking region, while the core promoter for *ABCC2* contained no variants. Surprisingly, there was greater mutation in the core promoter than upstream regions for *ABCC3-ABCC6* (Figure 5.2A). Values of θ for synonymous (syn) and nonsynonymous (n.s.) variants served as comparisons for the promoter values. The core promoter regions had greater heterozygosity than the coding regions for *ABCB1* and *ABCC5*, while the opposite was true for *ABCC2-ABCC4*. *ABCG2* and *ABCC6* demonstrated similar values for $\theta_{promoter}$ and θ_{coding}. There was no obvious trend as to whether the promoter or coding region was more variable in the xenobiotic ABC transporters. Additionally, the flanking intronic regions for the seven ABC genes had much lower degrees of mutation (θ) than the promoter regions (data not shown).

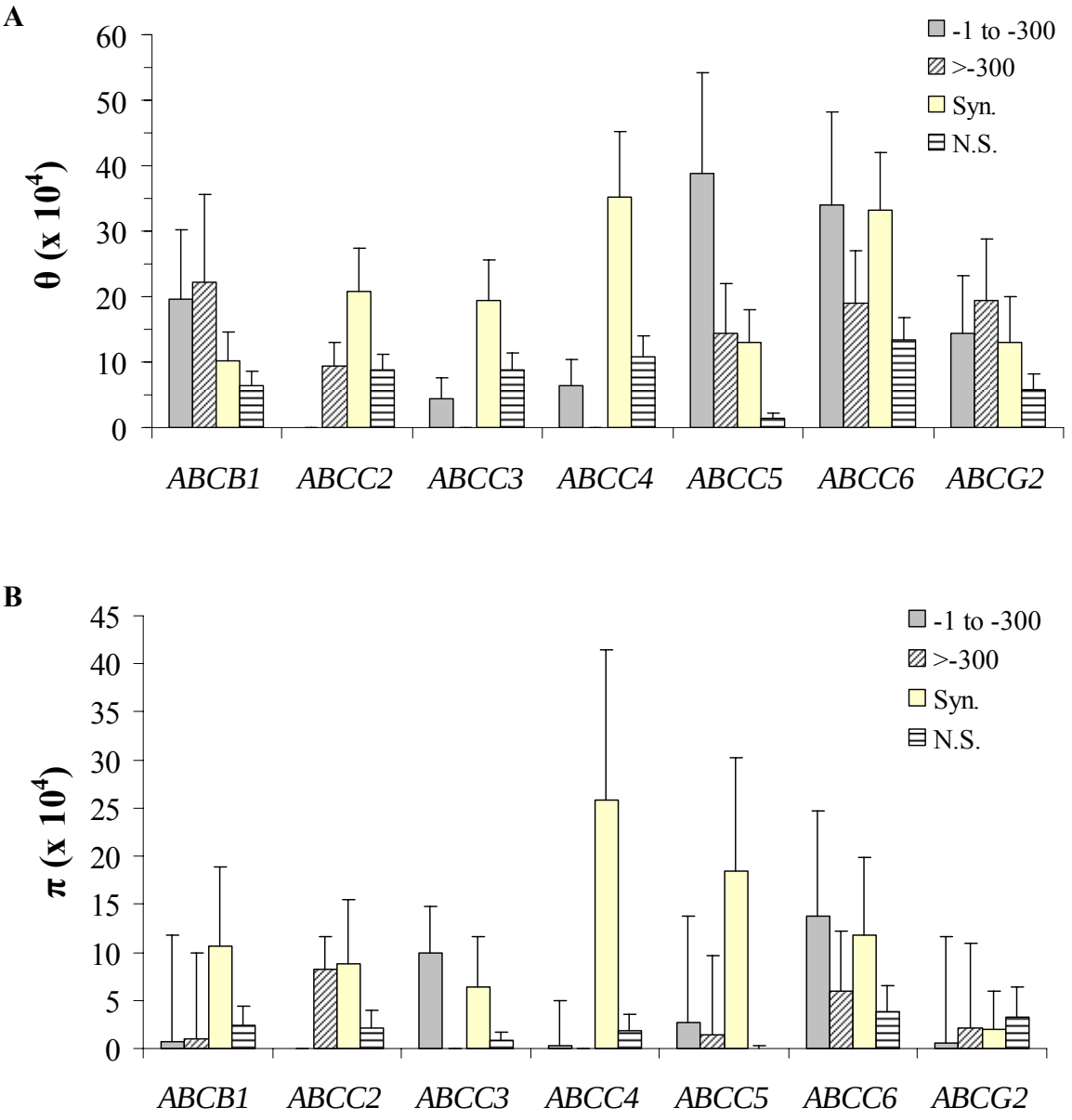


Figure 5.2. Nucleotide diversity of the promoter and coding regions in xenobiotic ABC genes. The population mutation parameter (θ) measures the degree of mutation in a given region of a gene (A). The average heterozygosity parameter (π) measures the proportion of nucleotide differences in a sample population (B). Promoter variants were grouped according to their position in the core promoter (-1 to -300) or flanking region (>-300). Coding region values for θ and π were calculated for synonymous (syn.) or nonsynonymous (n.s.) variants. Each bar represents the calculated θ ± s.e. or π ± s.e.

The average heterozygosity parameter (π) determines nucleotide diversity based on the allele frequencies of variants normalized to a given number of nucleotides and chromosomes screened. Among the promoter region variants, *ABCC2*, *ABCC3* and *ABCC6* had the largest π values, and *ABCB1*, *ABCC4*, *ABCC5* and *ABCG2* had the smallest π values. In general, synonymous variants generally had larger π values than nonsynonymous, and served as the primary comparison for $\pi_{promoter}$. *ABCB1*, *ABCC3-ABCC6* promoter regions had lower average heterozygosity than synonymous variants, while there was similar heterozygosity for *ABCC2* and *ABCG2* (Figure 5.2B).

5.3.3 Xenobiotic ABC Promoter Sequence Conservation

The goal of these studies is to predict the functional significance of promoter region variants on transcription. One predictive method is to identify conserved regions between the human, mouse and rat promoter sequences. An approximately 1000 bp region encompassing the promoter and TSS was obtained for human, mouse and rat, and aligned using Clustal W (ver. 1.83). There was a range of 46-59% sequence identity when comparing each human ABC promoter to either the mouse or rat homolog (Table 5.3). Conservation was generally the highest within ~400 bp of the TSS and more distal promoter regions showed decreased conservation. As expected, the mouse and rat sequences consistently showed the highest similarities (65-78%). An alignment was not determined for *ABCG2* because neither the human, mouse or rat sequences matched. It is possible the chromosomal sequences from the UCSC genome browser are inaccurate for mouse or rat. In addition, human *ABCG2* has more than one TSS [13].

Table 5.3
Promoter region sequence conservation for *ABCB1* and *ABCC1 -ABCC6*

Gene	Hu-Mo	Hu-Ra	Mo-Ra
ABCB1	57	52	75
ABCC1	48	49	78
ABCC2	47	47	65
ABCC3	49	46	74
ABCC4	59	50	71
ABCC5	59	58	77
ABCC6	47	48	74

Percent identity determined from Clustal W alignments of human (Hu), mouse (Mo) and rat (Ra) promoter regions (~1000 bp).

5.3.4 Promoter Variants in Predicted Regulatory Elements

Polymorphisms were considered functionally relevant if they were part of a conserved region with 70% sequence identity and/or were in a TFBS. Conservation was defined as seven out of ten bp surrounding each variant matching the mouse and/or rat sequence. There were at least 100 TFBSs found in ~1000 bp for each promoter, but only those containing a polymorphism were characterized. The other TFBSs should be investigated since little has been done to understand these ABC promoters, but the work is beyond the scope of these studies.

There were three *ABCB1* promoter variants in conserved regions (−413C>T, −336T>C and −5C>G) and two variants in putative TFBSs (−336T>C and −231A>G; Figure 5.3). The −336T>C variant may be the most interesting because it is in a conserved region and a putative PITX2 binding site. There is a putative CTF (CCAAT-

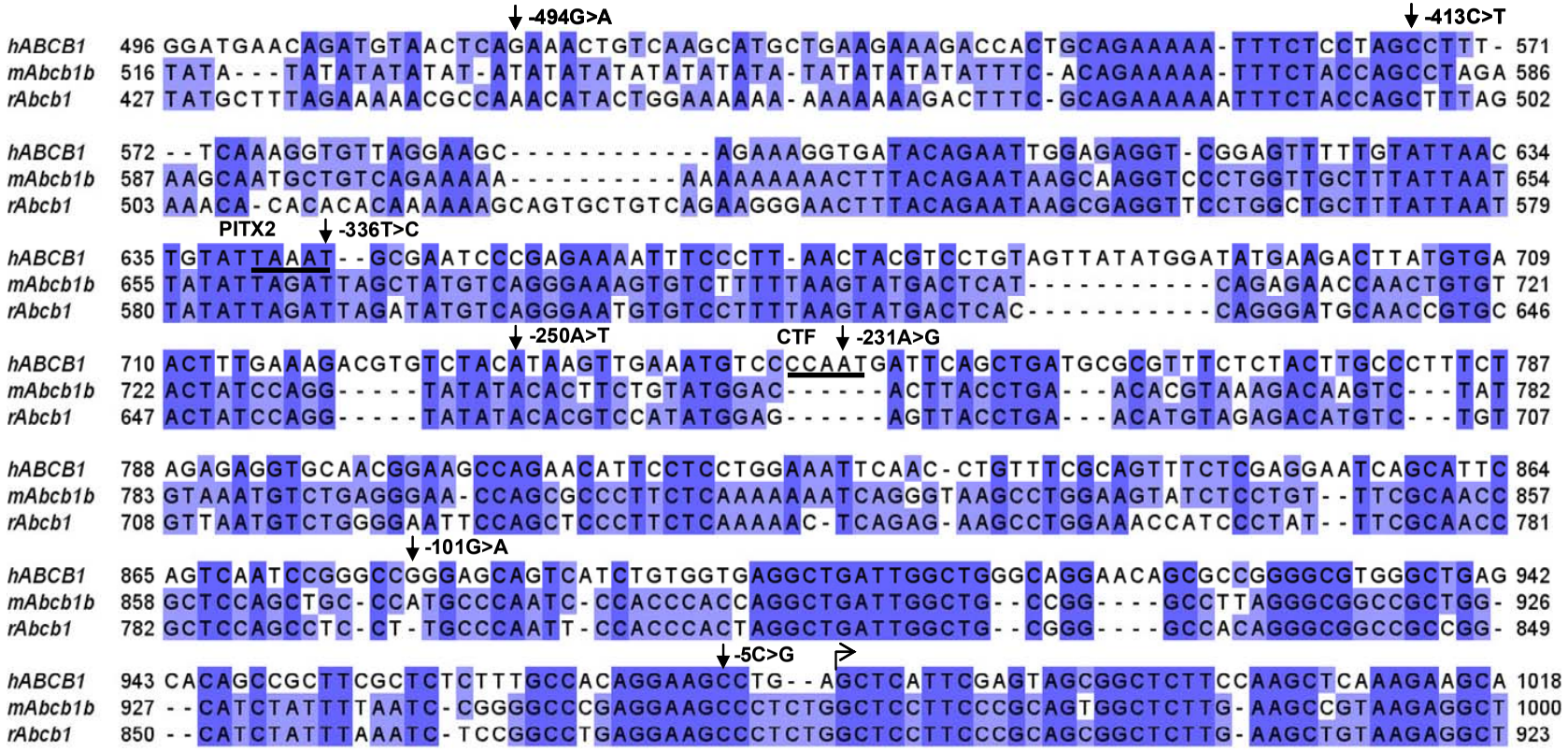


Figure 5.3. *ABCB1* promoter region alignment with selected mammalian species. Clustal W was used to align ~1000 bp of the human, mouse and rat promoter sequences and a ~500 bp segment from the alignment is shown. Conservation is represented by dark and light shading to show complete or partial homology, respectively. Polymorphisms are indicated by vertical arrows and putative TFBSs specific to a polymorphism are underlined. The human TSS is denoted by a horizontal arrow.

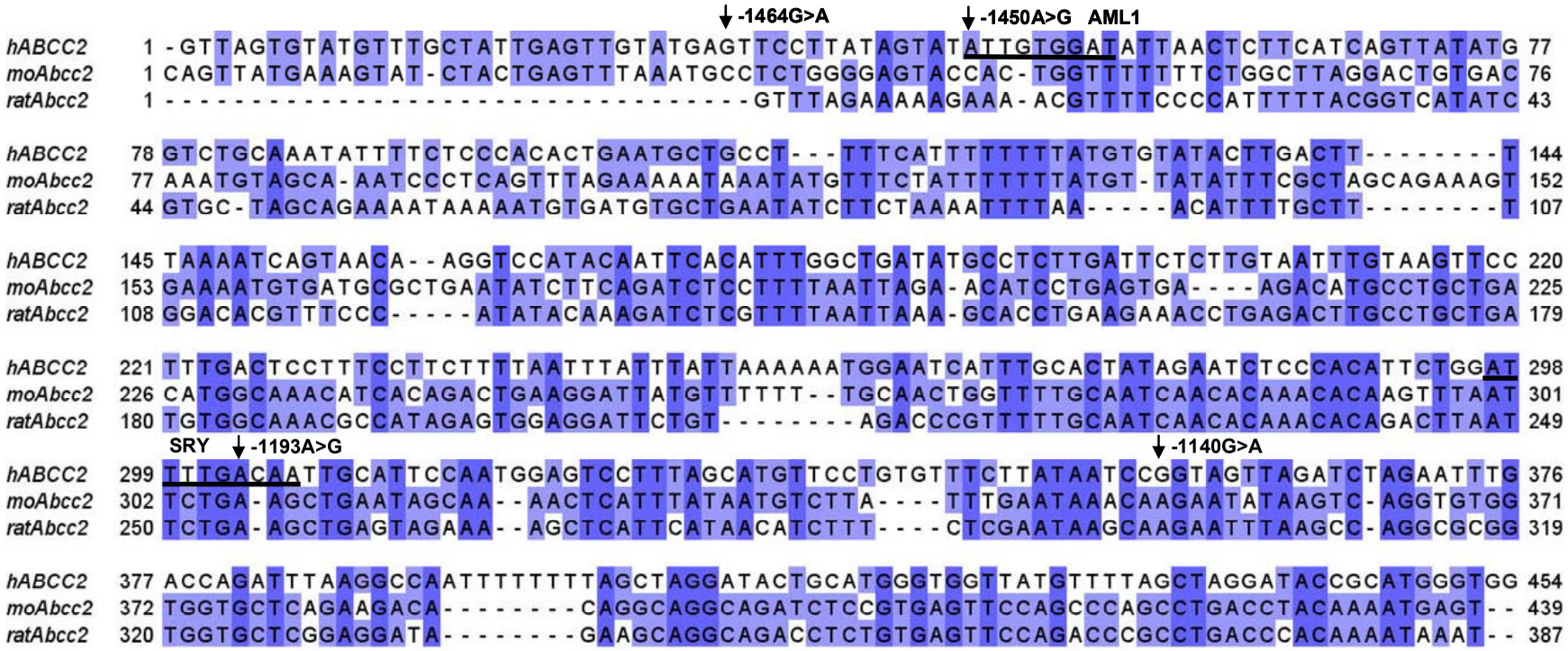
binding transcription factor) site encompassing −231A>G, however, there is no sequence conservation in this region. The −494G>A, −250A>T and −101G>A *ABCB1* variants were not in conserved regions or putative TFBSs.

The *ABCC1* multispecies alignment is not shown because variants have yet to be identified. Figure 5.4 shows *ABCC2* promoter variants and the sequence alignment with mouse and rat. Compared to other ABC genes, there was low conservation for the *ABCC2* promoter region including the pairwise comparison between mouse and rat. The −1193A>G and −634G>A variants were part of conserved regions, and −1193A>G was in a putative SRY motif. *ABCC2* −1450A>G, −960C>CG and −920A>G were located in putative AML1, PITX2 and PU.1 binding sites, respectively.

The human *ABCC3* promoter region had one of the lowest sequence identities with mouse and rat, and neither of its two polymorphisms was located in conserved regions. The −190T>A and −140C>T polymorphisms may disrupt putative C/EBPα and Sp1 binding sites, respectively (Figure 5.5). The −190A variant matches the mouse and rat sequence but the surrounding sequence conservation is minimal.

There was strong conservation from −1 to −300 of the *ABCC4* promoter but the similarities decreased further upstream. The rat sequence had an incomplete section in the middle of the promoter that probably contributed to the 50% identity with human. In contrast, there was 59% identity between the human and mouse *ABCC4* promoters. The −174C>G polymorphism was located in a conserved region and in a putatitve SMAD-3 binding site (Figure 5.6). The −113A>G variant was also in a conserved region and located in a possible Sp1 sequence motif. *ABCC4* −174C>G and −113A>G may be

A


-1464G>A
-1450A>G AML1
hABCC2 1 -GTTAGTGTATGTTTGCTATTGAGTTGTATGAGTTCCTTATAGTATATTGTGGATATTAACTCTTCATCAGTTATATG 77
moAbcc2 1 CAGTTATGAAAGTAT-CTACTGAGTTTAAATGCCTCTGGGGAGTACCAC-TGGTTTTTTTCTGGCTTAGGACTGTGAC 76
ratAbcc2 1 ----------GTTTAGAAAAGAAA-ACGTTTTCCCCATTTTTACGGTCATATC 43
hABCC2 78 GTCTGCAAATATTTTCTCCCACACTGAATGCTGCCT---TTTCATTTTTTTTATGTGTATACTTGACTT--------T 144
moAbcc2 77 AAATGTAGCA-AATCCCTCAGTTTAGAAAAATAAATATGTTTCTATTTTTTTATGT-TATATTTCGCTAGCAGAAAGT 152
ratAbcc2 44 GTGC-TAGCAGAAAATAAAAATGTGATGTGCTGAATATCTTCTAAAATTTTAA-----ACATTTTGCTT--------T 107
hABCC2 145 TAAAATCAGTAACA--AGGTCCATACAATTCACATTTGGCTGATATGCCTCTTGATTCTCTTGTAATTTGTAAGTTCC 220
moAbcc2 153 GAAAATGTGATGCGCTGAATATCTTCAGATCTCCTTTTAATTAGA-ACATCCTGAGTGA----AGACATGCCTGCTGA 225
ratAbcc2 108 GGACACGTTTCCC-----ATATACAAAGATCTCGTTTTAATTAAA-GCACCTGAAGAAACCTGAGACTTGCCTGCTGA 179
hABCC2 221 TTTGACTCCTTTCCTTCTTTTAATTTATTTATTAAAAAATGGAATCATTTGCACTATAGAATCTCCCACATTCTGGAT 298
moAbcc2 226 CATGGCAAACATCACAGACTGAAGGATTATGTTTTTT--TGCAACTGGTTTTGCAATCAACACAAACACAAGTTTAAT 301
ratAbcc2 180 TGTGGCAAACGCCATAGAGTGGAGGATTCTGT--------AGACCCGTTTTTGCAATCAACACAAACACAGACTTAAT 249
SRY -1193A>G
-1140G>A
hABCC2 299 TTTGACAATTGCATTCCAATGGAGTCCTTTAGCATGTTCCTGTGTTTCTTATAATCCGGTAGTTAGATCTAGAATTTG 376
moAbcc2 302 TCTGA-AGCTGAATAGCAA--AACTCATTTATAATGTCTTA----TTTGAATAAACAAGAATATAAGTC-AGGTGTGG 371
ratAbcc2 250 TCTGA-AGCTGAGTAGAAA--AGCTCATTCATAACATCTTT----CTCGAATAAGCAAGAATTTAAGCC-AGGCGCGG 319
hABCC2 377 ACCAGATTTAAGGCCAATTTTTTTTAGCTAGGATACTGCATGGGTGGTTATGTTTTAGCTAGGATACCGCATGGGTGG 454
moAbcc2 372 TGGTGCTCAGAAGACA--------CAGGCAGGCAGATCTCCGTGAGTTCCAGCCCAGCCTGACCTACAAAATGAGT-- 439
ratAbcc2 320 TGGTGCTCGGAGGATA--------GAAGCAGGCAGACCTCTGTGAGTTCCAGACCCGCCTGACCCACAAAATAAAT-- 387

B

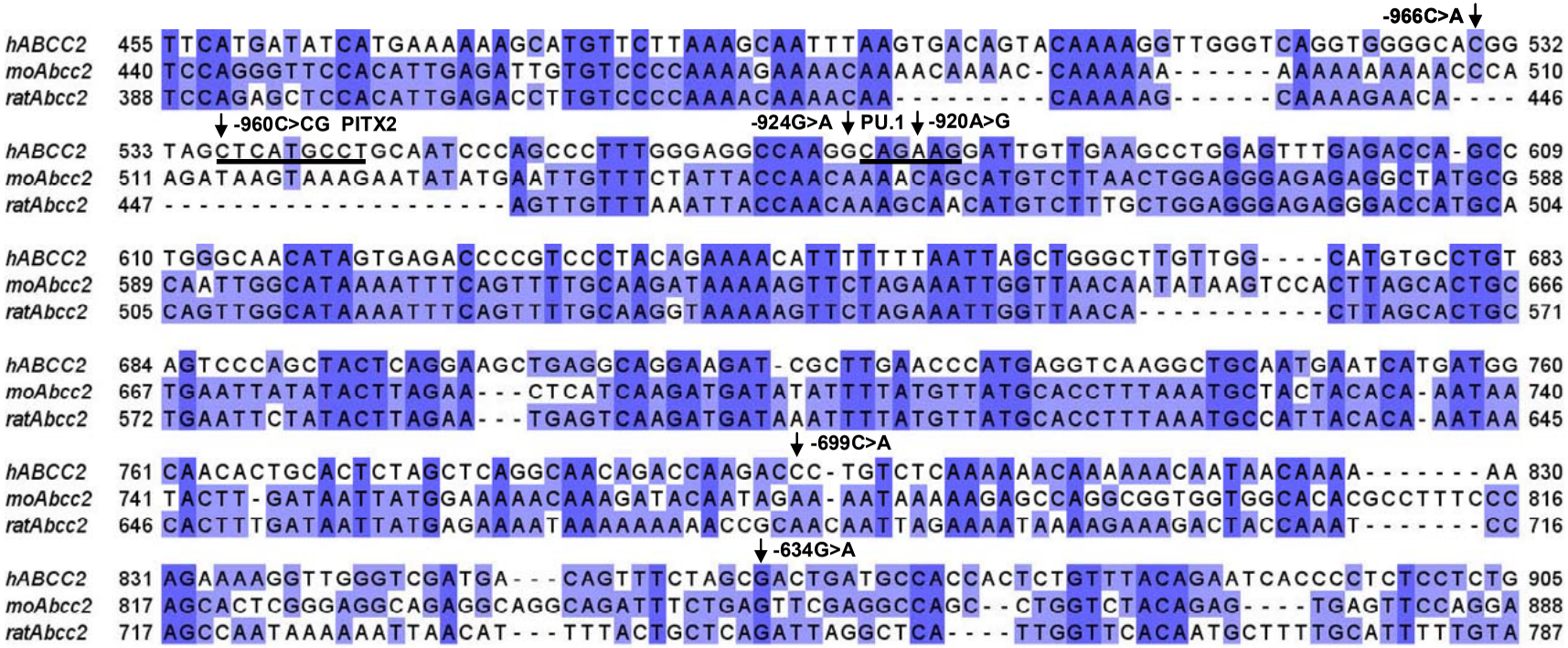


Figure 5.4. *ABCC2* promoter region alignment with selected mammalian species. Clustal W was used to align ~1000 bp of the human, mouse and rat promoter sequences, and the alignment was separated into two segments. Polymorphisms are indicated by vertical arrows in the promoter regions -1500 to -1000 (A) and -1000 to -500 (B). Conservation is represented by dark and light shading to show complete or partial homology, respectively.

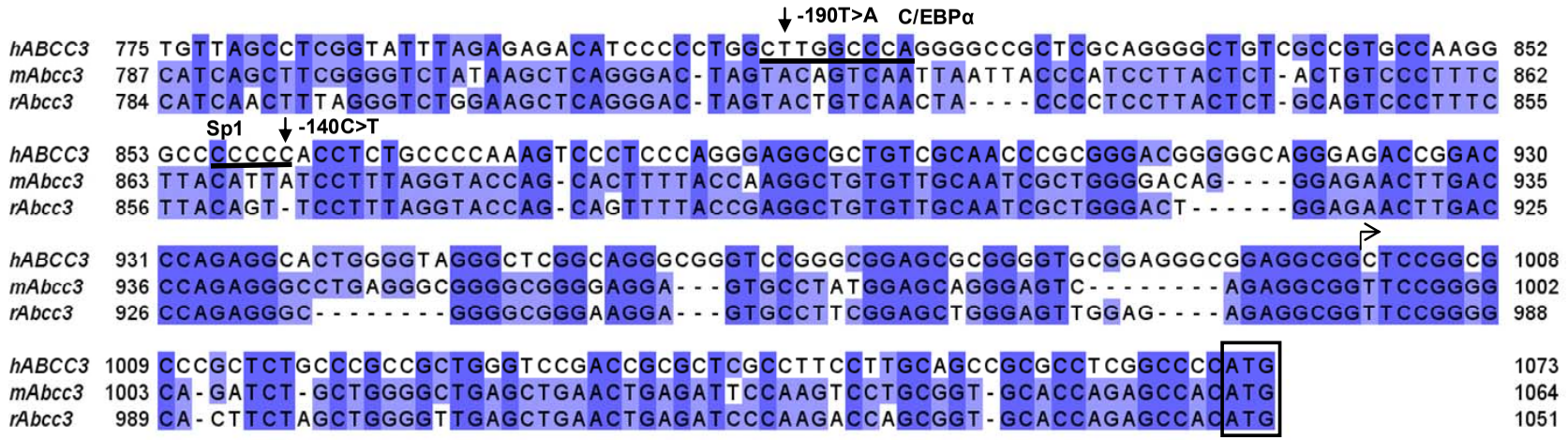


Figure 5.5. *ABCC3* promoter region alignment with selected mammalian species. Clustal W was used to align ~1000 bp of the human, mouse and rat promoter sequences and a ~300 bp segment from the alignment is shown. Conservation is represented by dark and light shading to show complete or partial homology, respectively. Polymorphisms are indicated by vertical arrows and putative TFBSs specific to a polymorphism are underlined. The human TSS is denoted by a horizontal arrow and the ATG start codons are outlined.

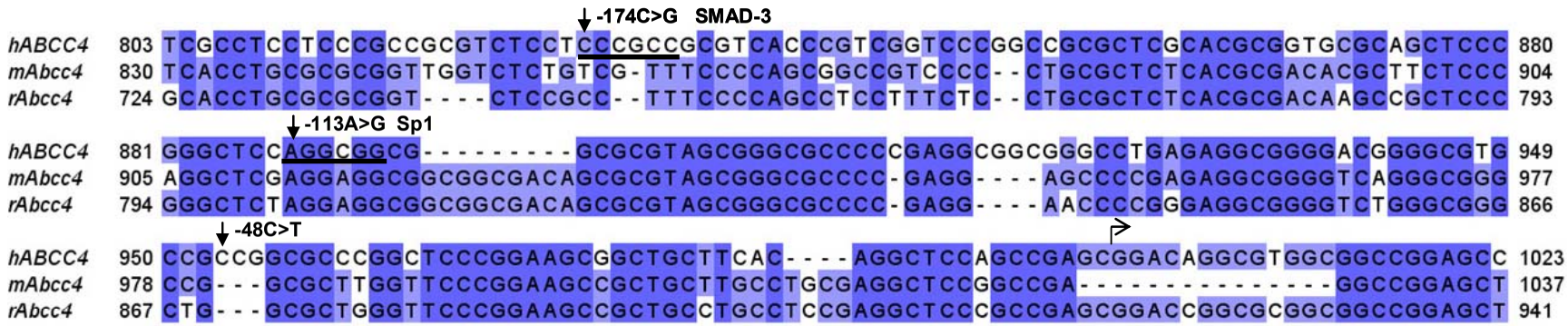


Figure 5.6. *ABCC4* promoter region alignment with selected mammalian species. Clustal W was used to align ~1000 bp of the human, mouse and rat promoter sequences and a ~200 bp segment from the alignment is shown. Conservation is represented by dark and light shading to show complete or partial homology, respectively. Polymorphisms are indicated by vertical arrows and putative TFBSs specific to a polymorphism are underlined. The human TSS is denoted by a horizontal arrow.

functionally important based on these computational predictions. There was minimal conservation and no putative TFBSs surrounding the −48C>T polymorphism.

The *ABCC5* promoter region was the most conserved over 1000 bp and there was significant conservation in the ~350 bp upstream of the TSS. As a result, there were eight variants in conserved regions: −494G>A, −255C>T, −182C>T, −163G>A, −127C>T, −124G>C, −65A>C and −9T>C (Figure 5.7). Putative Sp1 sites overlapped the −453G>A,−127C>T and −124G>C *ABCC6* variants while −163G>A and −110A>G may be in PEA3 binding domains. Another SMAD-3 motif was identified in the *ABCC5* promoter that contains the −494G>A polymorphism. The −65A>C *ABCC5* variant is contained in a putative c-ETS-1 transcriptional repressor site, which could disrupt protein binding in a similar manner as the −260G>C *ABCC1* variant (Chapter 1) [28]. A CREB sequence motif was predicted to contain the −367G>C SNP, although there was minimal sequence conservation in this region. *ABCC5* −494G>A, −163G>A, −127C>T, −124G>C and −65A>C are potentially the most important *ABCC5* variants to investigate because of their species conservation and presence in TFBSs.

There were 14 SNPs identified in the promoter region of *ABCC6*, which has below average conservation with mouse and rat. Multiple putative Sp1 sites were identified that contained five SNPs, but only *ABCC6* −62T>C displayed significant sequence identity in the surrounding region (Figure 5.8). The −509C>T variant may disrupt an AP-2 binding site while −471C>T is in a putative CREB transcription factor binding site. However, there was minimal conservation surrounding both variants. *ABCC6* −116C>T and −104C>T were in putative GCF and LUN-1 binding sites,

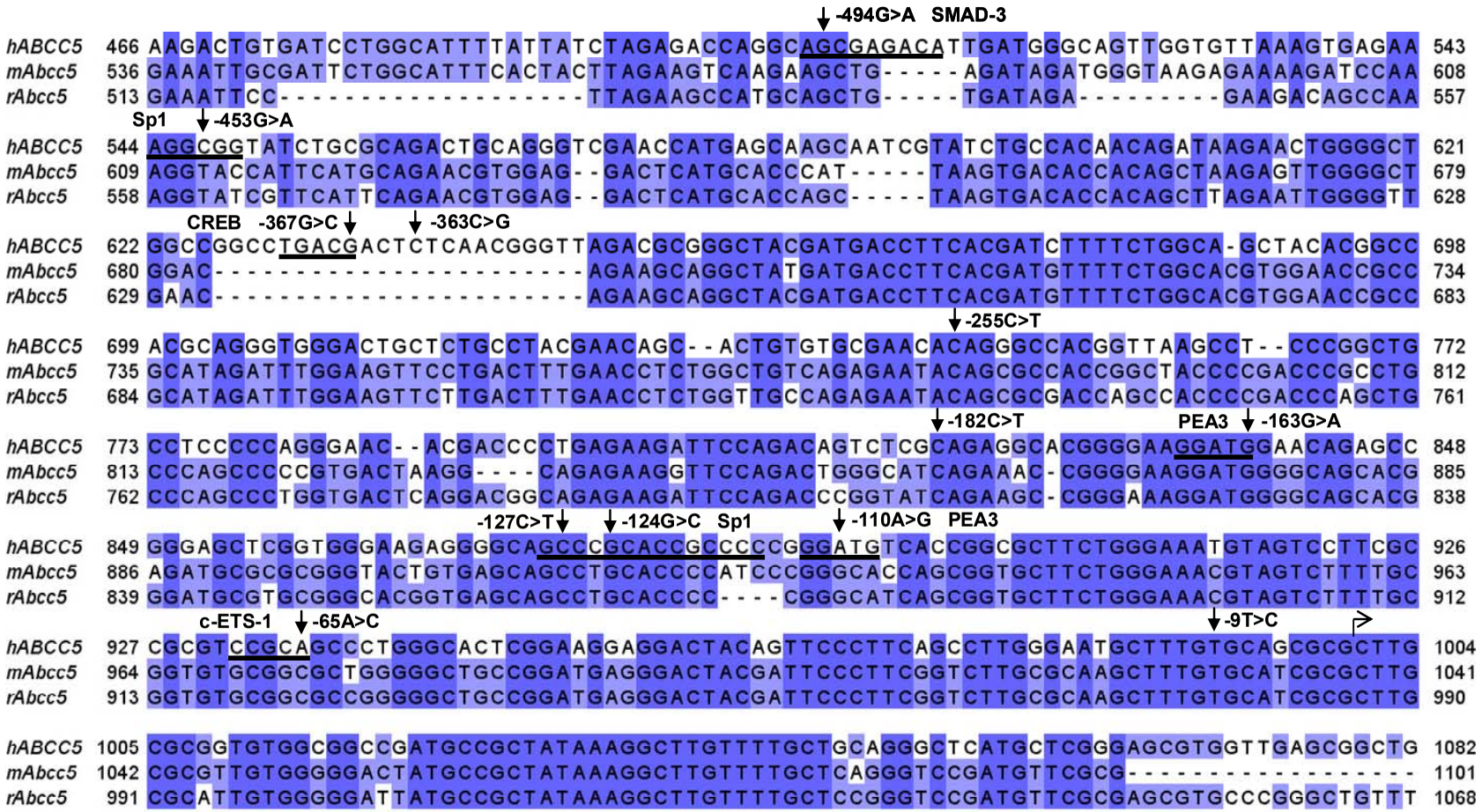


Figure 5.7. *ABCC5* promoter region alignment with selected mammalian species. Clustal W was used to align ~1000 bp of the human, mouse and rat promoter sequences and a ~600 bp segment from the alignment is shown. Conservation is represented by dark and light shading to show complete or partial homology, respectively. Polymorphisms are indicated by vertical arrows and putative TFBSs specific to a polymorphism are underlined. The human TSS is denoted by a horizontal arrow.

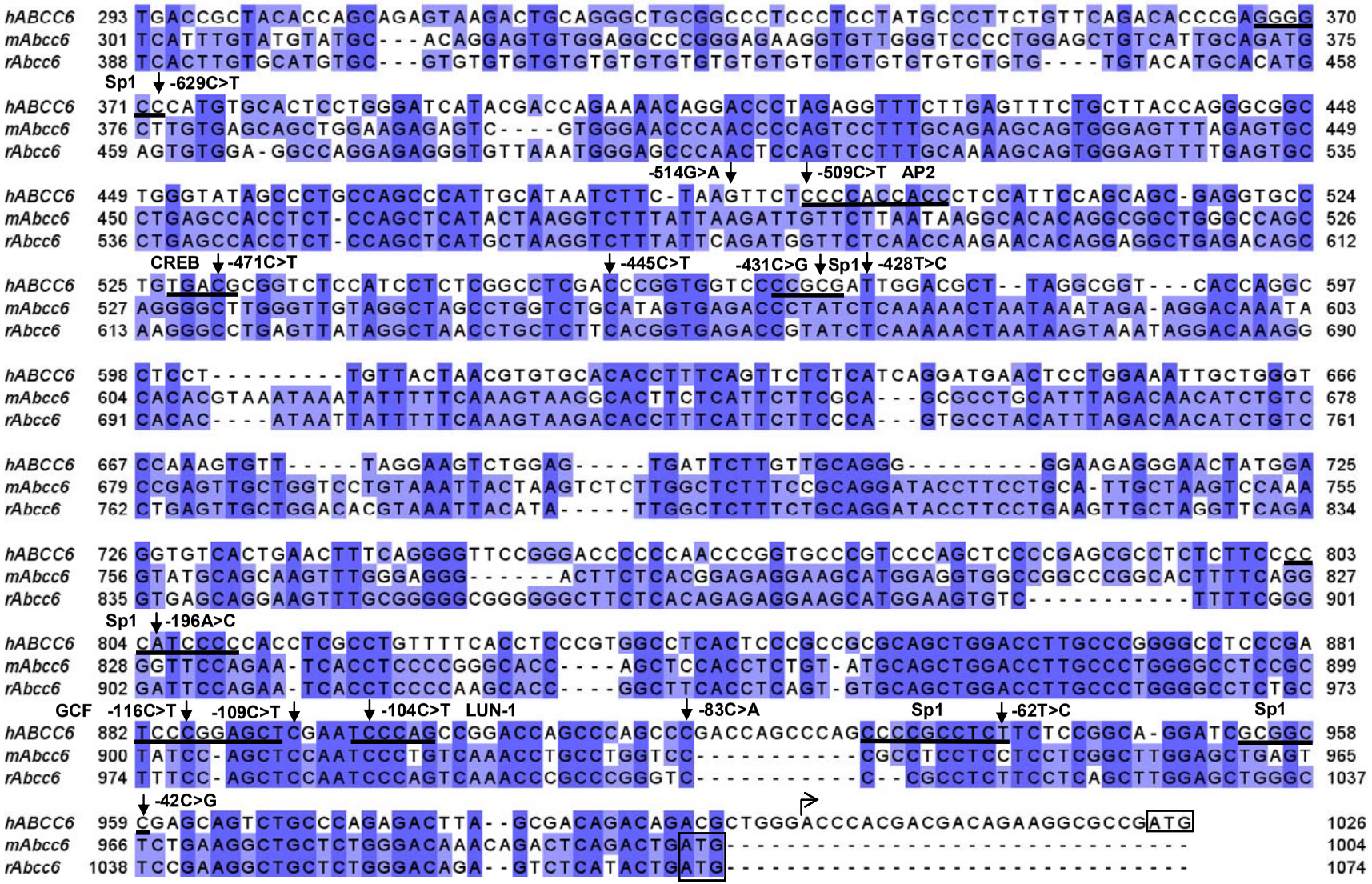

hABCC6 293 TGACCGCTACACCAGCAGAGTAAGACTGCAGGGCTGCGGCCCTCCCTCCTATGCCCTTCTGTTCAGACACCCGAGGGG 370
mAbcc6 301 TCATTTGTATGTATGC---ACAGGAGTGTGGAGGCCCGGGAGAAGGTGTTGGGTCCCCTGGAGCTGTCATTGCAGATG 375
rAbcc6 388 TCACTTGTGCATGTGC---GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG----TGTACATGCACATG 458
Sp1 -629C>T
hABCC6 371 CCCATGTGCACTCCTGGGATCATACGACCAGAAAACAGGACCCTAGAGGTTTCTTGAGTTTCTGCTTACCAGGGCGGC 448
mAbcc6 376 CTTGTGAGCAGCTGGAAGAGAGTC----GTGGGAACCCAACCCCAGTCCTTTGCAGAAGCAGTGGGAGTTTAGAGTGC 449
rAbcc6 459 AGTGTGGA-GGCCAGGAGAGGGTGTTAAATGGGAGCCCAACTCCAGTCCTTTGCAAAAGCAGTGGGAGTTTTGAGTGC 535
-514G>A -509C>T AP2
hABCC6 449 TGGGTATAGCCCTGCCAGCCCATTGCATAATCTTC-TAAGTTCTCCCCACCACCCTCCATTCCAGCAGC-GAGGTGCC 524
mAbcc6 450 CTGAGCCACCTCT-CCAGCTCATACTAAGGTCTTTATTAAGATTGTTCTTAATAAGGCACACAGGCGGCTGGGCCAGC 526
rAbcc6 536 CTGAGCCACCTCT-CCAGCTCATGCTAAGGTCTTTATTCAGATGGTTCTCAACCAAGAACACAGGAGGCTGAGACAGC 612
CREB -471C>T -445C>T -431C>G Sp1 -428T>C
hABCC6 525 TGTGACGCGGTCTCCATCCTCTCGGCCTCGACCCGGTGGTCCCCGCGATTGGACGCT--TAGGCGGT---CACCAGGC 597
mAbcc6 527 AGGGGCTTGGGTTGTAGGCTAGCCTGGTCTGCATAGTGAGACCCTATCTCAAAAACTAATAAATAGA-AGGACAAATA 603
rAbcc6 613 AAGGGCCTGAGTTATAGGCTAACCTGCTCTTCACGGTGAGACCGTATCTCAAAAACTAATAAGTAAATAGGACAAAGG 690
hABCC6 598 CTCCT---------TGTTACTAACGTGTGCACACCTTTCAGTTCTCTCATCAGGATGAACTCCTGGAAATTGCTGGGT 666
mAbcc6 604 CACACGTAAATAAATATTTTTCAAAGTAAGGCACTTCTCATTCTTCGCA---GCGCCTGCATTTAGACAACATCTGTC 678
rAbcc6 691 CACAC----ATAATTATTTTTCAAAGTAAGACACCTTTCATTCTTCCCA---GTGCCTACATTTAGACAACATCTGTC 761
hABCC6 667 CCAAAGTGTTT-----TAGGAAGTCTGGAG-----TGATTCTTGTTGCAGGG---------GGAAGAGGGAACTATGGA 725
mAbcc6 679 CCGAGTTGCTGGTCCTGTAAATTACTAAGTCTCTTGGCTCTTTCCGCAGGATACCTTCCTGCA-TTGCTAAGTCCAAA 755
rAbcc6 762 CTGAGTTGCTGGACACGTAAATTACATA-----TTGGCTCTTTCTGCAGGATACCTTCCTGAAGTTGCTAGGTTCAGA 834
hABCC6 726 GGTGTCACTGAACTTTCAGGGGTTCCGGGACCCCCCAACCCGGTGCCCGTCCCAGCTCCCCGAGCGCCTCTCTTCCCC 803
mAbcc6 756 GTATGCAGCAAGTTTGGGAGGG------ACTTCTCACGGAGAGGAAGCATGGAGGTGGCCGGCCCGGCACTTTTCAGG 827
rAbcc6 835 GTGAGCAGGAAGTTTGCGGGGGCGGGGGGCTTCTCACAGAGAGGAAGCATGGAAGTGTC----------TTTTCGGG 901
Sp1 -196A>C
hABCC6 804 CATCCCCCACCTCGCCTGTTTTCACCTCCCGTGGCCTCACTCCCGCCGCGCAGCTGGACCTTGCCCGGGGCCTCCCGA 881
mAbcc6 828 GGTTCCAGAA-TCACCTCCCCGGGCACC----AGCTCCACCTCTGT-ATGCAGCTGGACCTTGCCCTGGGGCCTCCGC 899
rAbcc6 902 GATTCCAGAA-TCACCTCCCCAAGCACC----GGCTTCACCTCAGT-GTGCAGCTGGACCTTGCCCTGGGGCCTCTGC 973
GCF -116C>T -109C>T -104C>T LUN-1 -83C>A Sp1 -62T>C Sp1
hABCC6 882 TCCCGGAGCTCGAATCCCAGCCGGACCAGCCCAGCCCGACCAGCCCAGCCCCGCCTCTTCTCCGGCA-GGATCGCGGC 958
mAbcc6 900 TATCC-AGCTCCAATCCCTGTCAAACCTGCCTGGTCC----------CGCCTCCTCCTCCTCGGCTTGGAGCTGAGT 965
rAbcc6 974 TTTCC-AGCTCCAATCCCAGTCAAACCCGCCCGGGTC----------C--CGCCTCTTCCTCAGCTTGGAGCTGGGC 1037
-42C>G
hABCC6 959 CGAGCAGTCTGCCCAGAGACTTA--GCGACAGACAGACGCTGGGACCCACGACGACAGAAGGCGCCGATG 1026
mAbcc6 966 TCTGAAGGCTGCTCTGGGACAAACAGACTCAGACTGATG---------- 1004
rAbcc6 1038 TCCGAAGGCTGCTCTGGGACAGA--GTCTCATACTGATG---------- 1074

Figure 5.8. *ABCC6* promoter region alignment with selected mammalian species. Clustal W was used to align ~1000 bp of the human, mouse and rat promoter sequences and a ~700 bp segment from the alignment is shown. Conservation is represented by dark and light shading to show complete or partial homology, respectively. Polymorphisms are indicated by vertical arrows and putative TFBSs specific to a polymorphism are underlined. The human TSS is denoted by a horizontal arrow and the ATG start codons are outlined.

respectively, and showed strong sequence conservation. The −62T>C, −104C>T, and −116C>T variants may be the most relevant to *ABCC6* transcriptional regulation.

A valid alignment with human, mouse and rat *ABCG2* was not produced, but six out of eight polymorphisms could reside in various TFBSs (Figure 5.9). The seven base pair deletion at −266 (AGTGTTT>−) shares five bases with a putative E47 binding site. The LUN-1 transcription factor may be disrupted by *ABCG2* −484T>A and −483C>T. Interestingly, the −424G>A variant may be present in an estrogen receptor α (ERα) binding site and could be related to altered expression levels in breast cancer [16, 29]. A potential GATA-1 site surrounds *ABCG2* −340G>C and −184C>T may exist in an AP-4 and/or a GCF binding site.

5.4 Discussion

The primary goal of this study was to predict the functional consequences of promoter region variants in xenobiotic ABC genes and the data are summarized in Table 5.4. Subsequent research can use these results to guide *in vitro* studies that measure transcriptional activity of reference and variant promoters. Transactivation assays use a reporter gene vector, such as luciferase or β-galactosidase, that is under the control of

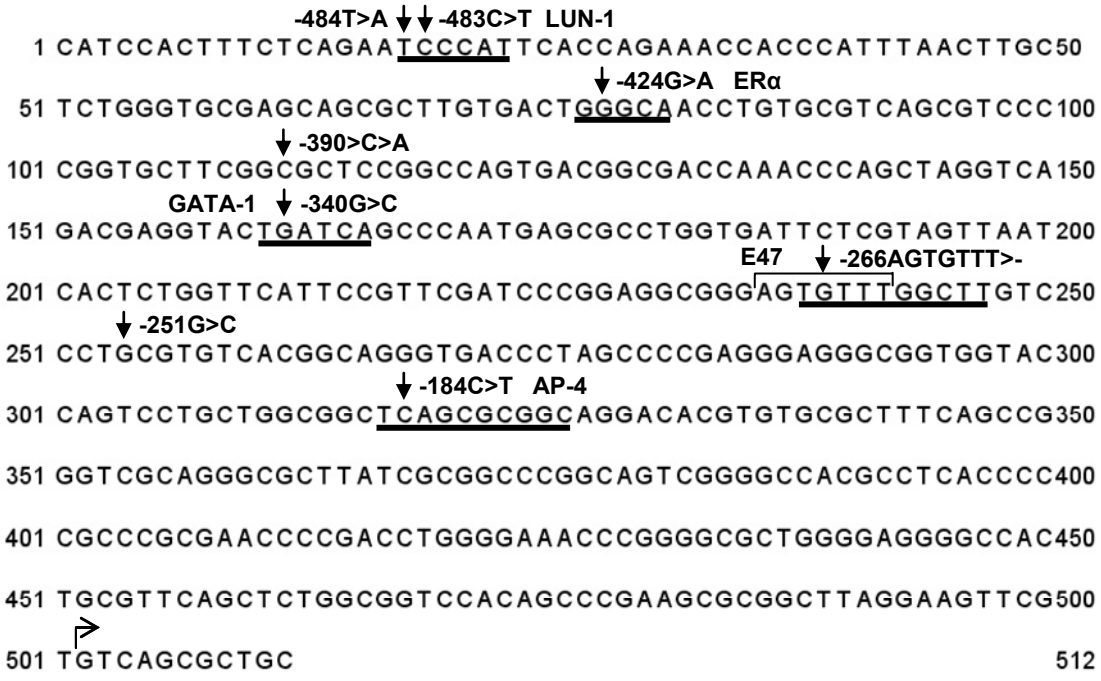


Figure 5.9. Genetic variation in the *ABCG2* promoter region. *ABCG2* promoter polymorphisms are indicated by vertical arrows and putative TFBSs specific to a polymorphism are underlined. The TSS is marked with a horizontal arrow.

reference and variant promoters. Transcriptional activity is determined by reporter gene levels, which are generally measured photometrically. The *in vitro* data will identify new polymorphisms of potential clinical significance.

Sequence data for *ABCB* and *ABCC* genes in other mammals, such as rhesus monkey, dog, and rabbit, were not always available. Thus, only rat and mouse were used for comparison in order to standardize the sequence alignments. The conservation surrounding a SNP was based on most transcription factors recognizing five to eight bases. Alternatively, an arbitrary region around a SNP could be compared to the average conservation of all possible regions across the promoter. This sliding entropy method utilizes a custom algorithm to determine conservation based on variable-length regions

Table 5.4
Xenobiotic ABC promoter polymorphisms with predicted functional importance

Gene	Polymorphism	Computational evidence[a]		Gene	Polymorphism	Computational evidence	
		Conserved	Putative TFBS			Conserved	Putative TFBS
ABCB1	-413 C>T	✓		*ABCC6*	-629 C>T		Sp1
	-336 T>C	✓	PITX2		-509 C>T		AP-2
	-231 A>G		CTF		-471 C>T		CREB
	-5 C>G	✓			-445 C>T	✓	
					-431 C>G		Sp1
ABCC3	-190 T>A		C/EBPα		-196 A>C		Sp1
	-140 C>T		Sp1		-116 C>T	✓	GCF
					-109 C>T	✓	
ABCC4	-174 C>G	✓	SMAD-3		-104 C>T	✓	LUN-1
	-113 A>G	✓	Sp1		-62 T>C	✓	Sp1
					-42 C>G		Sp1
ABCC5	-494 G>A	✓	SMAD-3				
	-453 G>A		Sp1	*ABCG2*	-484 T>A	N/A	LUN-1
	-367 G>C		CREB		-483 C>T	N/A	LUN-1
	-255 C>T	✓			-424 G>A	N/A	ERα
	-182 C>T	✓			-340 G>C	N/A	GATA-1
	-163 G>A	✓	PEA3		-266 AGTGTTT>-	N/A	E47
	-127 C>T	✓	Sp1		-184 C>T	N/A	AP-4, GCF
	-124 G>C	✓	Sp1				
	-110 A>G		PEA3				
	-65 A>C	✓	c-ETS-1				
	-9 T>C	✓					

[a] Conservation was based on 70% sequence identity as described in the Materials and Methods section. TFBS were determined from the TransFac and ConSite predictive algorithms. N/A = no multispecies sequence alignment available.

and it will provide better measures of conservation [30]. In addition, we can make use of all homologous, curated sequences because the sliding entropy method allows calculation of background probabilities for any multiple sequence alignment generated. As a result, it is not a requirement to have the same species for cross-gene comparisons. Efforts are being initiated to extend these investigations in this direction.

The TransFac and ConSite databases identified over 100 possible transcription factor sequence motifs in each ABC promoter. These predictive algorithms often detect false positives due to the short length of the TFBSs. One study identified the binding of 18 *ABCC6* transcription factors using a protein/DNA array, but out of the numerous sites predicted from TFBS databases only four matched the experimental data [18]. The multispecies sequence alignments of ABC promoters conducted in our studies provide insights as to which TFBSs are most likely relevant for gene regulation.

ABCB1 promoter research is ongoing and certain variants may alter transcription. A Japanese specific haplotype consisting of −1517T>C, −1017T>C, and −41A>G was shown to have increased luciferase activity when compared to *ABCB1* reference [31]. One study determined *ABCB1* −274A>G and −146C>T increased β-galactosidase activity but the results were cell-type dependent [32]. It should be noted that these studies numbered promoter variants relative to the start of exon 1a, while exon 1b is considered the transcriptional start site and is further downstream [12]. Our computational analysis is consistent with the observed functional effects of *ABCB1* −146C>T (−336), but not −274A>G (−464).

There are no available data for the functional effects of *ABCC3-ABCC6* promoter variants. The *ABCC3* −190T>A and −140C>T SNPs identified in this study were in

putative TFBSs but showed minimal conservation. Moreover, their relatively high allele frequencies may imply they are under minimal selective pressure. In contrast, there was little genetic variation in the *ABCC4* promoter and the −174C>G and −113A>G variants may be more likely to have a functional effect. High sequence identities with mouse and rat promoters suggest many *ABCC5* variants may be in regulatory elements not found in the TFBS search. *ABCC6* mutations in the coding region can lead to Pseudoxanthoma elasticum (PXE), a rare heritable disorder defined by the calcification of elastic fibers in the skin, retina and arteries [33]. The −104C>T, −109C>T and −196A>C promoter variants were found in PXE patients but none were associated with disease [34].

There were 18 putative transcription factor binding sites with diverse regulatory functions predicted to contain an ABC promoter variant (Table 5.4). Certain transcription factors are discussed below because of their possible relevance to ABC transporter expression and function. The putative transcription factor binding site containing the most ABC promoter SNPs was Sp1 (specificity protein 1), which is a versatile, transcriptional activator that controls the expression of thousands of genes [35]. The Sp1 binding site may be tolerant to changes and/or could be integral to xenobiotic ABC transporter expression. PEA3 and LUN-1 regulate certain oncogenes and lung cancer-associated genes, respectively, and putative binding sites were found in *ABCC5*, *ABCC6* and *ABCG2* [36, 37]. It is possible promoter variants in these genes may alter cancer-associated transcriptional activity. *ABCC4* and *ABCC5* contain putative SMAD-3 (similar to mothers against decapentaplegic 3) binding sites, which mediates expression of signal transduction genes in response to TGF-β [38]. Both genes may be involved in

signal transduction because their encoded proteins transport the signaling molecule cyclic AMP [39].

The next steps are to functionally test our predictions *in vitro* using a reporter gene assay. ABC promoter variants demonstrating altered transcriptional activity compared to reference should then be tested for changes in protein binding using electrophoretic mobility shift assays (EMSAs). Similar research was performed for *ABCC2* promoter variants, many of which were located in putative TFBSs. Luciferase activity in HepG2 cells for −1193A>G, −1140G>A, −966C>A and the −1450A>G/−1193A>G/−920A>G haplotype was shown to be 60-75% less than reference. Oligonucleotides spanning single variants were incubated with HepG2 nuclear extracts and protein binding was changed for the −1450A>G, −1193A>G and −966C>A variants [22]. Our predictions for the −1450A>G, −1193A>G and −966C>A variants agree with these results. In contrast, −1140G>A and −966C>A were not predicted to have functional effects, based on sequence conservation and TFBS predictions, demonstrating the current limitations of our computational methods.

The clinical importance of ABC promoter variants has been investigated for certain genes. The *ABCC3* promoter variant −211C>T was associated with decreased ABCC3 mRNA levels and MRP3 protein expression in the liver [40], although we did not detect this variant in our population. *ABCA1* encodes an efflux transporter involved in cholesterol homeostasis, and amino acid mutations have been linked to severely diminished HDL levels [41, 42]. Recently, *ABCA1* promoter variants exhibited significantly different allele frequencies in patients with aberrant HDL levels compared to healthy controls [43]. Future efforts will extend functional studies into the clinic

where the significance of xenobiotic ABC promoter variants on drug response can be tested.

5.5 References

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Chapter 6

Summary and Perspectives

6.1 Summary

A subset of ABC genes encode xenobiotic efflux transporters that limit toxin exposure and play a significant role in disease treatment by modulating drug disposition and response. Many were discovered in cell lines displaying resistance to multiple drugs and are also called multidrug resistance (MDR) transporters. *ABCB1*/P-gp was the first MDR transporter characterized for its overexpression in many tumor samples and cancer cell lines [1]. The role P-gp plays in drug disposition is apparent from investigations with *Abcb1a*(-/-) mice [2-5]. The MRP proteins (*ABCC* family) are involved in toxin and ion transport, but also confer resistance to many anticancer drugs [6]. The remaining xenobiotic ABC transporter, MXR (*ABCG2*), is a half transporter whose homodimer protein effluxes anticancer drugs [7-9]. The MDR transporters have a broad substrate specificity and tissue distribution in excretory and/or barrier sites, supporting their role in the elimination and tissue/cellular distribution of xenobiotics.

Differences in xenobiotic transporter expression and function can contribute to interindividual variation in drug response. Genetic variation in these transporters is documented and significant efforts have been made to understand their *in vitro* and clinical effects. Unfortunately, it is difficult to determine the functional and/or clinical significance of many ABC polymorphisms because of conflicting reports or insufficient data [10-12]. Therefore, our investigations were designed to better understand the

consequences of genetic variation on xenobiotic ABC transporter function and expression.

The overall goal of this dissertation was two-fold. The first was to determine how *ABCB1* variants modulate the cellular processes controlling P-gp expression and function. The second focus was to investigate variants in multiple ABC transporters for associations with either mRNA expression in colon cancer or their potential effects on transcription.

P-glycoprotein effluxes more diverse substrates than other xenobiotic transporters and plays a significant role in drug pharmacokinetics and pharmacodynamics [11, 13, 14]. Significant efforts have examined how *ABCB1* genetic variants influence P-gp expression and function, with the ultimate goal of translating this knowledge into improved drug therapy. The studies in Chapter 2 investigated how seven nonsynonymous variants and two common haplotypes altered P-gp function using a cell-based substrate accumulation assay. After an initial screen of six substrates, calcein-AM and bodipy-FL-paclitaxel were selected to test variant P-gp function in a cell-based transient expression assay. The Ala893Thr and Val125Ile variants showed a modest decrease of calcein-AM accumulation (increased P-gp function), and the Asn21Asp, Arg669Cys and Ala893Ser variants demonstrated increased inhibition of calcein-AM transport by cyclosporin A. The Val1251Ile and Ala893Ser variants and the Asp21/1236T/Ser893/3435T haplotype showed a modest increase in bodipy-FL-paclitaxel accumulation (decreased P-gp function). In the presence of cyclosporin A, Ala893Ser, Ala893Thr, Ser1141Thr, Val1251Ile, and the Asp21/1236T/Ser893/3435T haplotype showed decreased sensitivity to inhibition. These results demonstrate that

genetic variation in *ABCB1* alters function in the encoded P-gp in a substrate- and inhibitor-dependent manner. Future studies should explore the effects of *ABCB1* genetic variation on P-gp transport using a panel of diverse substrates.

Transport kinetics are dependent on the abundance of P-gp molecules present on the membrane and ABCB1 mRNA stability is an important cellular process that influences surface P-gp expression. Polymorphisms in the 3'-UTR of *ABCB1* could alter mRNA stability since this region contains elements that promote or repress mRNA degradation. Computational methods predicted the effects of *ABCB1* 3'-UTR variants on mRNA stability and a cell-based assay was developed to measure the mRNA half-life of ABCB1 reference and +89A>T, +146G>A and +193G>A 3'-UTR variants (Chapter 3). The mRNA half-life for the 3'-UTR variants was similar to the reference half-life of 9.4 h. Future studies should investigate the effect of other *ABCB1* 3'-UTR variants and haplotypes on mRNA stability.

Chemotherapy is a common treatment for colon cancer but many patients develop MDR after exposure to a single drug [15, 16]. There is also a variable response to chemotherapy, consistent with a role for genetic variation in the control of drug exposure. MDR transporter expression levels influence the MDR phenotype and drug response. In Chapter 4, variability in ABCB1 and ABCC1-ABCC3 mRNA expression was observed in matched normal and tumor colon tissue from 32 drug naïve colon cancer patients. This prompted a preliminary association analysis between genotypes/haplotypes and mRNA levels. Possible trends were identified for ABCB1, ABCC1 and ABCC2, although the results must be considered preliminary due to the small sample size. Interestingly, there was altered mRNA expression in tumor tissue compared to healthy for ABCB1, ABCC1

and ABCC3, suggesting differential regulation in normal and tumor tissue. The regulation of the ABC genes in tumors is important for drug therapy and requires further examination.

There is limited data on the functional effects of ABC promoter variants due to the exon-centric nature of previous studies. The promoter regions of many xenobiotic ABC transporters have been examined for regulatory domains, but the next step is to identify functionally relevant promoter variants. In Chapter 5, we screened for polymorphisms in the promoter regions of *ABCB1*, *ABCC2-ABCC6* and *ABCG2* using an ethnically diverse cohort of over 300 healthy volunteers. Possible regulatory elements were predicted based on sequence conservation and putative transcription factor binding sites. Promoter variants located in these regulatory elements may alter transcription and should be investigated in *in vitro* functional assays. Our predictions correctly identified three out of five *ABCC2* promoter variants (−1450A>G, −1193A>G and −920A>G) that previously demonstrated decreased luciferase activity [17], illustrating the potential utility of these computational predictions in guiding functional studies and follow-up clinical studies.

6.2 Perspectives

The xenobiotic ABC transporters mediate the efflux of endogenous and exogenous compounds. Research has focused on the *in vitro* and clinical effects of polymorphisms in xenobiotic ABC transporters in order to understand how they influence drug response. A review of the functional effects of promoter, coding region and UTR variants in *ABCB1*, *ABCC1-ABCC4* and *ABCG2* illustrates how multiple phenotypes,

such as mRNA levels, protein expression, transport function and membrane trafficking, can be altered (Chapter 1).

The dissertation work presented here investigated the effect of ABC polymorphisms on transporter expression and function. The lack of detailed mechanistic data on transporter variant function has hindered the interpretation of clinical association data. Consideration of possible functional effects of polymorphisms beyond transport function is relatively unexplored for the ABC proteins. As illustrated in Figure 6.1, gene expression starts with transcription where promoter variants can alter transcription factor binding and function. There is the potential for variants located in exon-intron boundaries to modify mRNA processing, although there are no examples for ABC transporters. At the mRNA level, 3'-UTR variants could influence regulatory protein binding that controls mRNA degradation machinery as well as secondary structure necessary for RNA/protein interactions. In a similar manner, variants in the 5'-UTR could alter the binding of translation initiation factors and the ribosome. Finally, nonsynonymous variants alter amino acid sequence, possibly resulting in different protein function. In order to better understand how ABC genetic variants influence drug response and disposition, future studies must recognize that multiple opportunities exist for polymorphisms to change gene expression and protein function.

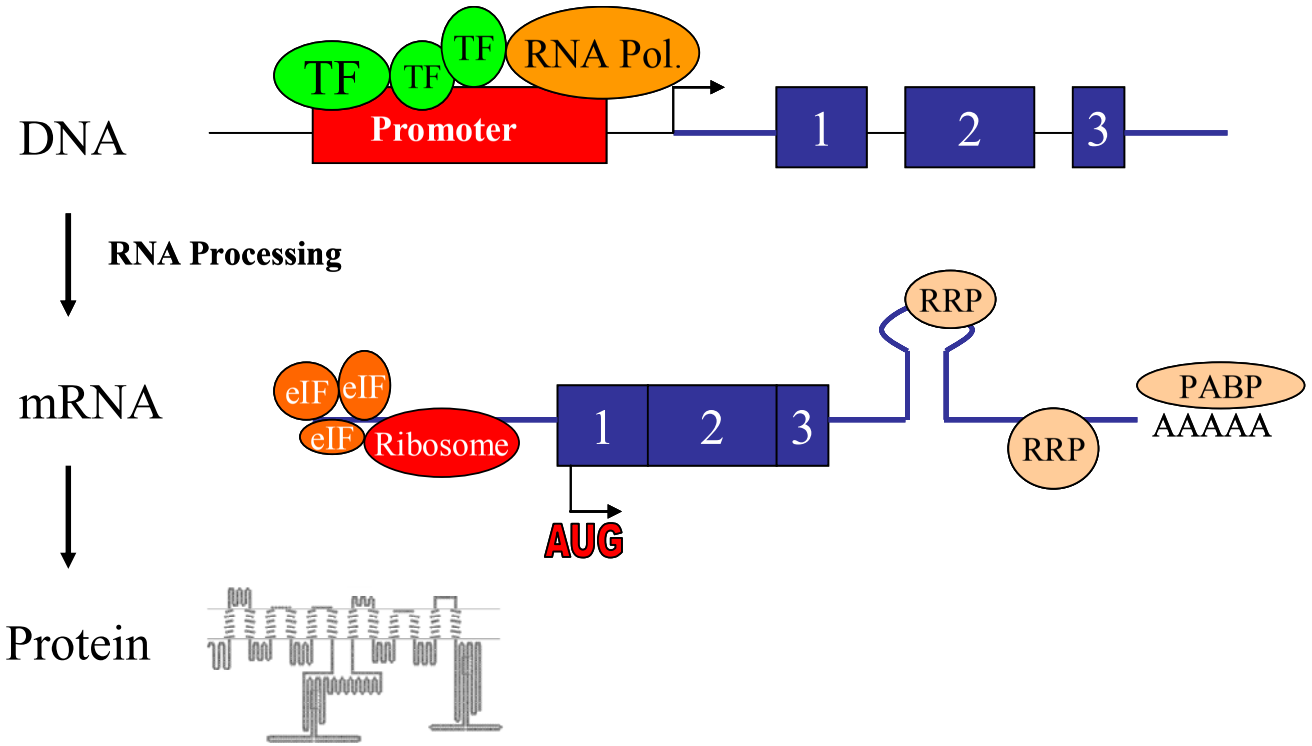

Figure 6.1. ABC transporter expression and function. There are multiple cellular processes in which polymorphisms can change transporter expression and function. Transcription factors (TF) bind the promoter and recruit RNA polymerase II (RNA Pol.) for transcription initiation. RNA recognition proteins (RRP) and poly-A binding protein interact with mRNA degradation machinery to promote or repress degradation. Translation intiation is controlled by initiation factors (eIF) and the ribosome binding the 5'-UTR. Exons (numbered boxes) dictate protein function according to the specific amino acid sequence they encode.

6.3 References

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