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Functional and clinical implications of genetic variation in ABCC2 and related ABC genes

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FUNCTIONAL AND CLINICAL IMPLICATIONS OF GENETIC VARIATION IN *ABCC2* AND RELATED ABC GENES

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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Science has always been of interest to me. Just tinkering and observing how life around us revolves has always intrigued me. Graduate school was just a logical step in the continuation of expanding my knowledge of the world around me. Little did I know what I was getting myself into. I did not anticipate so many frustrating days of trying to get an experiment to work. On the days that things did, in the mysterious ways that they do, the elation you feel for accomplishing a task is rewarding. This is not so much as finding the next breakthrough in science, as this I know now are rare occurrences, but the idea that you aren't hopelessly unskilled. For those graduate students who happen to read this, take note: 90% of the time, things won't work as they were intended.

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FUNCTIONAL AND CLINICAL IMPLICATIONS OF GENETIC VARIATION IN *ABCC2* AND RELATED ABC GENES

TAN DUY NGUYEN

The ABC superfamily of drug efflux transporters is an important class of proteins involved in drug absorption, disposition, and elimination. We hypothesize that genetic variations in *ABBC2* (MRP2) and related ABC genes may contribute to interindividual variability in drug response. Using an ethnically diverse set of human DNA sequences, the extent of genetic variation in the coding and non-coding regions of *ABCC2* was determined. Specifically exons, exon/intron boundaries, and the 5'-promoter region were screened for single nucleotide polymorphisms (SNPs). A total of 68 variant sites were identified, including 13 in the 5'-promoter region, 23 in the exon/intron boundaries, and 22 in the exons. Nucleotide diversity (π) in *ABCC2* was assessed for non-synonymous and synonymous sites and π_{NS}/π_{Syn} ratios show selective pressure against amino acid changes. Variants identified in the 5'-promoter region were assayed for promoter acitivity, and several were found to have decreased activity compared to a reference sequence. The most notable of these is the haplotype containing the -1549G>A, -1019A>G, and -24C>T variants. This haplotype was found to be in tight linkage disequilibrium with the synonymous variant 3972C>T, which had been associated with higher irinotecan exposure. Allele specific expression analysis of liver samples heterozygous for the 3972C>T variant showed a 10% bias towards the C allele in the mRNA transcript. The allelic imbalance increased to 14% when considering liver samples containing the -1549G>A/-1019A>G/-24C>T promoter haplotype. In a clinical study carried out to determine the genetic contribution to digoxin pharmacokinetics, a

one mg dose of digoxin, which undergoes minimal metabolism and is a substrate for transporters, was administered to monozygotic (MZ) and dizygotic (DZ) twin pairs. Oral clearance, $AUC_{0\text{-}3h}$, and apparent nonrenal clearance were found to have a high genetic component to their variability. Lastly, a broad genetic analysis was performed on 10 members of the ABC superfamily of drug efflux transporters. These transporters were found to be under selective pressure against amino acid changes, especially in the transmembrane domains, where substrate binding occurs. Results from these studies may help in understanding their cellular function and how they may contribute to interindividual variability.

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INTRODUCTION

1.1 GENETIC VARIATION IN DRUG RESPONSE

It is well known that individuals respond differently to drug treatments. In many patients, drug therapy can have the beneficial effects that were intended, while in a subset of patients the treatment is ineffective or leads to toxicity. Adverse drug reactions are the cause of over 100,000 deaths each year in the U.S. and their occurrence leads to financial burdens (1). Patient response to drugs varies greatly; for example the response rate is 80% for Cox-2 inhibitors, and often less than 25% for cancer chemotherapy (2). The cause of the interindividual variability in drug response can be genetic, physiological, or environmental.

The genetic influence in drug response can be attributed to enzymes, drug targets, and drug transporters (1). Both enzymes and drug transporters determine the drug levels in the body and changes in function and/or expression of these proteins can alter the way drugs are handled within the body. Pharmacogenetics, or the study of genetic variations that cause changes in drug response, has not been well understood. Most reports thus far have focused on drug metabolizing enzymes such as CYP2D6 and CYP2C19 (10-13). However, only recently has the role that genetic variation in transporters plays in drug response been considered (14-16).

Membrane transporters play an important role in drug absorption, distribution, and elimination from the body. They are found in organs of elimination such as the liver and kidneys, where they are responsible for the uptake and efflux of drug substrates (7, 8,

21). Transporters are also expressed at barrier sites such as the blood-brain barrier, blood-testis barrier, and intestinal lumen, where they limit drug access (26, 27). By controlling the amount of drug coming in and out of cells, membrane transporters play an important role in regulating drug concentrations at target sites.

Membrane transporters vary in function and mechanism of transport. They can transport compounds into a cell (uptake) or out of a cell (efflux) and can be energy dependent or independent. Some transporters transport substrates across the membrane going with or against the concentration or electrochemical gradient. Others require the coupling of a second compound to drive the transport, usually ions such as sodium and protons (28). There are two superfamilies of transporters that are able to transport compounds in one of these ways, the solute carrier (SLC) superfamily and the ATP-Binding Cassette (ABC) superfamily (29, 30). The latter family is the focus of this dissertation.

1.2 THE ABC FAMILY OF TRANSPORTERS

The ATP-Binding Cassette (ABC) transporters are a superfamily of proteins that use ATP hydrolysis to drive transport. ABC proteins contain at least two transmembrane domains (TMDs) and two nucleotide binding folds (NBFs). The TMDs contains between 6-11 membrane spanning alpha-helixes that provide the substrate specificity for these proteins. The NBFs contain two primary sequence motifs that are very well conserved among all ABC transporters (19, 20, 23-25, 34). These motifs, the Walker A and Walker B, are crucial for ATPase activity. The Walker A, with a consensus sequence of GxxGxGK[S/T], binds the β-phosphate group on ATP and the Walker B, having a hhhhD

sequence where h is a hydrophobic amino acid, binds magnesium (5, 25, 31-33). A signature C motif (LSGGQ[Q/R/K]QR) located upstream from the Walker B site distinguishes ABC transporters from other ATP binding proteins and its function is not yet known (23, 35, 39, 40). There are currently 48 known human ABC genes (23, 29, 36, 37) which are divided up into seven subfamilies: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG. The transporters characterized in the following studies have some therapeutic importance and belong to the ABCB, ABCC, and ABCG family of transporters (Table 1.1).

Table 1.1 ABC Transporters

Gene	Protein	Description	GenBank Accession	Nucleotide RefSeq
ABCB1, MDR1	P-gp	ATP-binding cassette, sub-family B (MDR/TAP), member 1	AF016535, M14758	NM_000927
ABCB4	MDR3	ATP-binding cassette, sub-family B (MDR/TAP), member 4	M23234, Z35284	NM_000443
ABCB11	BSEP	ATP-binding cassette, sub-family B (MDR/TAP), member 11	AF091582	NM_003742
ABCC1	MRP1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	L05628, U91318	NM_004996
ABCC2	MRP2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	U63970	NM_000392
ABCC3	MRP3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	AF009670, AF085690	NM_003786
ABCC4	MRP4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	AF071202	NM_005845
ABCC5	MRP5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	AF104942	NM_005688
ABCC6	MRP6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	AF076622	NM_001171
ABCG2	MXR	ATP-binding cassette, sub-family G (WHITE), member 2	AF103796	NM_004827

List of the ABC transporters being studied. Gene names, GenBank accession numbers and mRNA reference sequence numbers are given.

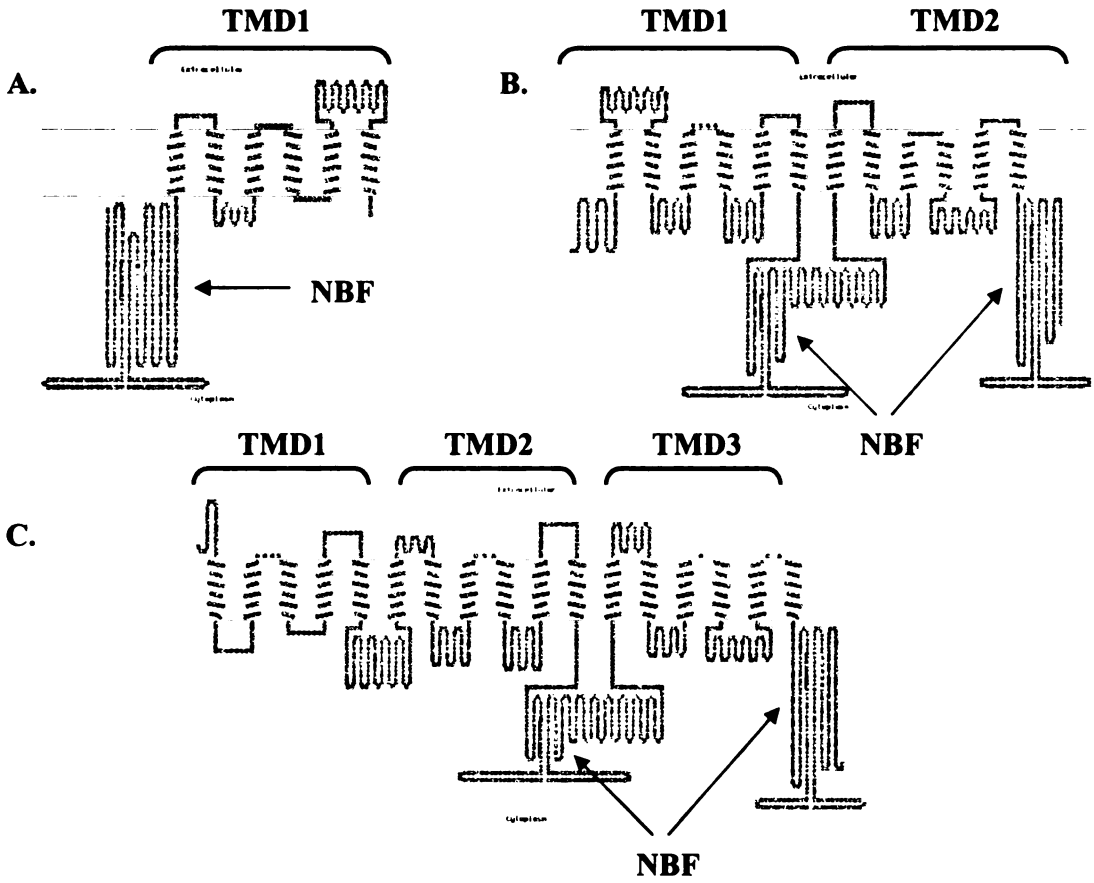

Figure 1.1 Secondary structure schematic of A) MXR with a 1 TMD – 1 NBF structure, B) P-gp with the 2 TMD – 2 NBF structure and C) MRP2 with the 3 TMD – 2 NBF structure. Secondary structures were predicted using TOPO (S.J. Johns, University of California, San Francisco, and R.C. Speth, Washington State University. Software is available at http://www.sacs.ucsf.edu/TOPO/topo.html). Double horizontal lines depict the membrane lipid bilayer. Each transmembrane domain (TMD) and nucleotide binding fold (NBF) are marked.

1.2.1 Multidrug Resistance Protein 1 (*MDR1*, P-glycoprotein; *ABCB1*)

P-glycoprotein (P-gp) is encoded by *ABCB1* (*MDR1*) and is the most extensively studied of the ABC transporters. It was first discovered by oncologists who found that cancers treated with multiple drugs developed cross-resistance (4, 44). P-gp was cloned from cells resistant to anti-cancer agents and is found on chromosome 7q21. MDR1

mRNA is roughly 4.8 kb and encodes a 1280 amino acid protein of 170 kDa in size. P-gp follows the two TMD structure (Figure 1.1) and is apically (Figure 1.2) expressed in epithelial cells in the intestinal lumen, liver hepatocyte, and renal tubules (4). P-gp is also expressed in the blood-brain barrier and blood-testis barrier where it limits the amount of toxins entering these sensitive organs (5). It has been shown that RARα regulates MDR1 expression in leukemia cells (22, 45) and mRNA is inducible by drugs through the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (46, 47).

The protein transports a wide variety of chemical compounds, as evident from studies in both transfected cells and *mdr1a*(-/-) & *mdr1b*(-/-) mice. P-gp substrates are typically hydrophobic cations or neutral compounds and range from anti-cancer drugs to HIV-protease inhibitors. P-gp substrates also span a large number of therapeutic classes including cardiac drugs (digoxin, verapamil), steroids (estradiol, dexamethasone, cortisol), H_2 receptor blockers (fexofenadine), anticancer agents (doxorubicin, vinblastine, etoposide) and protease inhibitors (indinovir, saquinavir, ritonavir) (4, 6, 43). Inhibitors of P-gp include verapamil, quinidine, and the cyclosporine analog PSC-833 (7). A summary of P-gp expression patterns and substrates are listed in Table 1.2. Based on its distribution it is evident that P-gp plays an important role in the elimination and distribution of numerous drugs. *Mdr1* mouse knockout models have provided crucial information on the protective nature of P-gp. Mice carry two genes that are homologous to human *MDR1*, *mdr1a* and *mdr1b*. Mice with genetic disruption of both *mdr1a* & *mdr1b* are healthy, but accumulate P-gp substrates in the brain and are unable to

eliminate these drugs from the circulation (48-50). For example *mdr1a/1b*(-/-) mice are 50-100 times more sensitive to toxicity from the pesticide ivermectin than wildtype mice.

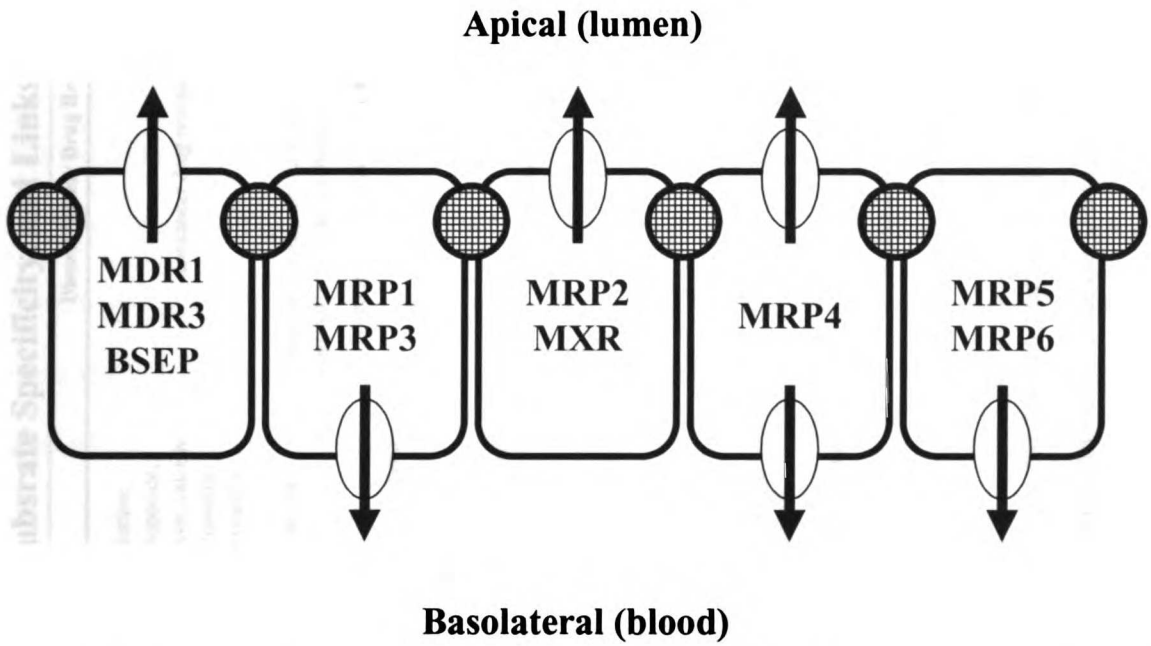

Figure 1.2 Diagram representing membrane localization of the ABC transporters. Cells are lined up in a polarized monolayer with tight junctions (hatched circles) separating apical and basolateral membranes. Transporter proteins are represented by an oval with an arrow pointing in the direction of transport.

Table 1.2 ABC Transporter Tissue Distribution, Subsrate Specificity, and Links to Disease

Gene	Name	Tissue Localization	Selected Substrates	Disease States / Drug Response	References
ABCB1, MDR1	**P-gp**	liver, kidney, blood brain barrier, adrenal gland, colon, small intestine, placenta, lymphocytes, pancreas	daunorubicin, doxorubicin, vinblastine, vincristine, irinotecan, topotecan, etoposide, colchicine, paclitaxel, ritonavir, indinavir, calcein-AM, rhodamine 123, digoxin, fexofenadine, cortisol, morphine, loperamide, ivermectin	anticancer drug resistance	(3-7)
ABCB4	**MDR3**	liver, adrenal gland, spleen	phosphatidylcholine, digoxin, vinblastine, paclitaxel, aureobasidin A	familial intrahepatic cholestasis of pregnancy; progressive familial intrahepatic cholestasis, type 3	(8, 9)
ABCB11	**BSEP**	liver	bile salts	Progressive intrahepatic cholestasis, type 2	(17-20)
ABCC1	**MRP1**	ubiquitous, high levels in brain, low in liver	glutathione-, glucuronate-, and sulfate-conjugated organic anions; estradiol 17-β-D-glucuronide ($E_2$17βG), LTC_4		(22-25)
ABCC2	**MRP2**	liver, small intestine, kidney	glutathione-, glucuronate-, and sulfate-conjugated organic anions; $E_2$17βG, LTC_4, bilirubin glucuronide, pravastatin, SN-38G, irinotecan	Dubin-Johnson syndrome	(5, 25, 31-33)
ABCC3	**MRP3**	adrenal gland, kidney, colon, pancreas, gallbladder, liver	epipodophyllotoxins, methotraxate, vincristine, etoposide, $E_2$17βG, DNP-SG, LTC_4, glycocholate, glycochenodeoxycholate, taurodeoxycholate, taurochenodeoxycholate, glucuronides		(22, 23, 34, 35)
ABCC4	**MRP4**	ubiquitous, high levels in prostate	steroid and bile acid conjugates, nucleoside analogs, 6-MP, PMEA, cAMP, cGMP		(22, 23, 36-38)
ABCC5	**MRP5**	ubiquitous, increased expression in cardiac tissue in ischemic cardiomyopathy	6-MP, PMEA, low affinity for cyclic nucleotides		(22, 23, 38, 41)
ABCC6	**MRP6**	liver, kidney	glutathione conjugates (LTC_4, NEM-GS)	Pseudoxanthoma elasticum	(21, 34, 42)
ABCG2	**MXR**	blood-brain barrier, placenta, liver, intestine, stem cells	anthracyclines, mitoxantrone, bisantrene, topotecan, SN-38		(34, 43)

Tissue distribution and substrate specificity of ABC transporters. Listed are the tissues that show detectable levels of transporter RNA or protein. Substrates were determined using in vitro assays or animal models. Disease states associated with a transporter are also shown.

1.2.2 Multidrug Resistance Protein 3 (MDR3, *ABCB4*)

ABCB4 is found on the same chromosome as MDR1, 7q21. It is believed that *ABCB4* arose from a gene duplication of *ABCB1* as they share 76% amino acid sequence identity (51). The mRNA for MDR3 is 3.9 kb in length and encodes a 1279 amino acid protein. MDR3 is highly expressed in the liver and is apically oriented (Figure 1.2) in the bile canaliculus. MDR3 can also be found in the adrenal glands and spleen (Table 1.2). Structurally, MDR3 is similar to MDR1 and has the two TMD structure (Figure 1.2) Recent studies show that MDR3 mRNA is transactivated through the nuclear orphan receptor, FXR (farnesoid X receptor), and can be modulated by the FXR agonist chenodeoxycholate (CDCA) (52).

In vitro studies have shown that MDR3 is able to transport xenobiotics such as digoxin, paclitaxel and vinblastine (8). MDR3 has low drug transport activity, however, and its main substrate is the endogenous lipid phosphatidylcholine (Table 1.2) (9). Transgenic expression of *MDR3* in *mdr2* deficient mice showed that these two genes were orthologs. Mice with genetic disruption of *mdr2* show normal bile salt excretion, although phospholipid secretion is abolished (53).

1.2.3 Bile Salt Export Pump (BSEP; *ABCB11*)

BSEP was formally known as the sister of P-glycoprotein (SPGP) and is localized to the canalicular (apical) membrane in the liver hepatocytes. Full length BSEP was cloned from the rat liver (54) and is located on chromosome 2q24 in humans. BSEP is a 1321 amino acid protein with a 4.7 kb transcript. Structurally, BSEP is composed of two TMDs and protein expression is exclusive to the liver (17). Functional studies of rat and

human BSEP in Sf9 insect cells have shown that BSEP is the major transporter for the excretion of monovalent bile salts (taurocholate, glycocholate, taurochenodeoxycholate; Table 1.2) into the bile (18-20). This movement of bile salts into the bile is important for the maintenance of bile flow. BSEP has also been shown to transport xenobiotics, such as vinblastine, cyclosporine, and taxol, and is inhibited by troglitazone, cyclosporine, rifampicin, and bosentan (8, 19, 55).

Mice with genetic disruption of *Abcb11* exhibit growth retardation and mild cholestasis. Bile flow is minimally reduced, and bile salt excretion is reduced to 30% of normal mice (56). Excretion of taurocholate is completely blocked in the *Abcb11*(-/-) mouse. In the mouse, cholic acid induces Bsep expression through the nuclear receptor farnesoid X-activated receptor (FXR) (57). Human *ABCB11* is also upregulated in an FXR-dependent mechanism (58).

1.2.4 Multidrug Resistance-associated Protein 1 (MRP1; *ABCC1*)

MRP1 is the most studied of the MRPs and was first discovered in multidrug resistant lung cancer cell lines which did not overexpress P-gp (34, 59). MRP1 is found on chromosome 16p13 and is expressed in most tissues in the body. The highest expression of MRP1 is found in the lung, testis, kidneys, and peripheral blood mononuclear cells (34), and MRP1 sorts to the basolateral membrane in polarized cells. MRP1 mRNA is 5 kb in length and encodes a 1531 amino acid protein. Structurally, MRP1 contains three TMDs and two NBFs. MRP1 transports organic anions that are conjugated to glutathione (GSH), glucuronate and sulfate moieties (22). Some examples are the anti-cancer drugs doxorubicin and vincristine, and the endogenous conjugated

compounds leukotriene C_4 (LTC_4), 17-β-D-estradiol glucuronide ($E_2$17βG) and estrone-3-sulfate (22-24). MRP1 can be inhibited with cyclosporine, rifampicin, probenecid, indomethacin, and the leukotriene D_4 receptor antagonist MK-571 (22).

A triple knockout mouse, with *mrp1*, *mdr1a*, and *mdr1b* genetically disrupted, shows higher sensitivity to etoposide (60). Immunohistochemistry identified Mrp1 on the choroid plexus epithelium and the *mrp1/mdr1a/mdr1b*(-/-) mouse shows a 10-fold increase of etoposide in the cerebrospinal fluid compared to the *mdr1a/mdr1b*(-/-) mouse. This suggests that MRP1 may be important in limiting drug and xenobiotic access to the brain. The proximal promoter of *ABCC1* is inducible by the PXR ligands tertiary butylated hydroquinone (tBHQ) and quercetin, however no known nuclear receptor binding sites have been identified in this region (61).

1.2.5 Multidrug Resistance-associated Protein 2 (MRP2; *ABCC2*)

MRP2 was first cloned from the rat hepatocyte and named the canalicular multispecific organic anion transporter (cMOAT) (62). MRP2 mRNA is 4.8 kb and the protein is 1515 amino acids in length. Like MRP1, MRP2 has the 3 TMD structure. MRP2 is found on chromosome 10q24 and is localized to the apical membrane of various tissues. MRP2 is highly expressed in the liver, where it can be found on the apically oriented bile canaliculus. There MRP2 drives the efflux of organic anions, mostly conjugated to GSH, glucuronic acid, or sulfate, into the bile for elimination. MRP2 can also be found in the kidney proximal tubules and the brush border membrane in the intestinal lumen (31, 32). MRP2 has similar substrate specificity as MRP1, however, their binding affinities are different. MRP2 can also transport neutral or basic

compounds like P-gp, but requires GSH for co-transport (33, 63). Some examples of MRP2 substrates are the HMG-CoA reductase inhibitor pravastatin, the anti-cancer drug irinotecan and its glucuronide metabolite SN-38G, cisplatin, and methotrexate. Some endogenous compounds for MRP2 are GSH, LTC_4, 17β-D-estradiol glucuronide and the heme metabolite bilirubin glucuronide (5). Inhibitors for MRP2 overlap with those of MRP1.

Most of the initial determination of substrate specificity for MRP2 was performed using transporter deficient (TR^-) rats and Eisei Hyperbilirubinemia rats (EHBR) which lack functional MRP2 protein (32, 64). EHBR rats were found to contain a single nucleotide change that resulted in a premature stop codon and truncated protein (65). In the TR^- rats, a single nucleotide deletion causing a frameshift and subsequent stop codon caused the absence of Mrp2 protein in the rat hepatocyte (31). In the rat, ligands for FXR, PXR, and CAR were found to induce mRNA expression. Human derived cell lines also showed mRNA induction by bile acid and xenobiotic ligands (66).

Because of the chemical properties of many MRP2 substrates, uptake transporters are often required in tissues that express functional MRP2. The glucuronate, sulfate, and glutathione moieties that are attached to many MRP2 substrates yield bulky polar compounds that do not pass through the membrane easily (67). Uptake transporters such as OATP1B1 and OATP1B3, which are exclusive to the liver, are needed to provide access of many MRP2 substrates to the hepatocyte (68, 69).

1.2.6 Multidrug Resistance-associated Protein 3 (MRP3; *ABCC3)*

MRP3 is located on chromosome 17q22 and has the highest amino acid identity to MRP1 at 58%. The 1527 amino acid protein is encoded by a 5.1 kb mRNA sequence. MRP3 has the same 3 TMD structural topology as MRP1 and MRP2 and is located on the basolateral membrane of polarized cells. MRP3 is predominantly expressed in the intestine, pancreas, placenta, and adrenal cortex. It can also be found in the liver, kidney and prostate at lower levels. It is thought that MRP3 acts as a compensatory transporter in organs such as the liver. MRP3 is induced if transport into the bile has been decreased, allowing the removal of compounds from the liver into the blood for subsequent urinary excretion (23). MRP3 is able to transport some of the same compounds as MRP1 and MRP2. MRP3 transport of anti-cancer drugs is not GSH dependent, and MRP3 has a higher affinity towards glucuronide metabolites (35). MRP3 can also transport monovalent bile acids such as cholate, taurocholate, and glycocholate (34). Sulfated bile salts were shown to be high-affinity competitive inhibitors of MRP3 transport (70).

The ability of MRP3 to transport bile components is important in cholestasis, where bile flow is perturbed. In cases where MRP2 and BSEP function has decreased, MRP3 plays a compensatory role by transporting the accumulating bile constituents into the blood for urinary excretion. MRP3, in fact, is induced during cholestatic conditions (71). MRP3 mRNA is also induced by PXR ligands such as clotrimazole, rifampicin, and PCN (72) but is not induced by CAR ligands (73).

1.2.7 Multidrug Resistance-associated Proteins 4 & 5 (MRP4, *ABCC4*; MRP5, *ABCC5)*

MRP4 and MRP5 are distinct from the MRPs discussed thus far, having a 2 TMD structure like that of MDR1. MRP4 is located on chromosome 13q32 and is found both apically and basolaterally oriented in polarized cells (36, 37). Its 4.2 kb mRNA encodes a 1325 amino acid protein. MRP5 is located on chromosome 3q27 and found on the basolateral membrane of polarized cells (27). It is 1437 amino acid in length, encoded by a 5.8 kb transcript. Both MRP4 and MRP5 are ubiquitously expressed, with high levels of MRP4 in the prostate and high levels of MRP5 in the brain, skeletal muscle, lung and heart (23, 41). Both transporters have been implicated in resistance and transport of purine and nucleotide analogs (36, 41) These include 9-(2-phosphonylmethoxyethyl) adenosine (PMEA), 6-mercaptopurine and 6-thioguanine. They both have also been shown to transport cyclic AMP and cyclic GMP (38). MRP4, but not MRP5, can transport 17-β-D-estradiol glucuronide, methotrexate and prostaglandins (74). MRP4 and MRP5 are both inhibited by probenecid, sulfinpyrazone, prostaglandin A_1, and progesterone. Prostaglandin E_1 inhibited MRP5 more effectively than MRP4, while bromophenol blue and bromosulfophthalein were more effective against MRP4 (38).

1.2.8 Multidrug Resistance-associated Protein 6 (MRP6; *ABCC6*)

MRP6 is localized to the basolateral membrane of the liver and kidneys (34, 42). The MRP6 transcript is roughly 4.5 kb in length and encodes a protein that is 1503 amino acids in length. MRP6 is located on chromosome 16p13 immediately 3' to MRP1 but in the opposite direction (34). Its 3'-end is almost identical to the anthracycline resistance-associated protein (ARA) (42). Less is known about substrates of MRP6 than other transporters. The rat protein transports the anionic cyclopentapeptide BQ123, but not

other typical MRP substrates. Human MRP6 transfected cell lines transport glutathione conjugates, and BQ123 but not the glucuronide conjugate $E_2$17βG (21). Human MRP6 also showed low-level resistance to anti-cancer drugs, but it was less effective than other MRPs (34).

1.2.9 Mitoxantrone Resistance Transporter (MXR; *ABCG2*)

MXR, also known as the Breast Cancer Resistance Protein (BCRP), was first cloned from a resistant breast cancer cell line (34). Unlike the other ABC transporters discussed so far, MXR is a half transporter containing only one TMD and one NBF. It is believed that MXR homodimerizes to fulfill the 2 TMD, 2 NBF characteristics of the ABC family. MXR is only 655 amino acids in length, encoded by a 2.7 kb transcript. MXR is located on chromosome 4q22 and is predominantly expressed on the apical membrane of polarized cells. MXR is expressed highly in placenta, with low levels in the liver, intestine, brain and breast (34). Along with mitoxantrone, MXR can transport a number of substrates that are transported by P-gp and other MRPs, such as polyglutamylated methotrexate, anthracyclines, topotecan and SN-38 (43). Unlike MRP1 or MRP2, MXR does not depend on GSH to transport drugs (34). MXR also shows a preference for sulfate conjugates. MXR transport is potently inhibited by the flavonoids 6-prenylchrysin and tectochrysin (75), fumitremorgin C (FTC) (76), and the FTC analog Ko143 (77). The recent development of the *abcg2*(-/-) mouse has been used to determine the function and substrates of MXR. Hepatobiliary excretion of nitrofurantoin (78) and the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (79) were

abolished in *abcg2(-/-)* mice. Interestingly, there was a decrease in the ability to extrude xenotoxins into milk as well (80, 81).

1.3 ABC TRANSPORTERS AND DISEASE / DRUG RESPONSE

Members of the ABC superfamily of transporters have been implicated in a number of different diseases that pertain to the transport of either endogenous or xenobiotic compounds. A change in function or expression can alter the way the body is able to handle toxic compounds. In the case of organs of elimination, a change in function or expression may mean a defect in the way compounds are eliminated from the body. This can lead to a buildup of toxins in the circulation and may lead to adverse reactions. In tissues that limit the access to sensitive organs, changes in function or expression may mean an increase in exposure to toxins.

1.3.1 Multidrug Resistance and Cancer Therapy

Resistance to chemotherapy is a common problem in the treatment of many diseases. During cancer chemotherapy, tumors often show little or no response to a variety of drugs that have different structures and targets. One mechanism for this multidrug resistance is overexpression of drug efflux transporters. The ABC transporters described above all transport a wide variety of chemical compounds, including lipids, bile salts and anti-cancer drugs. So far P-glycoprotein, MRP1, and MXR have been implicated in cancer resistance to a number of different anti-cancer agents and they account for a large percentage of multidrug resistance of tumor cells (15). For example P-gp is overexpressed in human KB carcinoma cells that have been selected with

colchicine, vinblastine or doxorubicin (82). P-gp is also associated with intrinsic drug resistance in human colon adenocarcinoma cells (83). MRP1 was shown to be overexpressed in doxorubicin-selected lung cancer cell lines (84) and MXR was identified as being the transporter involved in daunorubicin, mitoxantrone and topotecan resistance in MCF-7 cells (85). MXR was isolated from S1-M1-80 cells, which are derived from human colon carcinoma cells treated with mitoxantrone (86).

Overexpression of multidrug resistance transporters in tumor cells protects them from the toxic effects of cancer drugs by limiting intracellular drug concentrations. This reduces the ability of the drugs to effectively kill cancer cells. Although not proven, the other members of the ABC family could potentially confer resistance to cancer drugs as they are able to transport these compounds *in vitro* (23, 34, 43, 87). For example MRP2 transports many similar anti-cancer drugs as MRP1 (15, 62) and its overexpression could potentially lead to resistance to chemotherapeutic agents. Recently, MRP2 has been implicated in resistance to topoisomerase II inhibitors in human glioma cell lines (88).

1.3.2 Liver Disease

The ability of some hepatic ABC transporters to efflux endogenous compounds has led to their implication in liver disease. For instance, BSEP transports monovalent bile salts that are important for bile flow (20). A disruption of bile flow into the bile canaliculus can lead to cholestasis. One type of cholestasis is inheritable progressive familial intrahepatic cholestasis, type 2 (PFIC2). Patients with PFIC2 have severe pruritus, high serum primary bile acids, and low biliary primary bile acid concentrations (89). Patients suffering from this disease often develop the phenotype in early childhood,

which can lead to death due to liver failure. Positional cloning identified BSEP as the culprit for PFIC2 (89). In most cases, patients suffering from this disease were found to have no BSEP detectable at the canalicular membrane. An examination of PFIC2 patients has identified at least 10 single nucleotide polymorphisms thought to cause amino acid changes or premature truncation of the protein (90).

The second most important class of compounds in bile is lipids. Biliary lipids are mainly comprised of phosphatidylcholine (PC) and cholesterol. Defects in PC elimination into the bile leads to PFIC type 3. Patients who suffer from this disease don't present symptoms until later in life and, much like PFIC2, develop liver failure (91). These patients carry genetic variations in their *ABCB4* gene, causing premature truncation or amino acid changes in the MDR3 protein (92, 93). The affected protein is then no longer able to transport PC into the bile, leading to an accumulation in the liver. MDR3 has also been implicated in intrahepatic cholestasis of pregnancy (ICP). ICP is characterized by the temporary occurrence of cholestasis in pregnant women. Some women with ICP have children with PFIC3, and it is hypothesized that these women are heterozygous for MDR3 variants (94-96).

Bilirubin is a by-product of heme metabolism and is glucuronidated for elimination to the bile via active transport by MRP2 (69). A defect in the transport of conjugated bilirubin results in the hyperbilirubinemic disease, Dubin Johnson Syndrome (DJS) (97). Patients with DJS do not suffer from any severe phenotypes, but tend to be jaundiced due to the accumulation of bilirubin glucuronide in the blood. The lack of functional MRP2 protein has been identified as the molecular basis for DJS (98-100) and several variations in *MRP2* have been identified in DJS patients. A 2302C>T SNP was

found in the signature C motif in the first NBF and a 4175del6 variant deleted 2 amino acids from the second NBF. Both are predicted to affect ATP-binding but this has not been demonstrated. A third variant, a 3196C>T change, introduced a premature stop codon and removed the second NBF.

1.3.3 Other Diseases

MRP6 transports a number of different xenobiotic compounds in *in vitro* assays, including glutathione conjugates like LTC_4; however its function *in vivo* is still unclear. Recent studies have implicated genetic variations in MRP6 in a rare disease, pseudoxanthoma elasticum (PXE) (101, 102). PXE is an inherited systemic disease affecting connective tissue in the skin, retina and cardiovascular system (102). The disease is characterized by elastic tissue mineralization and fragmentation. Individuals affected by this disease carry genetic variations that alter amino acid sequence either by frameshift or substitutions, or produce a truncated protein. The connection between the disease and MRP6 is still unclear, as MRP6 is mainly expressed in the liver and kidneys. It is believed that there may be an unknown endogenous substrate of MRP6 that is needed for proper maintenance of the connective tissue.

1.3.4 Genetic Variation and Clinical Drug Response

Many of the ABC transporters are important in the absorption, distribution and elimination of drugs and their function can be important in therapeutic response. Along with anti-cancer drugs, ABC transporters are also able to transport antiarrhythmics, immunosuppressants, antihypertensives, antibiotics, HIV protease inhibitors, calcium

channel blockers, and steroid hormones (21, 23, 87). Changes in protein function and/or expression could potentially alter the pharmacokinetics of these substrate drugs. Currently, not much is known about genetic variation in these transporters and how they may affect drug response. A list of ABC transporters and their clinical implications are summarized in Table 1.3.

Genetic variations have been most extensively studied for *ABCB1*, encoding P-glycoprotein. One variant in particular, the 3435C>T synonymous variant, has been implicated in differential protein expression and response to drug therapy. In the most comprehensive analysis to date, more than 50 variants were identified in the coding and flanking intronic regions of *ABCB1* (103). Of these, only four common non-synonymous variants were identified: 61A>G (Asn21Asp), 1199G>A (Ser400Asn), 2677G>T/A (Ala893Ser/Thr), and 3421T>A (Ser1141Thr). With one exception, functional studies suggest that the Ala893Ser/Thr variant has no effect on P-gp function (104-108). Interestingly, the Asn400 P-gp variant exhibits substrate dependent differences in transport function relative to the reference protein (109). It is still unclear what function, if any, *ABCB1* variants have on clinical response.

Since P-gp is expressed in the gut, it is believed that alterations in P-gp function can change drug availability and plasma concentrations. For example, the 3435C>T *ABCB1* variant has been associated with increased plasma levels of the cardiac glycoside digoxin, and decreased expression of P-gp in the duodenum (110-112). Subjects homozygous for the 3435T allele had higher cyclosporine plasma concentrations than those homozygous for the C allele (113) and HIV-1 infected children heterozygous at the 3435 locus had higher nelfinavir plasma concentrations and better response to treatment

(114). However, the data regarding the *ABCB1* variants have been disputed. Others have shown that patients homozygous for the 3435T allele had decreased absorption of digoxin in the duodenum (115) or no difference between genotypes (116, 117), and studies using efavirenz and lopinavir showed no effect of the 3435C>T variant on plasma concentrations (118, 119). In a study in renal transplant patients, MDR1 haplotypes had no effect on cyclosporine steady-state pharmacokinetics (120), and MDR1 haplotypes containing the 2677G>T and 3435C>T had no influence on the pharmacokinetics of tacrolimus (118). MDR1 genotypes also had no effect on antiretroviral therapy response in HIV patients (121).

MDR1 is also expressed in the blood-brain barrier where it limits entry of toxins into the brain (122). In a study in 315 epilepsy patients, individuals homozygous for the 3435C allele were more likely to be resistant to drug treatment, suggesting that the variant 3435T was less functional and therefore allowed higher concentrations of epileptic drugs in the brain (123). A similar study in individuals with temporal lobe epilepsy found an association between those homozygous for the haplotype 1236C/2677G/3435C and higher resistance to drugs (124). A recent study, however showed no association between the 3435C>T variant and response to antiepileptic drug treatment (125) and a second study could not replicate the association between individuals homozygous for the 3435C genotype and drug resistance in epilepsy (126). Loperamide is a peripherally acting opiate agonist used in the treatment of diarrhea. The lack of typical opiate-associated central nervous system (CNS) effects following loperamide treatment is attributed to efflux of loperamide at the blood-brain barrier. Accordingly, loperamide caused respiratory depression in patients who took it with the P-

gp inhibitor quinidine (127). Patients homozygous for 3435T had the highest pupil constriction when given loperamide with quinidine compared to individuals homozygous for 3435C (128), consistent with higher brain concentrations of loperamide in the patients with the 3435TT genotype. However, a study of loperamide in healthy individuals revealed no effect of the 3435C>T variant on respiratory depression (129).

A study of P-gp function in $CD56^+$ natural killer cells from healthy Caucasians showed lower efflux of the substrate rhodamine in those carrying two copies of the T allele. ABCB1 mRNA expression was also lower in leukocytes from these patients, consistent with the decreased activity (130). However, a similar study found no difference in rhodamine efflux in peripheral blood lymphocytes carrying the 3435T variant in combination with the highly linked SNP 2677G>A/T (131).

MDR1 polymorphisms have also been associated with disease susceptibility and response to drug therapy. A study in healthy subjects and colon cancer patients associated those carrying at least one T allele at 3435 with a 1.7-fold increase in developing colon cancer (132) . Those carrying at least one T allele at 3435 were also at risk for renal tumors and those homozygous for 3435T had a 1.7-fold increased risk for clear cell renal cell carcinoma (133). Renal dysfunction was reduced in patients carrying the 2677TT genotype while undergoing treatment with calcineurin inhibitors (CI) after liver transplantation (134). These investigators postulated that the variant Ser893 P-gp expressed in these patients had increased function and pumped increased concentrations of nephrotoxic CI metabolites into the renal tubule. A study in Japanese patients, however, found no influence of *ABCB1* SNPs on mRNA expression in normal and tumor kidney cortex (135). The *ABCB1* variant 3435C>T has also been implicated in survival

in adult acute myeloid leukemia (AML). Those homozygous for the C allele at 3435 had decreased survival compared to those heterozygous at positions 1236, 3677, and 3435 (136).

Individuals carrying the 2677T and 3435T alleles were strongly associated with a reduced risk of Parkinson's disease in the Chinese population (137). An association was also made between the individual SNPs 1236C, 2677G, and 3435C with higher risk of developing Parkinson's disease in Chinese (138). However, the same group showed that *ABCB1* haplotypes in Caucasians did not modulate the risk of Parkinson's disease (139). Other studies were unable to associate *ABCB1* polymorphisms with Parkinson's disease, although the frequency of 3435T was highest in those with early-onset Parkinson's disease (140, 141). It was also noted that patients with Parkinson's disease who were exposed to pesticides had 3 to 5-fold increased risk of the disease when carrying at least one T at 3435 (141).

Limited information is available regarding variation in other ABC transporters and their impact on disease susceptibility or clinical response. Several variants in *ABCB4* were associated with low phospholipid-associated cholelithiasis, which is a subset of gallstone disease (142). The disease is characterized by cholesterol crystal deposits and bile duct inflammation but no presence of gallstones. In a retrospective study, the *ABCC2* synonymous variant 3972C>T was associated with higher irinotecan AUC and may affect the incidence of neutropenia (143). In *ABCC6*, a 3421C>T variant, which causes a premature stop codon, is associated with premature coronary artery disease (144).

High ABCG2 mRNA expression has been linked with poor response to therapy in acute myeloid leukemia (145), however the basis for the high expression has not been determined. *ABCG2* variants have been identified that lowered expression levels, and decreased drug resistance to anti-cancer drugs *in vitro* (146, 147). However a study on the *ABCG2* 421C>A variant exhibited no altered irinotecan pharmacokinetics (148). The *ABCG2* 421C>A variant also was not found to influence mRNA expression in human intestine (149). Increased mRNA expression for ABCC1 was associated with a decreased overall survival in adult AML (150). Although no genetic variation has been identified, it is reasonable to believe that variable gene expression may be due to genetic variation in gene regulatory regions.

ABC transporters are important proteins in the disposition of endogenous and xenobiotic compounds and changes in protein function due to genetic variations may be detrimental to patient response to drug treatment. Current data thus far on clinical implications of genetic variations are limited and at best controversial. Further studies are needed to understand the effects of single nucleotide polymorphisms on ABC transporter function and influence on clinical response.

Table 1.3 Summary of Clinical Implications of ABC Transporters

Gene	Polymorphism / Clinical Implications	References
ABCB1	Multidrug resistance	(4, 44)
	3435T / 3435TT	
	↑ digoxin plasma concentrations	(110-112)
	↓ digoxin absorption in the duodenum	(115)
	↔ digoxin absorption	(116, 117)
	↑ nelfinavir plasma concentrations	(114)
	↑ cyclosporine plasma concentrations	(113)
	↔ efavirenz plasma concentrations	(119)
	↔ lopinavir plasma concentrations	(119)
	↔ cyclosporine steady-state concentrations	(120)
	↓ resistance to drug treatment for epilepsy	(123)
	↔ response to drug treatment for epilepsy	(125, 126)
	↑ pupil constriction after loperamide/quinidine dose	(127, 128)
	↔ in respiratory depression after loperamide dose	(129)
	↓ efflux of rhodamine in $CD56^+$	(130)
	1.7-fold increased risk of developing colon cancer	(132)
	1.7-fold increased risk for clear cell renal cell carcinoma	(133)
	3-5-fold increased risk in Parkinson's disease when exposed to pesticides	(141)
	2677T	
	↓ risk of renal dysfunction with CI therapy	(134)
	2677T / 3435T	
	↔ tacrolimus PK	(118)
	↔ rhodamine efflux in PBMCs	(131)
	↓ risk in Parkinson's disease	(137)
	1236C / 2677G / 3435C	
	↑ resistance to drugs in temporal lobe epilepsy	(124)
	↑ risk for Parkinson's disease	(138)
	↔ risk in Parkinson's disease	(139)
ABCB4	Progressive intrahepatic cholestasis, type 3	(92, 93)
	Cholelthiasis	(142)

	Intrahepatic cholestasis of pregnancy	(93-96)
ABCB11	progressive intrahepatic cholestasis, type 2	(89, 90)
ABCC1	multidrug resistance	(34, 59)
	↓ survival in adult AML	(150)
ABCC2	Dubin-Johnson Syndrome	(98, 100, 151-153)
	3972C>T	
	↑ irinotecan AUC	(143)
ABCC6	pseudoxanthoma elasticum	(101, 102, 154)
	3421C>T	
	↑ risk of premature coronary artery disease	(144)
ABCG2	multidrug resistance	(85)
	↓ response to therapy in acute myeloid leukemia	(145)
	421C>A	
	↓ drug resistance *in vitro*	(146, 147)
	↔ irinotecan PK	(148)
	↔ mRNA expression in human intestine	(149)

Listed are diseases and clinical response implications associated with ABC transporters. An increase (↑), decrease (↓), or no change (↔) in drug response is given.

1.4 FOCUS OF DISSERTATION

With the sequencing of the human genome completed, genetic information will become more valuable in treating human diseases. The most abundant genetic variation in the human genome is the single nucleotide polymorphism (155) and these can drastically affect transporter function. ABC transporters are found in most tissues and control the absorption, distribution and elimination of drugs and xenobiotics. The goal of this dissertation research is to examine genetic variation in the ABC family of transporters and determine the functional and clinical implications of this variation. A long term goal is to use this knowledge to predict drug response.

The objectives of the studies are as follows:

Chapter 2. Identification and Genetic Analysis of Polymorphisms in *ABCC2*.

A large scale identification of genetic polymorphisms in human *ABCC2* was carried out using a set of 247 ethnically diverse DNA samples. All exons, 50-100 bases of flanking intronic sequence, and a 1.6 kb region proximal to the ATG start site were screened for single nucleotide polymorphisms. Genetic and haplotype analysis were performed to determine the diversity in *ABCC2* at the nucleotide level. Several heterologous assays were developed to characterize *ABCC2* coding variations. These include uptake studies with inside-out vesicles, flux assays in polarized MDCKII cells, and accumulation assays using fluorescence activated cell sorting (FACS).

Chapter 3. Characterization of 5'-Promoter Region Polymorphisms in *ABCC2*

The 5'-region of MRP2 was cloned into a reporter gene vector and polymorphisms were introduced either as single nucleotide polymorphisms or in

combination (haplotypes). Characterization of the SNPs was carried out in human hepatoma cell lines (HepG2). Altered promoter activity was further analyzed by electrophoretic mobility shift assay (EMSA) to determine if altered nuclear protein binding to SNP sites could explain the differences.

Chapter 4. Allelic Imbalance in ABCC2 mRNA

An association was made between a synonymous SNP in *ABCC2* (3972C>T) and irinotecan disposition. Allelic imbalance assays were performed to determine if transcript levels were altered between the C and T alleles, which could explain the clinical association.

Chapter 5. Impact of Genetics on Digoxin Pharmacokinetics

Many studies involving the pharmacogenetics of drug response have focused on drug metabolizing enzymes. Digoxin, which is minimally metabolized and a known substrate for several transporters, was used in a twin study to determine the heritability of pharmacokinetic variability associated with drug transport. Observed variability can be attributed to variation in transporters involved in digoxin disposition.

Chapter 6. Genetic Analysis of Members of the ABC Family of Transporters

A large scale genetic analysis was performed on members of the ABC family of transporters. Nucleotide diversity (π), neutral parameter (θ), linkage disequilibrium, and recombination events were analyzed for 10 members of the ABC family. Nucleotide variability was analyzed across exons and flanking intronic regions of each gene, and as a gene family, compared to analyses of other multi-gene families.

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CHAPTER 2

IDENTIFICATION AND GENETIC ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISMS IN *ABCC2*

2.1 INTRODUCTION

The body is able to protect itself from a wide variety of toxins that enter into the system either through oral intake or other routes. The first step in detoxification for many toxins introduced orally is metabolism in the liver. In the liver xenobiotics can undergo Phase I (oxidation, reduction, or hydrolysis), Phase II (conjugation) (1, 2), and/or phase III (transport) metabolism (3). There is interplay between all three metabolic groups, with the toxin first being bioactivated or inactivated by Phase I enzymes. These metabolites can further be transformed by Phase II enzymes, which can attach large hydrophilic groups such as glutathione, glucuronic acid, or sulfates. These biotransformed compounds can then be eliminated through the bile by transporters (4). Most studies regarding the detoxification of xenobiotics and inter-individual differences in response to oral drugs have focused on metabolizing enzymes. However there is an emerging interest in how transporters play a role in detoxification and response to xenobiotics. Recent studies have shown that transporters can precede metabolism by enzymes and therefore affect drug disposition (5, 6)

MRP2 is a 190 kDa (1545 amino acids) integral membrane transporter encoded by *ABCC2* and is a member of the ATP Binding Cassette (ABC) family of active efflux transporters, some of which have been shown to confer resistance to chemotherapeutic drugs. MRP2, also called the canalicular multispecific organic anion transporter

(cMOAT), was identified as an isoform of MRP1 and was cloned from the rat liver (7). MRP2 is believed to be one of the GS-X pumps that are able to transport glutathione conjugates during the detoxification process. The substrate specificity of MRP2 has been elucidated through the use of transport-deficient (TR^-) rats (8) and the Eisai-hyperbilirubinemic rat (EHBR) (9). These rats do not express the canalicular conjugate export pump and are deficient in the ability to transport glutathione and glucuronate conjugates (8-10). MRP2 mediates the unidirectional, ATP-dependent transport of a wide variety of endogenous and exogenous amphiphilic anions across membranes, which are often conjugated to glutathione, glucuronide, and sulfate moieties (11). In humans, MRP2 is expressed on the apical membrane of epithelial cells in the gut, kidney and liver (12). In the liver, where it is highly expressed, MRP2 extrudes endogenous and exogenous anionic compounds from the hepatocyte into the bile for elimination (7, 13) (Figure 2.1).

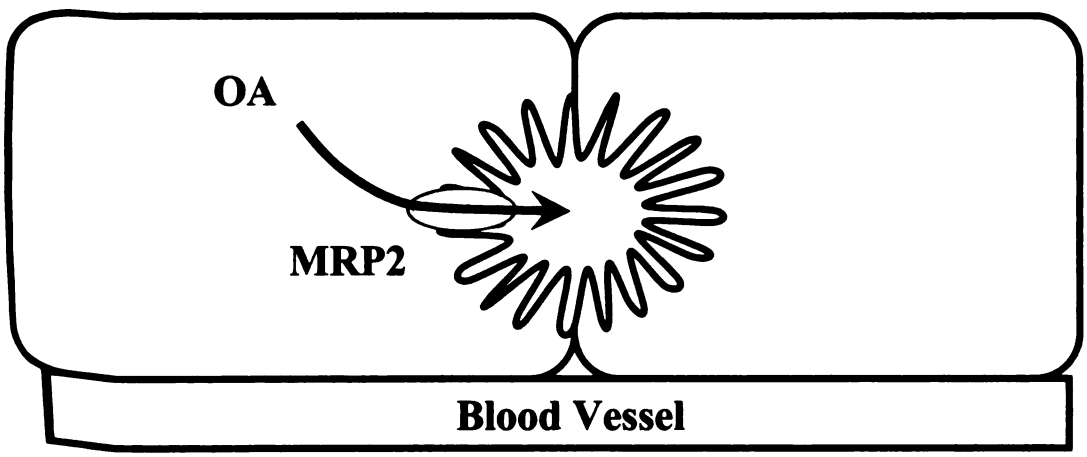


Figure 2.1 Schematic of a hepatocyte depicting the localization of MRP2 on the bile canaliculus. OA, organic anion; MRP2, multidrug resistance-associated protein 2.

The predicted topology of MRP2 is two nucleotide binding folds (NBF) where ATP is hydrolyzed, and three transmembrane domains (TMD) each consisting of 6

membrane spanning α-helices (Figure 2.2). The NBF contains characteristic motifs belonging to the ABC family, a Walker A, Walker B, and signature C motif (14). *ABCC2* is localized to chromosome 10q24 (15) and shares about 49% sequence identity with *ABCC1* (16).

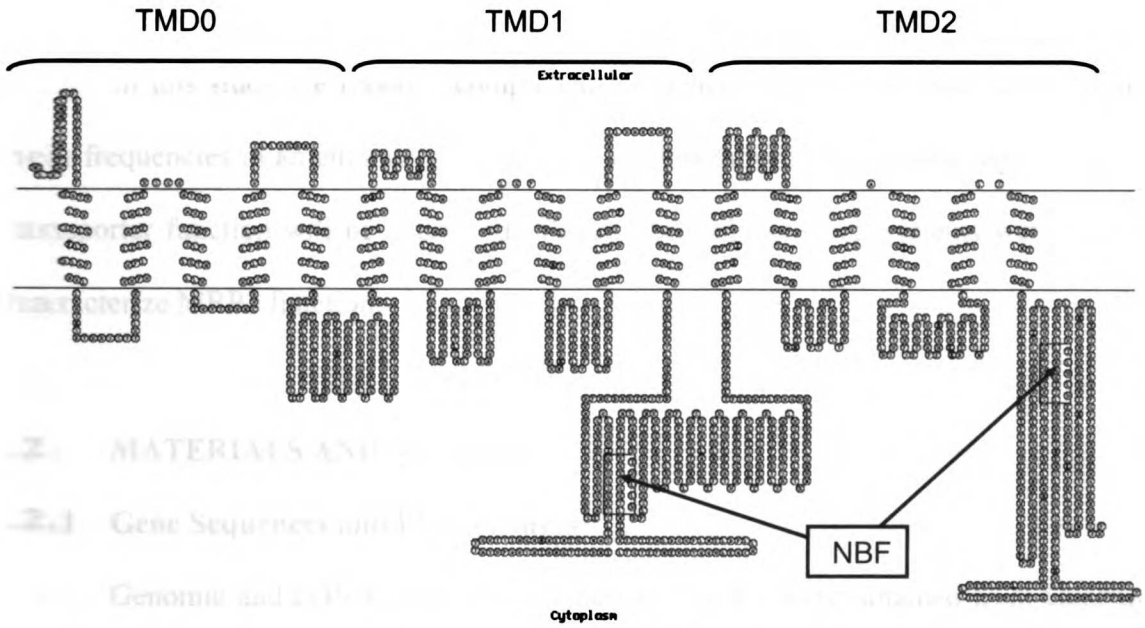


Figure 2.2 Secondary structure of MRP2 predicted using TOPO (S.J. Johns, University of California, San Francisco, and R.C. Speth, Washington State University. Software is available at http://www.sacs.ucsf.edu/TOPO/topo.html). Horizontal lines depict the membrane lipid bilayer. TMD, transmembrane domain; NBF, nucleotide binding fold.

Genetic variations in *ABCC2* can lead to altered transport function and hepatobiliary elimination of MRP2 substrates. One example is the endogenous substrate, conjugated bilirubin. Bilirubin is a by-product of heme metabolism and is glucuronidated for elimination to the bile via active transport by MRP2. A defect in the transport of conjugated bilirubin results in the hyperbilirubinemic disease, Dubin Johnson Syndrome (DJS). The lack of functional MRP2 protein has been identified as the molecular basis

for DJS (17, 18) and several variations in *ABCC2* identified in DJS patients are thought to be the cause of the impaired MRP2 protein (19-24). With its ability to transport a wide variety of compounds, both unconjugated and conjugated, and being highly expressed in the liver, genetic variation in *ABCC2* could potentially lead to a disruption in the biliary elimination of anionic drugs.

In this study we report a comprehensive genetic analysis of *ABCC2*. Variants, their frequencies in an ethnically diverse sample population, and possible significance to transporter function will be addressed. Several heterologous cell systems were used to characterize MRP2 function.

2.2 MATERIALS AND METHODS

2.2.1 Gene Sequences and PCR Primers

Genomic and cDNA sequences for human *ABCC2* were obtained from Genbank (http://www.ncbi.nlm.nih.gov). Accession numbers U63970.1 and AF144630.1 were used as a sequence base for determining single nucleotide polymorphisms (SNPs). The genomic structure of *ABCC2* was deduced by aligning the genomic and cDNA sequences using BLAST. Primers for genomic DNA were designed to include 30-50 bp of flanking intronic sequence. For closely spaced exons (4-5, 18-19, 20-21, 22-23), both exons and the complete spanning intronic sequence were included in a single amplicon. Three primer sets were used to sequence the 5'-promoter region. Primer sequences are available at http://www.pharmgkb.org and http://www.pharmacogenetics.ucsf.edu.

2.2.2 Variant Identification

A collection of 247 ethnically diverse genomic DNA samples were obtained from the Coriell Institute of Medical Research (http://coriell.umdnj.edu). This collection of samples was used to screen for genetic variations in *ABCC2*. Initial screening was done using PCR, denaturing HPLC (DHPLC) and direct sequencing as previously described (25). Briefly, PCR was carried out after optimization using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA). PCR samples from two to three individuals were then pooled and allowed to heteroduplex after denaturing and re-annealing. Heteroduplexed samples, 5-10 μL, were then injected into a WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE). All samples were visually inspected and samples with variant peaks were sequenced. Individual samples were amplified using PCR and sequenced in both directions using primers designed for DHPLC. Dye terminator sequencing was performed on an ABI 3700 automated sequencer using the ABI PRISM BigDye system. Sequences were analyzed by visual inspection and variants were identified if both traces showed deviation from the reference sequence. For promoter region variant identification, direct sequencing of genomic DNA was performed as described for the variant samples identified by DHPLC.

2.2.3 Population Genetics

The neutral parameter (θ), nucleotide diversity (π), and Tajima's D statistic were calculated as described by Tajima (26). Each parameter was calculated for a number of regions in the gene, including coding, non-coding, intron-exon boundaries, areas with synonymous and non-synonymous variations, evolutionarily conserved and non-conserved regions, and predicted extracellular and intracellular loops, and transmembrane

domains. Evolutionarily conserved sites were determined by aligning protein sequences of human, dog, rat, rabbit, mouse, and rhesus monkey using the ClustalX (27) and GeneDoc programs (28). Complete conservation across species was required for assignment of a site as evolutionarily conserved. Transmembrane domains and loops were determined based on prediction from the Swissprotein database (http://www.ebi.ac.uk/swissprot).

2.2.4 Haplotype Analysis

Haplotype analysis was performed using PHASE 2.0 (29). Singleton variant sites were removed from the data. Data was then segregated into ethnic groups, where unambiguous haplotypes were assigned. Ambiguous samples were run through PHASE ten times. A total of ten iterations were performed and a haplotype pair was assigned only if the haplotype pair occurred in seven of the ten iterations. Haplotype history for *ABCC2* was inferred by using a cladogram constructed from the number of haplotypes estimated for *ABCC2* in the study population. Cladograms were constructed using Network 4.0 (http://www.fluxus-technology.com/sharenet.htm) using the Reduced Median algorithm (30).

2.2.5 Linkage Disequilibrium and Intragenic Recombination

Linkage disequilibrium (LD) and recombination were quantified by the D' and r^2 statistics and significance of the associations was assessed by the Fisher's Exact Test. Analysis was performed using the program DnaSP (31).

2.2.6 Expression Plasmid Construction

An MRP2-pGEM3Z cloning plasmid was kindly provided by Marcel Kool (Netherlands Cancer Institute, Amsterdam, The Netherlands). For yeast expression plasmids, MRP2 was cloned into the yeast expression vectors pYES2 (Invitrogen, Carlsbad, CA), pRS416, and pRS426 (32) using homologous recombination (33-35). For pRS416 and pRS426 cloning, double stranded linker sequences were created for homologous recombination. The *MRP2* cDNA was cloned from MRP2-pYES2 into the mammalian vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA) using *HindIII* and *NotI* restriction enzyme sites. Variants, either as single variants or in haplotypes, were introduced using specific primers and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.2.7 Yeast Culture

The yeast strains NY605 (*MATa*; ura3-12Δ; leu2-3Δ; GAL), W303 (*MATa/MATα* ade2Δ/ADE2, can1-100Δ/CAN1, CYH2/cyh2Δ, his3-11,15Δ/his3-11,15Δ, LEU1/leu1-cΔ, LEU2/leu2-3,112Δ, trp1-1::URA3::trp1-3'D/trp1-1, ura3-1Δ/ura3-1Δ) and Ydr135c(-/-) (*MATa/MATα*, his3Δ/his3Δ, leu2Δ/leu2Δ, met15Δ/MET, lys2Δ/LYS, ura3/ura3, ycf1Δ/ycf1Δ) were used for protein expression. Prior to transformation with plasmids, yeast cells were grown at 30°C in SD-complete media (0.17% Yeast Nitrogen Base, 0.5% Ammonium sulfate, 2% glucose, 0.13% amino acids). Transformation of yeast strains was carried out by incubating the plasmid and yeast cells in transformation buffer (40% polyethylene glycol in TE buffer, 0.1 mM lithium acetate, 10 μg salmon sperm DNA) at

42°C for 40 min. Transformed cells were plated onto SD-Ura plates (plates containing no uracil) and incubated at 30°C overnight.

2.2.8 Growth Inhibition Assays

Yeast cells containing MRP2 plasmid were grown in SD-Ura, supplemented with 2% galactose and 1% raffinose. Cells were diluted in media to an OD of 0.1 (A600). Two hundred μL were spread onto SD-Ura/+Gal/Raf plates using glass beads. Filter discs were placed into one of four quadrants on the plate and 20 μL of 1-chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich, St. Louis, MO) at various concentrations (250 μM – 1 mM) was added. Plates were incubated at 30°C for 48 hours. For liquid cultures, 10 mL of yeast culture (OD = 0.1) were incubated either with 40-50 μM of CDNB or vehicle control (70% ethanol). At various time points, the OD was taken to measure yeast growth.

2.2.9 Mammalian Cell Culture

Human embryonic kidney epithelial cells (HEK-293) were cultured at 37°C in a humidified incubator at 5% CO_2 in EMEM (with EBSS, 10% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, and 1% penicillin/streptomycin). Cells were plated onto 24-well plates one day prior to transient transfection using the Lipofectamine Plus (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's protocol. Transfected cells were harvested 24 h later with 0.05% trypsin and 0.02% versene, or were supplemented with media containing 1000 mg/mL of the selection agent, geneticin (G418), for stable expression. Madine-Darby canine kidney II cells (MDCKII) and

MDCKII cells stably transfected with MRP2 cDNA (MDCKII-MRP2, a kind gift from Marcel Kool) were cultured at 37°C in a humidified incubator at 5% CO_2 DME-H21 (with 10% FBS and 1% penicillin/streptomycin).

2.2.10 Western Blot

Cell lysates from MDCKII, MDCKII-MRP2, HEK293 and HEK293-MRP2 cells were made by incubating cells in lysis buffer (10 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2 \cdot 2H_2O$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL aprotonin, 20 μg/mL leupeptin, 1 μg/mL pepstatin). Aliquots of 50-100 μg of protein (determined by the Pierce BCA Assay) were mixed with Laemmli buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol, 1 mM EDTA and 0.001% bromophenol blue) and run on an 8% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and MRP2 proteins were probed using the primary antibodies M_2-I-4 or M_2-III-6 (Alexis Biochemicals, Eugene, OR), which were diluted 1:50 in TBS-T buffer (10 mM Tris-base, 150 mM NaCl, 1% Tween-20) supplemented with 5% powdered milk, for one hour. Membranes were washed twice with TBS-T before incubating in the secondary antibody anti-mouse IgG-HRP (1:5000 dilution in 0.05% milk-TBS-T) for an hour. Membranes were again washed twice in TBS-T. Antibody detection was performed using ECL reagent (Amersham Life Science, Piscataway, NJ) according to the manufacturer's protocol.

2.2.11 Accumulation Assay Using Flow Cytometry

MDCKII and MDCKII-MRP2 were trypsinized, washed with cold PBS, resuspended in media and aliquotted into microcentrifuge tubes. Cells in suspension were incubated at 37°C with 5% CO_2 for 30 min with 5 μM 5-chloromethyl fluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR) in the presence or absence of the inhibitor cyclosporine. Cells were centrifuged at 500*g* for 2 min, aspirated, and resuspended in DME media and allowed to efflux accumulated CMFDA for 30 min at 37°C. Cells were centrifuged, aspirated, and resuspended in PBS supplemented with 2% FBS and placed on ice to stop efflux. Accumulation of CMFDA into cells was measured by multicolor flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) equipped with argon and crypton lasers. Data for experiments were acquired from 3,000 – 10,000 cells per sample.

2.2.12 Bidirectional Transport Assays

MDCKII and MDCKII-MRP2 cells were grown in 12-well transwell plates (Corning Costar, Acton, MA) until confluent. Cells were washed with PBS and the donor compartment was supplemented with media containing either vehicle control, 50 μM of CDNB used as a competitive inhibitor, 5 μM of the substrate CMFDA, or a combination of inhibitor/substrate. At various time points 100 μL aliquots were taken from the receiver side and replaced with receiver media solution to maintain the original starting volume. After the final time point, fluorescence was measured using a fluorescence plate reader.

2.2.13 Inside-Out Vesicle Preparation

Approximately 2 x 10^8 MDCKII or MDCKII-MRP2 cells were trypsinized, and centrifuged at 500*g* for 5 min at 4°C. Cells were washed in cold PBS, centrifuged, and aspirated. The cell pellet was resuspended in 40 volumes of hypotonic lysis buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 1 μg/mL pepstatin, and 5 μg/mL aprotonin). Cells were incubated on ice for 60 min with gentle stirring and then centrifuged in a Beckman SW28 rotor at 100,000*g* at 4°C for 40 min. The supernatant was aspirated and the pellet was resuspended in 10 mL of cold isotonic buffer (IB) (10 mM TrisHCl, 250 mM sucrose, pH 7.4). Cells were homogenized on ice using a Dounce B homogenizer using 30 strokes. Two mL of homogenate was layered over 5 mL of 38% w/v sucrose solution (in 5 mM Tris-HEPES pH 7.4) and centrifuged at 250,000*g* at 4°C for 60 min in a swing-out rotor. The turbid layer at the interface was collected and diluted to 35 mL in IB buffer. The solution was centrifuged at 100,000*g* at 4°C for 40 min. The supernatant was aspirated and the cell pellet was resuspended in 500 μL of IB buffer. Vesicle formation was performed by passing the suspension 30 times through a 27½ gauge needle. Newly formed vesicles were aliquotted and stored at -80°C.

2.2.14 Transport in Inside-Out Vesicles

The ATP-dependent transport of [^{3}H]LTC_4 into membrane vesicles prepared from MDCKII or MDCKII-MRP2 was measured by rapid filtration (36). Briefly, transport assays were performed at room temperature in a 110 μL total reaction volume containing 2.5 nM [^{3}H]LTC_4, 4 mM AMP or ATP, 10 mM $MgCl_2$, 100 μg/ml creatine kinase, 10 mM creatine phosphate and 20 μg of vesicle protein. Inhibition of uptake was performed

by incubating vesicles in the presence of 5 μM MK-571 (Axxora, LLC, San Diego, CA). Uptake was terminated at various time points by removing 15 μL of vesicles and rapidly diluting in ice-cold transport buffer and filtering under vacuum through filters using a Hoeffer filtration manifold (Hoeffer Scientific Instruments, San Francisco, CA). Filters were immediately washed twice and dried, and vesicle-associated radioactivity was quantified by scintillation counting.

2.3 RESULTS

2.3.1 Genetic Variation in *ABCC2*

Sequence spanning the 32 exons, flanking intronic regions and a 1.6 kb region upstream from the ATG start site were screened for genetic variation. Using DHPLC and direct sequencing, fifty-five variant sites were identified in the exon/intron regions, and thirteen variants were found in the 5'-promoter region. This equates to approximately 1 variant for every 142 base pairs screened. The location and frequencies of all variants identified are listed in Table 2.1 and have been posted to the Pharmacogenetics and Pharmacogenomics Knowledge Base (http://www.pharmgkb.org) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP). The observed allele frequencies were in Hardy-Weinberg equilibrium for all ethnic groups studied.

Table 2.1 Genetic Variation in *ABCC2*

SNP #	cDNA Position	Exon Position	Nucleotide Change	Amino Acid	Amino Acid Change	Allele Frequencies					
						Toal Freq n=494	AA Freq n=200	CA Freq n=200	AS Freq n=60	ME Freq n=20	PA Freq n=14
P.1*		(-48)	T→ C	-	-	**0.01**	**0.025**	0	0	0	0
P.2*	-1563	37	G→ A	-	-	0.002	0.005	0	0	0	0
P.3*	-1549	51	G→ A	-	-	**0.407**	**0.485**	**0.43**	**0.15**	**0.2**	**0.357**
P.4*	-1292	308	A→ G	-	-	0.006	**0.015**	0	0	0	0
P.5*	-1239	361	G→ A	-	-	**0.016**	**0.03**	0.005	0	**0.05**	0
P.6*	-1065	535	C→ A	-	-	0.004	0.005	0	0	**0.05**	0
P.7*	-1059	541	C→ +G	-	-	0.002	0.005	0	0	0	0
P.8	-1023	577	G→ A	-	-	**0.154**	**0.135**	**0.105**	**0.267**	**0.3**	**0.429**
P.9	-1019	581	A→ G	-	-	**0.36**	**0.365**	**0.43**	**0.167**	**0.2**	**0.357**
P.10*	-798	802	C→ A	-	-	0.002	0.005	0	0	0	0
P.11*	-733	867	G→ A	-	-	0.002	0	0	**0.017**	0	0
P.12	-24	1576	C→ T	-	-	**0.136**	**0.06**	**0.195**	**0.15**	**0.15**	**0.286**
P.13	-23	1577	G→ A	-	-	0.004	0	0	0	0	**0.143**
2.1*	Intron 1	(-8)	G→ A	-	-	0.008	**0.02**	0	0	0	0
2.2*	116	83	T→ A	39	Phe→ Tyr	0.008	**0.02**	0	0	0	0

2.3*	159	126	A → G	53	syn	0.008	**0.01**	**0.01**	0	0	0
3.1*	Intron 3	(+37)	C → G	-	-	0.004	0	**0.01**	0	0	0
6.1*	Intron 5	(-20)	G → A	-	-	**0.014**	**0.03**	0	0	**0.05**	0
7.1*	Intron 6	(-30)	G → T	-	-	0.008	**0.02**	0	0	0	0
7.2*	736	104	A → C	246	Met → Leu	0.002	0.005	0	0	0	0
8.1*	998	131	A → G	333	Asp → Gly	0.004	**0.01**	0	0	0	0
9.1	1058	27	G → A	353	Arg → His	**0.014**	**0.035**	0	0	0	0
10.1*	1219	10	C → T	407	syn	**0.016**	**0.036**	0.005	0	0	0
10.2	1249	40	G → A	417	Val → Ile	**0.158**	**0.17**	**0.17**	**0.117**	**0.15**	0
10.3*	1434	225	G → T	478	syn	0.002	0.005	0	0	0	0
10.4	1457	248	C → T	486	Thr → Ile	0.002	0	0	**0.017**	0	0
11.1*	Intron 10	(-25)	T → G	-	-	0.004	0.005	0	0	0	**0.083**
11.2*	1483	19	A → G	495	Lys → Glu	0.002	0	0.005	0	0	0
13.1*	1686	18	T → G	562	Phe → Leu	0.002	0.005	0	0	0	0
13.2*	Intron 13	(+27)	C → G	-	-	**0.04**	**0.095**	0	0	**0.05**	0
14.1*	Intron 14	(+22)	T → C	-	-	**0.02**	**0.025**	**0.025**	0	0	0
16.1*	2009	42	T → C	670	Ile → Thr	0.006	**0.015**	0	0	0	0
16.2*	2073	106	C → A	691	syn	0.002	0.005	0	0	0	0
17.1*	Intron 17	(+36)	G → T	-	-	0.002	0.005	0	0	0	0
19.1*	2546	107	T → G	849	Leu → Arg	0.006	0.005	**0.01**	0	0	0
20.1*	Intron 20	(+29)	C → A	-	-	0.002	0	0	**0.02**	0	0
21.1*	Intron 20	(-91)	C → T	-	-	0.004	**0.01**	0	0	0	0

21.2*	Intron 20	(-13)	T → G	-	-	0.002	0.005	0	0	0	0
22.1*	2901	18	C → A	967	Tyr → STOP	0.002	0.005	0	0	0	0
22.2	2934	51	G → A	978	syn	0.008	0	0	**0.067**	0	0
22.3*	2944	61	A → G	982	Ile → Val	0.002	0.005	0	0	0	0
23.1*	3107	4	T → C	1036	Ile → Thr	0.004	0.005	0.005	0	0	0
23.2*	3188	85	A → G	1063	Asn → Ser	0.002	0.005	0	0	0	0
24.1*	3396	138	T → C	1132	syn	0.004	0	**0.01**	0	0	0
25.1*	Intron 24	(-33)	G → A	-	-	0.004	0	**0.01**	0	0	0
25.2*	3542	128	G → T	1181	Arg → Leu	**0.034**	**0.085**	0	0	0	0
25.3*	3561	147	G → A	1187	syn	0.002	0.005	0	0	0	0
25.4	3563	149	T → A	1188	Val → Glu	**0.059**	**0.065**	**0.075**	0	0	**0.071**
25.5*	Intron 25	(+6)	G → C	-	-	0.002	0.005	0	0	0	0
26.1*	Intron 25	(-20)	C → T	-	-	0.006	0.005	**0.01**	0	0	0
26.2*	Intron 26	(+30)	G → A	-	-	0.002	0.005	0	0	0	0
27.1*	Intron 26	(-34)	T → C	-	-	**0.178**	**0.25**	**0.17**	**0.018**	**0.05**	**0.071**
27.2*	3817	76	A → G	1273	Thr → Ala	0.004	**0.01**	0	0	0	0
28.1*	3872	29	C → T	1291	Pro → Leu	**0.01**	**0.021**	0.005	0	0	0
28.2*	3972	129	C → T	1324	syn	**0.309**	**0.273**	**0.383**	**0.19**	**0.25**	**0.357**
28.3*	Intron 28	(+21)	C → T	-	-	0.007	0	**0.016**	0	0	0
29.1*	4062	75	C → T	1354	syn	**0.012**	**0.03**	0	0	0	0
29.2*	4110	123	C → T	1370	syn	**0.038**	**0.09**	0	0	**0.05**	0
29.3*	Intron 29	(+11)	G → C	-	-	**0.059**	**0.065**	**0.075**	0	0	**0.071**

30.1*	Intron 29	(-35)	G→ A	-	-	**0.079**	**0.144**	**0.027**	**0.017**	**0.2**	0
30.2*	4242	96	C→ T	1414	syn	0.002	0.005	0	0	0	0
30.3	4290	144	G→ T	1430	syn	**0.063**	**0.078**	**0.074**	0	0	**0.071**
30.4*	Intron 30	(+11)	A→ C	-	-	0.002	0.005	0	0	0	0
31.1*	4410	97	G→ A	1470	syn	**0.077**	**0.146**	**0.035**	**0.017**	**0.05**	0
31.2	4488	175	C→ T	1496	syn	**0.043**	**0.035**	**0.06**	**0.017**	0	**0.071**
31.3*	Intron 31	(+12)	G→ A	-	-	**0.061**	**0.071**	**0.07**	**0.017**	0	**0.071**
32.1*	4527	19	C→ T	1509	syn	0.006	0.005	**0.01**	0	0	0
32.2	4544	36	G→ A	1515	Cys→ Tyr	**0.116**	**0.196**	**0.081**	0	**0.05**	**0.071**

Variants are numbered sequentially in the promoter (P) and by exon. Novel variants are indicated by *

cDNA numbers are relative to the ATG start site and based on sequence from Genbank U63970.1 with a G as reference at position -1549 in the promoter. Intronic variants are labelled based on whether they are located 5' (-) or 3' (+) to an exon. The cDNA position for the promoter variants refers to the distance from the ATG, starting with -1. Promoter exon position denotes the position in the amplicon.

Amino acid change indicates whether a variant caused a synonymous change (syn) or non-synonymous change.

Allele frequencies were calculated for the total population and each ethnic group. CA, Caucasians; AA, African American; AS, Asian American; ME, Mexican American; PA, Pacific Islander

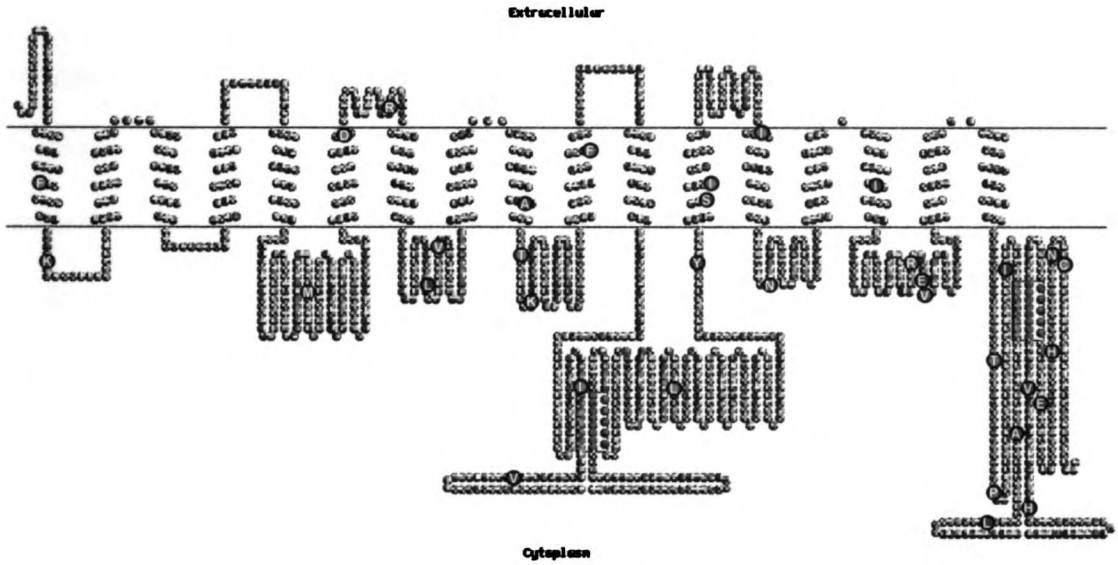


Figure 2.3 *ABCC2* cSNPs superimposed onto the predicted secondary structure. Non-synonymous changes are shown in red, synonymous changes in green, stop codon in blue, the Walker A motif in orange, the Walker B motif in violet, and the Signature C motif in cyan.

Of the fifty-five variants discovered in the exons and intronic boundaries, 34 were found in the coding region (cSNPs). Nineteen cSNPs caused an amino acid change (non-synonymous), with 10 found at > 1% allele frequency in any given ethnic group. Eight cSNPs were found in the transmembrane domain, one in the extracellular loops, while the majority of cSNPs were found in the intracellular loops (Figure 2.3). An alignment with MRP2 protein across species showed ten non-synonymous sites being evolutionarily conserved (Figure 2.4). Of the 5'-upstream SNPs, two were found in the 5'-untranslated region (5'-UTR), and one was found in the sequencing primer at the 5'-end. There was one single base insertion at position -1059 that was found in only one chromosome in the African American population. Nine promoter SNPs were found at an allele frequency > 1%. Sixty-six percent of all SNPs identified were transitions, and 26.5% of the variants

occurred at CpG dinucleotides. Twenty-one singletons were discovered in ~9.6 kb of sequence that was screened. A total of twelve variants were previously described in the literature and dbSNP (37-39). Genetic variation was not evenly distributed among the ethnic groups studied. Six variants were considered cosmopolitan, or were found in all ethnic groups. Twenty-nine variants were African American specific, five were Caucasian specific and four were Asian American specific.

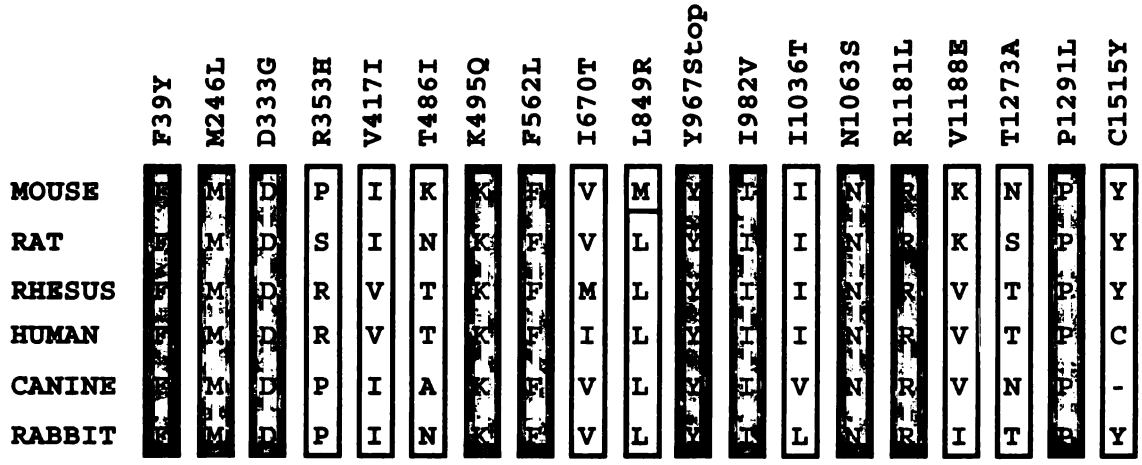
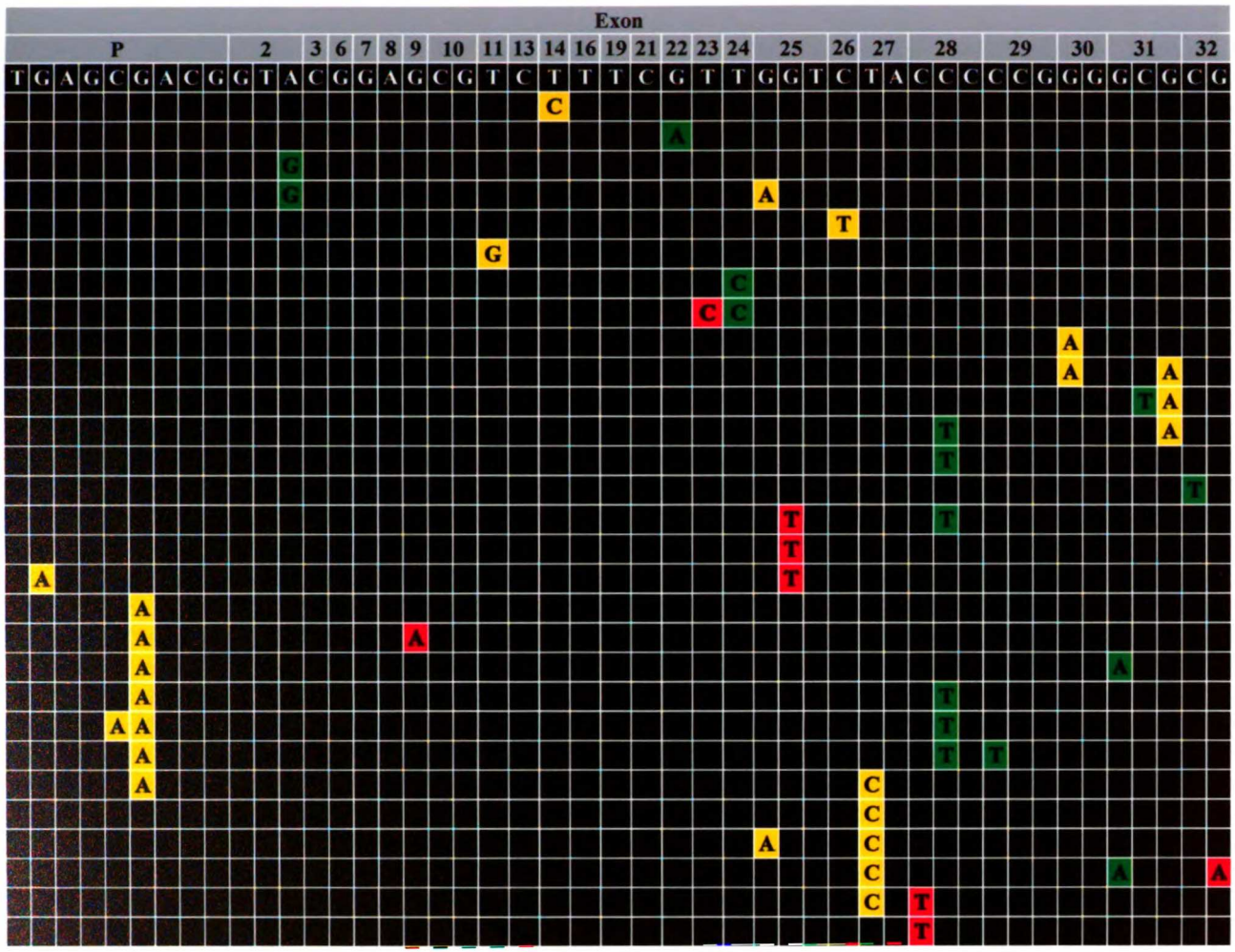


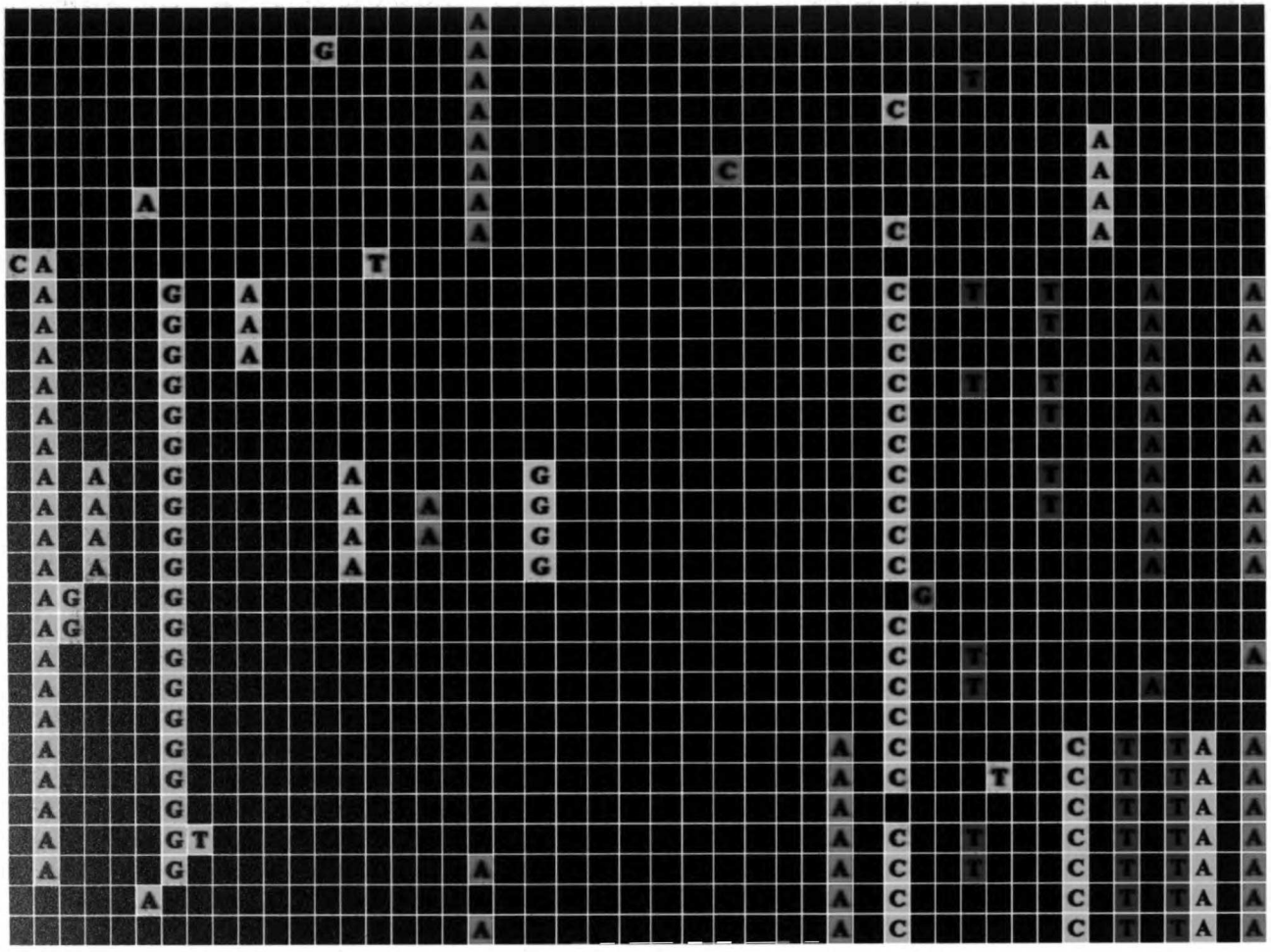
Figure 2.4 MRP2 species alignment across 6 mammalian orthologs. Shown are the non-synonymous variants and their location. Evolutionarily conserved sites are boxed in grey, evolutionarily unconserved sites are boxed in white, and partial conservation boxed in light grey.

2.3.2 Haplotype Analysis

Of the 247 individuals sampled, 11 individuals had their haplotypes inferred from at least 7 of 10 PHASE iterations, while the remaining were identified in all ten iterations. Haplotypes from 236 assignable samples were used in the estimation. Altogether 88 haplotypes were identified (Figure 2.5), of which 32 were found in 3 or more chromosomes and accounted for ~85% of the population. The 32 common



	Ethnicity				
	CA	AA	AS	ME	PA
*1	0.141	0.076	0.328	0.300	0.083
*44	0.025	0.022	0.000	0.000	0.000
*46	0.000	0.000	0.069	0.000	0.000
*47	0.005	0.011	0.000	0.000	0.000
*47A	0.005	0.000	0.000	0.000	0.000
*48	0.010	0.000	0.000	0.000	0.000
*49	0.000	0.000	0.000	0.000	0.167
*50	0.005	0.000	0.000	0.000	0.000
*50A	0.005	0.000	0.000	0.000	0.000
*51	0.000	0.000	0.000	0.050	0.000
*51A	0.005	0.000	0.000	0.000	0.000
*52	0.000	0.000	0.017	0.000	0.000
*36A	0.005	0.000	0.000	0.000	0.000
*36	0.045	0.000	0.052	0.000	0.000
*53	0.005	0.000	0.000	0.000	0.000
*7A	0.000	0.016	0.000	0.000	0.000
*7	0.000	0.060	0.000	0.000	0.000
*7B	0.000	0.005	0.000	0.000	0.000
*2	0.061	0.033	0.259	0.200	0.417
*2A	0.000	0.016	0.000	0.000	0.000
*2B	0.010	0.000	0.017	0.000	0.000
*3	0.010	0.022	0.000	0.050	0.083
*3B	0.000	0.000	0.000	0.050	0.000
*3A	0.000	0.005	0.000	0.000	0.000
*38	0.025	0.000	0.000	0.000	0.000
*37	0.040	0.011	0.000	0.000	0.000
*37B	0.005	0.000	0.000	0.000	0.000
*41	0.000	0.005	0.000	0.000	0.000
*37A	0.005	0.000	0.000	0.000	0.000
*45	0.000	0.022	0.000	0.000	0.000



*4	0.101	0.022	0.103	0.000	0.000
*4B	0.010	0.000	0.000	0.000	0.000
*4A	0.010	0.000	0.000	0.000	0.000
*6	0.010	0.000	0.000	0.000	0.000
*5	0.020	0.120	0.000	0.150	0.000
*5C	0.000	0.005	0.000	0.000	0.000
*5A	0.000	0.016	0.000	0.000	0.000
*5B	0.000	0.011	0.000	0.000	0.000
*35	0.000	0.022	0.000	0.000	0.000
*20A	0.000	0.005	0.000	0.000	0.000
*20	0.000	0.005	0.000	0.000	0.000
*19	0.000	0.005	0.000	0.000	0.000
*15A	0.000	0.005	0.000	0.000	0.000
*15	0.000	0.065	0.000	0.000	0.000
*14	0.000	0.016	0.000	0.000	0.000
*16	0.000	0.005	0.000	0.050	0.000
*16A	0.000	0.011	0.000	0.000	0.000
*18	0.000	0.011	0.000	0.000	0.000
*17	0.000	0.005	0.000	0.000	0.000
*30	0.000	0.011	0.000	0.000	0.000
*31	0.000	0.005	0.000	0.000	0.000
*22	0.005	0.000	0.000	0.000	0.000
*21	0.010	0.000	0.000	0.000	0.000
*13	0.000	0.011	0.000	0.000	0.000
*23	0.030	0.011	0.000	0.000	0.000
*23A	0.015	0.000	0.000	0.000	0.000
*23B	0.005	0.000	0.000	0.000	0.000
*25	0.000	0.000	0.000	0.000	0.083
*24	0.005	0.000	0.000	0.000	0.000
*39	0.000	0.016	0.000	0.000	0.000
*43	0.005	0.000	0.000	0.000	0.000

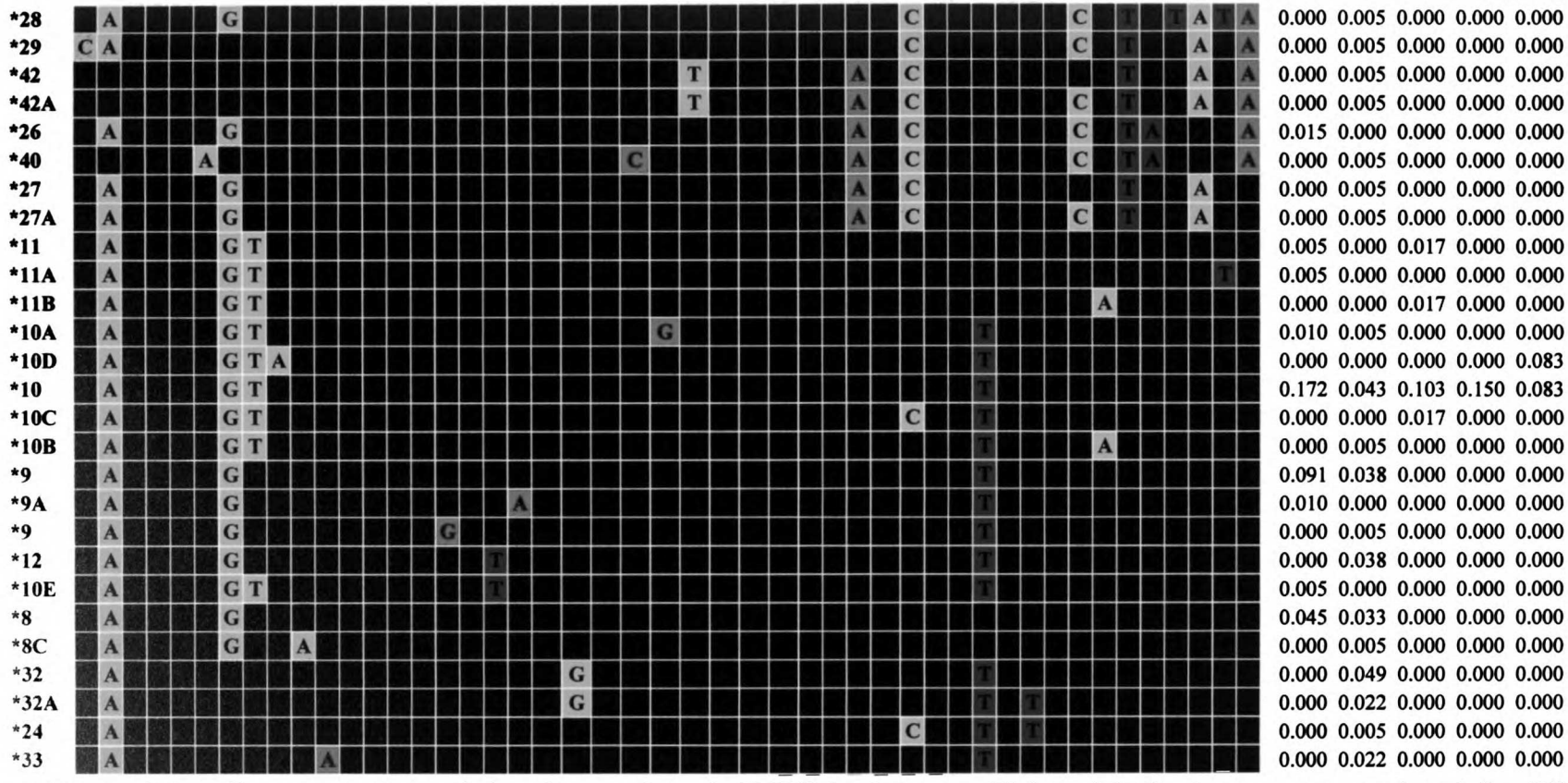


Figure 2.5 Alignment and population frequencies of *ABCC2* haplotypes. Eighty-eight haplotypes were assigned for 472 chromosomes using PHASE. Black boxes represent reference nucleotide, red boxes are non-synonymous, green boxes are synonymous, and yellow boxes are non-coding (intronic and promoter) variants. Haplotypes and population frequencies were called for 99 Caucasians (CA), 92 African Americans (AA), 59 Asian Americans (AS), 10 Mexican Americans (ME) and 6 Pacific Islanders (PA). Exon positions are listed at the top. Haplotype numbers were assigned based on haplotype frequency, starting with *ABCC2*1* (see Figure 2.7).

haplotypes (Figure 2.6) represented 30 segregating sites, including 8 non-synonymous sites, 9 synonymous sites, and 16 non-coding sites. The haplotype cladogram for the 32 common haplotypes of *ABCC2* is shown in Figure 2.7. Haplotype numbers were assigned based on haplotype clustering and frequency starting with the reference sequence, *ABCC2*1*. African Americans carried more haplotypes than Caucasians (53 vs. 39) and there were 13 haplotypes that were found in both populations (Figure 2.8). Figure 2.9 shows nine high frequency haplotypes that were either cosmopolitan or ethnic specific. Three haplotypes are present at high frequencies across all ethnic groups (*1, *11 and *2). There were two major haplotypes in the study population, the reference sequence *1 and haplotype *11, which contained a synonymous 3972C>T variant, a -24C>T 5'-UTR variant, and the -1549G>A and -1019A>G promoter variants. The frequency of *ABCC2*1* was 14.1% in Caucasians, 7.6% in African Americans, 32.8% in Asian Americans, 30% in Mexican Americans and 17.2% in Pacific Islanders. *ABCC2*10* was found at a frequency of 17.2% in Caucasians, 4.3% in African Americans, 10.3% in Asian Americans, 15% in Mexican Americans and 8.3% in Pacific Islanders. The two major haplotypes in African Americans were *ABCC2*5* followed by *ABCC2*1*. *ABCC2*1* and *ABCC2*10* have 2 and 4 times higher frequency, respectively, in Caucasians than in African Americans. *ABCC2*15* and *ABCC2*46* are African American and Asian American specific haplotypes. Forty-one haplotypes encode for the reference protein, but differ in intronic and promoter SNPs. There was also significant population substructure in the *ABCC2* haplotypes, given by Wright's F-statistic (F_{ST} = 0.039).

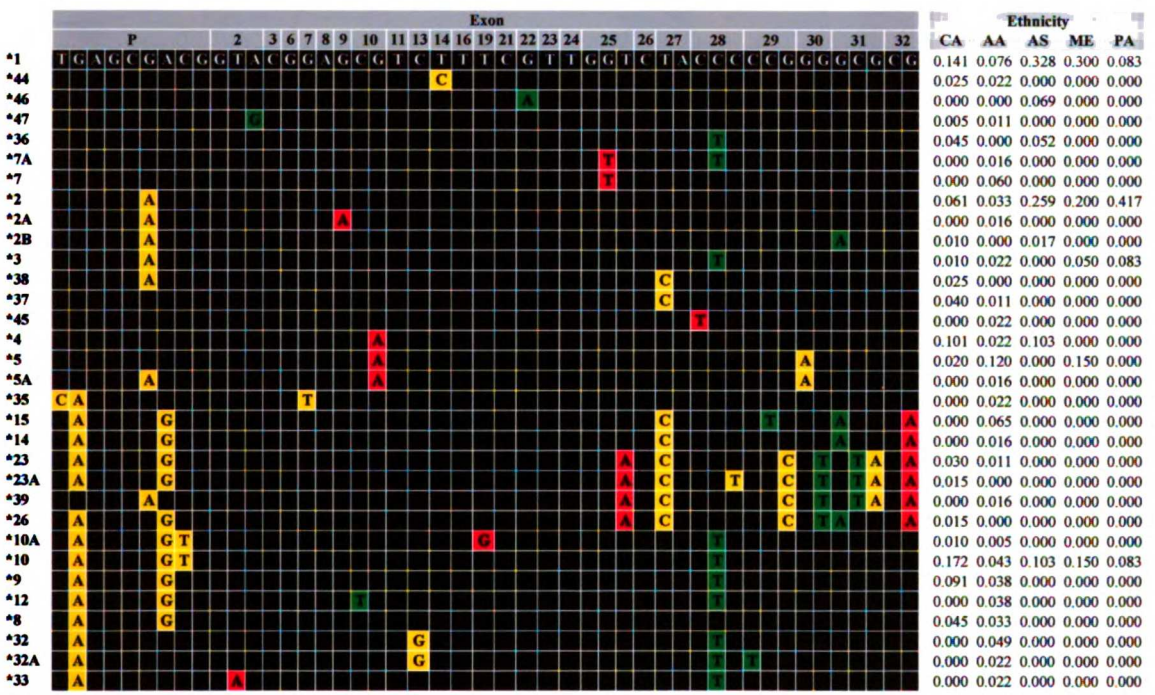


Figure 2.6 Alignment of the 32 *ABCC2* common haplotypes that were found in 3 chromosomes or more. Black boxes represent the reference nucleotide, red boxes are non-synonymous, green boxes are synonymous, and yellow boxes are non-coding (intronic and promoter) variants. CA, Caucasians; AA, African Americans; AS, Asian Americans; ME, Mexican Americans; PA, Pacific Islanders. Exon positions are listed at the top. Haplotype numbers were assigned based on haplotype frequencies.

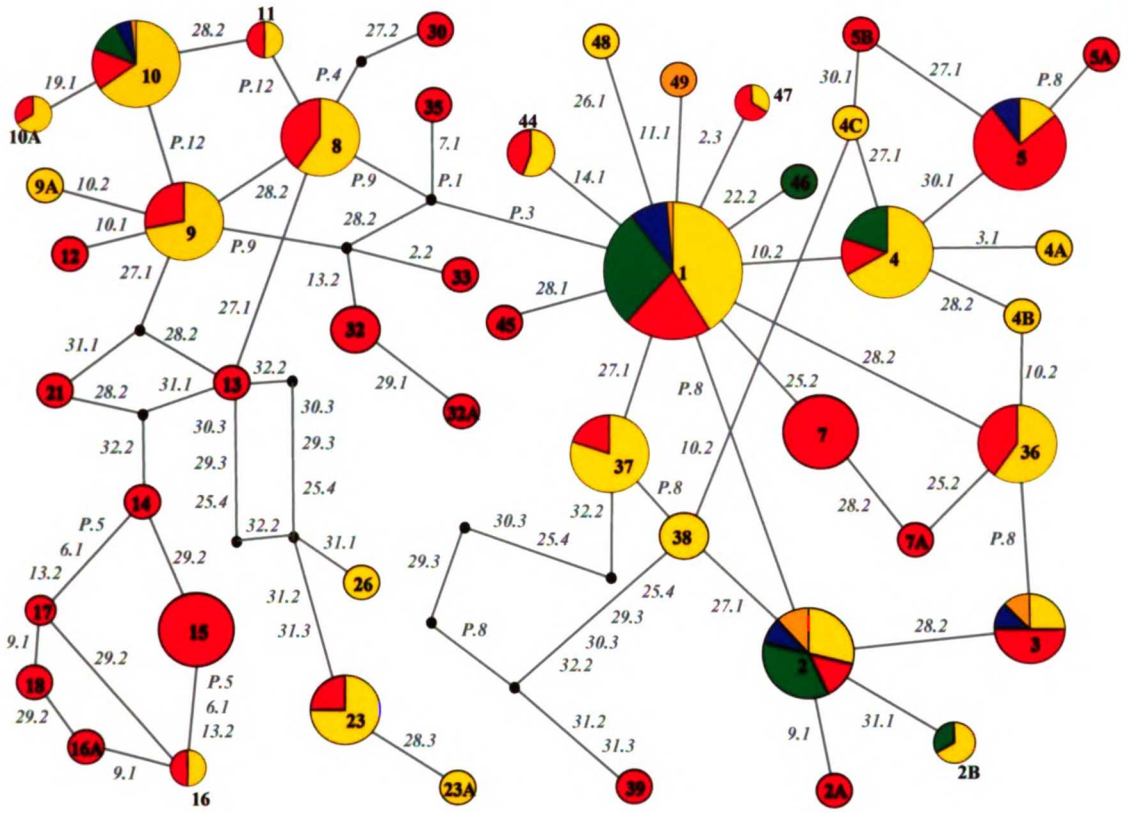
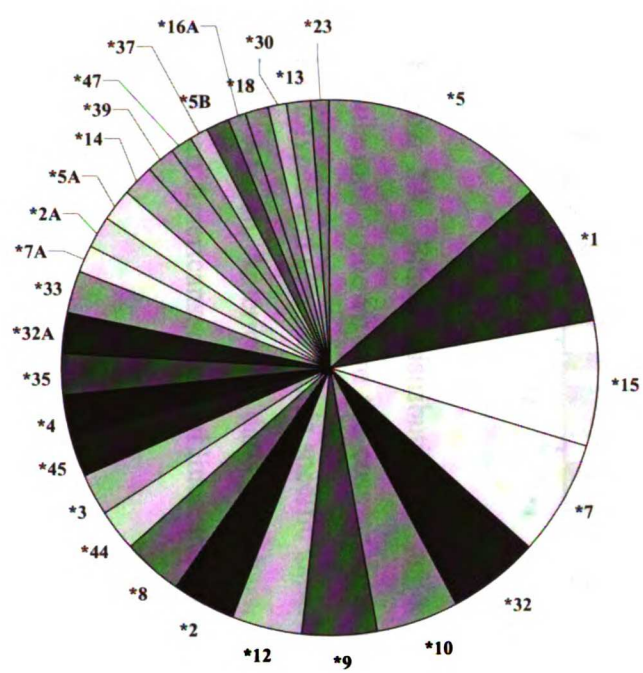
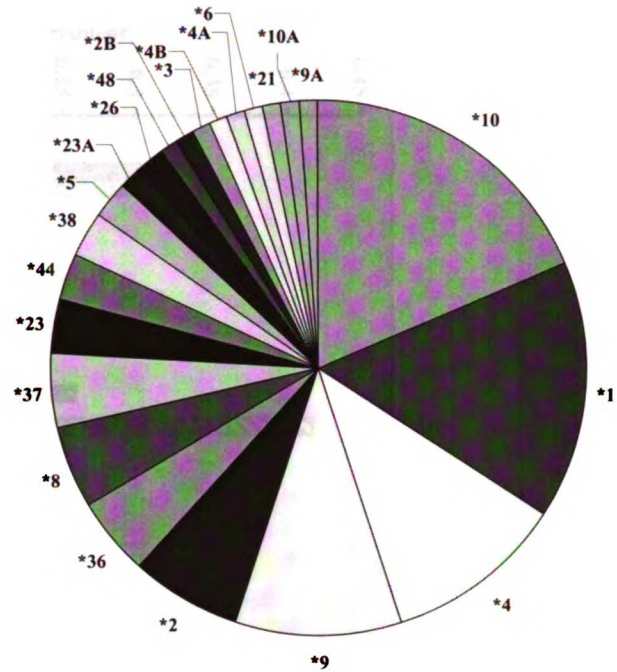


Figure 2.7 Cladogram depicting the evolution of *ABCC2* haplotypes. A haplotype network was constructed for 46 haplotypes found in 2 chromosomes or more using the Reduced Median algorithm in the Network4.0 program. These represent 91% of the chromosomes in the sample population. Ethnic makeup of each haplotype is represented by red, African Americans; green, Asian American; yellow, Caucasians; blue, Mexican Americans; orange, Pacific Islanders. Bolded labels represent haplotype number, and italicized labels represent the polymorphic change listed in Table 2.1. The size of the circles represents relative population frequencies.



African Americans



Caucasian Americans

Figure 2.8 *ABCC2* haplotype distribution in Caucasians and African Americans. Haplotypes found in two or more chromosomes are depicted. The African American population has 30 haplotypes while Caucasian Americans have 23 haplotypes. Fifty percent of the African American population is represented by seven haplotypes, while in Caucasians more than 50% is represented by four haplotypes.

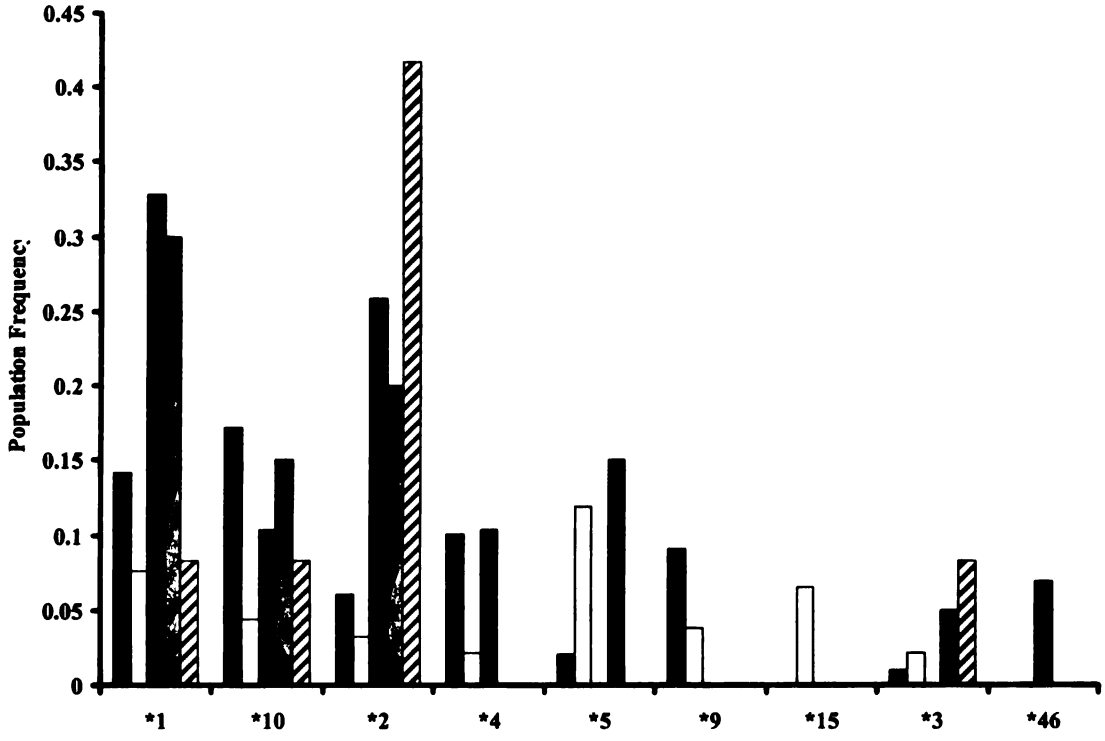


Figure 2.9 Ethnic distribution of major haplotypes among the five ethnic populations. Shown are the cosmopolitan haplotypes, several high frequency haplotypes and several ethnic specific haplotypes. Black bars, Caucasians; white bars, African Americans; dark grey bars, Asian Americans; light grey bars, Mexican Americans; diagonal lines, Pacific Islanders.

2.3.3 Recombination and Linkage Disequilibrium

Recombination was tested using the Hudson and Kaplan method of the four-gamete test (40). Any site pairs having four gametes must have had a recombination event occurring (assuming that repeated mutation does not occur). The four-gamete test was performed on the entire study population and separately for the two largest subpopulations. Four gametes were detected primarily at the 3'-end of *ABCC2* (Figure 2.10). In all there were 62 site pairs with four gametes in 351 pairwise comparisons in Caucasians, and 87 site pairs in 702 pairwise comparisons in African Americans. African Americans had a minimum of 7 recombination events occurring between the following SNP pairs: 6.1 and 9.1, 9.1 and 13.2, 16.1 and 25.4, 27.1 and 28.2, 28.2 and 29.2, 30.3 and 31.1, and 31.3 and 32.2. Caucasians also had a minimum of 7 recombination events, with recombination detected between sites P.9 and 10.2, 10.2 and 25.4, 25.4 and 27.1, 27.1 and 28.2, 28.2 and 29.3, 30.3 and 31.1, and 31.3 and 32.2. Linkage disequilibrium (Figure 2.11) was also determined for the African American and Caucasian populations. There was significant LD (Fisher's exact test) across the entire gene, and the two populations showed similar LD around the 3'-region.

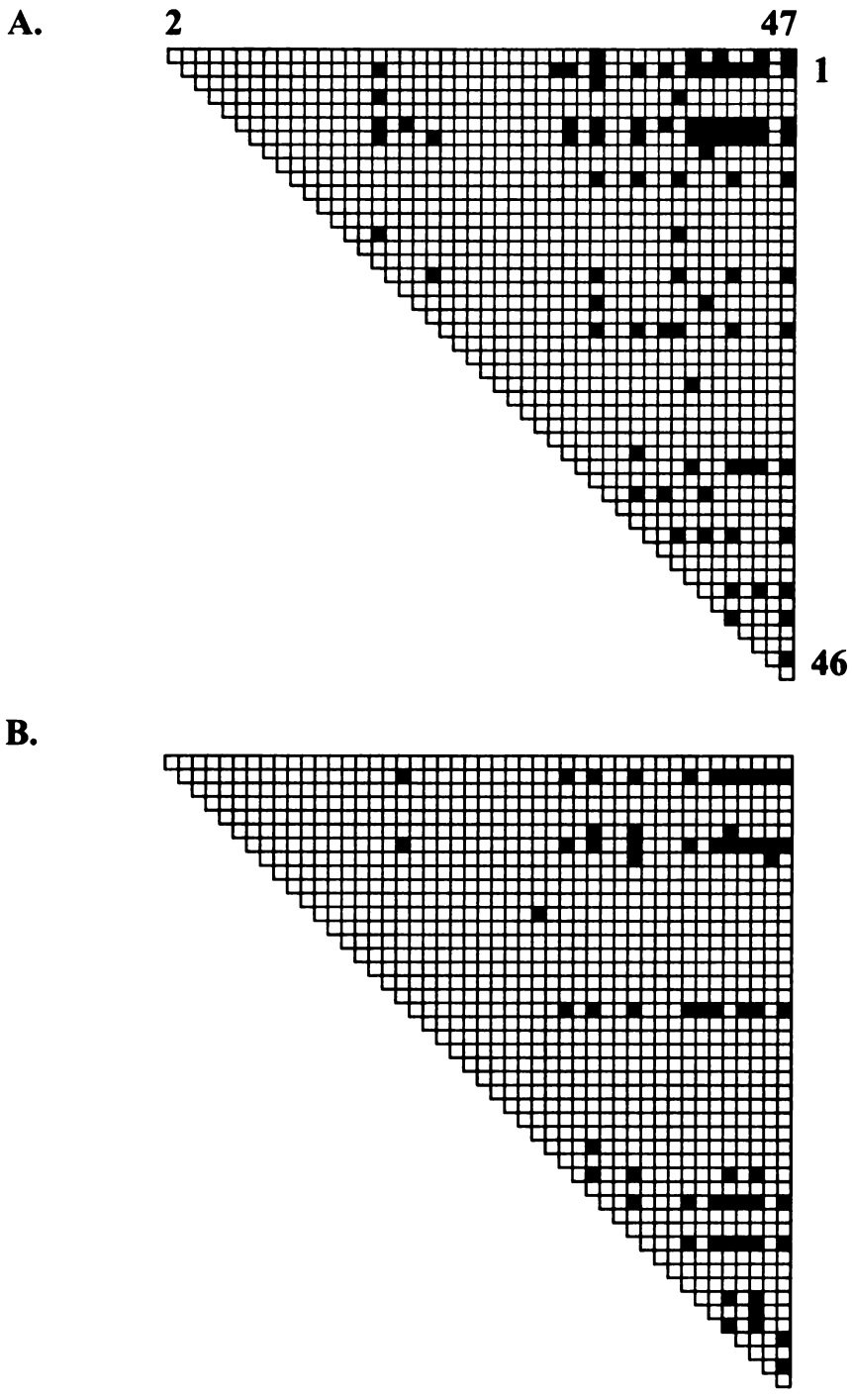


Figure 2.10 Triangle plot depicting the four-gamete test for intragenic recombination for A) African Americans and B) Caucasians. Site pairs with four gametes are shown in black. Segregating variant sites are labeled on the horizontal and vertical sides.

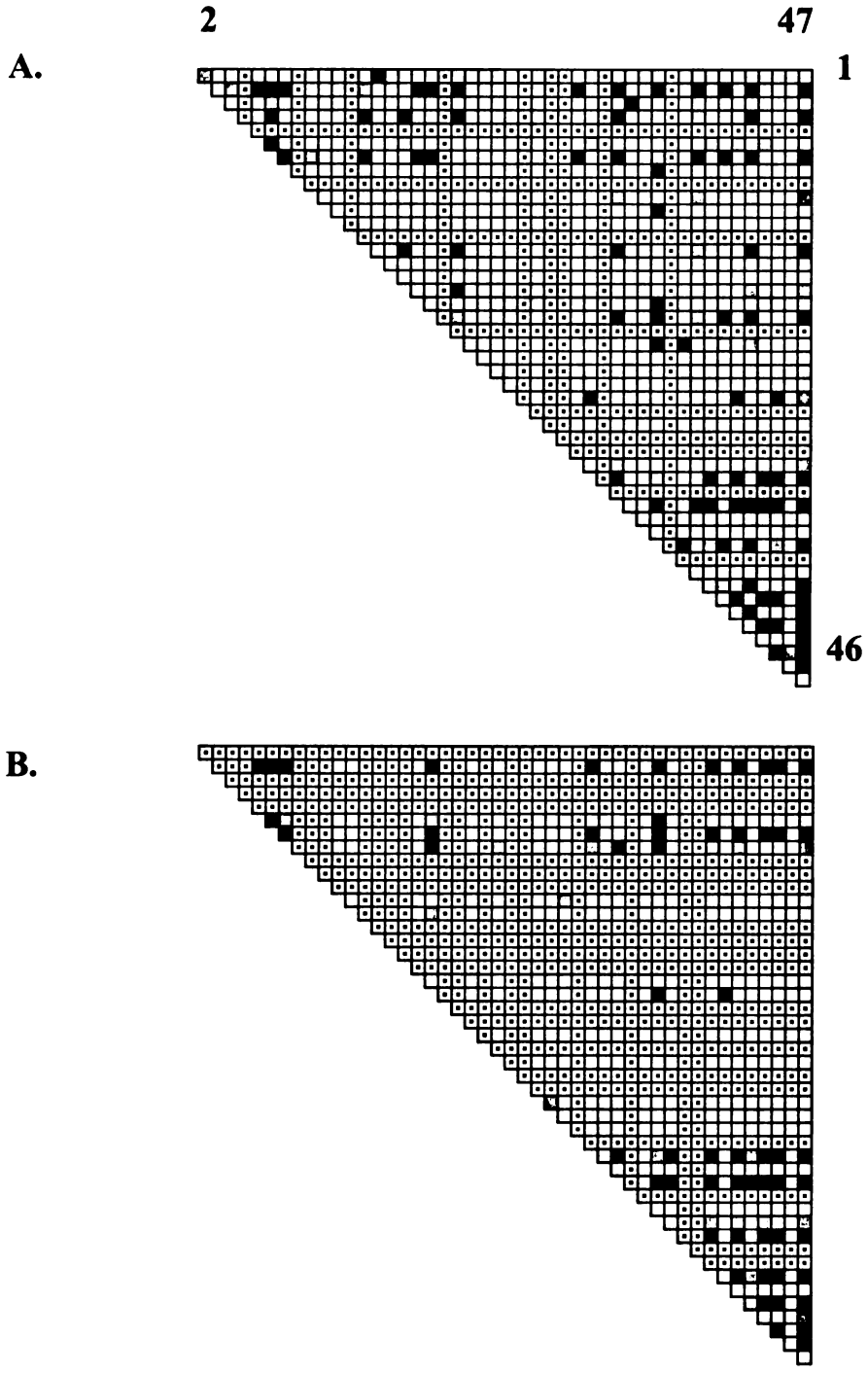


Figure 2.11 Triangle plot depicting pairwise linkage disequilibrium for A) African Americans and B) Caucasians. Statistical significance of LD was assessed by Fisher's exact test. Light grey squares, $0.05 > p > 0.01$; grey squares, $0.01 > p > 0.001$; and black squares, $p < 0.001$. Dots represent uninformative sites. Segregating variant sites are labeled on the horizontal and vertical sides.

2.3.4 Population Genetics

Two estimates of nucleotide diversity were calculated for our study population. The first was the neutral parameter (θ) that summarizes the rate at which mutation and genetic drift generate and maintain variations within a gene (41). The second estimate is the average heterozygosity, or nucleotide diversity (π). This measures the probability that an individual will be heterozygous at any given nucleotide site. A comparison of these two measures is done with Tajima's D test statistic, which measures deviation from a neutral mutation model (26). These data are shown in Table 2.2 for our study population. Mean values for θ ($10.20 \times 10^{-4} \pm 2.30 \times 10^{-4}$) and π ($4.28 \times 10^{-4} \pm 2.36 \times 10^{-4}$) were similar to values previously reported for other genes (25, 42-46). The ratio of θ for synonymous and non-synonymous sites varied from 1.26-8.97 across the ethnic groups. The synonymous to non-synonymous ratio for π was 4.04, suggesting that the non-synonymous sites are under selective pressure. An examination of the ratio of conserved and unconserved non-synonymous variations for π shows a range from 0.063 – 2.5, with the highest ratio found in the transmembrane domain. There was greater heterozygosity at conserved synonymous sites than conserved non-synonymous sites (12.58×10^{-4} vs. 0.60×10^{-4}) suggesting selective pressure against amino acid changes at conserved sites. Synonymous variations largely affected codons for an evolutionarily conserved amino acid (10/15). Interestingly, a majority of the non-synonymous variants identified were in evolutionarily conserved amino acids (10/19). However, nine had a frequency of $\leq 3\%$ with six being singletons. Nucleotide diversity was similar across all ethnic groups. However, the neutral parameter was 2-3 times higher in African Americans than the other

Table 2.2 Estimation of Population Neutral Parameter (θ), Nucleotide Diversity (π), and Tajima's D in the Study Population

SNP Type	Base Pairs Screened	N	S	θ (x 10^{-4})	π (x 10^{-4})	D
Total	9693	494	67	10.2 ± 2.30	4.28 ± 2.36	-3.07
Non-Coding	5055	494	33	9.63 ± 2.48	4.80 ± 2.88	-1.80
Coding	4638	494	34	10.8 ± 2.76	3.71 ± 2.40	-2.40
Intron-Exon Boundary	363	494	1	4.06 ± 4.14	0.11 ± 1.02	-0.35
Synonymous	1064	494	15	20.8 ± 6.65	8.81 ± 6.71	-1.31
Non-synonymous	3574	494	19	7.84 ± 2.33	2.18 ± 1.76	-1.89
Conserved (total)	2916	494	20	10.1 ± 2.96	3.41 ± 2.55	-1.79
Conserved (synonymous)	682	494	10	21.6 ± 7.97	12.6 ± 9.85	-0.74
Conserved (non-synonymous)	2234	494	10	6.60 ± 2.43	0.60 ± 0.99	-1.61
Unconserved (total)	1722	494	14	12.0 ± 3.93	4.22 ± 3.49	-1.41
Unconserved (synonymous)	382	494	5	19.3 ± 9.38	2.08 ± 4.39	-1.00
Unconserved (non-synonymous)	1340	494	9	9.91 ± 3.80	4.82 ± 4.15	-0.85
TMD (total)	1071	494	8	11.0 ± 4.42	0.64 ± 1.44	-1.44
TMD (synonymous)	259	494	3	17.1 ± 10.37	1.09 ± 3.78	-0.74
TMD (non-synonymous)	812	494	5	9.09 ± 4.41	0.50 ± 1.45	-1.06
TMD (non-synonymous, conserved)	469	494	4	12.6 ± 6.73	0.69 ± 2.24	-0.91
TMD (non-synonymous, unconserved)	343	494	1	4.30 ± 4.38	0.23 ± 1.52	-0.34
Loop (total)	3567	494	26	10.8 ± 2.93	4.63 ± 3.02	-1.79
Loop (synonymous)	805	494	12	22.0 ± 7.59	11.3 ± 8.69	-0.96
Loop (non-synonymous)	2762	494	14	7.48 ± 2.45	2.68 ± 2.20	-1.40

Loop (non-synonymous, conserved)	1765	494	6	5.01 ± 2.26	0.58 ± 1.08	-1.12
Loop (non-synonymous, unconserved)	997	494	8	11.8 ± 4.75	6.40 ± 5.53	-0.70
Extracellular (total)	432	494	1	3.41 ± 3.48	0.65 ± 2.26	-0.29
Extracellular (synonymous)	99	494	0	0.00 ± 0.00	0.00 ± 0.00	n/a
Extracellular (non-synonymous)	333	494	1	4.43 ± 4.51	0.84 ± 2.93	-0.29
Extracellular (non-synonymous, conserved)	197	494	0	0.00 ± 0.00	0.00 ± 0.00	n/a
Extracellular (non-synonymous, unconserved)	136	494	1	10.9 ± 11.07	2.06 ± 7.20	-0.29
Cytoplasm (total)	3135	494	25	11.8 ± 3.24	5.18 ± 3.39	-1.72
Cytoplasm (synonymous)	706	494	12	25.1 ± 8.65	12.9 ± 9.91	-0.96
Cytoplasm (non-synonymous)	2429	494	13	7.90 ± 2.65	2.93 ± 2.44	-1.31
Cytoplasm (non-synonymous, conserved)	1568	494	6	5.65 ± 2.54	0.65 ± 1.22	-1.12
Cytoplasm (non-synonymous, unconserved)	861	494	7	12.0 ± 5.07	7.08 ± 6.22	-0.58
Tail loop (total)	954	494	11	17.0 ± 6.05	11.3 ± 8.26	-0.63
Tail loop (synonymous)	219	494	8	54.0 ± 21.64	39.1 ± 30.63	-0.42
Tail loop (non-synonymous)	735	494	3	6.02 ± 3.66	3.05 ± 4.00	-0.39
Tail loop (non-synonymous, conserved)	535	494	1	2.76 ± 2.81	0.37 ± 1.52	-0.31
Tail loop (non-synonymous, unconserved)	201	494	2	14.7 ± 10.76	10.2 ± 13.91	-0.18
Internal loop (total)	2613	494	15	8.47 ± 2.71	2.19 ± 1.97	-1.68
Internal loop (synonymous)	586	494	4	10.1 ± 5.38	0.95 ± 2.37	-0.88
Internal loop (non-synonymous)	2027	494	11	8.01 ± 2.85	2.55 ± 2.38	-1.28
Internal loop (non-synonymous, conserved)	1230	494	5	6.00 ± 2.91	0.67 ± 1.39	-1.00
Internal loop (non-synonymous, unconserved)	796	494	6	11.1 ± 5.00	5.44 ± 5.43	-0.65
Globular loop (total)	2910	494	23	11.7 ± 3.28	5.51 ± 3.62	-1.54
Globular loop (synonymous)	653	494	11	24.9 ± 8.84	13.7 ± 10.58	-0.84
Globular loop (non-synonymous)	2257	494	12	7.84 ± 2.71	3.14 ± 2.62	-1.19

Globular loop (non-synonymous, conserved)	1462	494	5	5.05 ± 2.45	0.67 ± 1.28	-0.98
Globular loop (non-synonymous, unconserved)	795	494	7	13.0 ± 5.49	7.68 ± 6.74	-0.58
Short loop (total)	657	494	3	6.74 ± 4.09	0.73 ± 1.96	-0.71
Short loop (synonymous)	152	494	1	9.71 ± 9.88	1.05 ± 4.84	-0.32
Short loop (non-synonymous)	505	494	2	5.84 ± 4.28	0.63 ± 2.07	-0.53
Short loop (non-synonymous, conserved)	303	494	1	4.87 ± 4.95	0.13 ± 1.21	-0.35
Short loop (non-synonymous, unconserved)	202	494	1	7.30 ± 7.43	1.39 ± 4.84	-0.29

N is the number of chromosomes screened

S is the number of variant sites observed

Table 2.3 Estimation of Population Neutral Parameter (θ), Nucleotide Diversity (π), and Tajima's D Across Ethnic Groups

Population	SNP Type	Base Pairs	N	S	θ (x 10^{-4})	π (x 10^{-4})	Tajima D
Caucasian	Total	9693	200	29	5.09 ± 1.46	3.78 ± 2.13	-0.96
	Coding	4638	200	15	5.51 ± 1.86	3.13 ± 2.11	-1.10
	Non-synonymous	3574	200	7	3.34 ± 1.46	1.73 ± 1.52	-0.77
	Synonymous	1064	200	8	12.8 ± 5.32	7.80 ± 6.19	-0.68
	Non-Coding	5055	200	14	4.72 ± 1.63	4.39 ± 2.69	-0.17
	Intron-Exon Boundary	363	200	0	0.00	0.00	n/a
African American	Total	9693	200	57	10.0 ± 2.55	5.27 ± 2.84	-2.56
	Coding	4638	200	30	11.0 ± 3.13	5.01 ± 3.05	-2.07
	Non-synonymous	3574	200	17	8.10 ± 2.64	3.20 ± 2.31	-1.66
	Synonymous	1064	200	13	20.8 ± 7.34	11.1 ± 7.92	-1.09
	Non-Coding	5055	200	27	9.09 ± 2.64	5.50 ± 3.23	-1.42
	Intron-Exon Boundary	363	200	1	4.69 ± 4.8	0.28 ± 1.6	-0.39
Asian American	Total	9693	60	15	3.32 ± 1.25	2.15 ± 1.35	-1.09
	Coding	4638	60	6	2.77 ± 1.36	1.57 ± 1.32	-0.77
	Non-synonymous	3574	60	2	1.20 ± 0.91	0.68 ± 0.87	-0.37
	Synonymous	1064	60	4	8.06 ± 4.59	4.56 ± 4.42	-0.59
	Non-Coding	5055	60	9	3.82 ± 1.65	2.69 ± 1.87	-0.68

	Intron-Exon Boundary	363	60	0	0.00	0.00	n/a
Mexican American	Total	9693	20	15	4.36 ± 1.92	3.29 ± 1.98	-0.98
	Coding	4638	20	5	3.04 ± 1.74	2.08 ± 1.65	-0.66
	Non-synonymous	3574	20	2	1.58 ± 1.25	1.03 ± 1.15	-0.40
	Synonymous	1064	20	3	7.94 ± 5.39	5.59 ± 5.2	-0.45
	Non-Coding	5055	20	10	5.58 ± 2.65	4.39 ± 2.82	-0.67
	Intron-Exon Boundary	363	20	0	0.00	0.00	n/a
Pacific Islander	Total	9693	14	14	4.54 ± 2.16	3.96 ± 2.38	-0.54
	Coding	4638	14	5	3.39 ± 2.02	2.30 ± 1.81	-0.75
	Non-synonymous	3574	14	2	1.76 ± 1.42	0.80 ± 1.01	-0.71
	Synonymous	1064	14	3	8.86 ± 6.2	7.33 ± 6.36	-0.29
	Non-Coding	5055	14	9	5.60 ± 2.89	5.48 ± 3.46	-0.07
	Intron-Exon Boundary	363	14	0	0.00	0.00	n/a

N is the number of chromosomes screened

S is the number of variant sites observed

ethnic groups (Table 2.3). Tajima's D, and Fu and Li' test statistic, D* (47), yielded values of -1.16 and 0.34, respectively, neither of which were statistically significant.

2.3.5 Expression and Function of MRP2 in Yeast

A yeast expression system was initially investigated for the analysis of MRP2 amino acid variants. CDNB is conjugated to glutathione to produce dinitrophenol glutathione (DNP-SG) in the cell, which can be effluxed by MRP2. Cells containing expressed MRP2 are thus resistant to the toxic effects of DNP-SG. Plates containing either empty pYES2 vector or pYES2-MRP2 in the NY605 yeast strain showed no difference in growth when exposed to CDNB (Figure 2.12). The same was true when grown in the Ydr135c(-/-) strain (Figure 2.13). The Ydr135c(-/-) strain, which lacks the endogenous DNP-SG transporter YCF1, is theoretically more sensitive to CDNB, and all growth should be inhibited. The "halos" (areas of no growth) surrounding the discs were identical between plates transformed with the plasmid pYES2 and plates transformed with the inserted MRP2 cDNA, even with varying CDNB concentrations. Similar results were obtained when transformed yeast cells were grown in liquid cultures in the presence of the xenobiotic. For both NY605 (Figure 2.14) and Ydr135c(-/-) (Figure 2.15) yeast, there was no difference between the empty vector and MRP2 transformed cells. There was inhibition of growth for both strains in the presence of CDNB. However, expression of MRP2 did not reverse the growth inhibition. Switching to the single copy (centromeric vector pRS416) or the multicopy vector (2 micron vector pRS426) showed variable results. In W303 cells there appeared to be little or no difference between control and MRP2 expressing cells when grown in the presence of the xenobiotic CDNB

(Figure 2.16). The use of the single copy and multi-copy plasmids showed no difference between empty vector and *MRP2* transformed cells (Figures 2.17 & 2.18). pRS plasmids transformed into the YCF1 knockout strain showed that the yeast transformed with MRP2 cDNA had slightly lower survival than the control plasmid (Figure 2.19).

Western blots of yeast transformed with the MRP2 plasmid detected immunoreactive proteins with the MRP2 antibody M_2-III-6 (Figure 2.20), suggesting that MRP2 was expressed in the yeast. MRP2 protein in yeast, however, appeared to be smaller in molecular weight compared to liver and kidney microsomes and this may be due to a difference in glycosylation.

2.3.6 Transient Expression of MRP2 in Mammalian Cells

Several assays were developed to measure the function of MRP2 in mammalian cells. For both accumulation and flux assays, CMFDA was used as an MRP2 substrate. CMFDA passively diffuses into cells where esterases cleave the diacetate functional group, producing a fluorescent dye. Once cleaved, the dye becomes hydrophilic and cannot pass through the membrane, allowing it to accumulate within the cell. Glutathione S-transferases, in a nucleophilic reaction, can then attach intracellular glutathione making the dye susceptible to MRP2 transport. Cells expressing MRP2 are expected to have reduced intracellular fluorescence. FACS analysis showed that untransfected MDCKII cells had higher fluorescence than those stably transfected with MRP2 cDNA (Figure 2.21).

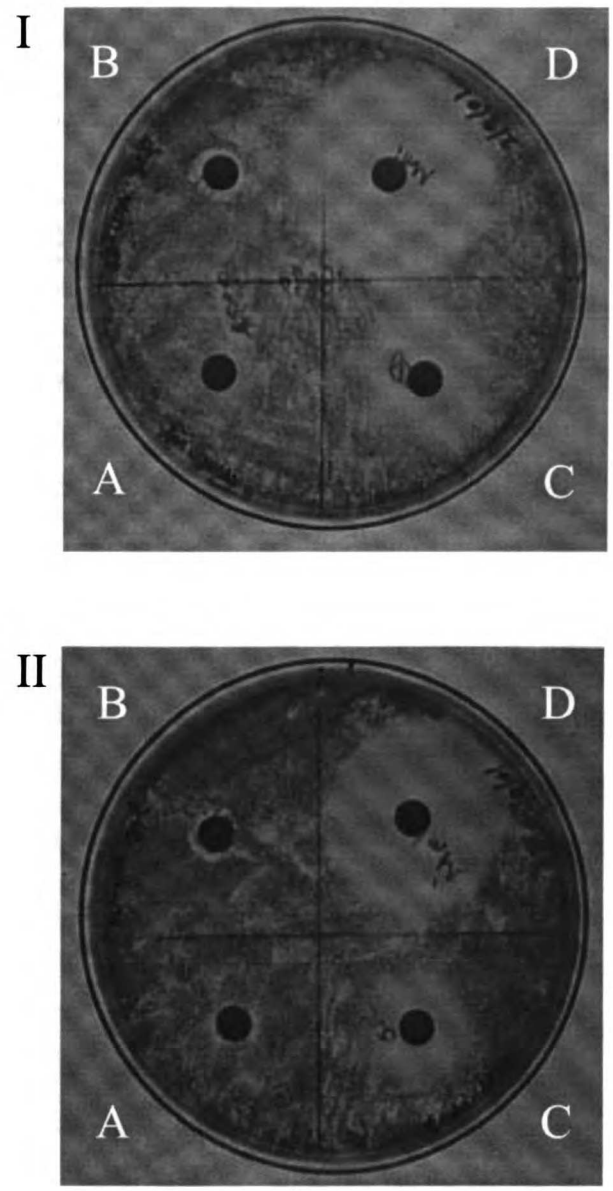


Figure 2.12 Plates showing growth inhibition of NY605 yeast by CDNB treatment. The plates are divided into quadrants containing A) 0 μM, B) 250 μM, C) 500 μM, and D) 1 mM of CDNB. Panel I contains the NY605 yeast strain transformed with the empty vector pYES2. Panel II contains yeast transformed with pYES2-MRP2 plasmid. There is no discernable difference in growth between the two vectors.

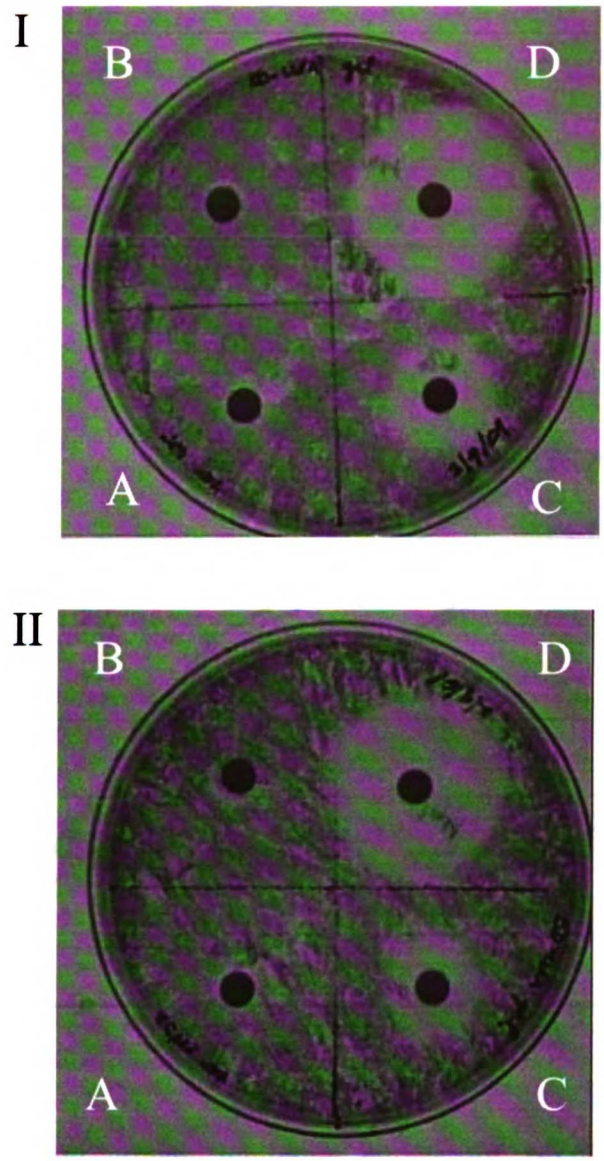


Figure 2.13 Plates showing growth inhibition of the Ydr135c(-/-) strain by CDNB treatment. The Ydr135c(-/-) strain lacks the endogenous pump YCF1 for DNP-SG. The plates are divided into quadrants containing A) 0 μM, B) 250 μM, C) 500 μM, and D) 1mM of CDNB. Panel I contains the Ydr135c(-/-) yeast strain transformed with the empty vector pYES2. Panel II contains yeast transformed with pYES2-MRP2 plasmid. There is no discernable difference in growth between the two plates.

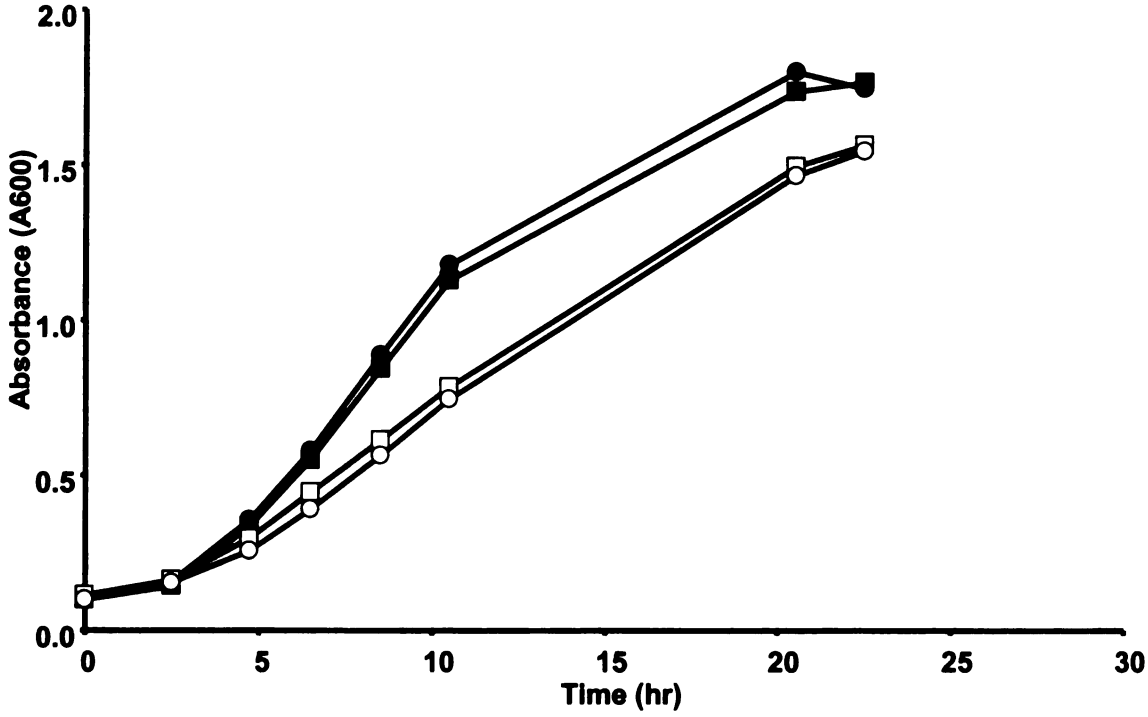


Figure 2.14 Liquid growth inhibition curves for NY605 yeast. Yeast were transformed with the empty pYES2 vector (circles) or pYES2-MRP2 (squares) and grown in the presence (white) or absence (black) of 50 μM CDNB. Absorbance measurements were taken over 24 hours to determine yeast viability. There was no difference between empty vector and MRP2 trasnformed cultures.

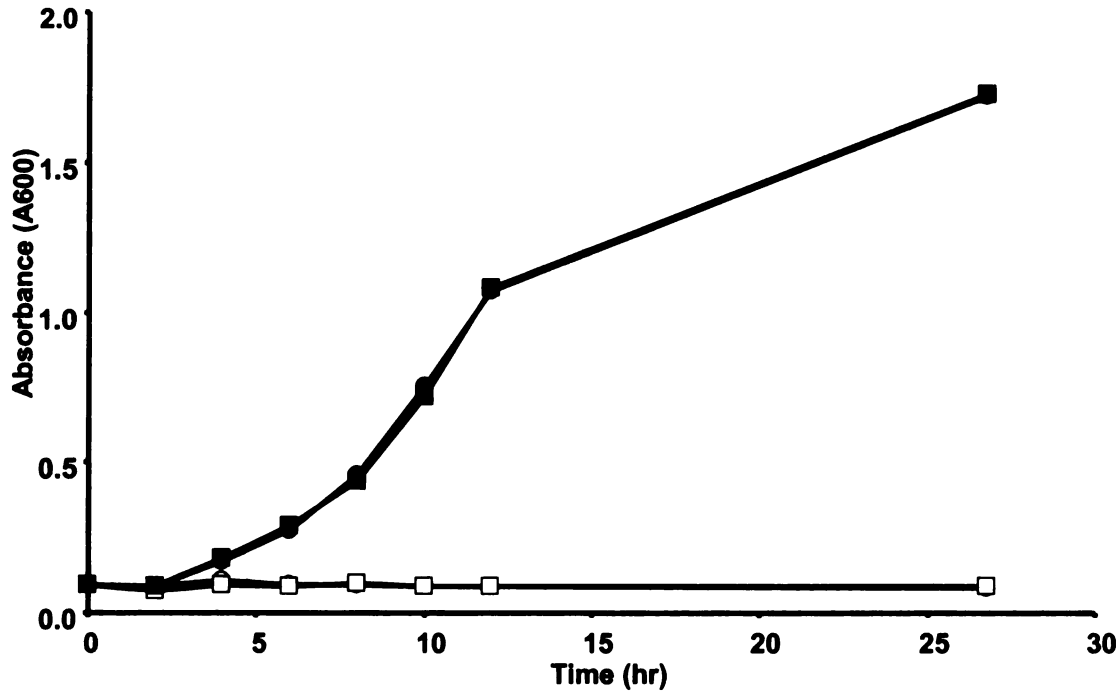


Figure 2.15 Liquid growth inhibition curves for the Ydr135c(-/-) strain. Yeast were transformed with the empty pYES2 vector (circles) or pYES2-MRP2 (squares) and grown in the presence (white) or absence (black) of 40 μM CDNB. Absorbance measurements were taken over 24 hours to determine yeast viability. There was no difference between empty vector and MRP2 transformed cultures.

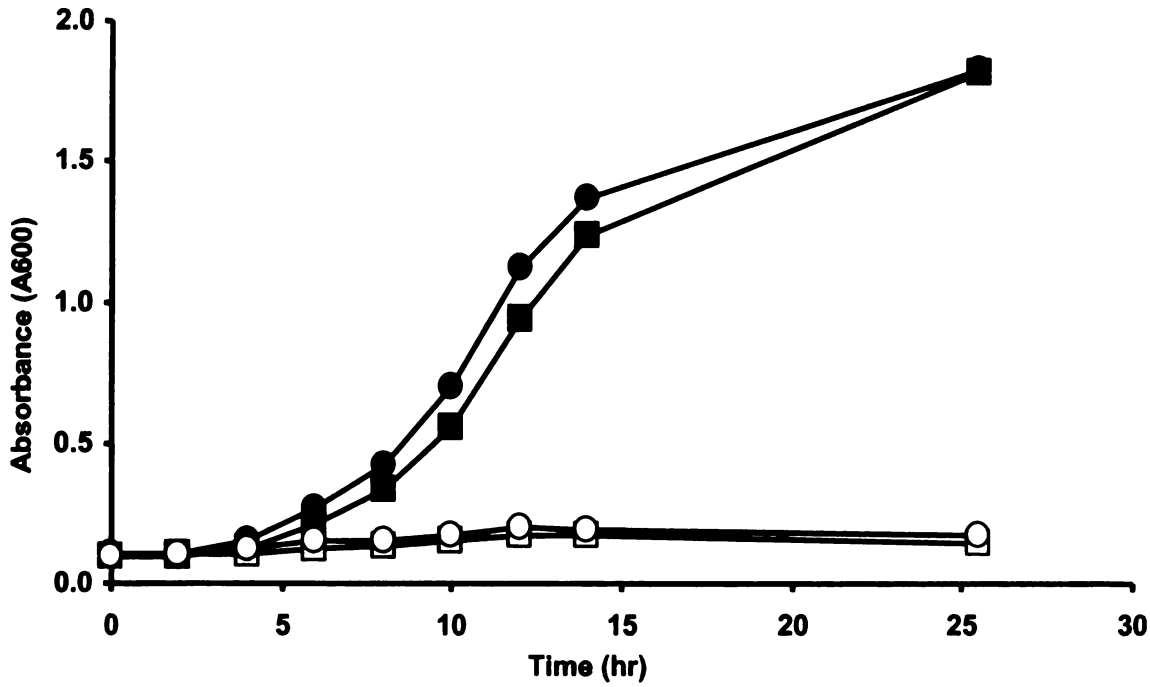


Figure 2.16 Liquid growth inhibition curves for the W303 yeast strain. Yeast transformed with the empty pYES2 vector (circles) or pYES2-MRP2 (squares) were grown in the presence (white) or absence (black) of 50 μM CDNB. Absorbance measurements were taken over 24 hours to determine yeast viability. There was no difference between control and MRP2 expressing yeast cells.

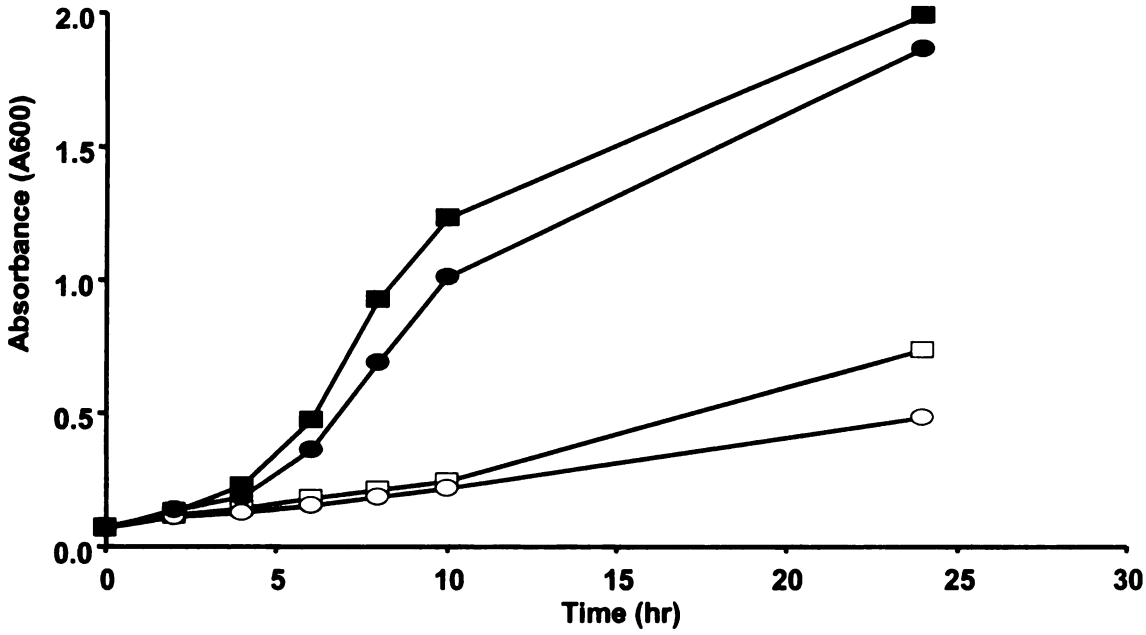


Figure 2.17 Liquid growth inhibition curves for W303 yeast strain transformed with the single copy plasmid pRS416. Yeast transformed with the empty single copy vector pRS416 (circles) or pRS416-MRP2 (squares) were grown in the presence (white) or absence (black) of 50 μM CDNB. Absorbance measurements were taken over 24 hours to determine yeast viability. Yeast cells transformed with the MRP2 plasmid show very little improvement in survival in the presence of CDNB.

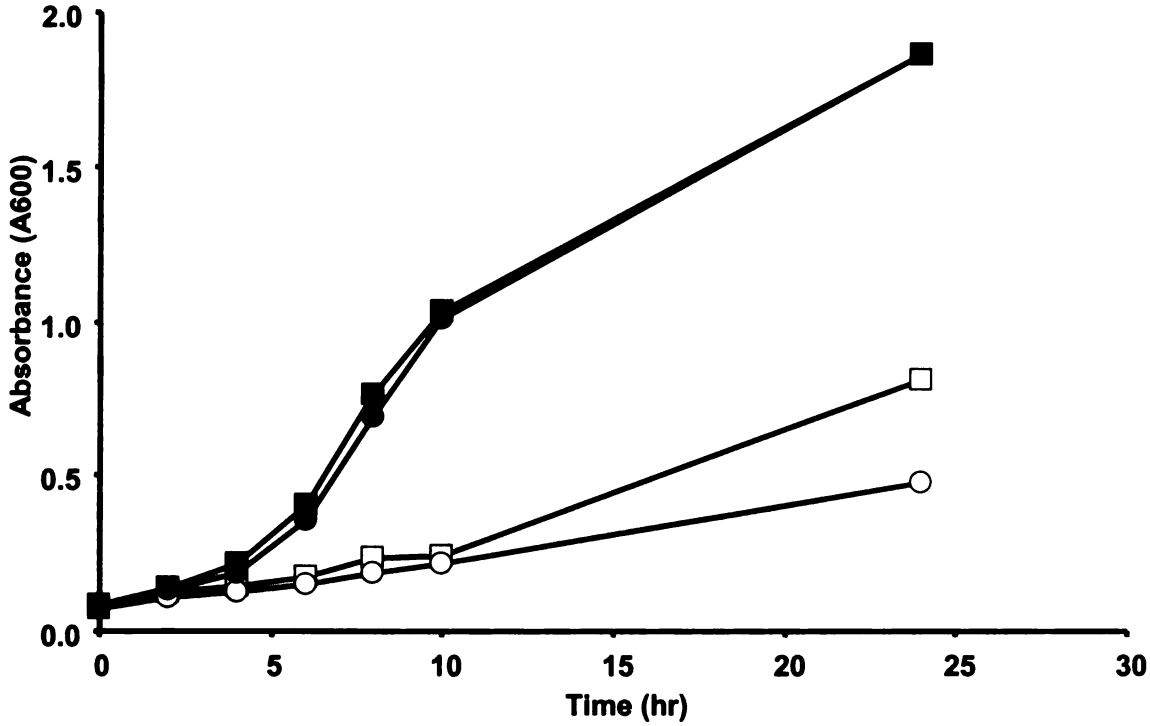


Figure 2.18 Liquid growth inhibition curves for W303 yeast strain transformed with the multicopy plasmid pRS426. Yeast transformed with the empty single copy vector pRS426 (circles) or pRS426-MRP2 (squares) are grown in the presence (white) or absence (black) of 50 μM CDNB. Absorbance measurements were taken over 24 hours to determine yeast viability. Yeast cells transformed with the MRP2 plasmid show very little improvement in survival in the presence of CDNB.

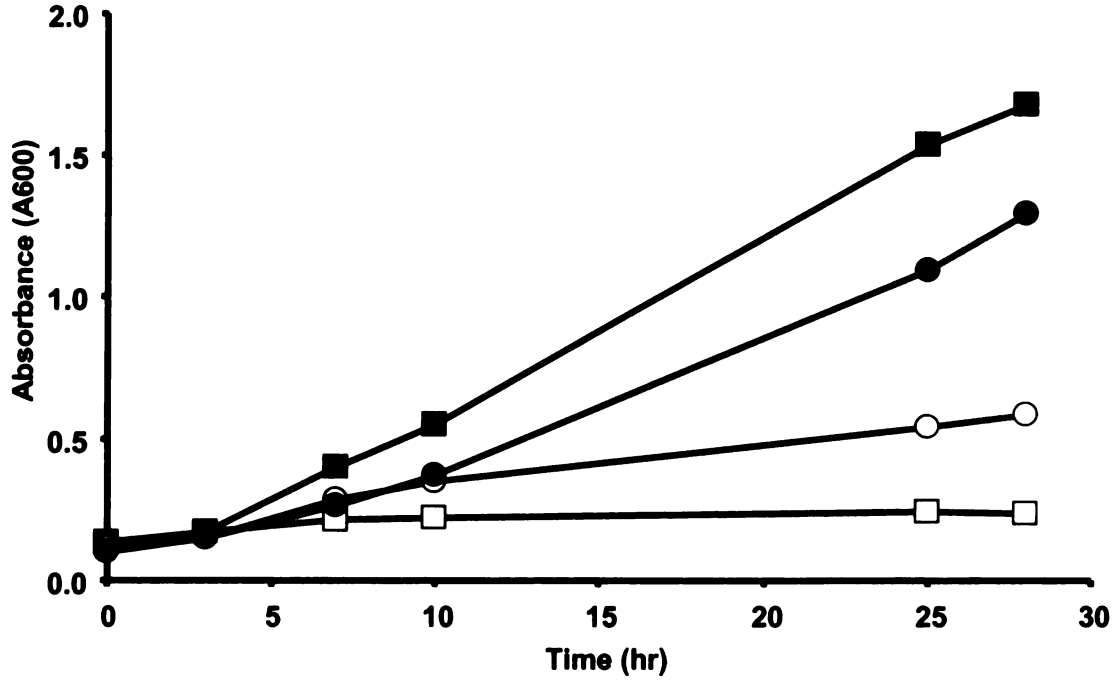


Figure 2.19 Liquid growth inhibition curves for yeast strain Ydr135c(-/-) using the pRS vectors. Yeast transformed with empty pRS416 vector (black squares), pRS416-MRP2 (white squares), empty pRS426 vector (black circles), or pRS426-MRP2 (white circles) were grown in the presence of 50 μM CDNB for up to 28 hours. Yeast transformed with MRP2 had slightly lower survival in the presence of CDNB.

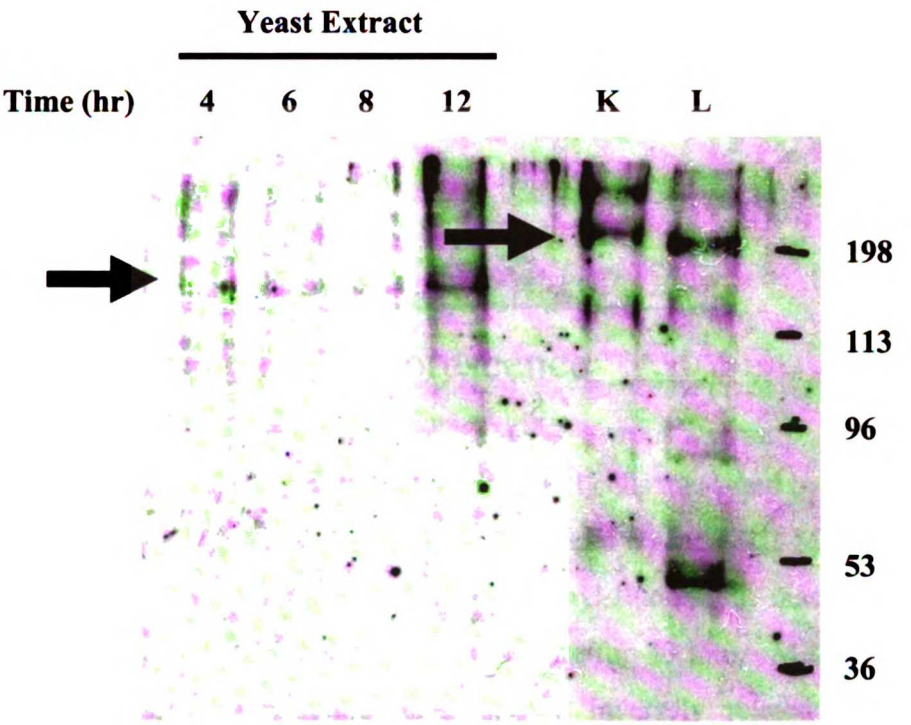


Figure 2.20 Western blot of proteins isolated from NY605 yeast transformed with pYES2-MRP2. Yeast were grown overnight in SD-ura media at 30°C. Ten mL of SD-ura media supplemented with 2% galactose for induction were inoculated with yeast and grown for 4, 6 , 8 or 12 hours. Protein bands were detected using the primary antibody M2-III-6 and compared to human kidney microsomes (K) and liver microsomes (L). Hash marks represent protein ladder in kDa.

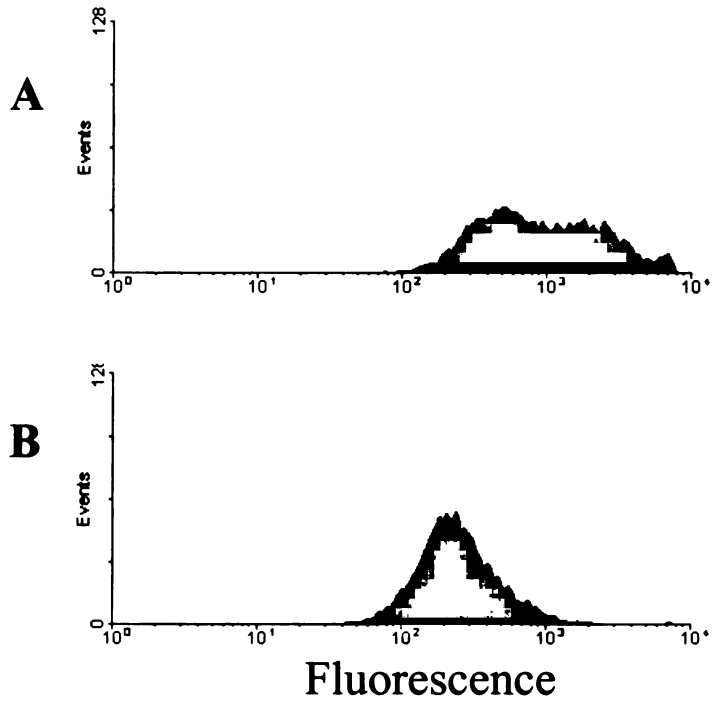


Figure 2.21 CMFDA uptake and efflux by MDCKII cells measured by flow cytometry. Median fluorescence was measured in A) MDCKII and B) MKCKII-MRP2 cells. Cells were incubated with non-fluorescent 5-chloromethyl-fluorescein diacetate (CMFDA) for 30 min at 37°C, washed, and allowed to efflux for 30 min. MRP2 efflux of the conjugated fluorescent glutathione metabolite is inversely related to intracellular fluorescence.

For flux assays MDCKII cells were grown on transwell inserts and allowed to polarize. Substrates were added to either the apical or basolateral donor compartment and transport was measured from the basolateral compartment (B) into the apical compartment (A) and vice-versa. When grown on transwell inserts, untransfected cells showed no difference in directional transport (A→B, B→A) (Figure 2.22A) while MRP2 transfected cells showed a preference for the B→A direction (Figure 2.22B). The

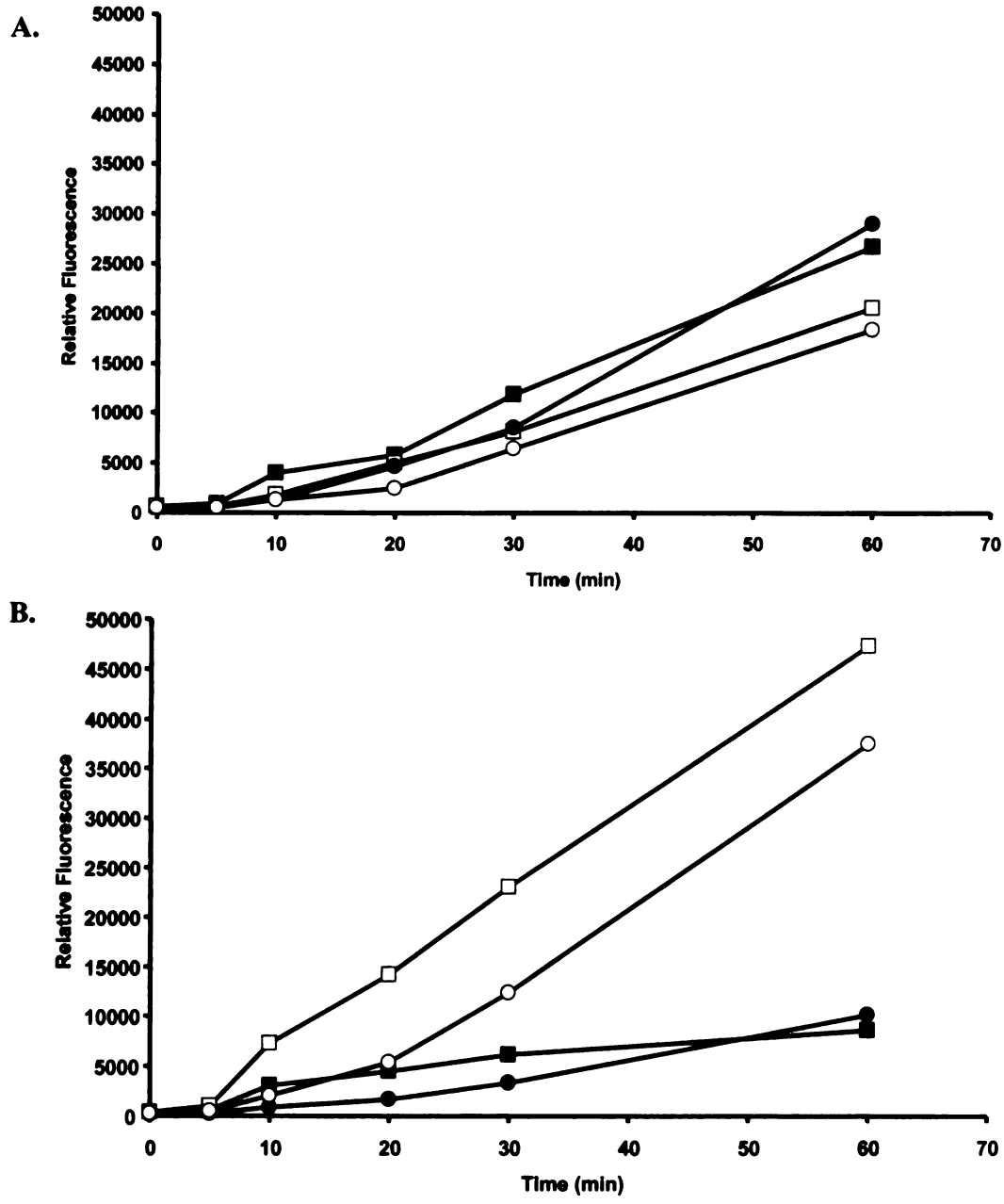


Figure 2.22 Bidirectional transport of 5 μM CMFDA across A) untransfected MDCKII and B) MDCKII-MRP2 cells. CMFDA was added to the donor compartment of a transwell system and the glutathione conjugate was measured on the receiver compartment. Transport was measured in the presence (circles) or absence (squares) of the competitive inhibitor CDNB, in either the B to A direction (white) or A to B direction (black).

competitive inhibitor CDNB was able to inhibit the transport of CMFDA for up to 30 min.

Uptake assays using inside-out vesicles bypass the conjugation step needed for many MRP2 substrates. Substrates such as leukotriene C_4 (LTC_4) are already conjugated and their addition in the presence of ATP allows the vesicles to take up the substrate and accumulate in the cell. In the inside-out vesicle assay, there was about a 2-fold increase in LTC_4 uptake in transfected cells compared to untransfected MDCKII cells. The inhibitor MK-571 eliminated transport of LTC_4 into the vesicles (Figure 2.23).

2.3.7 Stable Transfection of MRP2 in HEK293 Cells

Attempts to create stably transfected cell lines proved to be a difficult task. Although transient expression showed high protein levels in MDCKII cells (Figure 2.24A), stably transfected cells were not able to be isolated in the presence of the selection agent G418. HEK293 cells, which have higher transfection efficiency, were also used to create stable cell lines. Again, transient transfections showed high levels of MRP2 protein (Figure 2.24B). Three cell lines thought to be stably expressing MRP2 protein were produced, HEK293-MRP2Ref, HEK293-MRP2-3542, and HEK293-MRP2-2901 (Figure 2.25A, 2.25B Western blots). The 2901C>A variant introduces a premature stop codon in the mRNA resulting in a truncated protein. Western blot analysis shows a half sized protein, as well a larger protein, which could be due to dimerization.

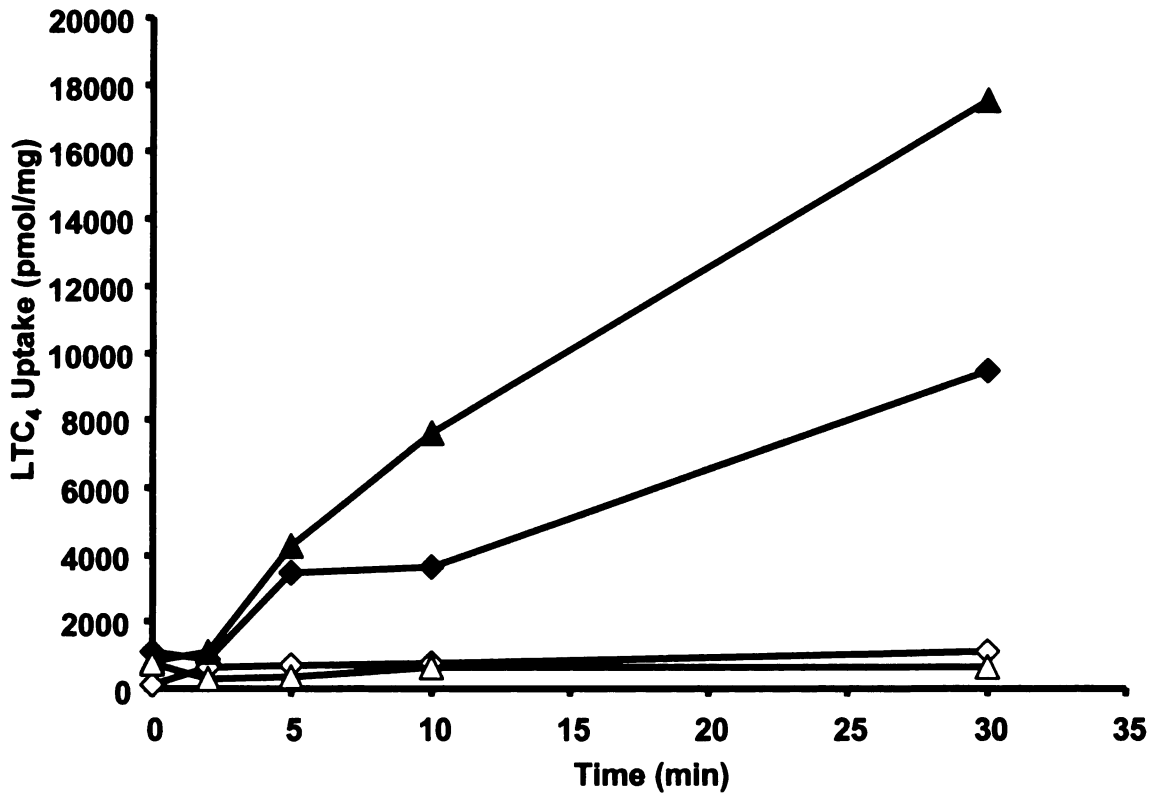


Figure 2.23 Uptake studies using inside-out vesicles created from MDCKII (diamond) or MDCKII-MRP2 (triangle) cells. Vesicles were incubated with 2.4 nM [^{3}H]LTC_4 in the presence (white) or absence (black) of the inhibitor MK-571. Samples were taken between 0-30 minutes, and aliquots were measured for radioactivity.

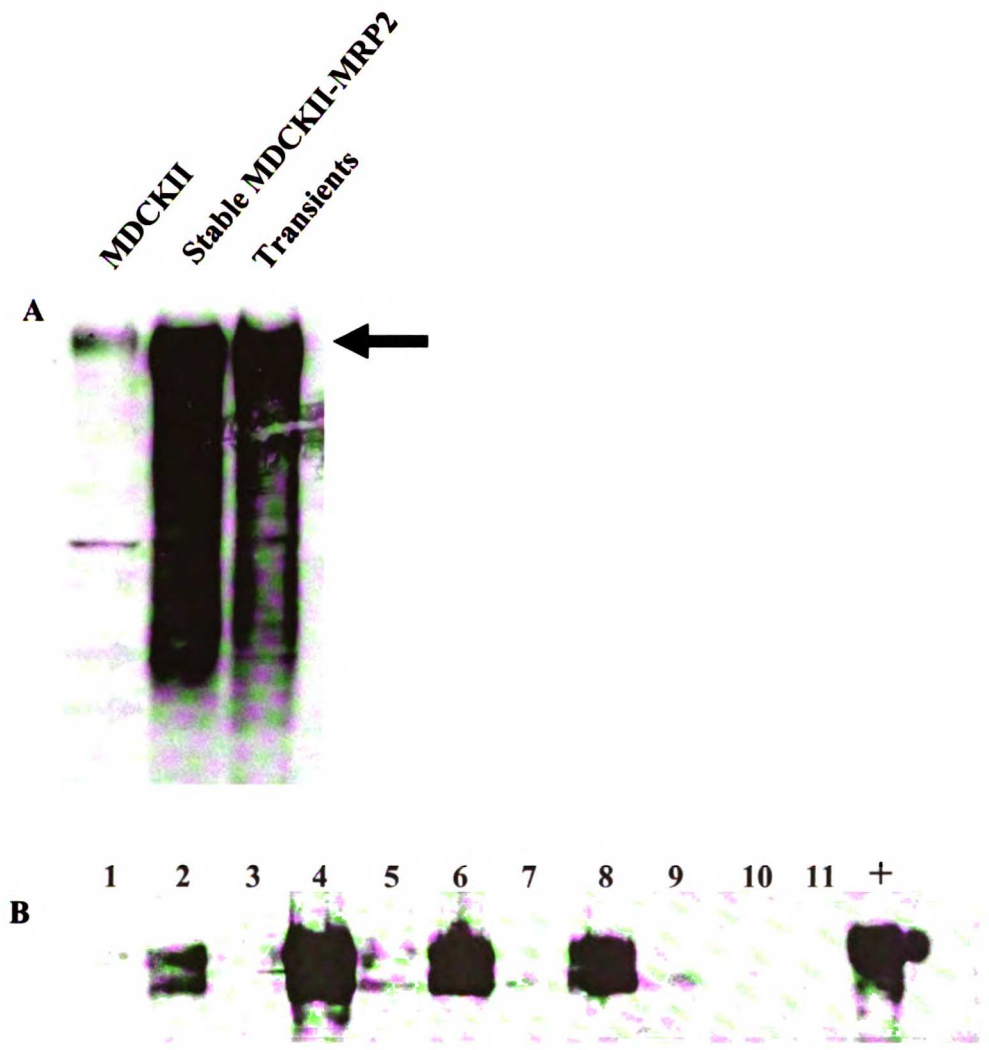


Figure 2.24 Western blot of MRP2 protein transiently expressed in MDCKII and HEK293 cells. A) MDCKII cells were transiently transfected with pcDNA3.1-MRP2 plasmid and proteins were detected using the antibody M2-III-6. Proteins from MDCKII (lane 1), MDCKII-MRP2 stables (lane 2), and MDCKII-pcDNA-MRP2 transients (lane 3) are shown. B) Transient transfections of HEK293 cells with pcDNA3.1-MRP2 carrying reference sequence (lane 2), 116A>G variant (lane 4), 1058G>A variant (lane 6), and 2546T>G variant (lane 8) compared to lysates from MDCKII-MRP2 as a positive control (lane 12).

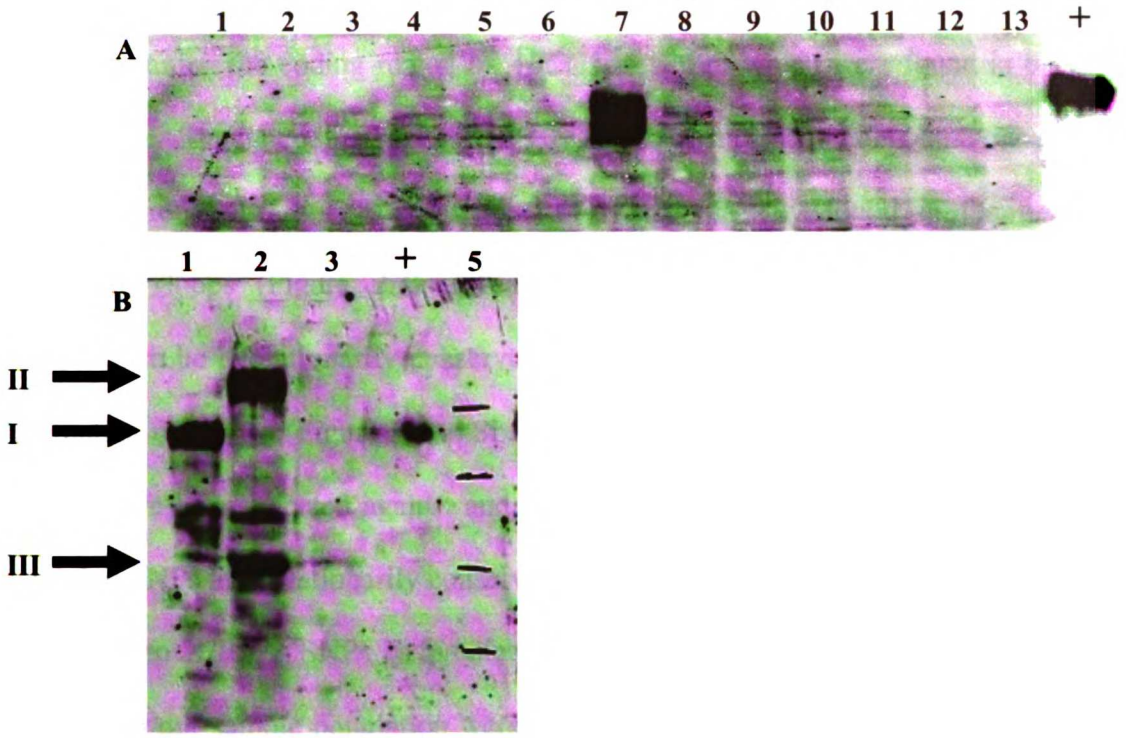


Figure 2.25 Isolation of stably transfected cell lines. A) Stably transfected HEK293 cells with reference plasmid. A positive clone is found in lane 7, with a positive control in lane 14. B) Isolation of stably transfected HEK293 cells carrying the 3542G>T variant (lane 1) and the 2901C>A variant (lane 2). A positive control is found in lane 4. Band I shows the typical protein band for MRP2. Band III shows a truncated MRP2 with band II being a possible dimer of the truncated protein.

2.4 DISCUSSION

2.4.1 Genetic Variation in *ABCC2*

This study provided a comprehensive examination of genetic variation in *ABCC2*. In all, 68 variants sites were identified in over 9.6 kb of sequence screened. Fifty-six of the 68 variants had not been identified previously. There were 19 non-synonymous variants spanning the coding region of *ABCC2*, seven of which were found in only one chromosome. The 34 coding-region SNPs, both synonymous and non-synonymous, were scattered throughout the protein structure, with a majority found in the predicted intracellular loops (Figure 2.3). Ten non-synonymous variants occurred at sites considered to be evolutionarily conserved based on protein sequence alignments across mammalian orthologs (Figure 2.4). Six of these were singletons, suggesting that these variants have been selected against and may have a deleterious effect. Shu *et al.* (48) found that SNPs in evolutionarily conserved sites can predict function in OCT1. This can be applied to *ABCC2* SNPs to predict function. Interestingly, none of the variants identified in our sample population were known Dubin Johnson Syndrome variants (17, 18, 21, 22, 49-52), however DJS variants are found at low frequencies One variant, 670I>T, is one amino acid away from the Walker A sequence and could potentially affect ATP binding.

There was substantial variation in allele frequencies of variants among the different populations that were studied (Table 2.1). The total number of segregating sites differed among the ethnic groups, with more variants identified in the African American population. This is consistent with the theory that the African population is the oldest human population and therefore has accumulated more variation (53). The African

American and Caucasian populations share common SNPs, which is consistent with, but by no means proof of, population admixture (41, 54).

Overall nucleotide variation, estimated by both the neutral parameter (θ) and nucleotide diversity (π), were similar to estimates of 24 members of the *ABC* and *SLC* gene families (25). When examining the transmembrane domain of MRP2, there were higher π values in the conserved sites than the unconserved sites, unlike the *SLC* family where the opposite is true. A possible explanation for this is that the substrate specificity for MRP2 (and other ABC transporters) is believed to lie in the TMD. Variability in this area may aid in the protein's ability to adapt to exposure to a diverse and changing array of toxins.

Haplotypes were statistically inferred using PHASE for 236 of the 247 DNA samples. Theoretically, the maximum number of haplotypes is 2^n, where the 2 represents the two alleles and n equals the number of variant sites. In the absence of intragenic recombination, repeated mutations, and back mutations, this maximum is $n + 1$ haplotypes (41). We were able to infer 88 haplotypes from 47 non-singleton sites and 32 were found in at least three chromosomes. A number of variable sites are in tight linkage disequilibrium with other variant sites making it possible to predict haplotypes from a smaller number of tagging SNPs.

2.4.2 Heterologous Assays

Several heterologous cell assays were tested to determine their ability to detect differences in MRP2 function. MRP2 was expressed in yeast although it was not clear whether it was being expressed at the membrane or in the appropriate orientation.

Immature protein or intracellular accumulation of protein could explain why a difference between vector transfected and MRP2 transfected cells was not observed, despite detection of MRP2 immunoreactive protein. MDCKII-MRP2 cell lines can likely be used in both flux and inside-out vesicle assays. Cells expressing MRP2 had higher transport of the substrates CMFDA and LTC_4. However, untransfected MDCKII cells transported both compounds, consistent with a functional endogenous transporter. Low levels of immunoreactive MRP2 were detected in MDCKII cell lysates and A→B transport was higher than B→A transport in these cells. This suggests that a basolateral membrane transporter, possibly MRP1, may be the rate limiting step in transport of CMFDA in these cells.

With several mammalian assays developed, expressed MRP2 variants can be analyzed for their function. Comparisons can be made between variant and reference MRP2 in their ability to transport MRP2 substrates. By using a wide array of MRP2 substrates and inhibitors, the effect of genetic variation on substrate specificity can be examined. A polarizable cell line will be necessary to study trafficking of mature protein.

2.5 PERSPECTIVES

The findings presented here represent the most extensive study to date of *ABCC2* genetic variation. With the number of variable sites discovered, it is likely that genetic variation may contribute to inter-individual variability in MRP2 function. The biggest impact in function would occur in the liver, where MRP2 is highly expressed and plays an important role in xenobiotic detoxification and bile flow. Studies using MRP2 deficient rats and cells overexpressing ABCC2 mRNA have shown the ability of MRP2

to transport a wide variety of endogenous and exogenous compounds, ranging from bile salts to chemotherapeutic agents (8-10, 13, 55-58). A better understanding of how genetic variations, both as single variants and in haplotypes, can affect transporter function is necessary. A disruption in MRP2 function could lead to changes in drug disposition, therapeutic response, and toxicity. Genetic variations in *ABCC2* can then be used to predict clinical response to administered drugs.

2.6 REFERENCES

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CHAPTER 3

CHARACTERIZATION OF 5'-PROMOTER REGION POLYMORPHISMS IN *ABCC2*

3.1 INTRODUCTION

Drug response can be dependent on the genetics of transporters and enzymes involved in drug absorption, distribution, and elimination. Most genetic studies involving these proteins have examined how coding region variants affect protein function and trafficking. Little is known about the non-coding regions of a gene (5'-region, 3'-region, and intronic sequences) and how genetic variations there can affect gene and protein expression. It is believed that with the low number of protein encoding genes in the human genome, that polymorphisms affecting gene expression may play a more prominent role (1). Further studies on gene expression profiles may help explain the variability seen in drug response.

Gene expression can be regulated by a number of factors such as chromatin condensation, DNA methylation, and mRNA stability (2). The level of mRNA expression is regulated by both cis-acting and trans-acting elements, which control and maintain cellular homeostasis. Cis-acting factors are the DNA sequences that contain gene regulatory regions, which bind trans-acting proteins. The most basic of the cis-acting elements is the minimal or core promoter, which contains a conserved DNA sequence motif called the TATA box. The TATA binding protein can bind this sequence and initiate the binding of RNA polymerase II and lead to transcription. Upstream of the minimal promoter is the proximal promoter and regulatory sequences that bind

transcription factors that either upregulate (enhance) or downregulate (repress) transcriptional activity (3).

Cis-acting elements can be methylated, buried within the DNA structure, or contain polymorphisms that change the sequence of binding sites. These elements can control gene expression levels in healthy or diseased tissues, determine where the proteins are expressed, and regulate protein expression at different stages of development (2). There is very little known about regulatory sequences of most genes. Genetic variations in the regulatory regions can be important in modulating the expression of the gene product and may help explain interindividual variability in expression.

Currently, there is very little information about the regulation of human *ABCC2*. Two studies have attempted to characterize the 5'-region of human *ABCC2*. In these studies, varying lengths of a 2 kb region of the upstream sequence were cloned into a reporter gene plasmid and promoter activity was tested. In two independent studies, it was determined that the region between 200 and 500 bases upstream from the translation start site was important for basal promoter activity (4, 5). This region contains consensus sequences for a number of transcription factors, including the TATA box, and the liver abundant CCAAT-enhancer binding protein β (C/EBPβ).

Studies characterizing the 5'-region of rat *Abcc2* have shown that basal expression of rat *Abcc2* is mediated by CBF/NF-Y and Sp1 transcription factors (6). Rat *Abbc2* is also inducible by ligands for the farnesoid X-activated receptor (FXR), pregnane X receptor (PXR), and the constitutive androstane receptor (CAR) (7). These include phenobarbital, dexamethasone, ethinyl estradiol, and a number of anti-cancer drugs (7-9). In another study it was demonstrated that bile acids and rifampicin are able

to induce rat and human ABCC2 mRNA levels (10, 11). These ligands and their interactions with the orphan nuclear receptors help maintain bile flow homeostasis in the liver by upregulating MRP2 and other proteins in the presence of bile acids and other MRP2 substrates. An ER-8 (everted repeat with an eight bp spacer) response element (RE) has been identified in rat *Abcc2* that is able to bind all three nuclear orphan receptors. However, it has not been found in humans and differs from the known RE found in CYP3A4 (7)

In this study, we examined whether genetic variations in the promoter region of *ABCC2* can alter promoter activity. We focused mainly on the core promoter of *ABCC2* and DNA sequence proximal to this region. Genetic variations in this region can alter transcriptional activity leading to changes in MRP2 expression and altering the elimination of MRP2 substrates in the liver.

3.2 MATERIALS AND METHODS

3.2.1 Genetic Analysis of the 5'-region of *ABCC2*

The identification of polymorphisms in *ABCC2* was described in the previous chapter. A 1.6 kb upstream region of *ABCC2* was sequenced for single nucleotide polymorphisms. This region was also compared to rat and mouse sequences (Genbank accession numbers AF261713 and NT039692) to determine nucleotide conservation. Using ClustalX and GeneDoc, the sequences were aligned to identify regions of conservation. Single nucleotide polymorphisms were also superimposed on putative transcription factor binding sites reported in the literature for human *ABCC2* (4, 5).

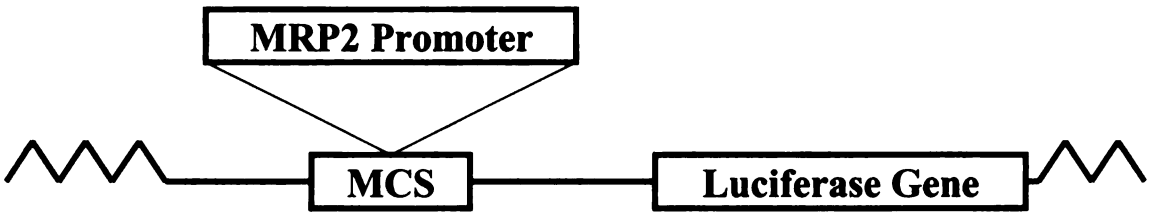

Figure 3.1 Schematic of the reporter gene expression plasmid. The 1.6 kb region of the MRP2 promoter was cloned into the *NheI* and *XhoI* sites. The MRP2 promoter will drive the expression of the downstream luciferase gene. MRP2, multidrug resistance-associated protein 2; MCS, multiple cloning site.

3.2.2 Plasmid Construction

A 1.6 kb region of the 5'-region of *ABCC2* was PCR amplified from genomic DNA and subcloned into pCR2.1 using the standard protocol from the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The amplified promoter DNA was digested with *NheI* and *XhoI* (New England Biolabs, Beverly, MA) and was cloned into the reporter gene expression vector pGL3-basic (Promega, Madison, WI), which contains a luciferase gene downstream from the multiple cloning site (Figure 3.1). Individual non-singleton variants (Table 3.1) or variants in haplotypes (Table 3.2) were introduced using specific primers (Table 3.3) and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids were sequenced by the Human Genetics core facility to verify the correct introduction of variants.

Table 3.1 5'-Promoter Region Variants Identified in *ABCC2*

Position	Nucleotide Change	Allele Frequency					
		Total n=494	CA n=200	AA n=200	AS n=60	ME n=20	PA n=14
Primer	**T→ C**	**0.010**	0.000	**0.025**	0.000	0.000	0.000
-1563	**G→ A**	0.002	0.000	0.005	0.000	0.000	0.000
-1549	**G→ A**	**0.407**	**0.430**	**0.485**	**0.150**	**0.200**	**0.357**
-1292	**A→ G**	0.006	0.000	**0.015**	0.000	0.000	0.000
-1239	**G→ A**	**0.016**	0.005	**0.030**	0.000	**0.050**	0.000
-1065	**C→ A**	0.004	0.000	0.005	0.000	**0.050**	0.000
-1059	**C→ +G**	0.002	0.000	0.005	0.000	0.000	0.000
-1023	**G→ A**	**0.154**	**0.105**	**0.135**	**0.267**	**0.300**	**0.429**
-1019	**A→ G**	**0.360**	**0.430**	**0.365**	**0.167**	**0.200**	**0.357**
-798	**C→ A**	0.002	0.000	0.005	0.000	0.000	0.000
-733	**G→ A**	0.002	0.000	0.000	**0.017**	0.000	0.000
-24	**C→ T**	**0.136**	**0.195**	**0.060**	**0.150**	**0.150**	**0.286**
-23	**G→ A**	0.004	0.000	0.000	0.000	0.000	**0.143**

Variants were previously reported in Chapter 2, Table 2.1. Nucleotide positions are numbered based on nucleotide distance from the ATG (+1) start site. Frequencies were calculated using the total population as well as for each ethnic group. The (+) in the nucleotide change column for -1059 represents an insertion. CA, Caucasians; AA, African Americans; AS, Asian Americans; ME, Mexican Americans; PA, Pacific Islanders

3.2.3 Reporter Gene Assay

Plasmids containing either reference or variant sequence were transiently co-transfected into HepG2 (human hepatoma) cells with pCMV-β-gal using the Lipofectamine Plus protocol (Invitrogen, Carlsbad, CA). pCMV-β-gal is used as a transfection efficiency control and all activity is normalized to β-gal activity. Twenty-four hours after transfection, cells were washed with calcium- and magnesium-free phosphate buffered saline and incubated in 150 μL of lysis buffer (Promega, Madison, WI). Cells were subjected to a freeze/thaw cycle, and aliquots were analyzed for luciferase activity using luciferase substrate (Promega, Madison, WI) and an Optocomp I

luminometer (MGM Instruments, Hamden, CT). β-gal activity was determined from the same lysate using a β-gal assay kit (Promega, Madison, WI) and a VERSAmax microplate reader at 420 nm (Molecular Devices, Sunnyvale, CA).

3.2.4 Transcription Factor Binding Sites

In order to identify potential regulatory SNPs that result in a change in transcription factor binding, possible alterations of transcription factor binding sites due to SNPs were examined from the vertebrate matrices in the TRANSFAC database (TFBlast, http://www.gene-regulation.com) and the Transcription Element Search System (http://www.cbil.upenn.edu/tess). Roughly 30 bases flanking the SNP site were analyzed for potential binding sites for the variant and reference allele.

3.2.5 Preparation of Nuclear Extracts

Cultured HepG2 cells were collected (1 x 10^7 cells) and washed with calcium- and magnesium-free PBS and centrifuged at 450*g*. Nuclear extracts were isolated via a modified protocol (12). Briefly, the pellet was resuspended in 400 μL of Nuclear Preparation Buffer (NPB) which contains 10 mM Tris/HCl pH 7.4, 2 mM $MgCl_2$, 140 mM NaCl, 0.5 mM DTT, and 0.5 mM PMSF. The mixture was subjected to a single freeze/thaw cycle and mixed gently. The mixture was then layered over 600 μL of 50% sucrose in NPB and spun at 12,000*g* for 10 min at 4°C. The solution was aspirated leaving only a pellet that was then resuspended in 175 μL of Dignum C buffer (25% glycerol, 20 mM HEPES/KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF). The mixture was rotated for 30 min at 4°C and subsequently

Table 3.2 Ethnic Distribution of Promoter Haplotypes

	-1549G>A	-1292A>G	-1239G>A	-1065C>A	-1023G>A	-1019A>G	-24C>T	-23G>A	CA	AA	AS	ME	PA	*ABCC2* Haplotypes
1	G	A	G	C	G	A	C	G	**0.46**	**0.39**	**0.57**	**0.50**	**0.25**	***1, *4, *4A, *4B, *5, *5B, *5C, *6, *7, *7A, *36, *36A, *37, *37A, *37B, *41, *42, *42A, *43, *44, *45, *46, *47, *47A, *48, *49, *50, *50A, *51, *51A, *52, *53***
2	A				G				**0.23**	**0.26**	0.00	0.00	0.00	***8, *8C, *9, *9A, *9B, *12, *13, *14, *15, *15A, *19, *20, *20A, *21, *22, *23, *23A, *23B, *24, *26, *27, *27A, *28***
3	A				G	T			**0.20**	**0.05**	**0.16**	**0.15**	**0.17**	***10, *10A, *10B, *10C, *10E, *11, *11A, *11B, *25***
4	A				G	T	A		0.00	0.00	0.00	0.00	**0.08**	***10D***
5	A		A		G				0.00	**0.03**	0.00	0.05	0.00	***16, *16A, *17, *18***
6	A	G			G				0.00	**0.02**	0.00	0.00	0.00	***30, *31***
7	A								0.00	**0.13**	0.00	0.00	0.00	***7B, *24, *29, *32, *32A, *33, *35***
8				A					**0.11**	**0.11**	**0.28**	**0.25**	**0.50**	***2, *2A, *2B, *3, *3A, *5A, *38, *39, *40***
9			A	A					0.00	0.00	0.00	**0.05**	0.00	***3B***

Haplotypes were estimated for eight non-singleton 5'-variants using Phase. Shown are the estimated haplotypes and their frequencies among the ethnic populations. The first row is the promoter reference haplotype with nucleotide changes listed below each variant site. *ABCC2* haplotypes are the corresponding promoter + coding region haplotypes described in Chapter 2. CA, Caucasians; AA, African Americans; AS, Asian Americans; ME, Mexican Americans; PA, Pacific Islanders

Table 3.3 Site Directed Mutagenesis Primers

Variant	Forward Primer	Reverse Primer
-23G>A	GAAGAGTCTTC**A**TTCCAGACGCAGTCC	GGACTGCGTCTGGAA**T**GAAGACTCTTC
-24C>T	GAAGAGTCTT**T**GTTCCAGACGCAGTCC	GGACTGCGTCTGGAAC**A**AAGACTCTTC
-733G>A	CGATGACAGTTTCTAGC**A**ACTGATGCC	GGCATCAGT**T**GCTAGAAACTGTCATCG
-1019A>G	GGCCAAGGCAG**G**AGGATTGTTGAAGCC	GGCTTCAACAATCCT**C**CTGCCTTGGCC
-1023G>A	GGAGGCCAAG**A**CAGAAGGATTGTTGAAGC	GCTTCAACAATCCTTCTG**T**CTTGGCCTCC
-1065C>A	GGTCAGGTGGGGCA**A**GGTAGCTCATGC	GCATGAGCTACC**T**TGCCCCACCTGACC
-1239G>A	GTGTTTCTTATAATCC**A**GTAGTTAGATCTAG	CTAGATCTAACTAC**T**GGATTATAAGAAACAC
-1292A>G	CCACATTCTGGATTTTG**G**CAATTGCATTCC	GGAATGCAATTG**C**CAAAATCCAGAATGTGG
-1549G>A	CCTTATAGTAT**A**TTGTGGATATTAACTC	GAGTTAATATCCACAA**T**ATACTATAAGG

Forward and reverse primers used for site-directed mutagenesis to introduce variant alleles. The nucleotide corresponding to the polymorphism is indicated in bold and underlined.

centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was removed and dialyzed in Dialysis Buffer (10% glycerol, 25 mM HEPES/KOH pH 7.9, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF) overnight at 4°C. Extracts were aliquotted and stored at -80°C until ready for use.

3.2.6 Electrophoretic Mobility Shift Assay (EMSA)

Probes for EMSA were synthesized from complementary oligonucleotides (Invitrogen, Carlsbad, CA) that were used for mutagenesis and were annealed at 100°C for 5 min in TE buffer at a concentration of 2 μM. Five pmols of probes were radiolabeled with 10 Units of γ^{32}P-ATP (3000 Ci/mmol, Perkin Elmer, Foster City, CA) using T4 Kinase (Invitrogen, Carlsbad, CA) at 37°C for 10 min after which the enzyme was inactivated at 65°C for 10 min. Probes were purified using microSpin G25 columns (Amersham Bioscience, Piscataway, NJ) and counted for radioactivity. For EMSA,

approximately 5 fmols of radiolabeled probe (~25,000 cpm) was incubated with 10 μg of nuclear extract in binding buffer (10 mM HEPES pH 7.9, 40 mM KCl, 6% glycerol, 0.5 mM DTT, 5 mM $MgCl_2$, and 1 μg poly dI-dC).

For competition assays, nuclear extracts were incubated in the presence of 50- to 200-fold excess of unlabeled annealed oligonucleotides for 15 min on ice in binding buffer before the addition of radiolabeled probe. For supershift experiments, antibodies for specific nuclear proteins were added to the reaction prior to the addition of radiolabeled probe. Consensus sequences and antibodies for putative transcription factors were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Complexes were loaded onto a 5% polyacrylamide gel and resolved using 0.5X TBE running buffer (2 hours, 200 V, 4°C). Gels were dried on 3mm Whatman paper, exposed to phosphor screens (Amersham Bioscience, Piscataway, NJ) overnight and visualized on a Storm 800 Imager (Amersham Bioscience, Piscataway, NJ) using ImageQuant software.

3.2.7 Affinity Bead Preparation

Probes, similar to those used for EMSA, were synthesized (Invitrogen, Carlsbad, CA) containing a biotin conjugate on the 5'-end of the main strand. Biotin-labeled main strand and unlabelled complementary strand oligos were annealed to produce double stranded probes. The affinity beads were prepared per the manufacturer's protocol. Briefly, 1 mg of Dynabeads™ M-280 streptavidin (Dynal Biotech, Brown Deer, WI) was washed with binding buffer, pulled down with a magnet, and the supernatant was aspirated. Beads were then incubated with 50 μg of biotinylated probes diluted in 200 μL of TE buffer supplemented with 0.5 M NaCl. When decreasing DNA concentrations

plateaued, measured by the absorbance at 260 nm, beads were pulled down with a magnet. The bead/probe complexes were washed with binding buffer before use for protein isolation.

3.2.8 Protein Isolation Studies

Nuclear extracts were made from 5 mL of packed HepG2 cells as described above. Extracts were then run through a size exclusion column (100 mL volume) containing a Sephacryl S-300 High Resolution matrix (Amersham Bioscience, Piscataway, NJ). Nuclear extracts were applied to the matrix and allowed to diffuse into the matrix before the addition of elution buffer (20 mM HEPES/KOH pH 7.6, 20% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium metabisulphite) (13). Two mL fractions were collected from the column and protein was detected at 280 nm on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Fractions that contained protein were then subjected to EMSA to determine which contained the protein of interest. Fractions were pooled and incubated with affinity beads for 20 min on ice. Beads were pulled down using a magnet and the extract was carefully removed. Beads were then washed twice with binding buffer before proteins were eluted (10 mM HEPES/KOH pH 7.9, 400 mM KCl). Eluted protein was then precipitated by adding 0.25 volumes of ice cold trichloroacetic acid (TCA). This removes chloride salts, which can interfere with subsequent analysis. After 15 min, the reaction is centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was aspirated without disturbing the pellet, and the pellet was resuspended in 1 mL acetone.

The mixture was then centrifuged at 12,000*g* for 5 min at 4°C, aspirated, and the pellet dried under a vacuum.

3.2.9 SDS-PAGE

Samples were prepped for blotting by adding Laemmli buffer and boiling for 5 min at 100°C. Samples were then loaded onto a 5% polyacrylamide gel and run at 45 mAmp for 6 hours. Protein bands were visualized using the Silver Stain Plus Kit (BioRad, Hercules, CA). Briefly, the gel was fixed in buffer containing 50% methanol, 10% acetic acid, and 10% Fixative Enhancer Concentrate (BioRad, Hercules, CA) for 20 min, followed by rinsing in deionized water. Gels were stained in 50 mL of staining solution (5% Silver Complex Solution, 5% Reduction Moderator Solution, 5% Development Reagent, 50% Development Accelerator Solution; BioRad, Hercules, CA) until desired staining. Staining was stopped by incubating the gel in a 5% acetic acid solution for 15 min.

3.3 RESULTS

3.3.1 Polymorphisms in the Proximal Promoter Region

To identify polymorphisms in the MRP2 promoter region, 1.6 kb of genomic DNA upstream from the ATG start site was sequenced from 247 ethnically diverse DNA samples from the Coriell Institute. Thirteen polymorphisms were identified with one found in the sequencing primer (primer SNP in Table 3.1). Two SNPs were found in the 5'-untranslated region, which is between -1 bp and -247 bp from the translation start site (4, 5). All other SNPs were found more than 700 bp upstream from the translation start

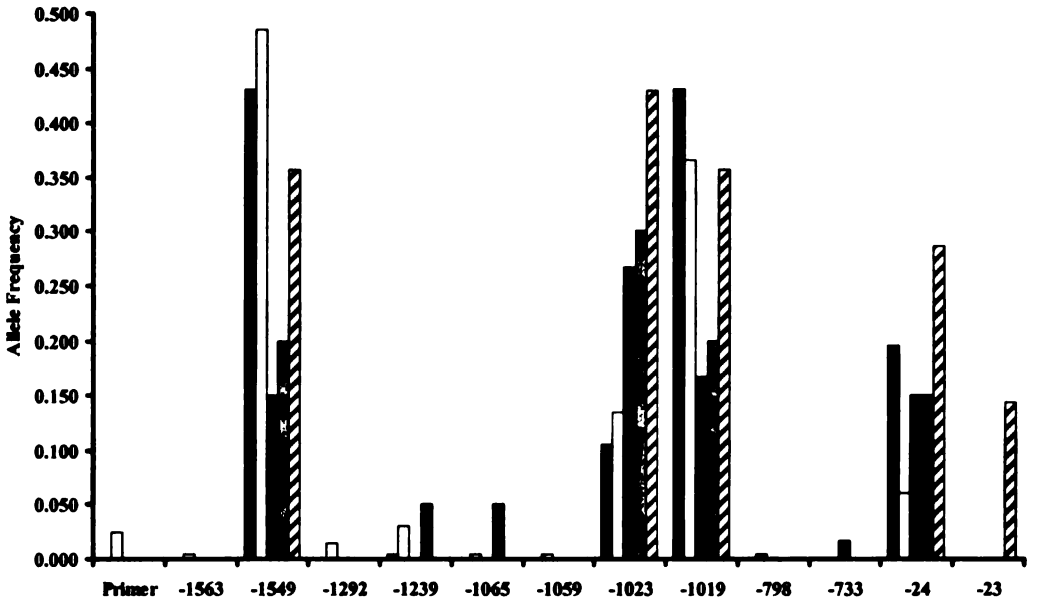

Figure 3.2 Distribution of promoter variants among five ethnic populations. Shown are cosmopolitan SNPs, high frequency SNPs, and ethnic specific SNPs. Black bars, Caucasians; white bars, African Americans; dark grey bars, Asian Americans; light grey bars, Mexican Americans; diagonal lines, Pacific Islanders.

site. A single insertion was discovered at -1059 that was found in a single chromosome in the African American population. There were no variations found between -247 and -500 base pairs, which was shown to be required for basal expression (5). Four polymorphisms were considered cosmopolitan and were found at greater than 5% frequency in each ethnic population (Figure 3.2). The -798C>A, -1059+G, -1292A>G, -1563G>A, and the T>C SNP found in the sequencing primer were African American specific. The -733G>A SNP was specific to the Asian American population, and the -23G>A UTR variant was Pacific Islander specific. The non-singleton variants were organized into 9 different promoter haplotypes (Table 3.2).

Sequence alignments with both rat and mouse sequences showed some similarity between the human and rodent sequences. The overall sequence similarity was 42% and

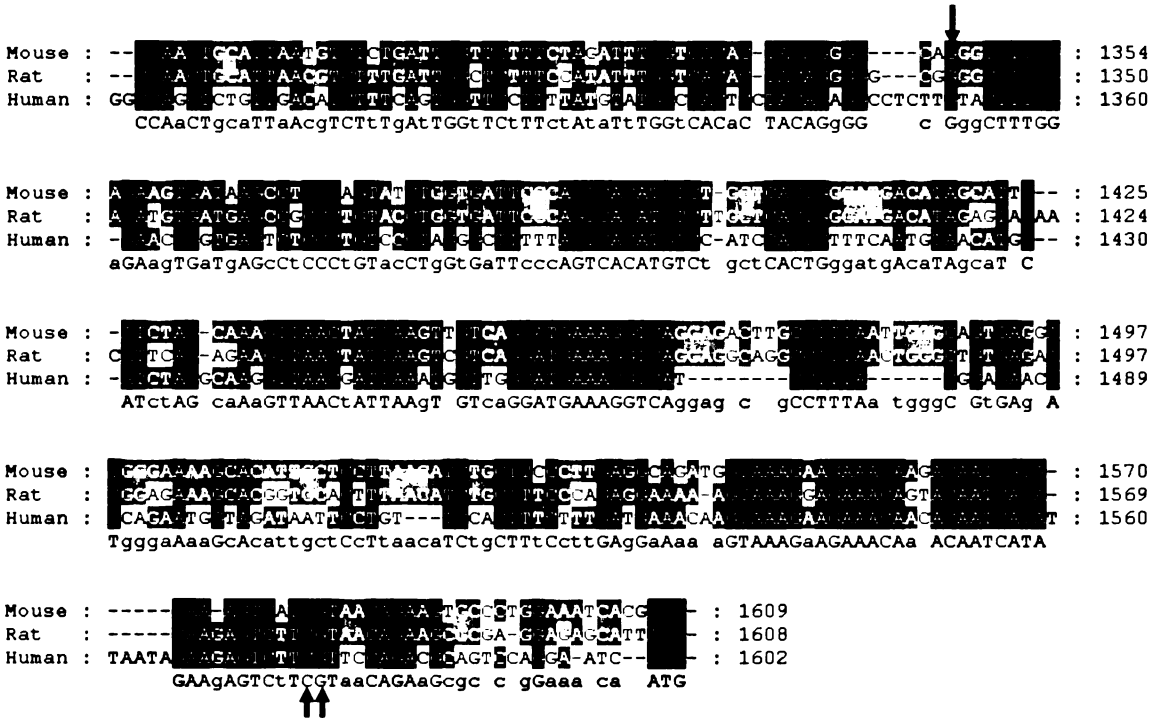

Figure 3.3 Alignment of the 5'-flanking region of rat, mouse, and human *ABCC2*. Depicted here is roughly a 300 bp region proximal to the ATG start site that was highly conserved across the three species. The arrow above the sequences indicates the transcription start site. Arrows below indicate the location of the -24C>T and -23G>A variants found in the 5'-UTR.

37% for the mouse and rat, respectively. The sequence similarity increased to 59% and 58% in the 5'-untranslated region (Figure 3.3). Of the variants identified, only the two 5'-UTR variants were found in a conserved region.

3.3.2 Promoter Activity *In Vitro*

The 1.6 kb promoter fragment was amplified from genomic DNA and cloned into the multiple cloning site of the promoterless luciferase plasmid, pGL3-basic (Promega, Madison, WI). Single variant or promoter haplotypes were introduced by site-directed mutagenesis. A plasmid containing the reference control showed approximately 277-fold

higher activity than the promoter-less pGL3-basic plasmid. Reporter activity of some of the promoter constructs, which were all normalized to β-gal activity, showed decreased activity compared to the reference sequence. Statistical analysis indicates that plasmids carrying the single variants -1549G>A, -1292A>G, -1239G>A, and -1065C>A (Figure 3.4A) and the haplotype plasmids -24C>T/-1019A>G/-1549G>A, -1292A>G/ -1019A>G/ -1549G>A, and -1065C>A/-1023G>A (Figure 3.4B) showed significant decreases ($p<0.05$, t-test) in promoter activity compared to the reference control.

3.3.3 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed using double stranded oligonucleotides spanning the polymorphic site. Three radiolabeled probes spanning the -1065, -1292, and -1549 loci showed differential binding of proteins with the reference and variant sequences (Figure 3.5). To determine the specificity to which these proteins bound the variant probes, competition assays were performed using unlabeled probes carrying the variant or reference allele. Although the probe containing the reference allele at position -1065 showed no protein binding, it was able to compete for protein binding with the radiolabeled variant allele to a similar degree as the variant sequence (Figure 3.6A). Competition for the -1292 (Figure 3.6B) and -1549 (Figure 3.6C) positions showed better specificity as increasing concentrations of unlabeled variant oligos showed higher binding inhibition compared to the reference oligos.

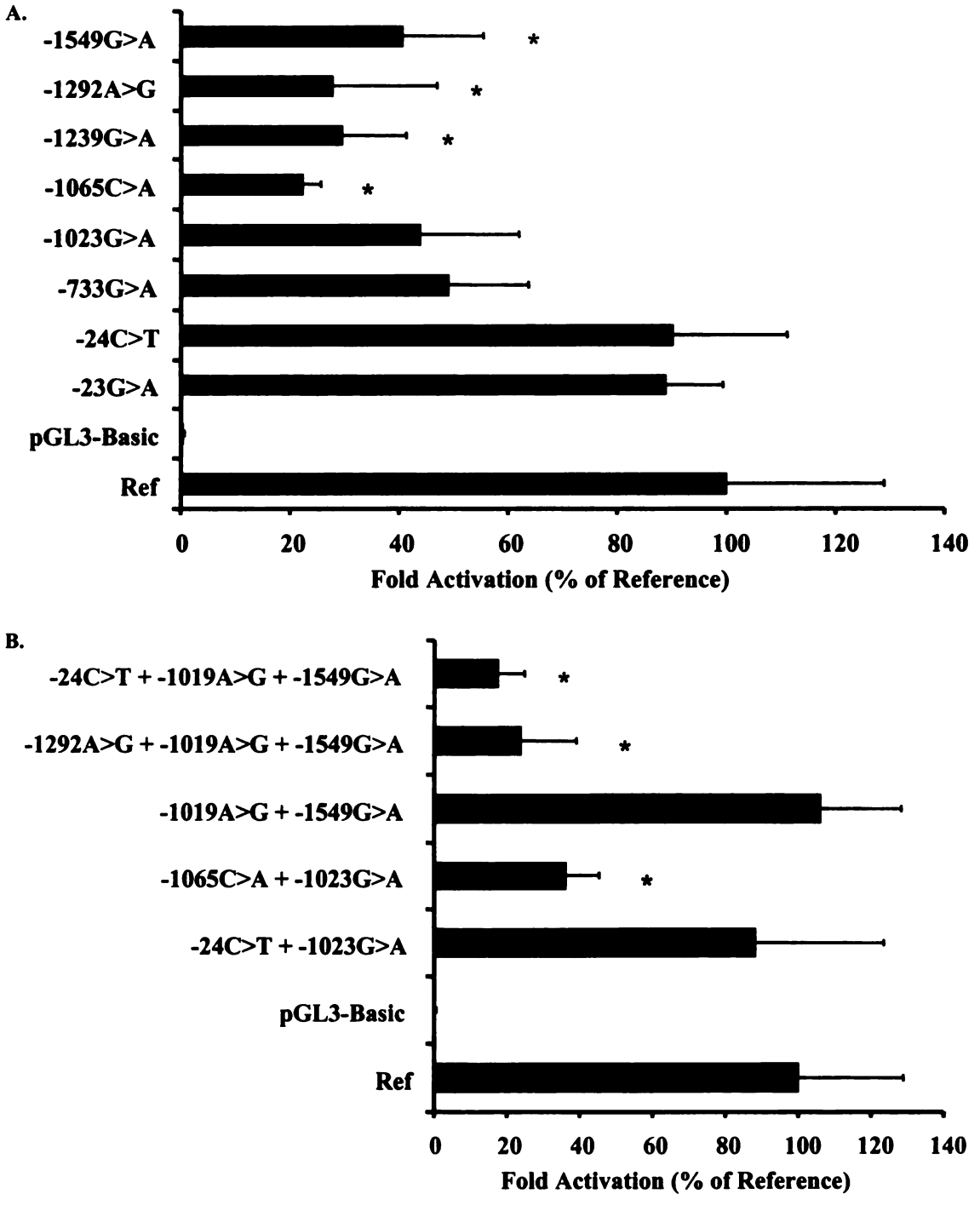

Figure 3.4 Activity of different MRP2 promoter constructs in HepG2 cells. A) Polymorphisms with a frequency of >1% and B) common haplotypes were tested for promoter activity. Each experiment was run in triplicate and the means (± SD) shown are representative of all experiments. * Significant difference in promoter activity compared to reference control ($p<0.05$, t-test).

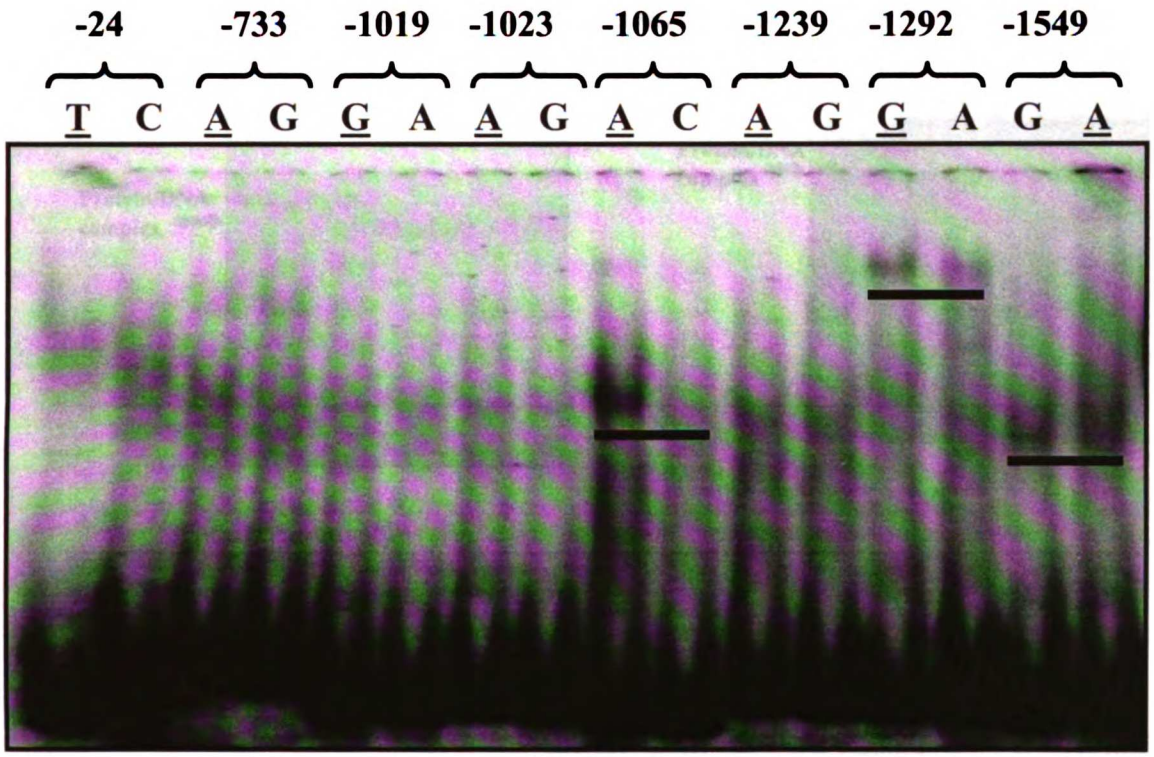

Figure 3.5 Electrophoretic mobility shift assay for each SNP site. The location of the polymorphic site is listed at the top of the gel. Radiolabeled probes containing the variant (underlined) or reference allele were tested for each variant site. DNA-protein complexes with differential binding are underlined.

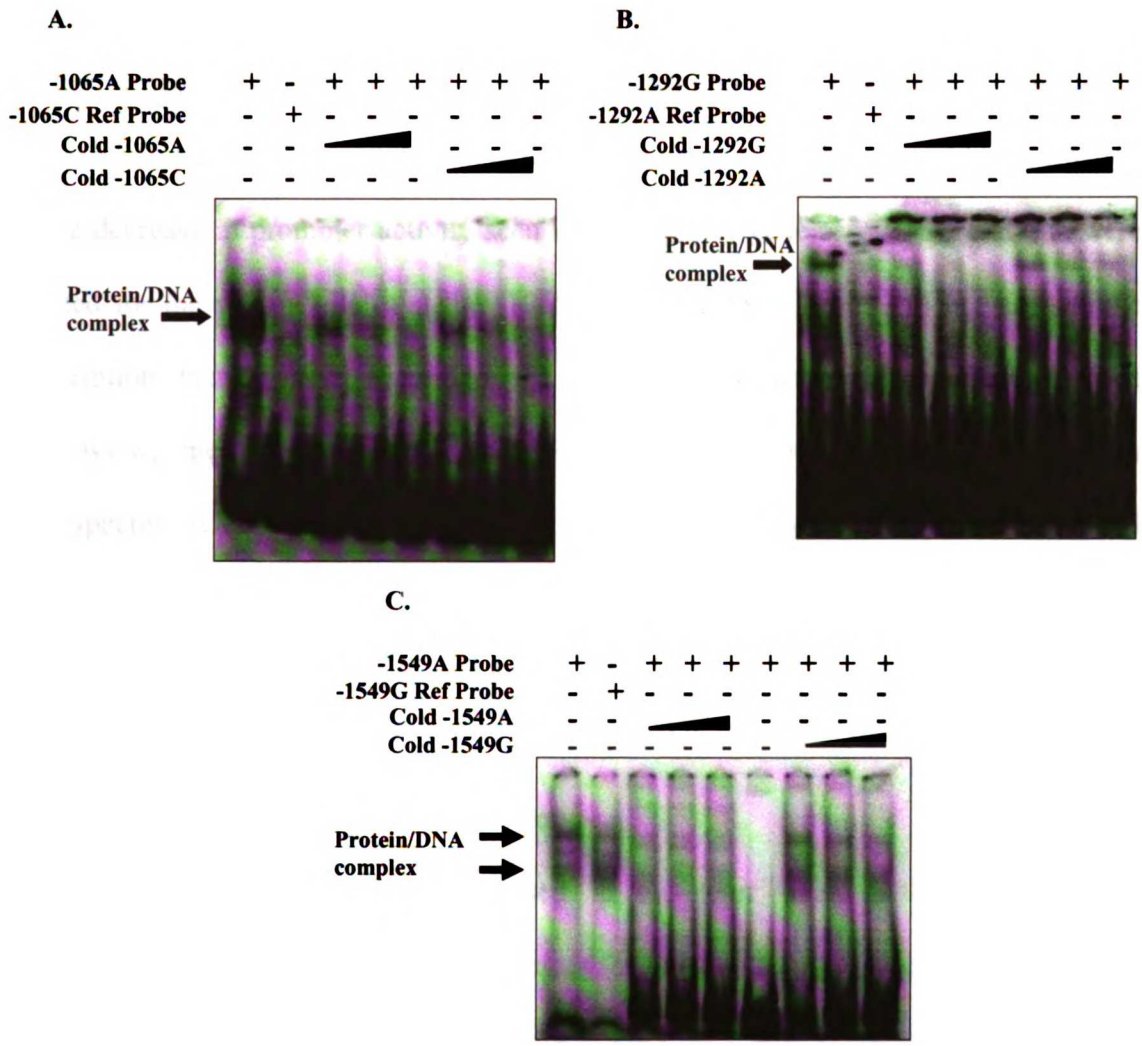

Figure 3.6 Specificity of nuclear protein binding. A 50- to 200-fold excess of unlabeled oligos containing either variant or reference sequences were incubated with probes carrying the variant allele at the A) -1065 locus, B) -1292 locus, and C) -1549 locus. DNA-protein complexes are indicated by an arrow.

3.3.4 Transcription Factor Binding Sites

Putative transcription factor binding sites have been identified for the 5'-region of *ABCC2* (4, 5). However, none of the identified polymorphisms in our study population were found at any of these sites. An updated search of transcription factor binding sites was performed using the web based matrices TFBlast and TESS to probe the identities of the bound proteins. Differential binding of nuclear proteins can be a possible explanation for the decrease in promoter activity seen in the reporter gene assay. Several hits were reported for the variant alleles at the -1065 and -1292 loci (Table 3.4) but no putative transcription factors were identified for the -1549 sequence when using TFBlast (http://www.gene-regulation.com) or TESS. Probe sequences were also run in MatInspector (Genomatix SoftwareGmbH; http://www.genomatix.de), which found similar putative transcription factor binding sites for the -1292 and -1065 sites, and came up with a single hit for -1549A (Table 3.4).

3.3.5 Protein Identification and Isolation

Nuclear proteins highly expressed in the liver were further analyzed by competition assays using consensus sequences for putative transcription factors. Nuclear protein bound to the -1065 variant probe was competed with consensus sequences for Nuclear Factor 1 (NF-1) and C/EBP (Figure 3.7A). The consensus sequence for C/EBP was shown to bind the α, β, and δ isoforms (14). Competition with these sequences did not inhibit binding of the protein to the -1065 variant probe. The -1292 variant probe was competed with consensus sequences for NF-1, C/EBP, and Activator Protein 3 (AP3). Of these three proteins, C/EBP appeared to decrease binding of the nuclear protein to the

Table 3.4 Predicted Transcription Factors

Nucleotide Locus	Allele	Putative Transcription Factor
-1065[≠]	A	C/EBPβ
	A	C/EBPα
	A	MAF
	A	NF-1
-1292[≠]	G	MBF-1
	G	C/EBPα
	G	NF-1
	G	NF-IL6
	G	AP-3
	G	C/EBP
	G	NF-1/L
-1292[§]	A	TG-interacting factor belonging to TALE class of homeodomain factors
	A	Meis1b and Hoxa9 form heterodimeric binding complexes on target DNA
-1549[§]	A	FAST-1 SMAD interacting protein

[≠]Putative transcription factor binding proteins were predicted using TFBlast and TESS

[§] Putative transcription factor binding proteins predicted by MatInspector

C/EBPα/β, CCAAT-enhancer binding protein α/β; MAF, musculoaponeurotic fibrosarcoma; NF-1, nuclear factor 1; MBF-1, multiprotein bridging factor 1; NF-IL6, nuclear factor interleukin 6; AP-3, activator protein 3; NF-1/L, nuclear factor 1-L; FAST-, foxhead activin signal transducer 1; SMAD, mothers against DPP

-1292 variant probe (Figure 3.7B). To further test this result, primary antibodies for C/EBP α, β, δ and γ were incubated with nuclear extracts prior to the addition of the radiolabeled -1292 or C/EBP consensus probes. Of the four antibodies, the antibody for the β isoform of C/EBP showed a supershift in the protein-DNA band in the C/EBP consensus sequence (Figure 3.8). In contrast, there was no supershift detected when antibodies were incubated with the -1292 variant probe.

To identify the protein binding to the -1549 variant probe, a different approach was taken after initial analysis found no putative transcription factor binding sites. An attempt was made to isolate and purify the bound protein through affinity bead

purification. Nuclear extracts were separated by size through a size exclusion chromatography column containing a Sephacryl S-300 matrix. Fractions were monitored by spectrophotometry to determine eluates containing protein (Figure 3.9). Fractions containing proteins were tested by EMSA to determine which fractions contained proteins that were bound to the -1549A probe (Figure 3.10). These fractions were combined and incubated with magnetic beads conjugated with the -1549A probe. Bound protein was pulled down using a magnet and run on an SDS-PAGE gel. The size of the bound protein appeared to be around 10 kDa (Figure 3.11). Unfortunately, the low quantity of protein isolated using this method was not sufficient for use in sequence analysis by mass spectrometry.

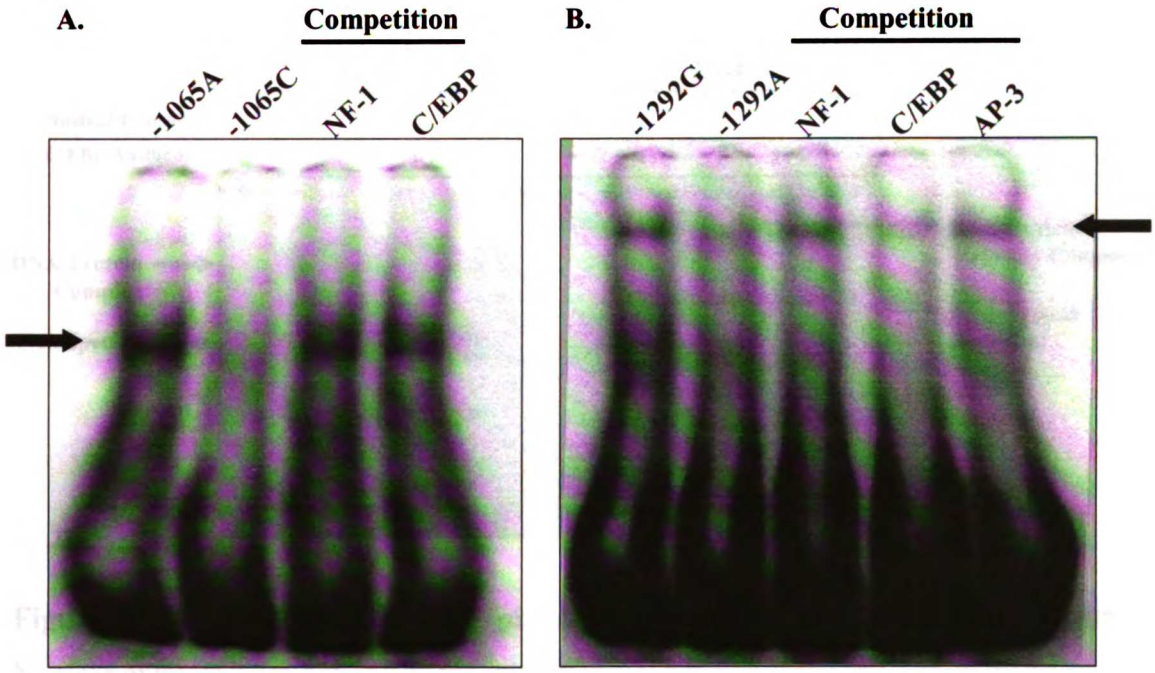

Figure 3.7 Competition studies using consensus sequences for putative transcription factors. A) Unlabeled consensus sequence oligos for NF-1, and C/EBP were used to compete against the -1065A variant probe to probe the identity of the bound nuclear protein. B) Unlabeled oligos for NF-1, C/EBP, and AP-3 were used to compete against the -1292G variant probe to probe the identity of the bound nuclear protein. The arrows indicate the respective DNA-protein complexes.

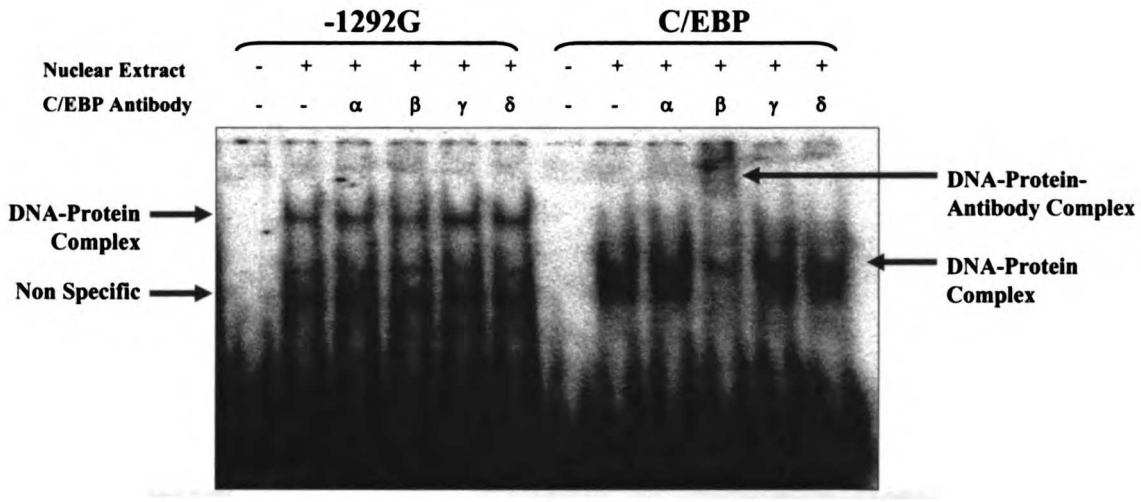

Figure 3.8 Supershift assays using primary antibodies for four isoforms of C/EBP. Nuclear extracts were pre-incubated with primary antibodies for the different isoforms of C/EBP before the addition of the radiolabeled -1292G variant probe or the consensus sequence for C/EBP. DNA-protein complexes are indicated by a black arrow. A supershift band representing the DNA-protein-antibody complex is indicated by an arrow in the C/EBP lane. Non-specific binding of proteins to the -1292G probe is indicated.

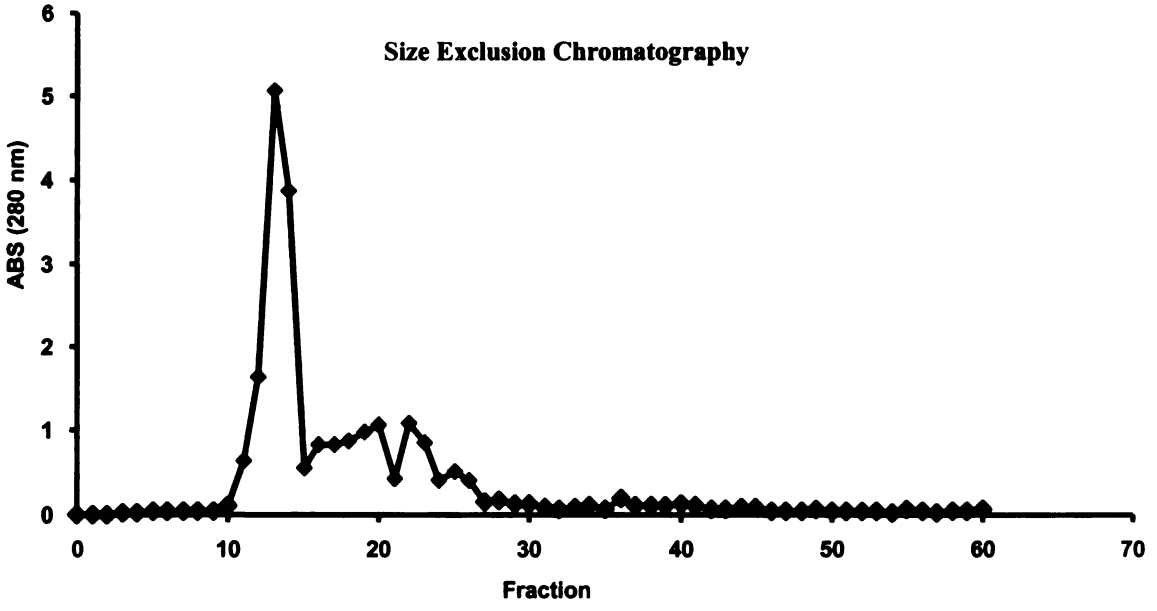

Figure 3.9 Chromatogram showing the protein content of fractions collected from a size exclusion column. Protein abundance was measured by absorbance at 280 nm. Each fraction contained 2 ml in volume.

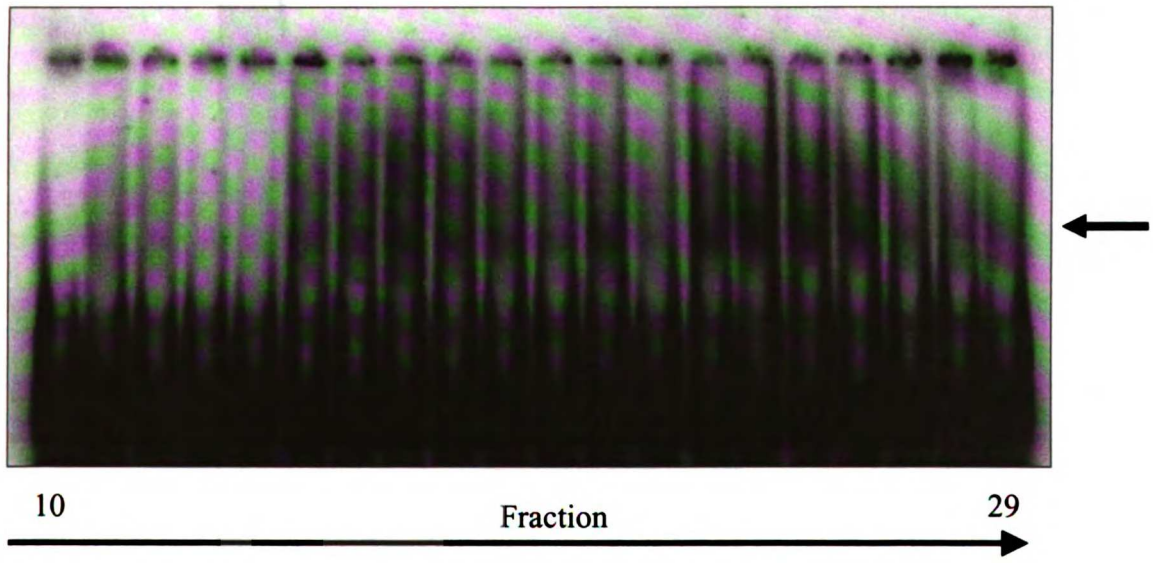

Figure 3.10 Binding proteins in column fractions. Fractions that showed a UV absorbance at 280 nm were probed with the radiolabeled -1549A oligo to determine which could bind to the DNA. Fractions 15-26 gave a DNA-protein complex indicated by the arrow.

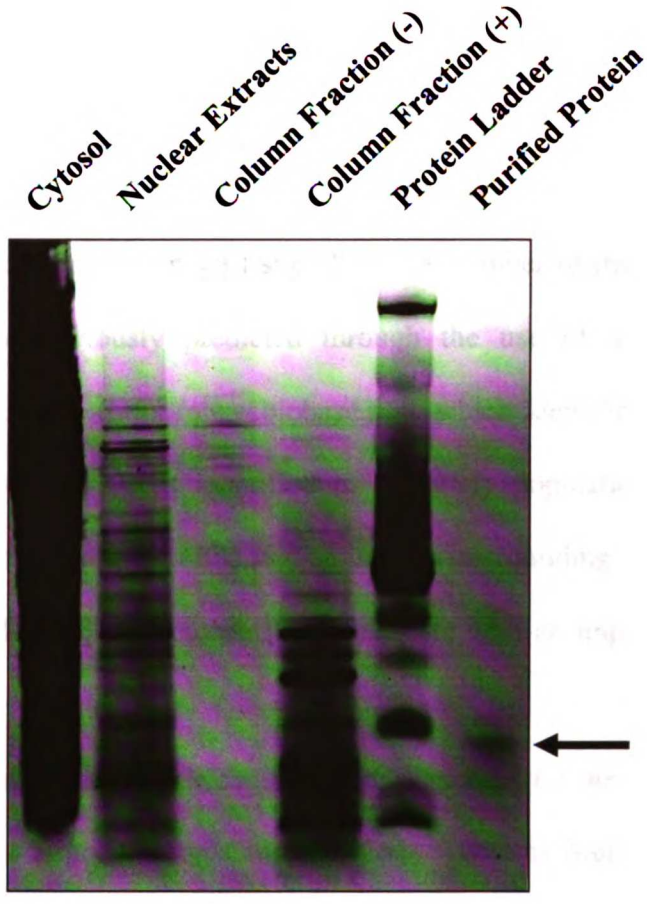

Figure 3.11 Western blot of proteins visualized by silver staining. Each lane contains proteins from 1) cytosolic extracts, 2) nuclear extracts, 3) column fractions negative for the -1549A DNA-protein complex, 4) column fractions positive for the -1549A DNA-protein complex, 5) protein ladder, and 6) affinity purified protein. The arrow indicates the isolated protein believed to be binding the -1549A sequence.

3.4 DISCUSSION

3.4.1 Characterizing Promoter Polymorphisms

This study closely examined the variations discovered in the 1.6 kb region of *ABCC2*. This region contains what is believed to be the core promoter and possible proximal regulatory sequences. Previous work characterizing the 5'-region of *ABCC2* had identified what is believed to be the potential core promoter, roughly 300-500 bases upstream from the translation start site (4, 5). A number of transcription factor binding sites were also previously predicted through the use of algorithms based on the TRANSFAC database (http://www.biobase.de/), which identifies predicted binding sites (15). There were 13 SNPs identified in the study population, with a few showing possible transcription factor hits. The region surrounding the core promoter was completely void of any polymorphisms, consistent with an important functional role for this area.

To determine nucleotide conservation, sequences for the 5'-regions for human, rat and mouse were aligned using ClustalX (16). There is high nucleotide conservation around the 5'-UTR. This region is transcribed into the mRNA and may be important for mRNA stability and/or protein translation. Less sequence conservation, however, was found in the rest of the 1.6 kb promoter sequence. The chimpanzee sequence for *ABCC2* was obtained by a BLAT search (http://www.genome.ucsc.edu) to determine the ancestral allele. There were thirteen mismatches between the chimpanzee and human sequences, three of which were polymorphic in the human sequence (Figure 3.12). Of the three human polymorphisms, the -1549G>A and -1019A>G revert back to the

chimpanzee allele indicating that the A and G alleles are the ancestral alleles at these two sites.

3.4.2 Promoter Activity Analysis

To characterize the promoter polymorphisms, a reporter gene assay was used to detect promoter activity. Single variant plasmids that showed decreased activity, also showed decreased activity when they were analyzed in haplotype context. The haplotype containing the -1549G>A, -1019A>G, and -24C>T variants had more than a 50% decrease in activity compared to the reference sequence. Individually, only the -1549G>A SNP showed decreased activity suggesting this variant may be the cause of the altered promoter activity in this common haplotype. To date, only the -24C>T variant has been studied in real tissues, and duodenal enterocytes showed no difference in mRNA expression between samples that were homozygous reference, heterozygous, and homozygous variant (17). This was similar to our *in vitro* data when examining the -24C>T variant alone. Data presented here suggest that single nucleotide polymorphisms can affect promoter activity in an artificial system. This could lead to decreased protein expression and decreased elimination of substrates. Several reduced function SNPs and haplotypes are found at high frequency in the population and can thus have an impact on response to MRP2 drug substrates. Individuals with reduced promoter activity variants, and thus less protein, will see an increase in substrate drug concentrations in the liver as fewer drugs are moved into the bile for elimination. With the accumulation of drug in the liver and systemically, these individuals will have an increased likelihood of adverse drug effects.

3.4.3 Transcription Factor Analysis

Of the SNPs that showed decreased function, three showed altered protein binding in gel shift assays. The -1065C>A, -1292A>G, and -1549G>A variant alleles showed altered protein binding compared to the corresponding reference alleles. Competition assays with both unlabelled variant and reference alleles revealed the specificity at which these proteins bind to the radiolabelled variant probe. In all three cases, unlabeled probes carrying the variant allele competed better with the radiolabelled probe than did the unlabeled probes carrying the reference allele.

To identify the bound protein, sequences flanking the variant site were analyzed for potential transcription factor binding sites through the use of TESS and TFBlast. These algorithms are rather limited in identifying putative transcription factors in humans, as they are based on data from other species. However, they provide a good starting point for trying to identify potential transcription factor binding sites. A number of hits were found using the TF search engines for the -1065C>A and -1292A>G variant sites, but none were found for the -1549G>A site. Use of a third search engine, MatInspector, found a single hit for the -1549A site.

For both the -1065C>A and -1292A>G variant sites, competitions were done with unlabelled consensus sequences for putative liver specific transcription factors. None of the consensus sequences tested were able to compete with the radiolabelled -1065C>A probe. However, for the -1292A>G probe the consensus sequence for C/EBP decreased binding of the probe to an unknown protein. Supershift assays using primary antibodies for the C/EBP isoforms did not support binding of the -1292G probe to any of the C/EBP

isoforms. There are several reasons for this discrepancy. Non-specific binding may account for the competition by C/EBP sequence with the -1292G probe. It also could be possible that the antibodies used were not working properly.

To further probe the identity of the protein bound to the -1549A probe, affinity chromatography was used to isolate protein for mass spectrometry. Using Dynabeads™, magnetic beads bound to streptavidin, biotinylated probes were constructed and conjugated to the beads. The conjugated beads were then used to pull down nuclear proteins bound to the -1549A probe. A small protein roughly 10 kDa in size was isolated and EMSAs using the isolated protein and radiolabelled probe showed the same banding pattern as before. Unfortunately, the amount of protein isolated, was not enough for further analysis using mass spectrometry (MS). A larger scale isolation project needs to be performed so that more protein can be isolated for MS identification.

3.5 PERSPECTIVES

The findings here represent a small window into the role of how variants found in the 5'-region of *ABCC2* can affect gene expression. Although data in this study suggest that several promoter variants show decreased activity, these data must be viewed with caution. The use of an in vitro system may not represent the complexities of an in vivo system. There are numerous gene-gene, gene-protein, and/or gene-environment interactions that are not accounted for in a controlled *in vitro* system. In this study, human hepatoma cells (HepG2) were used for the *in vitro* assays. This cell line may provide results that differ from other cell lines due to variations in cell machinery. Nevertheless, the data presented suggest that non-coding SNPs in *ABCC2* may play an

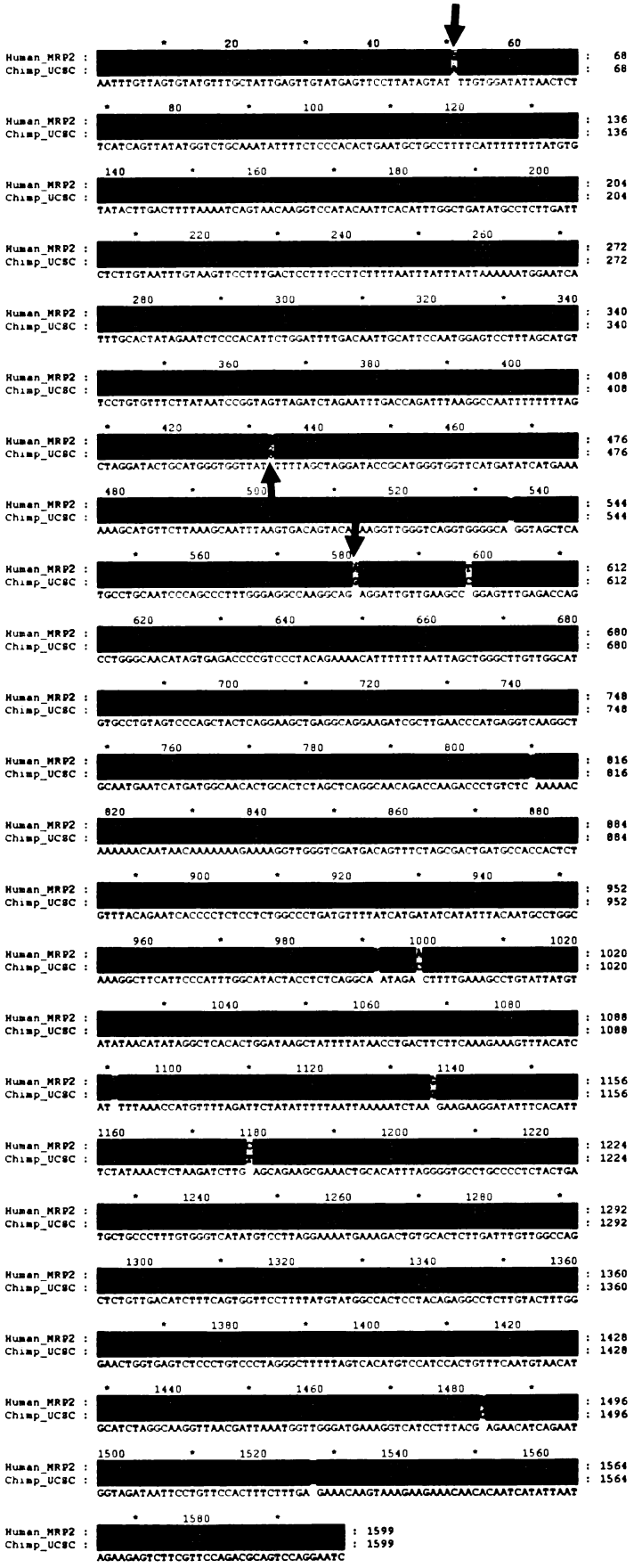

Figure 3.12 Alignment of 5'-region of *ABCC2* in human and chimp. There was high conservation in the nucleotide sequence between human and chimp. Shown in grey are differing nucleotides between the two sequences. Arrows indicate polymorphic sites in human *ABCC2*. Arrows above the alignment indicate polymorphic sites that revert back to the chimp allele.

important role in the variability in drug response. No variations were identified in the basal promoter of *ABCC2* suggesting the importance of the basal expression of MRP2 for detoxification. It is important to note that several SNPs had no effect on promoter activity individually, but showed decreased activity when in haplotypes. This emphasizes the importance of using haplotypes when studying the effects of genetic polymorphisms, as there may be interactions not fully understood that can produce an effect. For example, a non-functional coding region variant that has no known protein alteration may be explained by the fact that it is in a haplotype with promoter variants that show decreased function.

The identity of nuclear proteins bound to variant MRP2 promoters has yet to be elucidated. Further studies are needed to determine the identity of these proteins and to understand how they interact with DNA to regulate gene transcription. The C/EBP family of transcription factors is predicted multiple times as possible nuclear proteins binding to the variant sites and may play a role in *ABCC2* promoter activity. The C/EBP isoforms are expressed in many tissues, including the liver, and share substantial sequence identity (18). C/EBPβ had been shown to transactivate *ABCB1* (19), a member of the same gene superfamily as *ABCC2*.

Cis-acting elements can span the entire length of the gene, and this study only touched a small segment of the gene. This study focused on the core promoter and sequences proximal to this region. There are potentially thousands of nucleotides upstream that contain regulatory regions that have yet to be studied and there is increasing evidence that the 3'-UTR contains cis-acting elements that are important in post-transcriptional control of gene transcription (20).

The role of MRP2 is clear in the liver, acting as a detoxifying transporter to remove endogenous and exogenous compounds into the bile for elimination. A defect in this mechanism leads to Dubin-Johnson syndrome (21-25). MRP2 has also been implicated in cancer resistance. Recently, MRP2 was shown to be overexpressed in glioma cell lines leading to the resistance to topoisomerase II inhibitors (26). The mechanism of overexpression, however, has yet to be determined. Recent studies of promoter polymorphisms in *ABCB1* have associated them with resistant leukemia (27). It is possible for promoter polymorphisms in *ABCC2* to have a similar effect. Further analyses of polymorphisms in regulatory regions are necessary to fully understand how they may affect the expression of *ABCC2*, drug response in tumors, and drug disposition.

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CHAPTER 4

ALLELIC IMBALANCE OF *ABCC2* mRNA

4.1 INTRODUCTION

Irinotecan is a topoisomerase I inhibitor used in the treatment of colorectal cancer (1). Approximately 20-30% of patients receiving this drug suffer from severe diarrhea and myeloid suppression (2). Irinotecan is administered by IV infusion and can undergo metabolism in the bloodstream to form its active metabolite SN-38. SN-38 can then cause the destruction of white blood cells leading to myeloid suppression. Irinotecan, however, is mainly metabolized in the liver to its active metabolite SN-38. SN-38 can be further metabolized into its conjugated form, SN-38 glucuronide (SN-38G) by the uridine diphosphate glucuronosyltransferase 1A1 isoform (UGT1A1). Irinotecan and its metabolites SN-38 and SN-38G have been shown to be substrates for a number of efflux transporters in the liver, including P-glycoprotein and MRP2 (3-5) and are transported out into the bile where they can exert their gastrointestinal effects (Figure 4.1).

UGT1A1 polymorphisms have been identified and their functional implications have been described (6). Several variations have been reported in *UGT1A1* in patients with Gilbert's syndrome, a disorder that is characterized by the accumulation of serum bilirubin (7). A TA repeat in the promoter region of *UGT1A1*, $(TA)_7TAA$, showed lower glucuronidation rates than the reference $(TA)_6TAA$ in human liver microsomes (8). Further analysis showed that patients homozygous for the $(TA)_7$ repeat had a higher occurrence of grade 4 neutropenia following irinotecan treatment than those with the $(TA)_6$ repeat (9, 10).

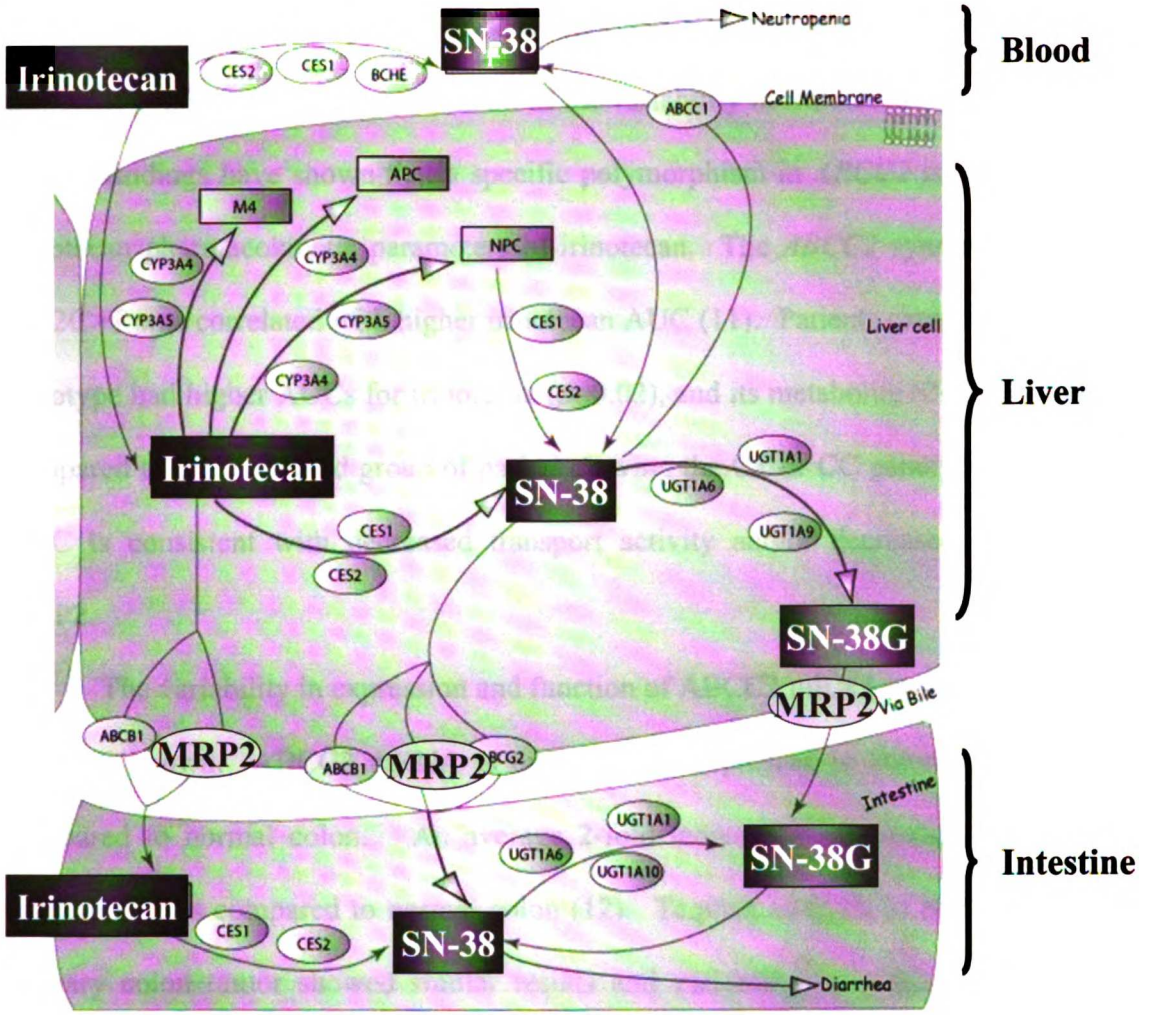

Figure 4.1 Irinotecan pathway. The diagram depicts irinotecan disposition in the blood stream, liver and intestine. Shown are enzymes and transporters involved in the metabolism and transport of irinotecan and its metabolites in the liver and intestine. CYP3A4, Cytochrome P450 3A4; CYP3A5, Cytochrome P450 3A5; CES1 & 2, carboxylesterase 1 and 2; BCHE, butyrylcholinesterase, UGT1A1, UGT1A6, UGT1A9, UGT1A10, isoforms of UDP-glucuronosyltransferase 1 family; ABCB1, ATP-binding Cassette subfamily B member 1 (P-glycoprotein); YEAR>, ATP-binding Cassette subfamily G member 2 (MXR); MRP2, multidrug resistance-associated protein 2; SN-38, M4, APC, & NPC are metabolites of irinotecan; SN-38G is the glucuronidated form of SN-38. This figure is reproduced from the Pharmacogenetics and Pharmacogenomics Knowledge Base (http://www.pharmgkb.org).

Although polymorphisms in *UGT1A1* are strongly associated with irinotecan disposition, this does not account for all of the variability in irinotecan pharmacokinetics. Recent findings have shown that a specific polymorphism in *ABCC2* is associated with irinotecan pharmacokinetic parameters of irinotecan. The *ABCC2* synonymous variant 3972C>T was correlated with higher irinotecan AUC (11). Patients carrying the 3972TT genotype had higher AUCs for irinotecan (p=0.02), and its metabolite SN-38G (p<0.001) compared to the combined group of patients having the CT or CC genotype. The higher AUC is consistent with decreased transport activity and/or decreased expression of MRP2.

The variability in expression and function of ABCC2 mRNA and MRP2 protein is not well known. ABCC2 mRNA is differentially expressed in colorectal carcinomas compared to normal colon. An average 2-fold increase in expression was found in cancerous cells compared to normal colon (12). Taqman analysis of healthy colon and primary colon tumor showed similar results and variability in ABCC2 mRNA levels within 34 healthy colon samples was more than 20-fold [Gow and Kroetz, unpublished results]. ABCC2 mRNA levels are also higher in term placenta compared to preterm (less than 37 weeks) placentas (13). Preterm placentas carrying either one or two copies of the *ABCC2* variant 1249G>A had reduced mRNA expression compared to placentas homozygous for the reference allele. Similar variability in MRP2 expression is predicted in the liver.

Allele specific expression, or allelic imbalance, is a method in which the influences of cis-acting elements can be detected by examining allelic abundance (14). An individual having no cis-acting regulatory variations will have both alleles of a gene

expressed equally. However, an individual who is heterozygous for a non-functional or reduced function cis-acting variation will have mRNA from each copy expressed at different levels (15). A polymorphism found in the mRNA transcript with no known regulatory effects can be used as an indirect marker for these cis-acting elements. mRNA abundance for each allele at the marker SNP is measured and normalized to genomic DNA, which theoretically should have equal amounts of each allele. This method has been used to indirectly measure the effects of regulatory regions on mRNA abundance in a number of different genes (16-20).

ABCC2 5'-promoter region variants were described in Chapter 3. A genetic analysis of the 5'-promoter and coding regions revealed that there was substantial linkage disequilibrium between the 3972C>T SNP and other *ABCC2* polymorphisms. These include the promoter SNPs -1549G>A, -1023G>A, -1019A>G and the 5'-UTR polymorphism -24C>T. Interestingly, several of these SNPs were associated with decreased promoter activity in vitro either as single nucleotide polymorphisms or in haplotypes. It is possible that the irinotecan pharmacokinetic changes associated with the 3972C>T SNP may be due to its linkage to the promoter SNPs.

In this study, the 3972C>T SNP in *ABCC2* was used as a marker SNP to determine if one allele is differentially expressed. We predict that the C allele will have greater expression than the T allele, since the T allele is in linkage disequilibrium with a reduced function promoter haplotype. Human livers heterozygous for the 3972C>T SNP were used to determine allele specific expression. In addition to the promoter SNPs, a highly linked SNP in intron 26 (-34T>C) and the coding SNP 1249G>A, which was

associated with reduced expression of ABCC2 mRNA in preterm placentas (13), were analyzed.

4.2 MATERIALS AND METHODS

4.2.1 Sample Population

Genomic DNA and mRNA from 200 liver samples were provided by Dr. Mary Relling at St. Jude Children's Research Hospital (Memphis, TN).

4.2.2 Genotyping

Genotyping was performed using a 5'-nuclease assay (21) and direct sequencing. The 5'-promoter variants -1549G>A, -1019A>G, and the UTR variant -24C>T, the coding region variant 1249G>A, the intron 26 variant -34T>C, and the synonymous variant 3972C>T were genotyped in the 200 liver samples. Briefly, PCR for direct sequencing was carried out after optimization using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA). Genomic DNA was denatured at 94°C for 20s, annealed at 61°C for 20s, and extended at 72°C for 45s. Ten cycles were performed, with the annealing temperature decreasing by 0.5°C after each cycle. This is followed by 35 cycles of 94°C for 20s, 56°C for 20s, and 72°C for 45s. PCR extension was done at 72°C for ten min followed by a 4°C hold. Dye terminator sequencing was performed using a 25 cycle reaction at 96°C for 10s, 50°C for 5s, and 60°C for 4 min. Analysis was done on an ABI 3700 automated sequencer using the ABI PRISM BigDye system. For Taqman 5'-nuclease sequencing, PCR and sequencing were performed in one reaction using specific primers for the variant of interest, either by Assays-on-Demand (-24C>T

and 3972C>T) (Applied Biosystems, Foster City, CA) or Assays-by-Design (primer/probes in Table 4.1). The Taqman reaction was started with a 10 min denaturation step at 95°C, followed by 40 cycles of a 2-step cycling at 92°C for 15s and 60°C for 1 min.

Table 4.1 Genotyping Primers and Probes

			Primer/Probe Sequences	
Variant	**Location**	**Fluor**	**Taqman Probe**	**Forward/Reverse Primer**
-1549G>A	Promoter	VIC	CTTATAGTAT**G**TTGTGGATATT	AGTGTATGTTTGCTATTGAGTTGTATGAGTT
		FAM	CCTTATAGTAT**A**TTGTGGATATT	TGTGGGAGAAAATATTTGCAGACCAT
-34C>T	Intron 26	VIC	TGAAAATCATCAT**A**GGCACAG	TGGTTTGAGTGGTTGAGTTGGTTT
		FAM	AAAATCATCAT**G**GGCACAG	GTTCAGGGTTTGTGTGATCTACAGA
1249G>A	Exon 10	VIC	CTGTTTCTCCAA**C**GGTGTA	CCAACTTGGCCAGGAAGGA
		FAM	ACTGTTTCTCCAA**T**GGTGTA	GGCATCCACAGACATCAGGTT
-24C>T	Promoter		Assay on Demand	
3972C>T	Exon 28		Assay on Demand	

		Sequencing Primers	
		Forward	**Reverse**
-1019A>G	Promoter	TCTCAGGCAAATAGAACTTTTGAA	TGTAATTTCTCACCCAAAAAGTAGA

Sequences are in the 5' to 3' direction. Bold, underlined letters represent the variant site. Probes were either fluorescently labeled with 6-FAM or VIC. Assays on Demand primer/probe sets were designed by Applied Biosystems and not disclosed.

4.2.3 Haplotype Analysis

Haplotype analysis for the five SNP sites was performed using PHASE 2.0 (22). Unambiguous haplotypes were first assigned before ambiguous haplotypes were estimated with PHASE. A haplotype was assigned if it occurred in seven of ten PHASE runs.

4.2.4 Reverse Transcription, PCR and Single Base Extension

RNA from human liver samples heterozygous for the 3972C>T SNP were selected for reverse transcription and further analysis. One microgram of RNA was reverse transcribed using the SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Following reverse transcription, approximately 100 bp surrounding the 3972C>T SNP site was PCR amplified from the resulting cDNA and genomic DNA (gDNA) using specific primers (Table 4.2). Excess dNTP and primers were removed from the amplified product by incubating in shrimp alkaline phosphatase (Promega, Madison, WI) and *ExoI* (New England Biolabs, Beverly, MA). Cleaned PCR products then underwent single base extension (SBE) using the SNaPshot mix (Applied Biosystems, Foster City, CA) and extension primers (Table 4.2). The dNTPs used in SBE contained a different fluorophore for each base. Allele abundance was measured by an ABI 3700 sequencer. Peak heights of each allele were recorded from the resulting chromatogram (Figure 4.2).

Table 4.2 Single Base Extension Primers

	Forward	Reverse
cDNA	CCTGGGTGACTGATAAGAGG	GATTCTGAAGAGGCAGTTTGTGAGG
gDNA	CCTGGGTGACTGATAAGAGG	CTCTGTCCAATTGTTGTTTGATC
SBE	CCTCAGAGGGATCACTTGTGACAT	

Forward and reverse primers amplified a 100 bp region surrounding the 3972C>T site. The reverse primer for gDNA is located in intron 28, while the reverse primer for cDNA is located in exon 29. The SBE primer is one base short of the 3972C>T site. cDNA, complementary DNA; gDNA, genomic DNA; SBE, single base extension.

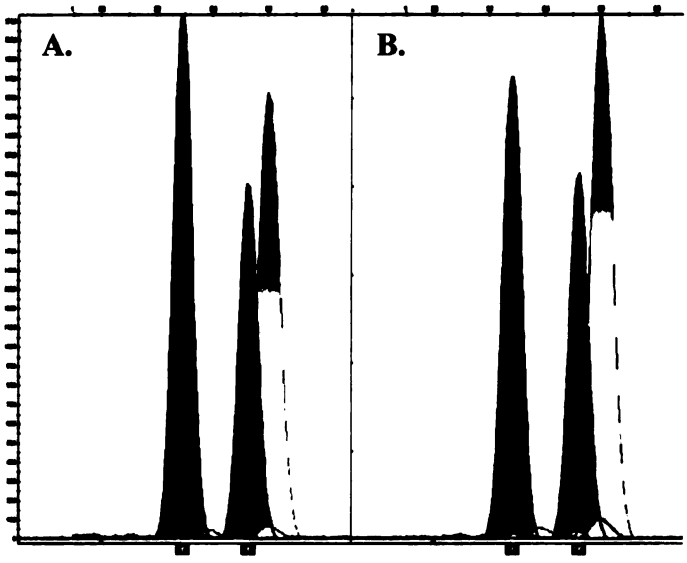

Figure 4.2 Sample chromatograms of single base extension analysis. Shown are allele traces for A) cDNA and B) gDNA. Black peaks represent the C allele at 3972, grey peaks represent the T allele, and gradient peaks are the internal standard. cDNA, complementary DNA; gDNA, genomic DNA.

4.2.5 Allele Specific Expression

An allelic imbalance ratio was quantified by the following equation:

$$R = \frac{C_1}{C_2} \Big/ \frac{G_1}{G_2}$$

where C_1 and G_1 are the peak heights of one allele in cDNA and gDNA respectively, and C_2 and G_2 are the peak heights of the other allele. The genomic DNA ratio was used as an internal normalization control to account for any fluorophore differences between the two alleles. To equalize the ratios in graphical space, the values were log-transformed. Log-transformed values were defined as follows:

$\log R = 0$; no preference for either allele

$\log R > 0$; preference for allele 1

$\log R < 0$; preference for allele 2

Table 4.3 Inferred Haplotypes

Number	Haplotype	Occurrence	Frequency
1≠	GACGTC	137	0.3425
2≠	AGTGTT	81	0.2025
3≠	GACATC	72	0.18
4≠	AGCGTT	53	0.1325
5≠	AGCGTC	19	0.0475
6≠	AGCGCC	14	0.035
7≠	GACGTT	12	0.03
8≠	GACGCC	5	0.0125
9	AGTGTC	2	0.005
10	AACGTC	2	0.005
11≠	GACATT	1	0.0025
12	AACGTT	1	0.0025
13	AACGCC	1	0.0025

Haplotypes inferred using PHASE2.0 for 200 liver samples. Listed are the haplotypes identified in the 200 liver samples and their frequencies. Haplotype numbers marked with ≠were found in the 86 heterozygous samples.

4.2.6 Statistical Analysis

Statistical analysis was done using GraphPad Prism Software (San Diego, CA) using a t-test when comparing log ratios to zero. Values were significant if $p<0.05$.

4.3 RESULTS

4.3.1 Genotyping and Haplotype Analysis

Of the 200 liver samples screened, 86 were heterozygous at the 3972 polymorphic site. Haplotype analysis of these 200 samples revealed that they were composed of 13 different haplotypes, 9 of which were found in the heterozygous population (Table 4.3). The heterozygous samples were comprised of 53 Caucasians, 11 African Americans, 9 Mexican Americans, and one Asian American. Twelve samples had no ethnic description. Samples were further segregated into haplotype pairs. There were 25

samples that carried haplotypes 1 and 2, twenty carried haplotypes 1 and 4, thirteen carried haplotypes 2 and 3, ten carried haplotypes 3 and 4, and seven carried haplotypes 3 and 7. Eleven samples carried lower frequency haplotype pairs and were not included in further analysis. Seven of those samples were of Caucasian American descent. Sample demographics and genotypes are listed in Table 4.4 for the 46 Caucasian liver samples that carried the more abundant haplotype pairs. These samples were used for allele specific expression. Five different haplotypes were represented in the 46 liver samples used for this analysis. Haplotype 2 was the only haplotype to carry the three promoter SNPs that have decreased promoter activity (Chapter 3, Figure 3.4B). Haplotype 7 differed from the reference haplotype 1 at only the marker SNP. Haplotype 3 differed from reference by carrying the 1249G>A SNP. Haplotype 4 differed from haplotype 2 at the -24C>T position in the 5'-UTR (Figure 4.3).

4.3.2 Allele Specific Expression Analysis

To assay for allelic imbalance, preliminary studies utilized only the 53 Caucasian samples. Of these, 46 carried one of the haplotype pairs mentioned above. The remaining 7 livers carried low frequency haplotypes. Five liver RNA samples could not be reverse transcribed and they were removed from allelic imbalance analysis. Overall, there was a statistically significant preference for the 3972C allele compared to the 3972T allele (log ratios > 0, $p < 0.0001$) (Figure 4.4, Table 4.5). When examining haplotype pairs, haplotype 1/2 and haplotype 2/3 showed a significant preference for the C allele. Samples carrying haplotypes 2/3 showed the highest mean C/T ratios of all haplotype combinations ($p<0.0001$). In these samples, the C allele was 14% higher in

abundance than the T allele. There was no statistically significant difference between any of the haplotype groups when analyzed by the student Newman-Keuls multiple comparison test.

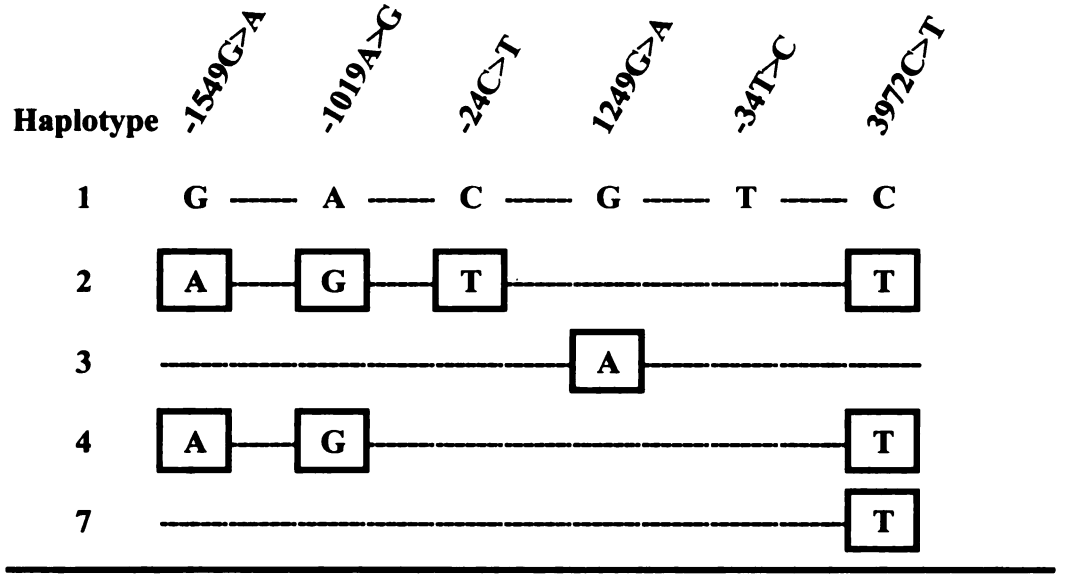

Figure 4.3 The 5 haplotypes represented in the 41 Caucasian liver samples used for allele specific expression. Haplotype 1 represents the reference sequence. Boxed letters represent the nucleotide change at that position.

Table 4.4 The Genotypes of the 46 Caucasian Liver Samples Used for Allele Specific Expression.

Sample	-1549G>A	-1019A>G	-24C>T	1249G>A	-34T>C	3972C>T	Haplotypes		Gender	Age
1	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	16
2	G/A	G/A	C/T	G/G	T/T	C/T	1	2	female	3
3	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	63
4	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	63
5	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	37
6*	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	28
7	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	72
8	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	54
9	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	22
10	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	44
11	G/A	G/A	C/T	G/G	T/T	C/T	1	2	female	62
12	G/A	G/A	C/T	G/G	T/T	C/T	1	2	female	7
13	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	48
14	G/A	G/A	C/T	G/G	T/T	C/T	1	2	female	66
15	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	56
16	G/A	G/A	C/T	G/G	T/T	C/T	1	2	male	17
17	G/A	G/A	C/C	G/G	T/T	C/T	1	4	female	8
18	G/A	G/A	C/C	G/G	T/T	C/T	1	4	female	4
19*	G/A	G/A	C/C	G/G	T/T	C/T	1	4	male	30
20	G/A	G/A	C/C	G/G	T/T	C/T	1	4	female	75
21	G/A	G/A	C/C	G/G	T/T	C/T	1	4	male	62
22	G/A	G/A	C/C	G/G	T/T	C/T	1	4	female	66
23	G/A	G/A	C/C	G/G	T/T	C/T	1	4	female	56
24*	G/A	G/A	C/C	G/G	T/T	C/T	1	4	male	6

25	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	70
26	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	59
27	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	43
28	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	60
29	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	21
30	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	73
31	G/A	G/A	C/T	G/A	T/T	C/T	2	3	female	6
32	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	53
33	G/A	G/A	C/T	G/A	T/T	C/T	2	3	male	46
34*	G/A	G/A	C/T	G/A	T/T	C/T	2	3	female	64
35	G/A	G/A	C/C	G/A	T/T	C/T	3	4	male	2
36	G/A	G/A	C/C	G/A	T/T	C/T	3	4	male	66
37	G/A	G/A	C/C	G/A	T/T	C/T	3	4	male	29
38	G/A	G/A	C/C	G/A	T/T	C/T	3	4	female	61
39	G/A	G/A	C/C	G/A	T/T	C/T	3	4	male	28
40	G/A	G/A	C/C	G/A	T/T	C/T	3	4	male	62
41	G/G	A/A	C/C	G/A	T/T	C/T	3	7	female	59
42*	G/G	A/A	C/C	G/A	T/T	C/T	3	7	female	32
43	G/G	A/A	C/C	G/A	T/T	C/T	3	7	female	43
44	G/G	A/A	C/C	G/A	T/T	C/T	3	7	male	46
45	G/G	A/A	C/C	G/A	T/T	C/T	3	7	female	68
46	G/G	A/A	C/C	G/A	T/T	C/T	3	7	male	63

Shown are the base pairs at each polymorphic site along with the inferred haplotype. Haplotype numbers correspond to those in Table 4.3. Gender and age are presented for each liver sample. Asterisks represent samples that were removed from analysis because of poor quality RNA. -1549G>A and -1019A>G are in the 5'-promoter region, -24C>T is in the 5'-UTR, -34T>C is in intron 26, and 1249G>A and 3972C>T are in exons 10 and 28, respectively.

Table 4.5 Statistical Analysis of the Allele Specific Expression Data.

	All	Hap 1/2	Hap 1/4	Hap 2/3	Hap 3/4	Hap 3/7
Number of samples	41	15	5	9	7	5
Minimum	-0.062	-0.013	-0.062	0.032	-0.048	-0.013
25% Percentile	0.007	0.005	-0.035	0.038	0.004	-0.007
Median	0.041	0.023	0.008	0.051	0.064	0.063
75% Percentile	0.068	0.067	0.078	0.076	0.072	0.101
Maximum	0.150	0.150	0.103	0.095	0.074	0.134
Mean	0.041	0.038	0.019	0.056	0.038	0.050
Std. Deviation	0.045	0.045	0.062	0.023	0.046	0.059
Lower 95% CI of mean	0.027	0.013	-0.059	0.038	-0.005	-0.024
Upper 95% CI of mean	0.055	0.063	0.096	0.074	0.080	0.124
One sample t test						
Theoretical mean	0.00	0.00	0.00	0.00	0.00	0.00
Actual mean	0.041	0.038	0.019	0.056	0.038	0.050
95% CI of discrepancy	0.027 to 0.055	0.013 to 0.063	-0.059 to 0.096	0.038 to 0.074	-0.005 to 0.080	-0.024 to 0.124
t	5.908	3.31	0.6731	7.228	2.181	1.887
df	40	14	4	8	6	4
P value (two tailed)	<0.0001	0.0052	0.5378	<0.0001	0.0719	0.1323

All haplotypes were compared to the theoretical value of zero, representing no difference in abundance for either allele. P values < 0.05 were considered significant.

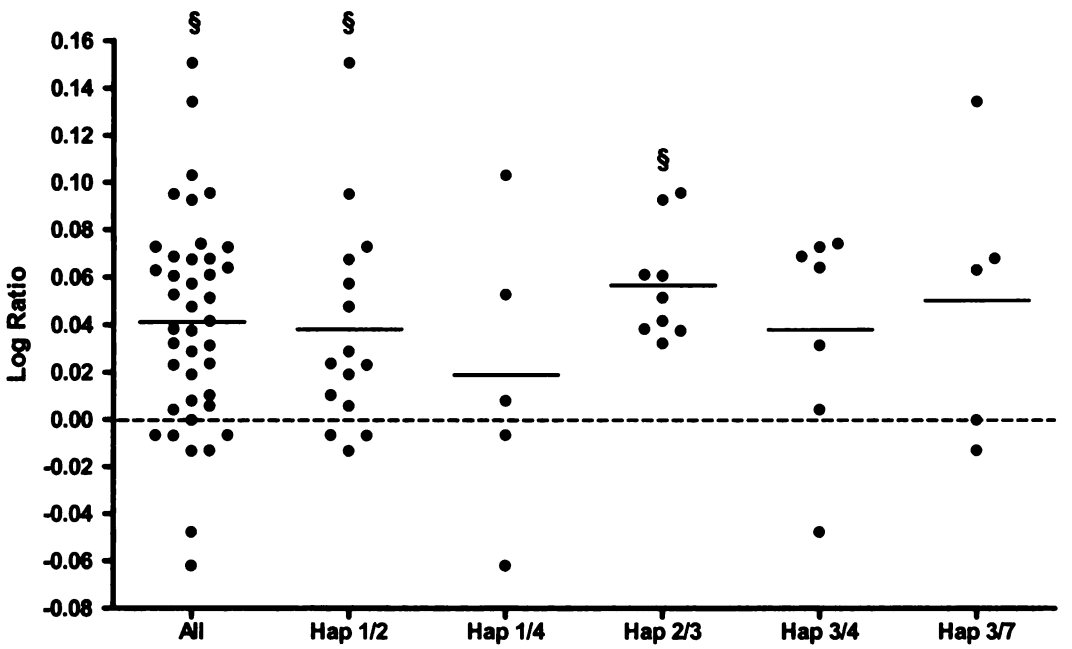

Figure 4.4 Allele specific expression ratios for 41 Caucasian liver samples. The dashed line represents a ratio where the C allele and T allele are equal in abundance. Lines for each group represent the mean values. All, all 41 liver samples. Hap X/Y represents liver samples carrying one copy of each haplotype. Columns with § were found to be significantly different from 0 using a t-test ($p<0.05$).

4.4 DISCUSSION

The *ABCC2* synonymous SNP 3972C>T was associated with higher AUC in patients receiving the anti-cancer drug irinotecan (11). This altered AUC would suggest that these individuals carried reduced function MRP2 or were expressing less of the transporter in the liver where irinotecan is mainly metabolized. The 3972C>T SNP had previously been shown to be in linkage disequilibrium with several cis-acting *ABCC2* promoter SNPs. These SNPs showed lower promoter activity compared to a reference sequence (Chapter 3).

An allelic imbalance occurs when one allele in a heterozygous sample is in higher abundance than the other. There are several possible scenarios that can account for this finding. mRNA from one allele can be degraded faster than the mRNA from the other allele. Alternatively, there can be a difference in transcription between the two alleles. In this study the 41 liver DNA and RNA samples showed that at the mRNA level, there were differences in transcript abundance. mRNA containing the 3972C allele was approximately 10% higher in abundance than the 3972T allele. When the data was parsed into haplotypes, all haplotype pairs showed a preference for the 3972C allele. The highest ratio was found in samples containing the haplotype 2/3 pair. These samples showed a 14% difference between the C and T allele. There was no statistically significant difference between any of the groups studied, but this is likely due to the small number of samples per group. Increasing the sample size for each haplotype group may provide a clearer answer.

Liver samples carrying the reduced activity promoter SNPs (haplotype 2) showed a higher preference for the C allele than samples carrying no promoter SNPs (haplotypes 1 & 3). This supports the idea that the decreased promoter activity SNPs are affecting gene transcription and irinotecan disposition. There was also preference for the 3972C allele in haplotypes 3/7, in which haplotype 3 carries the C allele at 3972 and the variant A allele at position 1249. This contradicts data that the 1249G>A variant causes reduced expression of ABCC2 mRNA in preterm placentas (13). However, the sample size is small, and there may be other cis-acting elements in linkage disequilibrium with the 3972C>T site that may cause the C allele to be more abundant. These can be polymorphic sites further upstream from the promoter, in intronic sequences, or found in

the 3'-end of the gene. Recent studies suggest that polymorphisms in the 3'-UTR are important for mRNA localization and translation (23).

The 3972C>T SNP could itself be affecting mRNA translation or stability. The 3972C>T synonymous SNP affects the third position of a codon, which encodes for the amino acid isoleucine. It is possible that translation is affected based on the abundance of the anti-codon carrying the amino acid. For isoleucine, the most abundant anti-codon is UAG (24), which corresponds to an AUC codon (the C allele at 3972) on the mRNA. The major nucleotide at the third position in a codon has been shown in *E. coli* to be translated more quickly (25) resulting in higher expression of that allele. The level of mRNA stability could also be affected by having the C nucleotide, which contains an extra hydrogen bond compared to the T nucleotide.

4.5 PERSPECTIVES

The data presented here explored the mechanistic basis for the association between the *ABCC2* 3972C>T SNP with lower AUC for irinotecan and SN-38G. The data supported the hypothesis that the *ABCC2* promoter SNPs may result in decreased irinotecan and SN-38G transport, leading to higher AUC values. The C allele was shown to be 10% more abundant than the T allele in the sample population and was statistically significant for liver samples carrying haplotype 2. However, all haplotypes analyzed showed higher preference for the C allele compared to the T allele at nucleotide position 3972.

Further studies need to be done to determine if a 10% increase in abundance would account for the differences in irinotecan disposition and whether there are other

cis-acting elements contributing to the allelic imbalance. The 10% increase in allele abundance is lower than what has been seen in literature. Other studies have shown as much as a 4-fold increase in abundance for one allele over the other (15, 20), although the number of samples having that magnitude was low. Other studies showed no preference for either allele or a moderate 50% increase in abundance (26-28).

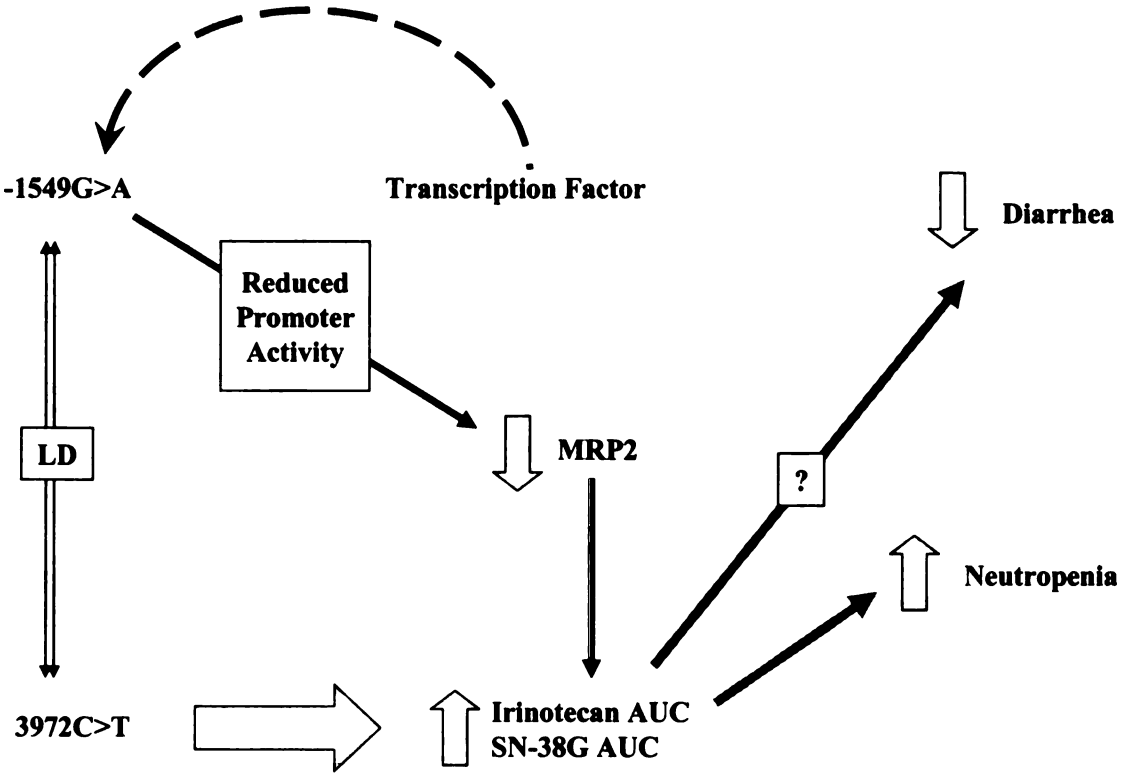

Figure 4.5 Schematic diagram linking irinotecan disposition and toxic effects to *ABCC2* SNPs. LD, linkage disequilibrium; MRP2, multidrug resistance-associated protein 2; AUC, area under the curve; SN-38G, glucuronide metabolite of irinotecan.

The connection between the promoter SNPs, the 3972C>T synonymous SNP and irinotecan disposition may be useful information for the clinical setting. Data presented

earlier in Chapter 3 showed that the -1549G>A variant binds an unknown nuclear protein. It has also been shown that this variant has decreased promoter activity in vitro. The nuclear protein binding -1549A thus is acting as a repressor of gene transcription. Less mRNA is transcribed that leads to less protein being made. Individuals who carry this variant and are given MRP2 substrates that are metabolized in the liver are then less able to excrete the drug into bile. In the case of irinotecan, less of the drug is eliminated into the bile leading to the accumulation of the drug in the liver and circulation, and increased incidence of neutropenia (Figure 4.5). Theoretically, there would be less incidence of diarrhea since less of the drug is being transferred into the intestine through the bile (Figure 4.3). This information may be useful for proper irinotecan therapy of patients carrying reduced function *ABCC2* SNPs, although larger studies are first needed to confirm these findings.

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CHAPTER 5

THE INFLUENCE OF GENETICS ON DIGOXIN PHARMACOKINETICS

5.1 INTRODUCTION

Drug response varies among individuals taking the same medication. A fraction of patients will experience the intended therapeutic response to the drug. However, there is a subset of people who will not respond to the drug or will suffer from an adverse reaction. Such variation in drug response can be contributed to by many factors controlling drug levels, including a person's genetics and the environment. Limited exposure to the drug leads to diminished response, while increased exposure can lead to toxic events (1).

The pharmacogenetics of drug disposition is currently an area of intense research. It is believed that as much as 20-95% of variability in drug disposition may be due to genetics (2). Most studies to date have focused on the effects of genetic polymorphisms on drug metabolizing enzymes. For example CYP2C9 genotypes have been associated with altered clearance of warfarin (3, 4) and genotype information has been used to manage the dosing of warfarin. Those with reduced function CYP2C9 are given lower doses of warfarin to compensate for the slower metabolism. Similarly, individuals carrying reduced function TPMT alleles have higher risk of severe toxicity when undergoing purine therapy (5). Limited information is currently available about the influence of genetic variations in drug transporters and their effects on drug disposition, and the relative contributions of genetics and environment to transporter regulation have not been demonstrated.

The classical method to determine whether the genetics of transporters contribute to variability in drug response is to study twins (6). Studies in identical twins (monozygotic, MZ) and fraternal twins (dizygotic, DZ) provide a unique resource for studying the relative importance of heredity and environment to a particular phenotype. MZ twins arise from a single fertilized egg and thus share 100% of their genes. DZ twins only share 50% of their genes since they develop from two fertilized eggs. If genetics plays an important role in the variability in drug response, then for MZ twins there is expected to be little variation, while the DZ twins will show higher variation. If the environment has a greater influence, then MZ twins would show the same variability in drug response as DZ twins. To determine the contribution of genetics to variability, the heritability index, which is a ratio that describes the amount of phenotypic variation that can be attributed to genetics, is calculated from data from both sets of twins (7).

Digoxin (Figure 5.1), a cardiac glycoside used in the treatment of heart failure and atrial fibrillation (8, 9), is a drug that is used to study the influence of transporters. Digoxin is minimally metabolized by cytochrome P450 enzymes, and is excreted unchanged into the urine (10). Recent studies have shown that the efflux transporter P-glycoprotein (P-gp) plays an important role in digoxin absorption, disposition and elimination. Digoxin is a substrate for P-gp (11-13) and P-gp disruption is associated with increased digoxin bioavailability in mice (14). Similar results were shown for other P-gp substrates in humans (15, 16). Digoxin is also a substrate for the human hepatic uptake transporter OATP1B3 (17) and the kidney transporter OATP4C1 (18). Rat studies show that Oatp1a4 is a high affinity transporter for digoxin (19). Given orally, 50-90% of the drug enters the systemic circulation depending on the formulation (20).

Digoxin treatment efficacy is maximal at a serum concentration of 0.8 – 2.0 ng/mL (20, 21), however toxicity has been seen at lower concentrations. Digoxin pharmacokinetics have been widely studied. For a digoxin dose between 0.25-0.5 mg, maximal plasma concentrations of digoxin were 1.8-2.5 ng/mL, renal clearance was 1-2.7 mL/min/kg, and oral clearance was 2.5-6 mL/min/kg (22-26). Linearity is observed between the dose and AUC, CL/F and C_{max}. Digoxin pharmacokinetics can also be influenced by drug-drug interactions and patient health (20).

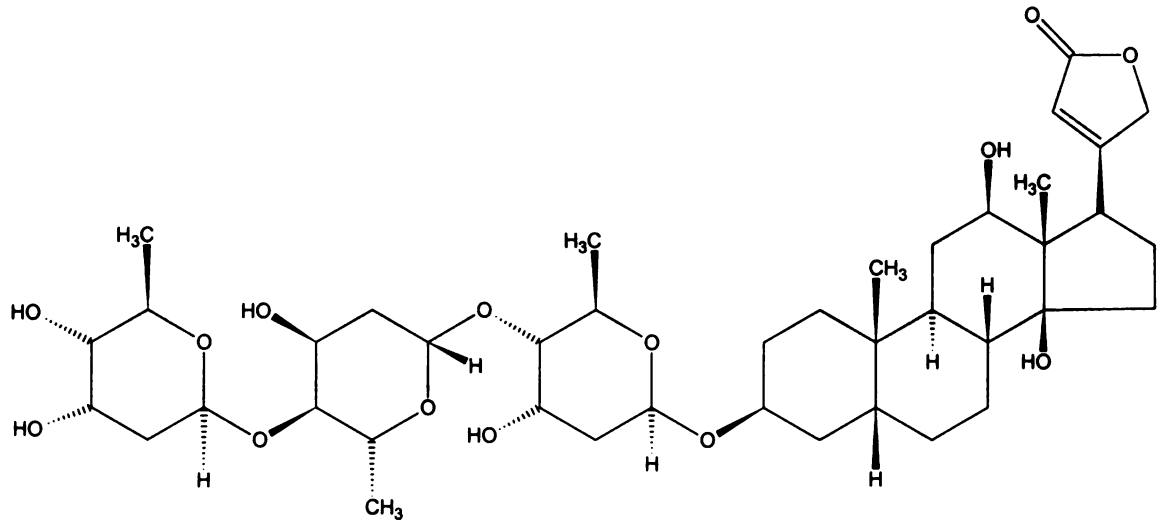

Figure 5.1 Chemical structure of digoxin.

The aim of the study presented here is to answer the basic question: How much does genetics contribute to digoxin pharmacokinetics? Since digoxin is not significantly metabolized and is a substrate for both uptake and efflux transporters in the intestine, liver and kidney, its pharmacokinetic variability may be the result of variability in

transporters. Digoxin will be used as a probe drug to determine the contribution of genetics to pharmacokinetic variability.

5.2 MATERIALS AND METHODS

5.2.1 Study Population

Ten monozygotic and ten dizygotic twin pairs were recruited by the Stanford Research Institute from the Northern California Twin Registry. All twins included in the study were in the range of 18-60 years old and provided written informed consent. All volunteers were in good health, as indicated by medical history and questionnaire, and normal kidney function was confirmed by measurement of plasma electrolytes and blood urea nitrogen (BUN). All participants abstained from medications, herbal remedies such as St. John's Wort, orange and grapefruit juice, alcohol, and caffeinated beverages throughout the study. Pregnant women and individuals with abnormal renal function or chronic disease were excluded from the study.

5.2.2 Study Design

After approval by the Committee on Human Research at the University of California San Francisco, the study was conducted at the UCSF GCRC in accordance with all local legal and ethical requirements. Within one week of the study, blood was drawn for BUN and electrolyte analysis and for DNA isolation. After an overnight fast twin pairs arrived at the General Clinical Research Center at Moffitt Hospital and female participants provided urine samples for a pregnancy test. Before the start of the study a catheter was placed into the forearm and a baseline blood sample was collected into five

10 mL vacutainer tubes. Lymphocytes were isolated from 40 mL of blood and plasma was separated from the remaining sample for baseline digoxin measurements. A baseline electrocardiogram (ECG) was performed by an attending physician to confirm normal PR interval. Afterwards a 1 mg dose of digoxin (four 0.25 mg tablets) was given to each twin. A physician was on hand to monitor the subjects' ECG and blood pressure for the first 2 hours post dose to assess any adverse reactions. After the 12 hour study period, the catheter was removed and subjects were allowed to go home. Blood and urine samples were collected at 24, 48, and 72 hours as described below.

5.2.3 Lymphocyte Isolation

A 40 mL blood sample from each subject was diluted 1:2 in calcium-magnesium free phosphate buffered saline. Aliquots of diluted blood (20 mL) were layered over 8 mL of room temperature Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) in a plastic 50 mL conical (Fisher Scientific, Fairlawn, NJ). Conicals were centrifuged in a Sorvall Legend (Thermo Electron Corp., Asheville, NC) at 400*g* for 20 minutes at 10°C. From each conical the buffy coat layer, the fuzzy layer between the red blood cells and plasma that contains the lymphocytes, was removed with a Pasteur pipette and placed into a new 50 mL conical. Isolated lymphocytes were diluted 1:2 with room temperature PBS and centrifuged again at 400*g* for 20 minutes. The PBS was carefully aspirated, and the lymphocyte pellet was washed a second time with 5 mL of PBS and centrifuged. The resulting pellet was resuspended in 8 mL of PBS supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Isolated lymphocytes were stored in 2 mL cryo-vials (Fisher Scientific, Fairlawn, NJ) and stored in the vapor phase of liquid nitrogen.

5.2.4 Blood Sampling and Urine Collection

After digoxin dosing, blood samples (10 mL) were obtained at 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 hours from the forearm catheter. The 24, 48, and 72 hour samples were collected by venipuncture at the subject's home or work by a phlebotomist. Blood samples were spun at 1500 rpm for 2 minutes within 2 hours of collection, and plasma was collected into 2 mL tubes. Urine was collected into sterile containers during the 0-12, 12-24, 24-48, and 48-72 hour intervals after digoxin dosing. Samples were kept cool either by refrigeration or placement in a cool dark space until they were aliquotted or picked up by the phlebotomist. Urine volume was recorded and 10 mL aliquots were taken from each fraction. Both plasma and urine samples were stored at -20°C until analysis.

5.2.5 Digoxin Concentration Analysis in Urine and Plasma Samples

Digoxin analysis was done by the Drug Studies Unit (University of California, San Francisco) using LC/MS methods. Briefly, samples were run on an LC/MS Micromass Quattro LC (Micromass, Manchester, UK) fitted with a 4.6 x 50 mm, 5 μm particle size C18 column (Thermo Electron, San Jose, CA). Samples were subjected to negative mode electrospray ionization (ESI) with a capillary voltage at -3.5 kV. Collision voltage and cone voltage were set at -48 V. The mobile phase was composed of 62:38 (v/v) methanol:water and the run time was 4.1 minutes. Unchanged digoxin, D3, was monitored from m/z 779.4 to 84.9. Oleandrin, the internal standard, was measured from m/z 575.40 to 531.20.

5.2.6 Pharmacokinetic Analysis

Noncompartmental analysis was performed using WinNonLin 4.0 (Pharsight Corporation, Mountain View, CA). The area under the plasma concentration-time curve (AUC) was calculated using linear/log trapezoidal rule over the 0-3, 0-72, and 0-infinity intervals. The extrapolated AUC was calculated as C_{last}/λ_z where C_{last} was the last measured concentration and λ_z represents the terminal elimination rate constant calculated as the slope of the terminal phase. Digoxin oral clearance (CL/F) was estimated individually as dose/$AUC_{0-\infty}$ The apparent volume of distribution (V_z/F) was calculated as the ratio of CL/F to λ_z. The amount excreted into urine (Ae_{0-72h}) was calculated individually as the digoxin concentration in urine times the volume of urine collected. The total excretion of unchanged digoxin, $fe_{0-\infty}$ was estimated as fe_{0-72h} • $AUC_{0-\infty}/AUC_{0-72h}$. Digoxin renal clearance (CL_R) was calculated by the ratio Ae_{0-72h}/AUC_{0-72h}. Time to maximal concentration (T_{max}) and the maximal concentration (C_{max}) were visually estimated from the concentration-time curve. Apparent nonrenal clearance was calculated by subtracting renal clearance from total oral clearance.

5.2.7 Concordance

A semi-quantitative measure of concordance was made by examining the coefficient of variation (CV) for a given PK parameter between twin pairs. As a more quantitative measure, the correlation coefficient (r) was used. Pharmacokinetic parameters from each twin pair were plotted against each other and the correlation coefficient was estimated from these plots by linear regression.

5.2.8 Heritability Calculations

The heritability for each pharmacokinetic parameter was calculated using the following formula:

$h^2 = (Variance_{DZ} - Variance_{MZ}) / Variance_{DZ}$ (27, 28)

Variance was calculated as the square of the standard deviation estimated for each twin pair. As a comparison, heritability was also calculated using the intraclass correlation. Heritability using intraclass correlation (r_{MZ} and r_{DZ}) can be calculated by Holzinger's formula:

$h^2_H = (r_{MZ} - r_{DZ}) / (1 - r_{DZ})$ (7)

or Falconer's equation:

$h^2_F = 2*(r_{MZ} - r_{DZ})$ (7, 29).

5.2.9 Statistical Methods and Power Analysis

Data resulting from the noncompartmental analysis are presented as mean ± SEM. Comparisons between mean values for MZ and DZ pharmacokinetic parameters were analyzed by ANOVA. Power analysis was calculated with the assumption that the means between MZ and DZ twin groups were equal but that the standard deviations between the two groups varied. Based on this assumption, the ten MZ and ten DZ twin pairs would have the power to detect a heritability index of 0.60.

5.3 RESULTS

5.3.1 Study Population

Ten monozygotic and ten dizygotic twin pairs were recruited for the twin study. No serious adverse events were reported although one subject experienced nausea and vomiting within the first hour after dosing. One MZ twin pair was non-compliant with urine collection, and their data was omitted from renal clearance calculations. One dizygotic twin pair was an outlier and as a precaution, PK parameters were calculated with and without their data. A demographic profile of the subjects is shown in Table 5.1. Overall, DZ twins were approximately 10 kg heavier and 10 years older than the MZ twins. A majority of the twins recruited were of Caucasian descent and female. The MZ twin group was comprised of 16 Caucasians and 4 Mexican Americans. The DZ twin group included 14 Caucasians, 2 Mexican Americans and 2 African Americans.

Table 5.1 Demographics of Twin Study Population

	Mean (± SD)	
	MZ	**DZ**
Age (yr)	32 (± 11)	41 (± 13)
Weight (kg)	61.8 (± 9.25)	71.9 (± 11.3)
Gender		
Male	2	5
Female	18	13
Ethnicity		
Mexican American	4	2
Caucasian	16	14
African American	0	2

Population demographics of monozygotic and dizygotic twins recruited for the study. A majority of participants were Caucasian. Age and weight are given as mean ± standard deviation. MZ, monozygotic; DZ, dizygotic

5.3.2 Pharmacokinetics of Oral Digoxin

Figure 5.2A depicts the mean digoxin concentration-time profiles of MZ and DZ twins after a single 1 mg oral dose of digoxin. There was no significant difference in the mean concentration-time profiles between MZ and DZ twin groups (unpaired t-test). A closer examination of the concentration-time profiles from zero to three hours showed similar results (Figure 5.2B). However, differences were noted in several individual twin pairs (Figure 5.3). Digoxin pharmacokinetic parameters (T_{max}, C_{max}, $AUC_{0\text{-}3h}$, $AUC_{0\text{-}72h}$, $AUC_{0\text{-}\infty}$, CL/F, $Ae_{0\text{-}72h}$, CL_R, λ_z, CL_{nr}, and $t_{1/2}$) for the MZ and DZ twin pairs are summarized in Table 5.2. The terminal elimination rate constant for DZ twins was lower than that of MZ twins and this was reflected in the lower oral clearance and longer half life, although this did not reach statistical significance.

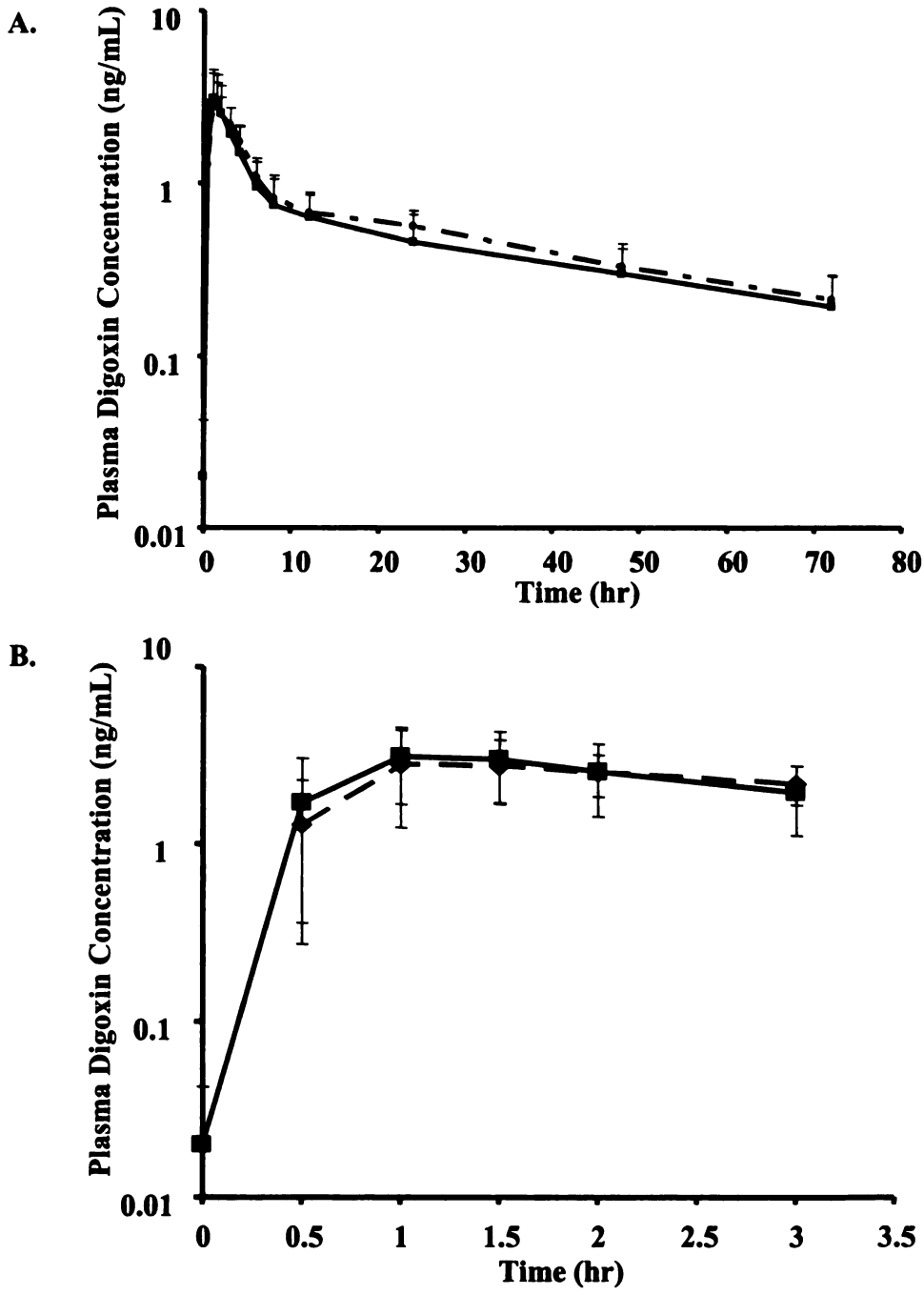

Figure 5.2 Semi-logarithmic plot of mean (± SD) plasma digoxin concentration-time profiles for monozygotic (dashed lines) and dizygotic (solid lines) twins after a 1 mg oral dose. Profiles were plotted over a A) 72 hour period and B) 3 hour period.

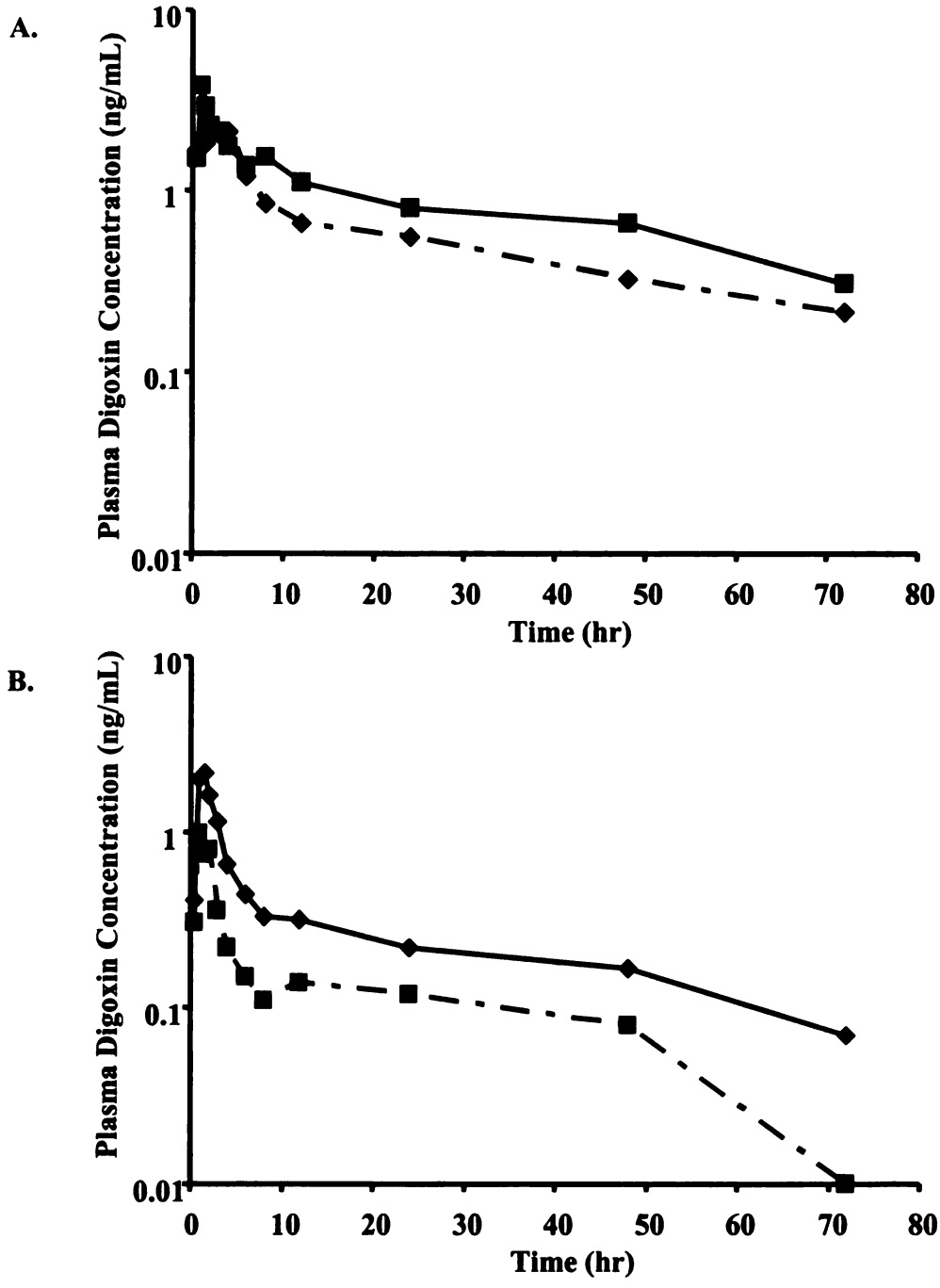

Figure 5.3 Discordant concentration-time curves between twin pairs. Semi-logarithmic plot of plasma digoxin concentration-time profiles for a single A) monozygotic and B) dizygotic twin pair after a 1 mg oral dose.

Table 5.2 Digoxin Pharmacokinetic Parameters

		Monozygotic Twins (Mean ± SD)	Dizygotic Twins (Mean ± SD)
CL/F	**(mL/min/kg)**	5.69 (± 1.36)	5.04 (± 1.93)
fe_{0-72h}	**(%)**	30.2 (± 9.7)	27.8 (± 5.9)
$fe_{0-\infty}$	**(%)**	38.2 (± 12.6)	36.8 (± 9.9)
T_{max}	**(hr)**	1.60 (± 0.84)	1.47 (± 0.70)
C_{max}	**(ng/mL)**	3.33 (±1.23)	3.65 (± 1.28)
CL_R	**(mL/min/kg)**	2.15 (± 0.51)	1.73 (± 0.49)
AUC_{0-3h}	**(hr•ng/mL)**	6.33 (± 2.06)	7.28 (± 2.37)
AUC_{0-72h}	**(hr•ng/mL)**	40.1 (± 9.93)	39.4 (± 10.7)
$AUC_{0-\infty}$	**(hr•ng/mL)**	51.0 (± 13.9)	52.3 (± 17.4)
V_z/F	**(mL/kg)**	16600 (± 4600)	15700 (± 4430)
λ_z	**(hr^{-1})**	0.031 (± 0.04)	0.019 (± 0.01)
Half Life	**(hr)**	32.6 (± 10.3)	40.9 (± 14.0)
CL_{nr}	**(mL/min/kg)**	3.70 (± 1.42)	3.31 (± 1.66)

Noncompartmental analysis of digoxin pharmacokinetic parameters after a 1 mg oral dose. CL/F, oral clearance normalized to subject weight; fe_{0-72h}, fraction of digoxin exreted into urine from 0-72 hours; $fe_{0-\infty}$, extrapolated total digoxin excreted unchanged in urine; T_{max}, time to maximal concentration; C_{max}, maximum concentration; CL_R, renal clearance from 0-72 hours normalized to weight; AUC, area under the curve from time 0 to 3 hours, 0 to 72 hours and 0 to infinity; V_z/F, volume of distribution normalized to weight; λ_z, terminal elimination rate constant; CL_{nr}, apparent nonrenal clearance. The non-compliant MZ twin pair was removed before calculating CL_R and CL_{nr}.

5.3.3 Heritability of Digoxin Pharmacokinetics

Twin pair concordance was analyzed for $AUC_{0\text{-}3h}$, oral clearance, renal clearance, apparent nonrenal clearance and C_{max}. Overall, MZ twin pairs were more concordant in their $AUC_{0\text{-}3h}$, oral clearance, apparent nonrenal clearance and C_{max} than DZ twin pairs. Seven of the MZ twin pairs had less than a 25% coefficient of variance for their $AUC_{0\text{-}3h}$ and C_{max} values, while there were only two and three pairs, respectively, found in the DZ twin group (Figures 4 & 5). A similar trend was also found in oral clearance with 9 of 10 MZ and 4 of 10 DZ twin pairs having a coefficient of variance of less than 25% (Figure 5.6). Renal clearance was highly concordant between both MZ and DZ twin pairs (Figure 5.7). Among MZ twin pairs, apparent nonrenal clearance was more concordant than in DZ twin pairs (Figure 5.8).

Heritability values were calculated for several pharmacokinetic parameters using three different equations for heritability. These values are summarized in Table 5.3A and 3B. Heritability based on Holzinger and Falconer's equations was calculated using intrapair correlation values (Figures 9 & 10). There was no genetic contribution to renal clearance calculated with any of the three heritability formulas (0.02 – 0.08). In contrast, there was a high genetic contribution to $AUC_{0\text{-}3h}$ (0.52-1.00), and moderate contributions to oral clearance (0.32 – 0.67) and to C_{max} (0.26 – 0.60). Apparent nonrenal clearance was highly heritable (0.61-1.00). Inclusion of the DZ outlier twin pair has its greatest effect on oral clearance and apparent nonrenal clearance, with heritability estimates close to 1.0 if this twin pair is included.

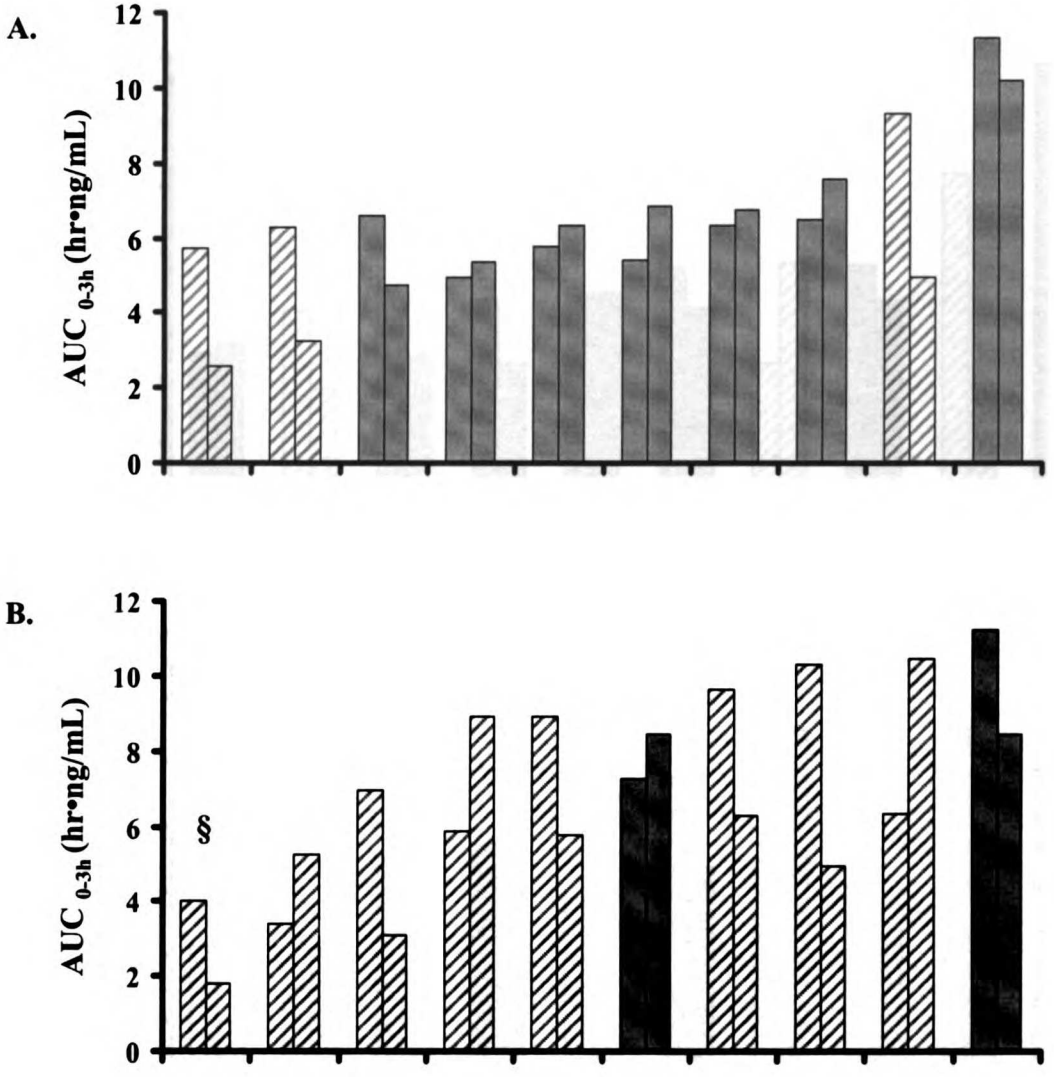

Figure 5.4 $AUC_{0\text{-}3h}$ concordance for A) monozygotic and B) dizygotic twins. Each group represents a twin pair. Grey bars represent twin pairs with less than a 25% coefficient of variance. $AUC_{0\text{-}3h}$ is more concordant in monozygotic twin pairs than in dizygotic twin pairs. § represents data from the DZ twin outlier.

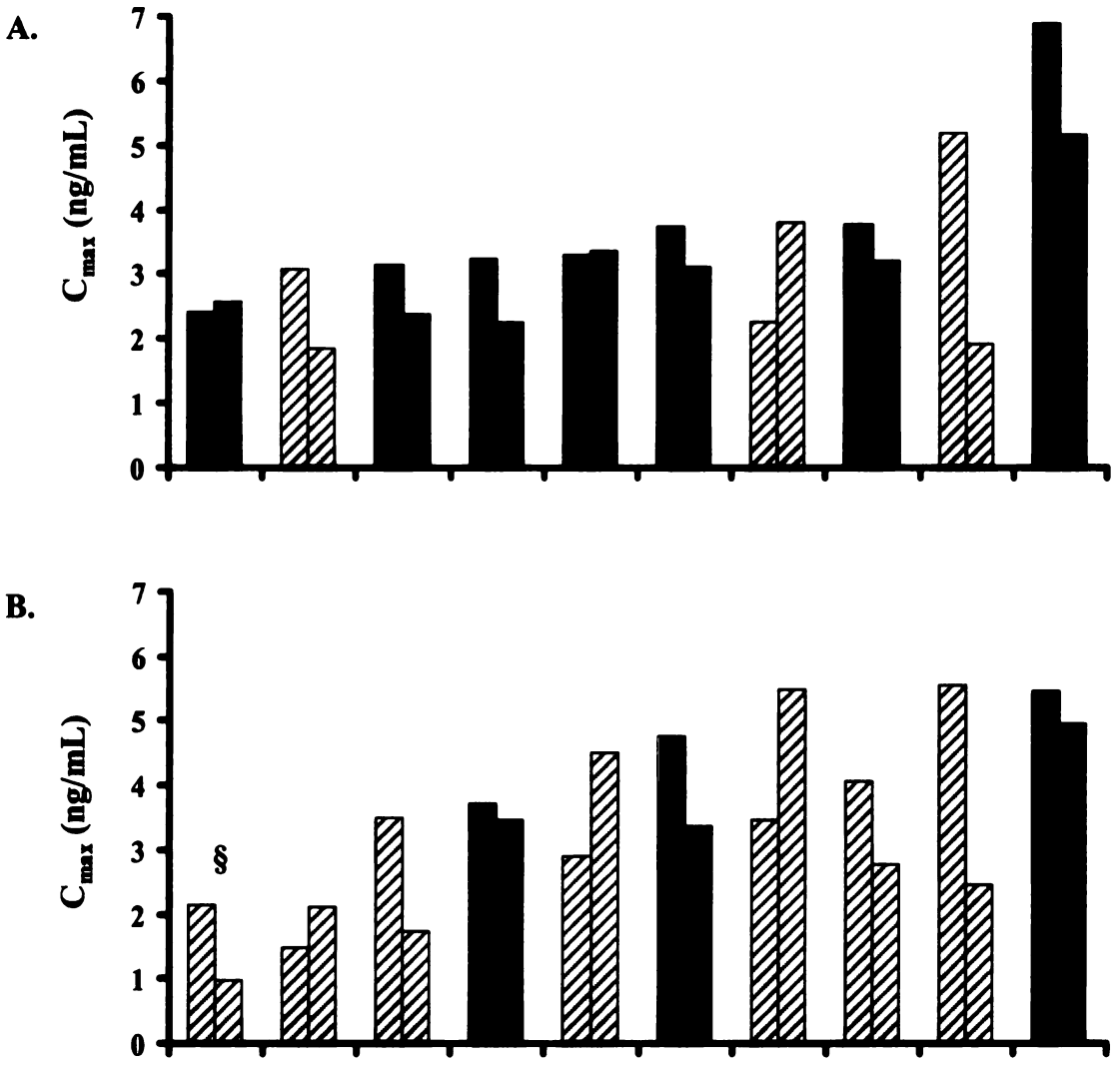

Figure 5.5 C_{max} concordance for A) monozygotic and B) dizygotic twins. Each group represents a twin pair. Grey bars represent twin pairs with less than a 25% coefficient of variance. There is very little concordance of C_{max} among dizygotic twin pairs. § represents data from the DZ twin outlier.

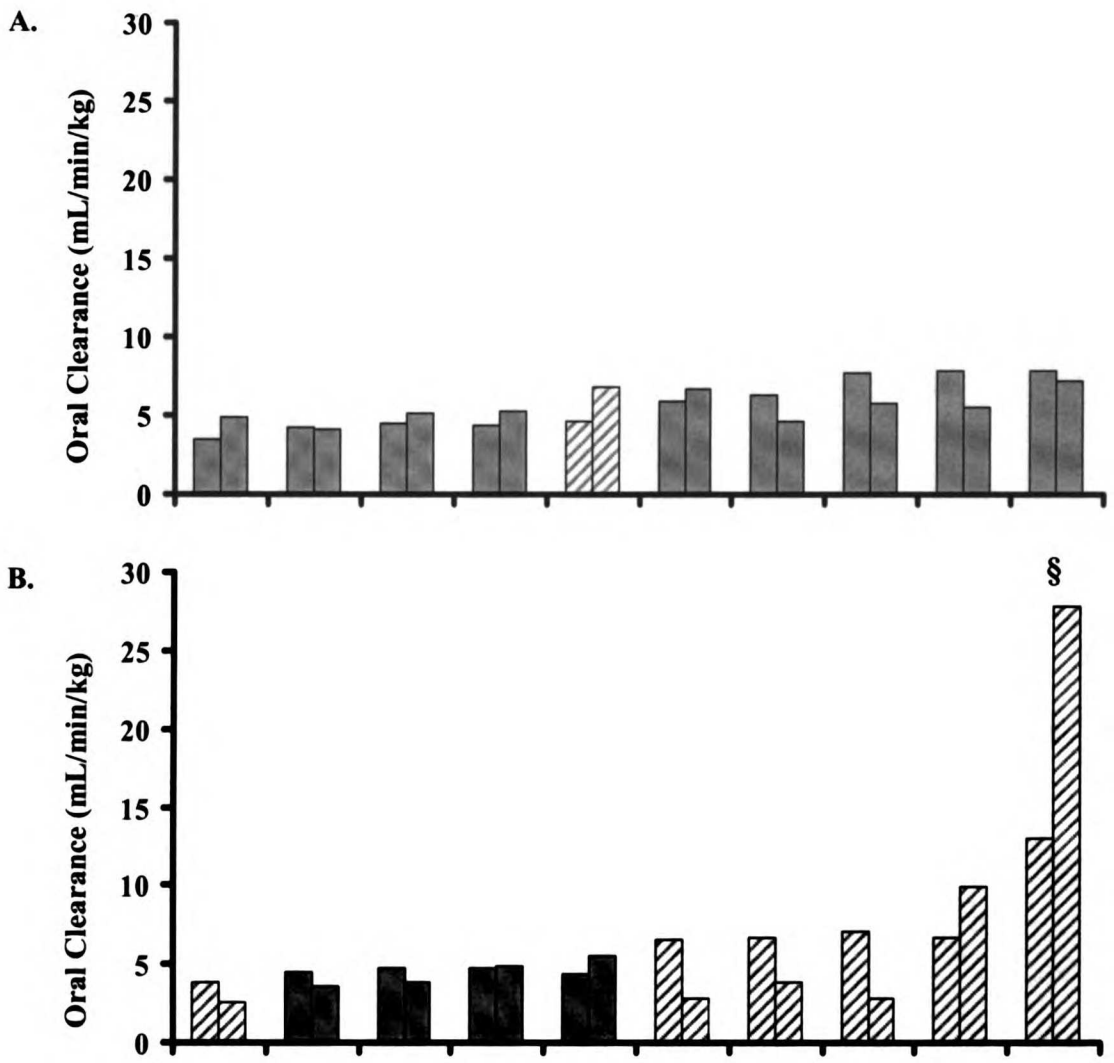

Figure 5.6 Oral clearance concordance for A) monozygotic and B) dizygotic twins. Each group represents a twin pair. Grey bars represent twin pairs with less than a 25% coefficient of variance. Oral clearance is more concordant in monozygotic twin pairs than in dizygotic twin pairs. § represents data from the DZ twin outlier.

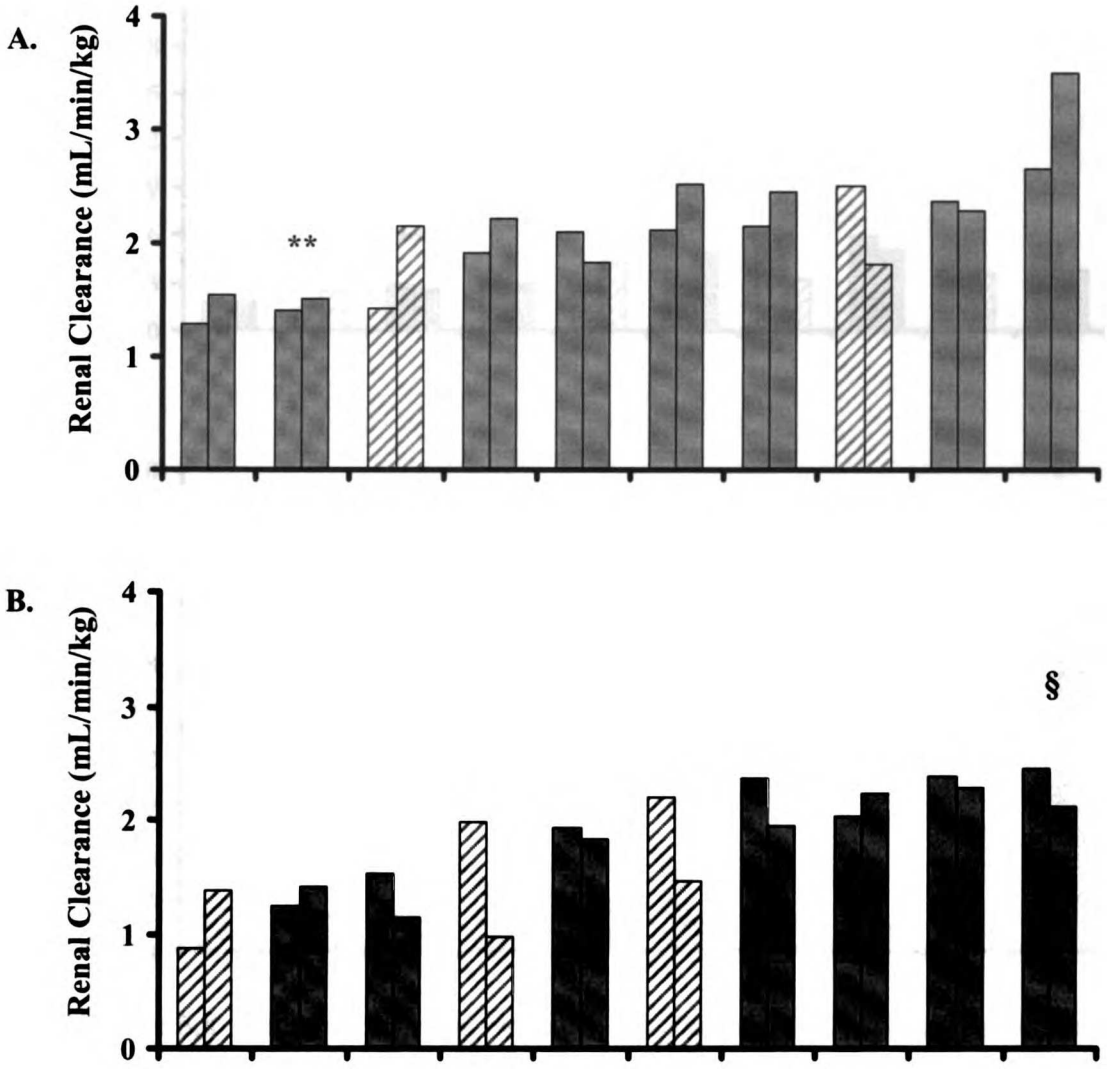

Figure 5.7 Renal clearance concordance for A) monozygotic and B) dizygotic twins. Each group represents a twin pair. Grey bars represent twin pairs with less than a 25% coefficient of variance. Renal clearance is highly concordant in both monozygotic twin pairs and dizygotic twin pairs. § represents data from the DZ twin outlier. ** represents data from the non-compliant MZ twin pair that was not used in calculations.

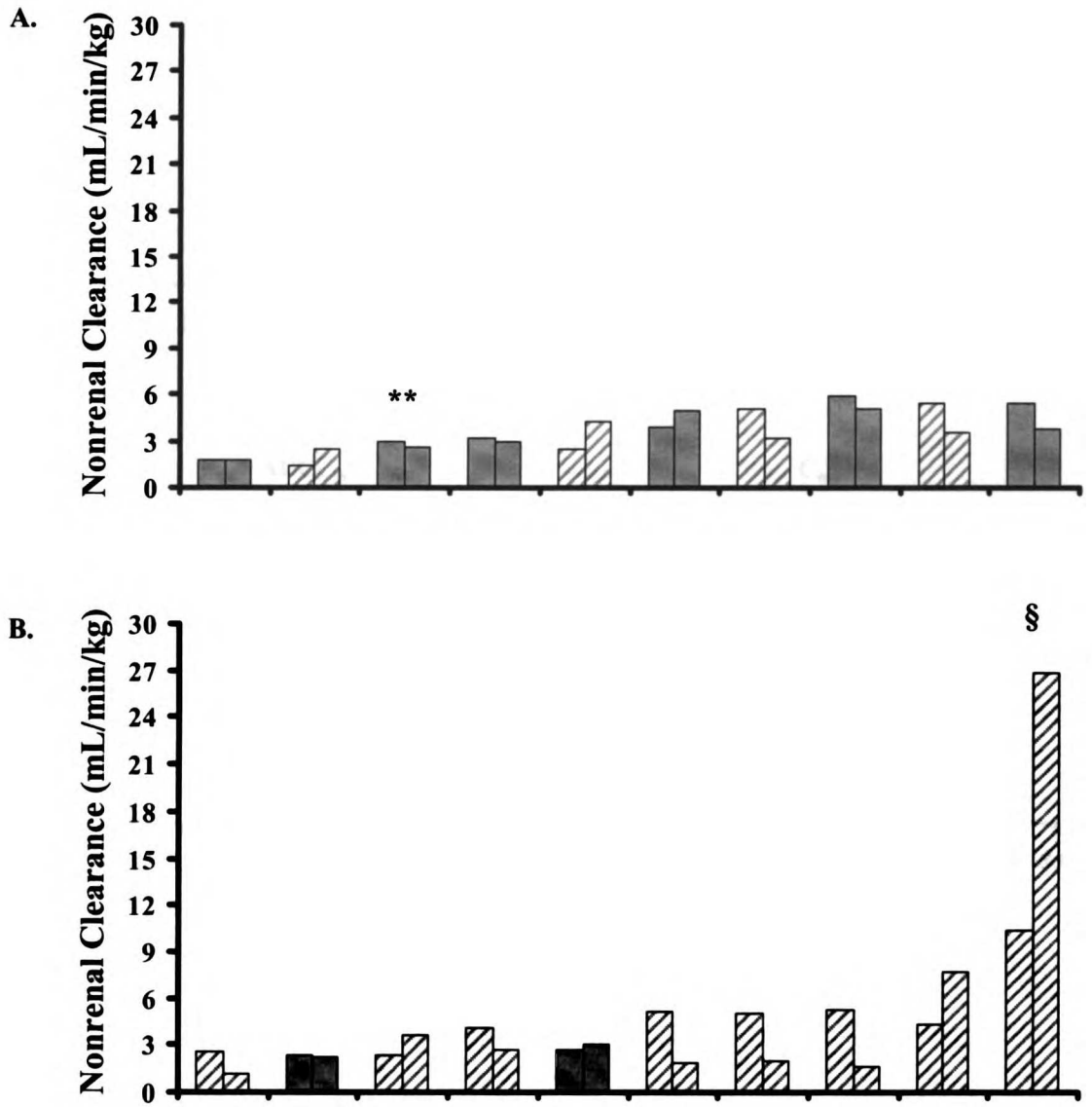

Figure 5.8 Apparent nonrenal clearance concordance for A) monozygotic and B) dizygotic twins. Each group represents a twin pair. Grey bars represent twin pairs with less than a 25% coefficient of variance. Apparent nonrenal clearance is more concordant in monozygotic twin pairs than in dizygotic twin pairs. § represents data from the DZ twin outlier. ** represents data from the non-compliant MZ twin pair that was not used in calculations.

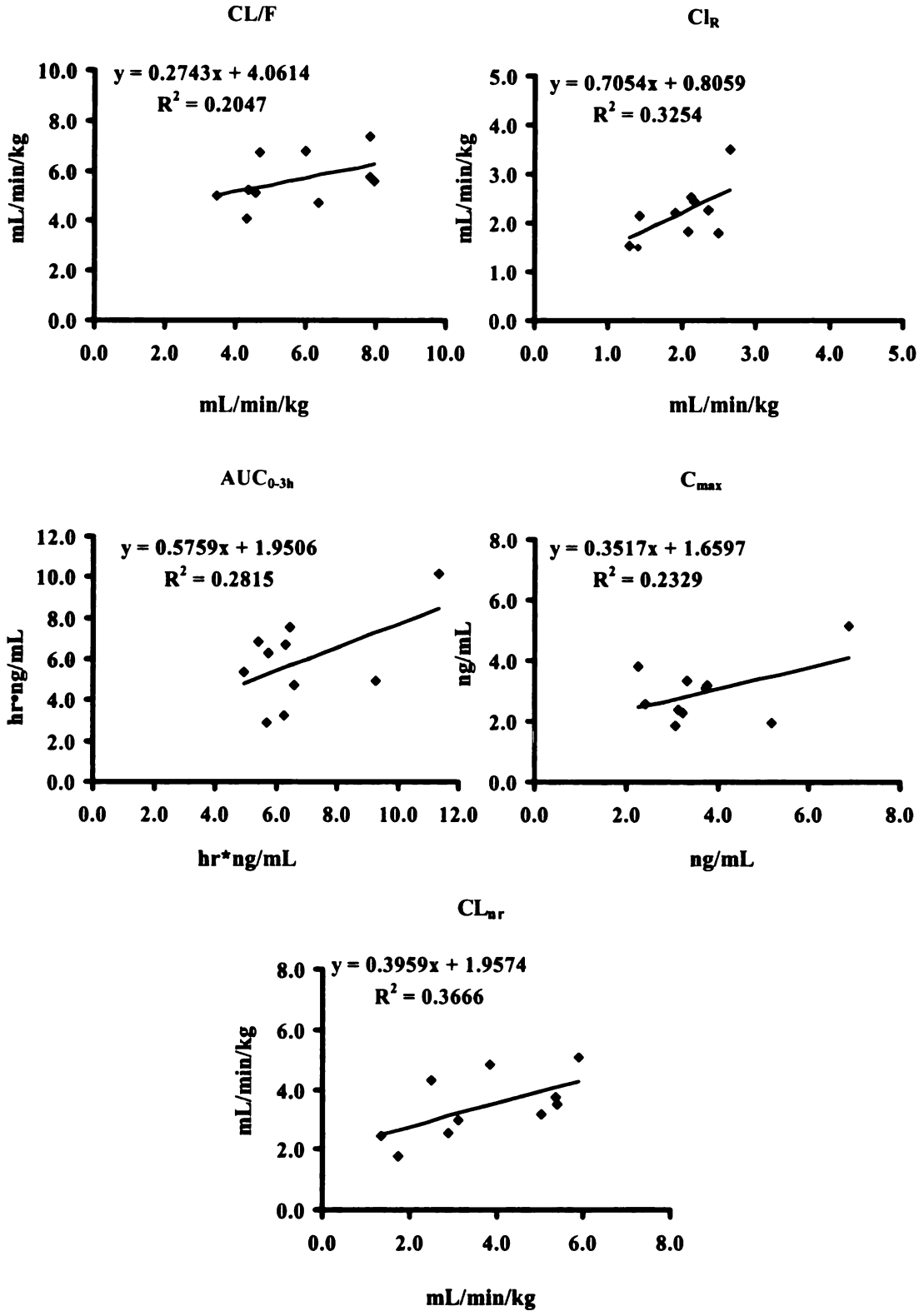

Figure 5.9 Intrapair correlation in monozygotic twins for digoxin pharmacokinetic parameters. Pharmacokinetic parameters in Twin 1 were plotted against those of Twin 2. The line represents the regression line.

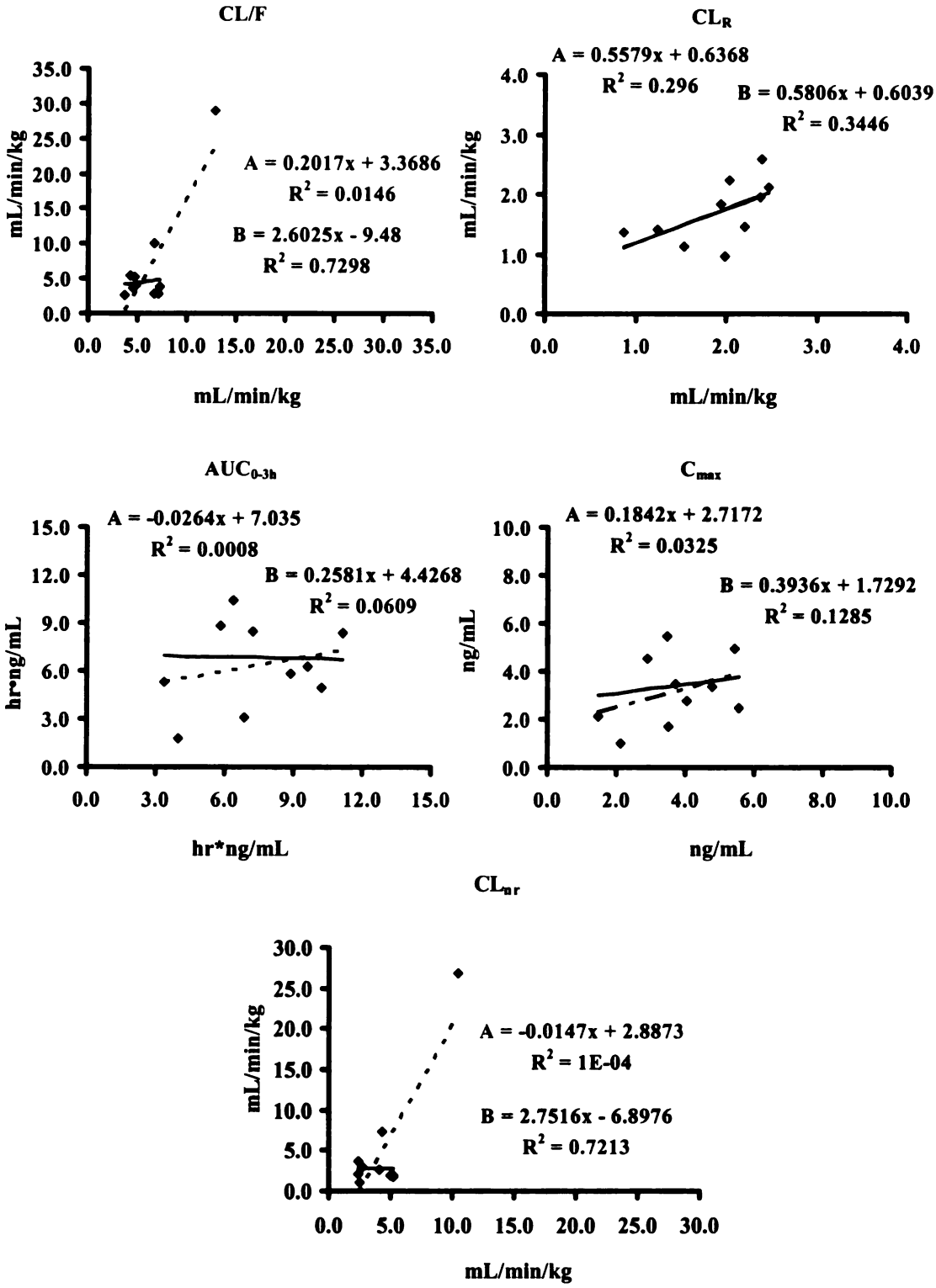

Figure 5.10 Intrapair correlation in dizygotic twins for digoxin pharmacokinetic parameters. Pharmacokinetic parameters in Twin 1 were plotted against Twin 2. The solid line and equation A represent the regression line with the outlier removed and the dotted line and equation B represent the regression which includes the outlier.

Table 5.3 Heritability Index for Digoxin Pharmacokinetic Parameters

A.

		CL/F	Cmax	CL_R	AUC_{0-3h}	CL_{nr}
h^2	$(V_{MZ} - V_{DZ}) / V_{DZ}$	0.69	0.26	0.12	0.60	0.69
h^2_H	$(r_{MZ} - r_{DZ}) / (1 - r_{DZ})$	0.33	0.37	0.05	0.52	0.59
h^2_F	$2 \bullet (r_{MZ} - r_{DZ})$	0.54	0.60	0.05	1.00	1.00

B.

		CL/F	Cmax	CL_R	AUC_{0-3h}	CL_{nr}
h^2	$(V_{MZ} - V_{DZ}) / V_{DZ}$	0.93	0.22	0.07	0.58	0.95
h^2_H	$(r_{MZ} - r_{DZ}) / (1 - r_{DZ})$	0.00	0.19	0.14	0.38	0.00
h^2_F	$2 \bullet (r_{MZ} - r_{DZ})$	0.00	0.24	0.11	0.57	0.00

Heritability estimates for digoxin pharmacokinetic parameters A) without the DZ outlier twin pair or B) with the outlier. V_{MZ}, variance calculated from the mean values in monozygotic twins; V_{DZ}, variance derived from dizygotic twins; r, correlation coefficient in MZ or DZ twins; h^2_H, heritability value calculated using Holzinger's equation; h^2_F, heritability calculated from Facloner's equation.

5.4 DISCUSSION

Twins are a useful tool to examine the relative contributions of environment and genetics on a given phenotype. Heritability indices calculated from twin studies are used to determine the contribution of genetics to a phenotype. To gain insight into the heritability of transporter-mediated drug disposition, heritability of digoxin pharmacokinetics was estimated.

Digoxin bioavailability is estimated to be 70%, however it can range from 50-90% (20). Digoxin is not extensively metabolized and is eliminated through the kidneys (26) with little involvement of transporters as its renal excretion is proportional to glomerular filtration rate (9). Current studies suggest that P-gp is the major digoxin transporter in the intestine (30, 31) and P-gp limits drug bioavailability.

As there were more women then men participating in this study, there was a possibility that digoxin PK may be affected by female sex hormones. P-gp expression at the mRNA and protein level has been shown to be induced by estrone, estriol, and ethinyl estradiol *in vitro* (32). Digoxin PK parameters estimated from this study were different from those reported previously (10, 33, 34). Oral clearance was higher and renal clearance was lower in this study. Subsequently, apparent nonrenal clearance made up a large proportion of total oral clearance. The fraction of unchanged digoxin that was excreted was close to 38% in this study, well below what has been reported in the literature (35). A likely explanation is that earlier reports used a non-specific radioimmunoassay for digoxin quantification (10, 25, 33). That assay measures unchanged digoxin plus a significant percentage of active metabolites, particularly the bisdigitoxoside primary metabolite. It is likely that digoxin is being removed through

biliary and intestinal excretion, based on previous studies in P-gp knockout mice (12) and studies in rats administered digoxin and P-gp inhibitors such as quinidine (36-38). P-gp is expressed in the hepatocyte on the canalicular membrane, where it can extrude compounds into the bile for elimination (39). If digoxin is being excreted into the bile, then P-gp and/or organic anion transporters may have a major impact on digoxin elimination in the liver.

Heritability in digoxin pharmacokinetics was calculated using data from MZ and DZ twins and oral clearance, apparent nonrenal clearance, C_{max} and $AUC_{0\text{-}3h}$ were identified as heritable. Both oral and apparent nonrenal clearance had high heritability values (both 0.69) compared to other PK parameters, and the heritability for these clearances was similar. Apparent nonrenal clearance was calculated to be 65% of total clearance. This indicates that the high heritability in oral clearance may be due to apparent nonrenal clearance such as active secretion in the liver and intestine. Variability in renal clearance had no genetic contribution ($h^2 < 0.15$), consistent with a genetic contribution estimate (r_{GC}) based on a repeated measures analysis (40). Lack of heritability in renal clearance is consistent with the fact that digoxin is not actively secreted by efflux transporters in the kidney.

Three different formulas were used to estimate heritability. An inherent weakness with these formulas is that each assumes that the environment between twin pairs is equal. These heritability estimates also do not account for environmental differences between MZ and DZ twins. Although all twins recruited were from the Bay Area, there may be subtle differences in geography, lifestyle, and health of each twin. Such as it is, the heritability index calculated from the three formulas will overestimate the

contribution of genetics to a given phenotype. Heritability by variance also suffers from the method of calculation. Variance is defined as the standard deviation squared, and in this study the standard deviation is calculated from two data points and does not accurately represent the true variation in the samples. Both estimates based on correlation rely on a large sample set to produce a regression line. A single outlier in a small population can thus skew the data, which is seen in the heritability estimates when including the outlying twin pair (Table 5.3B).

Since digoxin t_{max} is ~1.5 hours, $AUC_{0\text{-}3h}$ reflects both the rate of digoxin absorption and the rate of elimination. Digoxin plasma concentration after an oral dose is defined as:

$$C=\frac{ka \bullet F \bullet D}{V(ka-k)}(e^{-kt}-e^{-kat}).$$

The absorption rate constant (k_a) is not likely affected by genetics as it reflects diffusion of digoxin into the intestine. The elimination rate constant (k) reflects primarily glomerular filtration, which is also unlikely to be affected by genetics, and a nonrenal component, most likely biliary and intestinal excretion. Digoxin biliary excretion has not been well studied due to difficulties measuring bile in humans. Older studies have reported that as much as 10-30% of an intravenous dose of digoxin is excreted into the bile (41, 42). A recent study using an intestinal perfusion catheter technique was able to measure 0.8% of an i.v. dose of digoxin in the bile within 3 hours post administration (43). Extended over 72 hours, as much as 19% of an i.v. dose of digoxin could potentially be excreted through the bile. The study also estimated that as much as 11% of the i.v. dose of digoxin can be secreted through the intestinal wall for elimination. The apparent volume of distribution (V_z/F) was similar in both MZ and DZ twin populations.

This suggests that bioavailability (F) and nonrenal elimination are the most likely parameters to be variable and influenced by genetics. Since $AUC_{0\text{-}3h}$ reflects both absorption and elimination, the estimated heritability may be due to the involvement of P-gp or other uptake transporters in the intestine and liver.

5.5 PERSPECTIVES

The work presented here is one of the first attempts to determine the importance of transporter genetics in drug variability. Digoxin is a substrate of P-glycoprotein which is expressed in a number of tissues, including the intestine and liver (44). The results from this study support the idea that genetic variability in transporters contributes to the variability in digoxin pharmacokinetics, specifically that of bioavailability and apparent nonrenal clearance. It is possible that genetic variations in P-gp can alter the disposition of digoxin. P-glycoprotein can limit digoxin bioavailability by effluxing the drug back out into the intestinal lumen and, genetic variations in *ABCB1* may therefore affect the way P-gp interacts with digoxin. Once in circulation, digoxin can eventually be eliminated through the intestinal wall and bile canaliculus by P-gp. Uptake transporters in the intestine and liver may also be involved in digoxin disposition. OATP2B1 is expressed in the intestine (45), and OATP1B3 can transport digoxin (17). Associations between *SLCO1B3* (OATP1B3), *SLCO2B1* (OATP2B1), and *ABCB1* genotypes and digoxin bioavailability and nonrenal elimination need to be evaluated.

The heritability values presented here must be viewed with caution, however. The small number of twin pairs recruited provided enough power to detect a heritability value of 0.60. There may be a number of possible sources of variation that could skew

the data obtained from this study. Variance calculated for heritability estimates was determined from only two data points (each twin in a pair), and this can add to the variability of the parameter being tested. Similarly, intrapair correlations can be skewed by the small sample size. It is evident that additional twins must be studied to provide greater power. Further analysis should also consider the influence of gender, weight, and age on the variability in digoxin pharmacokinetics.

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CHAPTER 6

NUCLEOTIDE DIVERSITY IN THE ABC TRANSPORTER FAMILY

6.1 INTRODUCTION

Large scale single nucleotide polymorphism (SNP) discovery studies have been carried out to determine the level of variation in genes. These studies have focused on a single gene (1, 2) candidate genes for a disease phenotype (3, 4), or on a large sample set of unrelated genes (5). These studies sequenced less than 90 individuals, and therefore were able to accurately identify population variants of at least 2% frequency and ethnic specific variants of at least 5% frequency (5). One of the largest sampling efforts thus far has been in a recent study using 247 unrelated individuals. In that study, sequencing efforts were able to detect SNPs ($\geq$1%) and rarer variants (<1%) in 24 membrane transporter proteins (6). These transporters were membrane bound proteins, five of which were members of the ATP-Binding Cassette (ABC) superfamily of efflux transporters.

The ABC superfamily of transporters is a large family of membrane proteins that are able to transport a wide variety of compounds important for cellular processes or cell survival (7). Substrates range from bile constituents (8, 9), to drugs used in the treatment of cancer and HIV (10). These compounds can be hydrophobic, neutral, anionic, or cationic in nature. ABC transporters are expressed in barrier and eliminating tissues and may play a role in drug absorption, distribution, and elimination (11-14).

In this study, ten members of the ABC superfamily were screened for genetic variation. Because some ABC transporters are expressed in similar tissues, or are able to

transport similar compounds, the analysis of their nucleotide diversity may provide some insight into their importance in cellular function.

6.2 MATERIALS AND METHODS

6.2.1 Variant Identification of ABC Genes

Genomic and cDNA sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). DNA samples were obtained from an ethnically diverse collection from the Coriell Institute of Medical Research (http://coriell.umdnj.edu) for Set I genes and from the UCSF cohort of normal healthy volunteers, Study of Pharmacogenetics In Ethnically Diverse Populations (SOPHIE), for Set II genes (Table 6.1). Variant identification has been previously outlined in Chapter 2. Set I genes included the Bile Salt Export Pump (BSEP; *ABCB11*), the Multidrug Resistance Protein 1 (MDR1; *ABCB1*), the Multidrug Resistance Protein 3 (MDR3; *ABCB4*), and the Mulidrug Resistance-associated Proteins 1 & 2 (MRP1, ABCC1; MRP2, *ABCC2*). Set II genes included the Multidrug Resistance-associated Proteins 3, 4, 5 and 6 (MRP3, *ABCC3*; MRP4, *ABCC4*; MRP5, *ABCC5*; MRP6, *ABCC6*) and the half-transporter Mitoxantrone Resistance Transporter (MXR, *ABCG2*). These transporters are summarized in Table 6.2. Detection of single nucleotide polymorphisms was described earlier and in Chapter 2 (6).

Table 6.1 Sample Populations for Sequence Analysis

	Sequencing Population					
	AA	**CA**	**AS**	**ME**	**PA**	**Total**
Coriell Collection	100	100	30	10	7	247
SOPHIE Collection	80	80	60	50	6	276

Two sample sets were used for variant identification in the ABC transporters. Set I genes were sequenced using genomic DNA from the Coriell collection, while Set II genes were sequenced from the SOPHIE collection. The ethnic makeup of each dataset is listed.

Table 6.2 ABC Transporters

Gene	Protein	Genotyping Sample Set	Chromosome Location
ABCB1	P-gp	Coriell	7q21.1
ABCB4	MDR3	Coriell	7q21.1
ABCB11	BSEP	Coriell	2q24
ABCC1	MRP1	Coriell	16p13.1
ABCC2	MRP2	Coriell	10q24
ABCC3	MRP3	SOPHIE	17q22
ABCC4	MRP4	SOPHIE	13q32
ABCC5	MRP5	SOPHIE	3q27
ABCC6	MRP6	SOPHIE	16p13.1
ABCG2	MXR	SOPHIE	4q22

The ABC transporters characterized in this study. Listed are the 10 ABC transporters used for genetic analysis. Their chromosome location and DNA sample set are shown.

6.2.2 Population Genetic Statistics and Haplotype Analysis*

Genetic analysis was described in Chapter 2. Briefly the neutral parameter (θ), the average heterozygosity (π), and Tajima's D statistic were calculated as described by Tajima (15). Each parameter was calculated for the coding, non-coding, intron-exon boundaries, areas with synonymous and non-synonymous variations, evolutionarily conserved and non-conserved regions, and predicted extracellular loops, intracellular loops and transmembrane domains. Evolutionarily conserved sites were determined by aligning protein sequences of human, dog, rat and mouse orthologs using the GCG program PILEUP. Haplotype estimation was performed using PHASE 2.0 (16) and was determined for each gene.

6.3 RESULTS AND DISCUSSION

6.3.1 Genetic Variation in ABC Transporters

Two sample populations were used to identify single nucleotide polymorphisms in the membrane transporters. For Set I genes, the 247 sample Coriell Collection, which contained 100 Caucasians, 100 African Americans, 30 Asian Americans, 10 Mexican Americans, and 7 Pacific Islanders was used. For Set II genes, the SOPHIE collection, gathered at the University of California, San Francisco was used. This collection consisted of 80 Caucasians, 80 African Americans, 60 Asian Americans, 50 Mexican Americans, and 6 Pacific Islanders (Table 6.1). Exons and up to 100 bp of intronic

* Variant identification, haplotype analysis and genotyping were part of a collaborative effort by the UCSF Pharmacogenetics of Membrane Transporters network that included Doug Stryke, Conrad Huang, Travis Taylor, Elaine Carlson, and Michiko Kawamoto.

boundaries of nine ABC transporters have been fully sequenced. One transporter, *ABCC6*, has had 25 of its 31 exons sequenced. Sequencing efforts for MRP2 also included a 1.6 kb region of the promoter, as described in Chapter 2.

Using DHPLC and direct sequencing, 600 polymorphic sites were identified over 76 kb of genomic DNA. This equated to roughly one variant per 127 bases screened and this was similar to the frequencies of other large scale genetic studies that showed a frequency of 1 in 185 (5) and 1 in 217 (3) bases screened. These earlier studies screened over 720 kb and 190 kb of sequence, respectively, which included coding exons, 5'- and 3'-UTR, and intronic sequences. The former also screened the 5'-upstream region (5). Seventy one percent of the variants identified in the ABC transporters were transitions (C↔T, G↔A) even though transitions make up one third of all possible base changes. The large scale screening of variants allowed for the detection of rare variants. Of the rare variants identified, there were a total of 232 singletons and 66 doubletons (Table 6.3). Over 78% of the variants identified in the ten ABC transporters were considered low frequency variants (<5% allele frequency) and ranged from as low as 64% of *ABCG2* variants to as high as 89% of *ABCC1* variants. Only 13% of all variants were considered common (>15% allele frequency), with *ABCC1* having the lowest frequency of common variants (7.9%) and *ABCB11* the highest (20%) (Table 6.3).

The amount of non-coding and coding regions screened were equal in base length and the number of variants identified was equally divided between the two regions (295 non-coding variants vs. 272 coding variants). Of the coding variants, a little over half were found to be synonymous changes (Table 6.4). The non-synonymous, evolutionarily conserved variations, thought to have the most dramatic effect on proteins (4), were rare,

suggesting purifying selection. However, three evolutionarily conserved SNPs, a Gln141Lys in *ABCG2*, an Arg1268Gln in *ABCC6*, and Gly187Trp in *ABCC4* were found at an allele frequency of 0.167, 0.164, and 0.054, respectively and may have a clinical consequence.

SNP discovery screens were also able to detect insertions and deletions (indels) in the genes sequenced. In the 10 ABC transporters, there were 18 indels, the largest being a ten base deletion in *ABCC1* found in one chromosome in the Asian American population. One indel was found in the *ABCC6* coding region and resulted in a frameshift and subsequent truncation of the protein. This variant was found in only 2 chromosomes in the Asian American population. There were 3 incidences of triallelic nucleotides, where a variant site carried 2 alleles. Of these, only one was found in the coding region, and resulted in two different amino acids in P-glycoprotein (Ala893Ser/Thr). There was one variant in *ABCC2* that resulted in a premature stop codon and it was found in one chromosome in African Americans.

Table 6.3 Rare and Common Variants in ABC Transporters

	ABCB1	*ABCB4*	*ABCB11*	*ABCC1*	*ABCC2*	*ABCC3*	*ABCC4*	*ABCC5*	*ABCC6*	*ABCG2*
# Variants	48	34	41	63	68	70	99	43	101	33
Singletons	20 (42%)	13 (38%)	14 (34%)	30 (48%)	21 (31%)	31 (44%)	34 (34%)	19 (44%)	40 (40%)	10 (30%)
Doubletons	6 (13%)	5 (15%)	1 (2.4%)	2 (3.2%)	10 (15%)	8 (11%)	14 (14%)	2 (4.7%)	15 (15%)	3 (9.1%)
<5%	38 (79%)	27 (79%)	29 (71%)	56 (89%)	54 (79%)	59 (84%)	73 (74%)	31 (72%)	85 (84%)	21 (64%)
5-15%	3 (6.3%)	1 (2.9%)	4 (9.8%)	2 (3.2%)	8 (12%)	5 (7.1%)	10 (10%)	4 (9.3%)	4 (4%)	6 (18%)
>15%	7 (15%)	6 (18%)	8 (20%)	5 (7.9%)	6 (8.8%)	6 (8.6%)	16 (16%)	8 (19%)	12 (12%)	6 (18%)
Transitions	37 (77%)	25 (74%)	30 (73%)	40 (63%)	45 (66%)	48 (69%)	73 (74%)	33 (77%)	74 (73%)	25 (76%)

Number of variants identified in ABC transporters. Listed are the number and percentage of variants identified in each gene, and the makeup of rare and common variants. The percentage of total variants is given in parentheses.

Table 6.4 Variants in Coding and Non-coding Regions

	Coding	Non-coding	Synonymous	Non-synonymous	Intron-Exon	Total
ABCB1	19	29	6	13	1	48
ABCB4	18	16	11	7	1	34
ABCB11	24	17	15	9	1	41
ABCC1	25	38	20	5	1	63
ABCC2	34	34	15	19	1	68
ABCC3	36	34	15	21	1	70
ABCC4	45	55	22	23	3	99
ABCC5	12	31	9	3	0	43
ABCC6	49	52	24	24	4	101
ABCG2	10	23	4	6	1	33
Total	272	329	141	130	14	600

Number of coding and non-coding variants in the ABC transporters. Coding variants are broken down into synonymous and non-synonymous variants, and the non-coding intron-exon boundary variants are listed.

African Americans carried the most variants and subsequently the most haplotypes of all ethnic groups. This is consistent with the theory that they are the oldest ethnic population (17). Of all genetic variations, 211 were African American specific, 77 were Caucasian specific, 57 were Asian American specific, 20 were Mexican American specific, and 7 were of Pacific Islander origin (Table 6.5). Ninety genetic variations were found across all ethnic groups (cosmopolitan). Parsed into haplotypes, 44% were specific to the African American population, 18% were specific to the Caucasian population, 8% were Asian American specific, 7% Mexican American specific and 2% were specific to the Pacific Islander population. Only 1.7% of the haplotypes were considered cosmopolitan haplotypes, being found in all five ethnic populations. Roughly 11% of the haplotypes

Table 6.5 Number of Ethnic Specific Variants

	AA	CA	AS	ME	PA	Cosmo
ABCB1	16	8	4	1	0	9
ABCB4	17	6	3	0	0	5
ABCB11	13	9	0	0	0	9
ABCC1	24	5	14	0	0	6
ABCC2	29	5	4	0	0	6
ABCC3	22	8	8	6	1	8
ABCC4	31	9	7	6	3	20
ABCC5	8	8	5	1	1	10
ABCC6	45	17	7	4	1	11
ABCG2	6	2	5	2	1	6
	211	**77**	**57**	**20**	**7**	**90**

Ethnic specific variants. Listed are the number of genetic variations identified that were ethnic specific or cosmopolitan. AA, African American; CA, Caucasians; AS, Asian Americans; ME, Mexican Americans; PA, Pacific Islanders; Cosmo, cosmopolitan. The number of base pairs screened for each gene are 7381 (*ABCB1*), 6066 (*ABCB4*), 6443 (*ABCB11*), 7296 (*ABCC1*), 9693 (*ABCC2*), 9127 (*ABCC3*), 8660 (*ABCC4*), 9478 (*ABCC5*), 7750 (*ABCC6*), and 4441 (*ABCG2*).

were shared between the African American and Caucasian populations. An estimate of the amount of population subdivision was given by Wright's F_{ST}. Population subdivision, possibly due to genetic drift, accounted for approximately 12% of the variation in the ABC transporters with the highest reported for *ABCB4* at 32% (Table 6.6). The subdivision seen in *ABCB4* was found in comparisons between Pacific Islanders (PA) and other populations. However, only 7 PA individuals were used in the analysis which could skew the data. The average F_{ST} values were similar to the range of other genes studied (1, 18, 19).

On average, there were 60 variant sites identified per gene. If genetic variations were random, there would be 2^{60} possible haplotypes for each gene studied. Without recombination, the number of haplotypes would equal the number of SNPs (1). Considering all non-singleton variant sites, there was a total of 845 haplotypes estimated for 9 of the 10 genes (analysis of *ABCC6* haplotypes is incomplete), with an average of 93 haplotypes per gene. The fact that there was almost twice the number of haplotypes compared to the number of SNPs suggests that there was some degree of recombination in the ABC transporter genes. *ABCC5* had the lowest number of haplotypes of the ABC transporters studied at 31, while *ABCC4* was the most diverse with 252 haplotypes (Table 6.7). Interestingly, for *ABCC4*, no more than 11 chromosomes were found to contain the same haplotype sequence, with the most abundant haplotype found at 2.3% in the population.

Table 6.6 ABC Transporter Haplotypes and Population Substructure

A.

	Cosmo	CA/AA	F_{ST}
ABCB1	0	16	0.062
ABCB4	1	8	0.319
ABCB11	2	11	0.108
ABCC1	1	8	0.129
ABCC2	4	13	0.039
ABCC3	1	14	0.056
ABCC4	0	5	0.054
ABCC5	2	8	0.148
ABCC6	N/A	N/A	N/A
ABCG2	3	9	0.16

B.

Pop 1	Pop 2	F_{ST}
CA	AA	0.075
CA	AS	0.037
CA	ME	-0.015
CA	PA	0.537
AA	AS	0.146
AA	ME	0.072
AA	PA	0.463
AS	ME	0.033
AS	PA	0.474
ME	PA	0.563

A) Listed are the number of haplotypes that were found in all ethnic groups (cosmopolitan) or in Caucasians and African Americans. Population substructure for each gene is given by Wright's F_{ST}. B) Population subdivision of *ABCB4* between Pacific Islanders and other ethnic groups for this gene. AA, African American; CA, Caucasian; AS, Asian American; ME, Mexican American; PA, Pacific Islander; Cosmo, cosmopolitan.

Table 6.7 Number of Ethnic Specific Haplotypes

	Total	AA	CA	AS	ME	PA
ABCB1	66	35	5	6	1	0
ABCB4	48	28	4	2	2	0
ABCB11	108	55	21	8	6	1
ABCC1	94	47	23	8	0	3
ABCC2	88	39	23	4	2	3
ABCC3	100	33	16	13	13	3
ABCC4	252	77	65	36	44	4
ABCC5	31	9	4	2	4	0
ABCC6	N/A	N/A	N/A	N/A	N/A	N/A
ABCG2	58	20	5	10	6	0
	845	**343**	**166**	**89**	**78**	**14**

Distribution of ethnic specific haplotypes among the ABC transporters. AA, African American; CA, Caucasian; AS, Asian American; ME, Mexican American; PA, Pacific Islander

6.3.2 Population Genetic Analysis

To further analyze the amount of variation in the ABC transporters, two measures of nucleotide diversity were calculated; the average heterozygosity (π) and the population neutral parameter (θ). Both parameters provide a measure of genetic variation that has been normalized to the number of chromosomes sampled and length of sequence screened. This allows the ability to compare the extent of variability among genes. In addition, Tajima's D statistic was calculated to determine the deviation from the neutral model. These parameters were calculated across the entire gene, for coding and non-coding regions, for non-synonymous and synonymous sites, across the TMD and NBF loops, and for conserved and unconserved sites. For the genes studied, the population neutral parameter (θ) ranged from 6.58 ($\times 10^{-4}$) to 18.35 ($\times 10^{-4}$), while the average

heterozygosity (π) ranged from 3.55 (x 10^{-4}) to 9.24 (x 10^{-4}) (Figures 6.1 and 6.2). The neutral parameter (θ) was highest in *ABCC4* and lowest in *ABCC5*, which closely followed the number of SNPs identified in each (Figure 6.2). The average heterozygosity, again, was highest for *ABCC4*, but the lowest value was in *ABCB4*. African Americans had the highest θ values among all ethnic groups for each gene. θ and π values were similar to large scale SNP discovery studies in the 313 genes studied by Stephens *et al.* (5), 75 blood pressure genes studied by Halushka *et al.* (3) and the 24 membrane transporter genes studied by Leabman *et al.* (6).

Values for π were significantly lower than θ for all genes studied ($p < 0.0001$). The difference between the two values is the basis for the Tajima's D statistic that detects the deviation from the neutral model (15). Under neutrality, π and θ values are equal and therefore D values are zero. A positive D value suggests that heterozygotes have a selective advantage, while a negative value provides evidence that one allele is favored over the other (5). A negative D value can also mean a recent population expansion that produces an excess of rare variants (15). For the members of the ABC transporters, total D values were negative suggesting population expansion or negative selection. However, D values were less than zero for different ethnic groups for each gene (Table 6.8). For example, African Americans had a positive D value for *ABCB11*, Caucasians had a postive D value for *ABCC5*, and Asian American had positive D values for *ABCB11*, *ABCC3*, *ABCC4*, *ABCC5*, and *ABCG2*. The negative values may be due to the large number of rare variants that were identified in these sample populations, while positive D values may mean a selective advantage for heterozygosity in these genes.

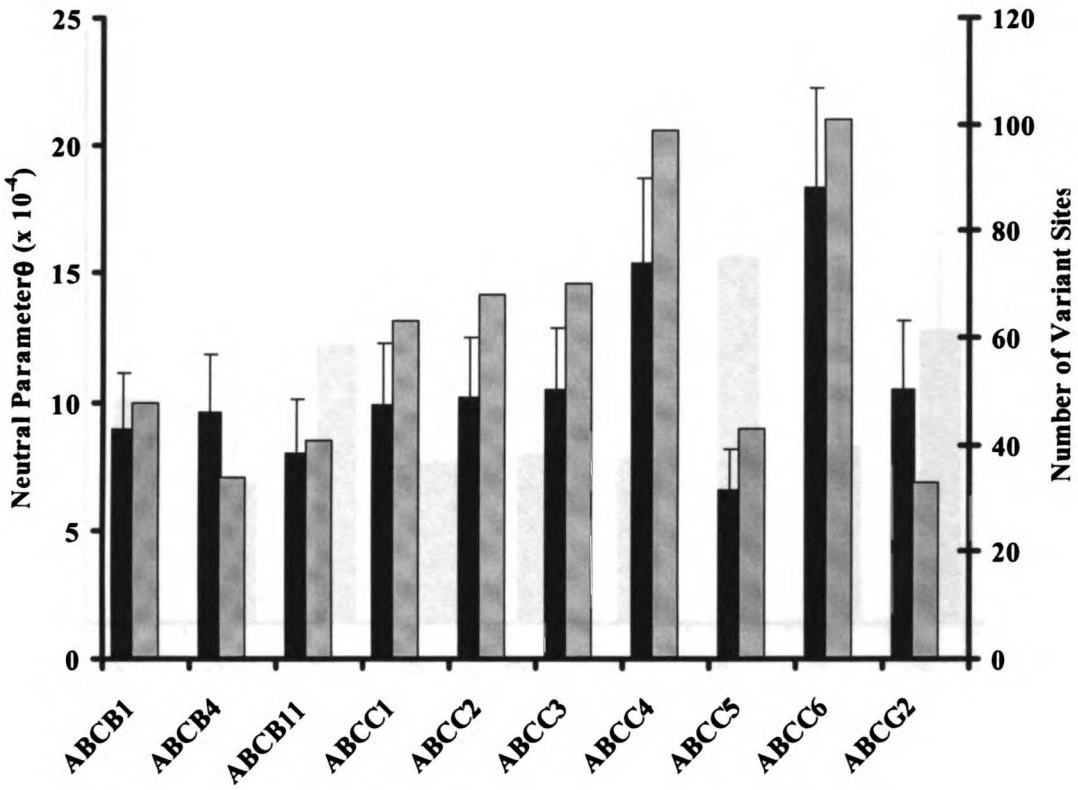

Figure 6.1 Neutral mutation parameter (θ) and number of variant sites identified across ABC transporters. Analysis of the neutral parameter shows that *ABCC4* and *ABCC6* contain the most variations among the ABC transporters, while *ABCC5* has the least. The neutral parameter pattern for each gene is consistent with the number of SNPs identified. Black bars, θ values; grey bars, variant number.

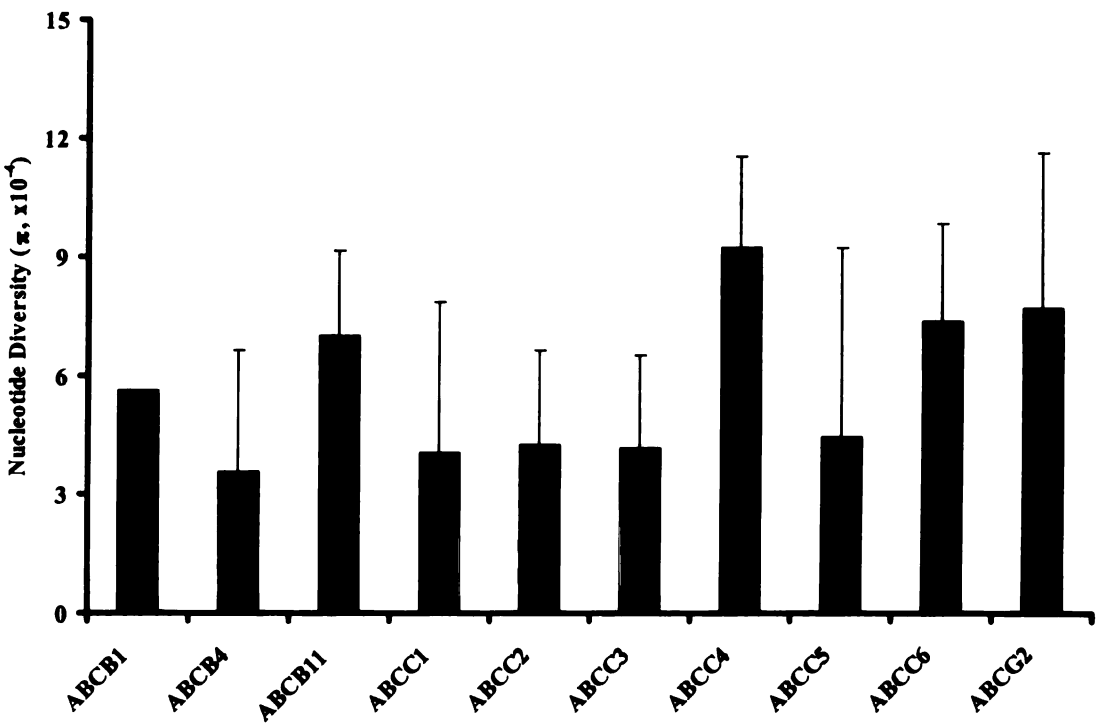

Figure 6.2 Nucleotide diversity (π) among ABC transporters studied. *ABCC4* has the highest heterozygosity values among ABC transporters, followed by *ABCG2*, *ABCC6*, and *ABCB11*.

Table 6.8 Summary of Population Statistics Across Ethnic Groups in ABC Transporters

Gene	Base Pairs	Ethnicity	Chromosomes	Segregating Sites	θ ($\times 10^{-4}$)	± SD ($\times 10^{-4}$)	π ($\times 10^{-4}$)	± SD ($\times 10^{-4}$)	Tajima D
ABCB1	7381	Total	494	48	9.59	2.28	5.63	3.10	-1.83
		African American	200	35	8.07	2.23	5.52	3.06	-1.31
		Caucasian	200	25	5.77	1.71	5.38	2.99	-0.229
		Asian	60	15	4.36	1.64	4.19	2.45	-0.118
		Mexican	20	13	4.96	2.24	5.93	3.42	0.711
		Pacific Islander	14	9	3.83	1.98	5.55	3.30	1.48
ABCB4	6066	Total	494	33	8.03	2.06	3.55	2.19	-2.00
		African American	200	25	7.02	2.08	4.44	2.63	-1.26
		Caucasian	200	13	3.65	1.29	2.38	1.61	-0.814
		Asian	60	10	3.54	1.48	2.52	1.70	-0.697
		Mexican	20	8	3.72	1.86	2.08	1.53	-1.21
		Pacific Islander	14	5	2.59	1.55	3.24	2.19	0.586

ABCB11	6443	Total	494	39	8.93	2.21	6.99	3.81	-0.85
		African American	200	30	7.93	2.25	8.17	4.39	0.115
		Caucasian	200	23	6.08	1.83	5.52	3.12	-0.303
		Asian	60	12	3.99	1.59	5.19	2.99	0.812
		Mexican	20	17	7.44	3.20	6.74	3.88	-0.397
		Pacific Islander	14	11	5.37	2.66	4.50	2.82	-0.596
ABCC1	7296	Total	494	49	9.91	2.35	4.07	2.36	-2.63
		African American	200	40	9.33	2.51	4.42	2.54	-2.34
		Caucasian	200	21	4.90	1.51	3.74	2.21	-0.737
		Asian	60	13	3.82	1.49	3.05	1.89	-0.576
		Mexican	20	8	3.09	1.55	3.35	2.12	0.235
		Pacific Islander	14	5	2.15	1.28	2.42	1.68	0.292
ABCC2	9693	Total	494	67	10.2	2.30	4.28	2.36	-3.07
		African American	200	57	10.0	2.55	5.27	2.84	-2.56
		Caucasian	200	29	5.09	1.46	3.78	2.13	-0.959
		Asian	60	15	3.32	1.25	2.15	1.35	-1.09
		Mexican	20	15	4.36	1.92	3.29	1.98	-0.975
		Pacific Islander	14	14	4.54	2.16	3.96	2.38	-0.544

ABCC3	9127	Total	552	66	10.5	2.34	4.16	2.32	-3.14
		African American	160	43	8.34	2.28	4.34	2.42	-2.29
		Caucasian	160	28	5.43	1.60	3.97	2.24	-1.02
		Asian	120	16	3.27	1.13	4.03	2.27	0.664
		Mexican	100	27	5.71	1.79	3.97	2.25	-1.21
		Pacific Islander	12	9	3.27	1.73	2.31	1.56	-1.02
ABCC4	8660	Total	552	92	15.4	3.29	9.24	4.76	-2.48
		African American	160	69	14.1	3.62	11.1	5.68	-1.30
		Caucasian	160	45	9.20	2.49	7.73	4.06	-0.783
		Asian	120	28	6.03	1.84	8.10	4.24	1.35
		Mexican	100	42	9.37	2.73	7.73	4.07	-0.882
		Pacific Islander	12	24	9.18	4.23	9.59	5.38	0.266
ABCC5	9478	Total	552	43	6.58	1.58	4.48	2.46	-1.32
		African American	160	28	5.23	1.54	4.14	2.31	-0.785
		Caucasian	160	21	3.92	1.23	4.30	2.39	0.311
		Asian	120	17	3.35	1.14	4.52	2.50	1.04
		Mexican	100	20	4.08	1.36	3.69	2.10	-0.317
		Pacific Islander	12	12	4.19	2.11	3.17	2.00	-1.00

ABCC6*	7750	Total	552	98	18.4	3.89	7.39	3.92	-3.83
		African American	160	67	15.3	3.94	8.27	4.36	-2.78
		Caucasian	160	40	9.14	2.52	7.50	3.99	-0.823
		Asian	120	24	5.78	1.81	4.87	2.74	-0.567
		Mexican	100	32	7.98	2.42	6.65	3.60	-0.725
		Pacific Islander	12	12	5.13	2.58	4.56	2.81	-0.456
ABCG2	4441	Total	552	32	10.5	2.68	7.70	4.36	-0.918
		African American	160	22	8.77	2.73	7.81	4.43	-0.362
		Caucasian	160	16	6.38	2.15	4.67	2.91	-0.731
		Asian	120	16	6.72	2.32	7.81	4.44	0.463
		Mexican	100	16	6.96	2.45	7.18	4.14	0.092
		Pacific Islander	12	6	4.47	2.60	4.88	3.27	0.249

Population genetic statistics for each gene broken down by ethnic group.

* *ABCC6* was analyzed with 28 of 31 exons sequenced.

6.3.3 Nucleotide Diversity in the Coding and Non-coding Region

In general, non-coding regions had higher π values compared to coding regions (Figure 6.3). When examining the coding region variants, there was a bias towards synonymous variants compared to non-synonymous variants. This was also true when examining the conserved variant sites (Table 6.9). Ratios of non-synonymous and synonymous π values provide a measure of selection in a given gene (6, 20). These ratios revealed that the ABC transporters are under selective pressure to reduce the variability in their coding sequence. *ABCC1* and *ABCC5* were the least tolerable of amino acid changes than other members of the family. The bias towards synonymous variants was more evident in evolutionarily conserved sites, where *ABCC1* and *ABCC5* had ratios of .002 and .001, respectively. Interestingly, *ABCG2* had a slight bias towards non-synonymous changes, with ratios of 1.66 for the total coding sequence and 1.02 for evolutionarily conserved sites. This may mean that *ABCG2* is under positive selection and is allowing variations to occur. With the wide range of substrates transported by MXR, allowing genetic variation to occur may be a mechanism to adapt its function to the ever-changing exposure to xenobiotics.

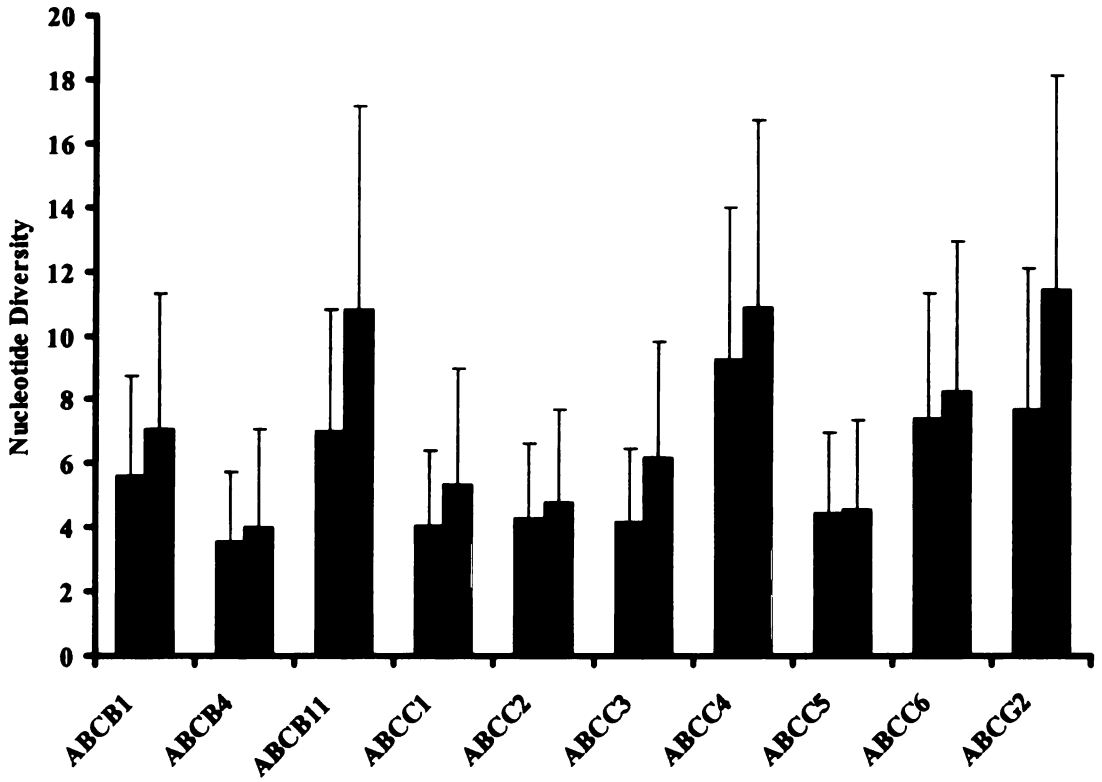

Figure 6.3 Nucleotide diversity (π) in coding and noncoding regions. Black bars represent coding sequences and grey bars represent non-coding sequences.

Table 6.9 Nucleotide Diversity (π x 10^{-4}) in the Coding Region

	Coding Region		Conserved Sites		Unconserved Sites		π_{NS} / π_{Syn} Ratios		
	NS	Syn	NS	Syn	NS	Syn	Coding	Conserved	Unconserved
ABCB1	2.41	10.7	0.540	13.9	8.16	0.000	0.230	0.040	N/A
ABCB4	1.43	9.60	0.350	11.5	5.78	1.01	0.150	0.030	5.71
ABCB11	2.30	12.6	2.37	14.4	2.08	7.37	0.180	0.170	0.280
ABCC1	0.150	13.6	0.030	14.0	0.690	11.9	0.010	0.000	0.060
ABCC2	2.18	8.81	0.590	11.5	4.99	3.59	0.250	0.050	1.39
ABCC3	0.780	6.37	0.350	3.74	1.84	13.6	0.120	0.090	0.130
ABCC4	1.88	25.9	0.680	32.1	6.47	0.990	0.070	0.020	6.52
ABCC5	0.030	18.5	0.040	18.8	0.000	15.4	0.000	0.000	0.000
ABCC6	4.24	13.3	2.51	14.8	7.75	9.89	0.320	0.170	0.780
ABCG2	3.30	1.99	2.71	2.650	4.78	0.290	1.66	1.02	16.5

Nucleotide diversity (π) values for the coding region across ABC transporters. Listed are the π values for the coding region, for conserved and unconserved sites, and non-synonymous to synonymous ratios. Evolutionarily conserved and unconserved sites were determined by protein alignment with human, rat, mouse, and dog orthologs. NS, non-synonymous; Syn, synonymous.

6.3.4 Nucleotide Diversity in Evolutionarily Conserved (EC) and Unconserved (EU) Sites

Evolutionarily conserved and unconserved sites were determined by aligning human, rat, mouse, and dog protein sequences. There were 178 SNPs that occurred in evolutionarily conserved sites, and 94 SNPs that occurred at evolutionarily unconserved sites. For the majority of transporters studied, π_{NS} at EC sites was lower than π_{NS} in EU sites, and π_{NS}/π_{Syn} ratios were lower at conserved sites than unconserved sites (Table 6.9). This suggests that EC sites may be important for protein function and that variations that cause protein changes in these conserved areas are selected against. For *ABCC1* and *ABCC5*, π_{NS} values at EC sites were the lowest, while *ABCB11*, *ABCC6* and *ABCG2* had the highest. *ABCC1*, *ABCC3* and *ABCC5* had extremely low π_{NS}/π_{Syn} ratios both in EC and EU nucleotides, suggesting that these three transporters do not tolerate amino acid changes. On the other hand, *ABCG2* showed that it was tolerating amino acid changes at both EC and EU sites with π_{NS}/π_{Syn} ratios of 1.02 and 16.5, respectively.

6.3.5 Nucleotide Diversity in Structural Regions

The secondary structure of ABC transporters is comprised of membrane spanning α-helices linked together by loops. One of the crucial areas is the transmembrane domains (TMDs) where it is believed substrate binding occurs. Nucleotide diversity was variable in the TMDs, and in general was lower than the loops (Table 6.10). *ABCB11*, *ABCB4*, *ABCC1*, *ABCC4*, *ABCC5* and *ABCC6* had relatively high π values in the TMDs among the ABC transporters studied while *ABCB1* had the lowest value for this region. However, the π values were biased towards synonymous SNPs for these transporters

(Table 6.11). An examination of π_{NS}/π_{Syn} ratios revealed that for BSEP, MDR3, MRP1, MRP4, and MRP5 amino acid changes were not tolerated as their ratios were less than 0.025 (Table 6.12). MDR1 had no synonymous changes in the TMD and therefore a ratio could not be calculated. However, the π value at non-synonymous sites was very low suggesting any variation in the TMD was not well tolerated.

The nucleotide binding folds (NBFs) are small regions where ATP binding and hydrolysis occur. They are comprised of the Walker A, Walker B and Signature C motifs and genetic variations in this region can affect protein function. Variation in this region was low and, of all the ABC transporters characterized, *ABCC6* and *ABCG2* were the only genes found to have a non-synonymous SNP inside this region. The *ABCC6* variant, a Pro664Ser, was found in one chromosome in Caucasians and Asian Americans, while the *ABCG2* variant, an Ile206Leu, was found in one chromosome in Caucasians. Other transporters carried SNPs surrounding the NBF, which potentially could affect structure.

The tail loops, which are defined as the N-terminal and C-terminal tails, had a broad range of nucleotide diversity across all genes studied (Table 6.10). Much like the TMD, there was more variability in synonymous sites than non-synonymous sites (Table 6.11) and π_{NS}/π_{Syn} ratios were low providing evidence that variations are kept low in this region possibly to reduce the likelihood of losing protein function (Table 6.12). This may be due to the presence of the NBF in the C-terminal tail or that they may have an effect on tertiary structure. The two exceptions to this were *ABCC6* and *ABCG2*, which had ratios of 3.84 and 1.68, respectively. There are no apparent reasons why these two transporters would tolerate variation in this region, especially since ATPase activity is found in the C-terminal tail and MXR only carries one NBF. Simple random genetic drift

could be a possible explanation for the higher non-synonymous variations for these two genes. In CFTR (cystic fibrosis transmembrane conductance regulator, *ABCC7*), the C-terminal tail loop carries a PDZ domain which controls its trafficking and recycling to the plasma membrane (21). Perhaps variability in this region may allow for changes in trafficking and expression of MRP6 and MXR.

The internal loops, or loops between the two tail loops, were just as variable as the tail loops when examining π values and there again was a trend towards synonymous sites being more variable (Table 6.11). *ABCC2* and *ABCG2* had higher π values at non-synonymous sites than synonymous sites. Only two genes had a π_{NS}/π_{Syn} ratio of below 0.025, *ABCC1* and *ABCC5* (Table 6.12). In fact *ABCC1* and *ABCC5* had low ratios across all structural regions, perhaps signifying the importance of their function.

Table 6.10 Population Genetic Statistics Across Structural Regions in ABC Transporters

Gene	Sequence Section	Base Pairs	Variant sites	θ ($\times 10^{-4}$)	$\pm$ SD ($\times 10^{-4}$)	π ($\times 10^{-4}$)	$\pm$ SD ($\times 10^{-4}$)	π_{NS} ($\times 10^{-4}$)	Tajima D
ABCB1	Total	7381	48	9.59	2.28	5.63	3.10	2.41	-1.83
	TMD	756	1	1.95	1.99	0.053	0.49	0.071	-0.352
	Tail loop	1014	7	10.2	4.30	6.50	5.56	2.42	-0.508
	Short loop	450	1	3.28	3.34	0.090	0.819	0.113	-0.352
	Loop	3087	18	8.60	2.60	5.33	3.48	2.96	-0.963
	Internal loop	2073	11	7.83	2.79	4.76	3.57	3.22	-0.736
	Globular loop	2637	17	9.51	2.92	6.22	4.07	3.45	-0.846
	Extracellular	306	1	4.82	4.91	0.132	1.20	0.166	-0.352
	Cytoplasm	2781	17	9.02	2.77	5.90	3.86	3.27	-0.846
ABCB4	Total	6066	33	8.03	2.06	3.55	2.19	1.43	-2.00
	TMD	753	5	9.80	4.76	5.70	5.71	0.071	-0.471
	Tail loop	1032	4	5.72	3.06	0.578	1.40	0.051	-0.870
	Short loop	435	2	6.78	4.96	0.278	1.47	0.00	-0.572
	Loop	3087	13	6.21	2.09	2.71	2.14	1.75	-1.17
	Internal loop	2055	9	6.46	2.48	3.78	3.06	2.59	-0.685
	Globular loop	2652	11	6.12	2.18	3.11	2.47	2.03	-0.922
	Extracellular	291	1	5.07	5.16	0.139	1.26	0.00	-0.352
	Cytoplasm	2796	12	6.33	2.18	2.98	2.36	1.93	-1.05
ABCB11	Total	6443	39	8.93	2.21	6.99	3.81	2.30	-0.855
	TMD	756	3	5.85	3.56	7.08	6.52	0.00	0.167
	Tail loop	1056	6	8.38	3.77	1.77	2.51	0.294	-1.00
	Short loop	279	2	10.6	7.74	0.865	3.25	0.894	-0.548
	Loop	3210	21	9.65	2.79	4.04	2.80	2.82	-1.61
	Internal loop	2154	15	10.3	3.29	5.16	3.74	4.06	-1.13
	Globular loop	2931	19	9.56	2.84	4.35	3.03	3.01	-1.43
	Extracellular	330	2	8.94	6.54	2.87	5.56	0.00	-0.405
	Cytoplasm	2880	19	9.73	2.89	4.18	2.96	3.15	-1.49

ABCC1	Total	7296	49	9.91	2.35	4.07	2.36	0.149	-2.63
	TMD	1071	4	5.51	2.95	3.45	3.66	0.050	-0.362
	Tail loop	906	7	11.4	4.82	4.58	4.65	0.00	-0.841
	Short loop	618	7	16.7	7.06	10.1	8.83	0.919	-0.552
	Loop	3477	21	8.91	2.57	3.24	2.34	0.179	-1.77
	Internal loop	2571	14	8.03	2.63	2.76	2.30	0.242	-1.43
	Globular loop	2859	14	7.22	2.36	1.74	1.65	0.018	-1.65
	Extracellular	393	6	22.5	10.13	15.0	13.4	0.265	-0.422
	Cytoplasm	3084	15	7.18	2.30	1.73	1.60	0.168	-1.72
ABCC2	Total	9693	67	10.2	2.30	4.28	2.36	2.18	-3.07
	TMD	1071	8	11.0	4.42	0.639	1.44	0.496	-1.44
	Tail loop	954	11	17.0	6.05	11.3	8.26	3.05	-0.629
	Short loop	657	3	6.74	4.09	0.731	1.96	0.634	-0.707
	Loop	3567	26	10.8	2.93	4.63	3.02	2.68	-1.79
	Internal loop	2613	15	8.47	2.71	2.19	1.97	2.55	-1.68
	Globular loop	2910	23	11.7	3.28	5.51	3.62	3.14	-1.54
	Extracellular	432	1	3.41	3.48	0.648	2.26	0.841	-0.293
	Cytoplasm	3135	25	11.8	3.24	5.18	3.39	2.93	-1.72
ABCC3	Total	9127	66	10.5	2.34	4.16	2.32	0.783	-3.14
	TMD	1071	4	5.42	2.89	0.135	0.653	0.091	-0.930
	Tail loop	951	9	13.7	5.24	5.23	4.95	1.30	-1.01
	Short loop	663	7	15.3	6.46	4.16	4.99	0.718	-1.01
	Loop	3513	32	13.2	3.39	2.77	2.08	0.991	-2.76
	Internal loop	2562	23	13.0	3.64	1.86	1.79	0.877	-2.48
	Globular loop	2850	25	12.7	3.48	2.44	2.05	1.05	-2.45
	Extracellular	438	6	19.9	8.92	6.22	7.49	0.973	-0.861
	Cytoplasm	3075	26	12.3	3.32	2.28	1.91	0.993	-2.52

ABCC4	Total	8660	92	15.4	3.29	9.24	4.76	1.88	-2.48
	TMD	756	10	19.2	7.04	4.33	4.83	0.324	-1.35
	Tail loop	1263	12	13.8	4.74	4.85	4.25	0.643	-1.27
	Short loop	483	5	15.0	7.28	3.90	5.52	4.92	-0.821
	Loop	3222	35	15.8	3.96	8.04	4.77	2.22	-1.80
	Internal loop	1959	23	17.0	4.76	10.1	6.33	3.25	-1.18
	Globular loop	2739	30	15.9	4.14	8.78	5.28	1.74	-1.51
	Extracellular	201	2	14.4	10.6	3.72	8.06	4.66	-0.436
	Cytoplasm	3021	33	15.9	4.04	8.33	4.97	2.06	-1.68
ABCC5	Total	9478	43	6.58	1.58	4.48	2.46	0.033	-1.32
	TMD	756	2	3.84	2.81	1.96	3.09	0.00	-0.288
	Tail loop	1407	3	3.09	1.88	2.82	2.91	0.034	-0.069
	Short loop	246	1	5.90	6.00	0.147	1.42	0.00	-0.348
	Loop	3558	10	4.08	1.50	4.85	3.13	0.039	0.328
	Internal loop	2151	7	4.72	1.99	6.17	4.25	0.043	0.425
	Globular loop	3312	9	3.94	1.51	5.19	3.36	0.042	0.517
	Extracellular	246	1	5.90	6.00	0.147	1.42	0.00	-0.348
	Cytoplasm	3312	9	3.94	1.51	5.19	3.36	0.042	0.517
ABCC6	Total	7750	98	18.4	3.89	7.39	3.92	4.24	-3.83
	TMD	756	9	17.3	6.60	4.44	4.91	1.88	-1.21
	Tail loop	855	15	25.5	8.10	4.69	4.82	5.76	-1.83
	Short loop	377	5	19.2	9.32	1.33	3.50	0.382	-1.03
	Loop	3104	40	18.7	4.57	7.05	4.32	4.79	-2.46
	Internal loop	2249	25	16.1	4.41	7.95	5.09	4.42	-1.54
	Globular loop	2727	35	18.6	4.68	7.84	4.83	5.40	-2.12
	Extracellular	248	4	23.4	12.49	1.88	5.12	0.390	-0.877
	Cytoplasm	2856	36	18.3	4.57	7.50	4.62	5.17	-2.20

ABCG2	Total	4441	32	10.5	2.68	7.70	4.36	3.30	-0.918
	TMD	378	2	7.68	5.61	0.381	1.85	0.372	-0.558
	Tail loop	1200	7	8.47	3.57	4.74	4.27	5.23	-0.610
	Short loop	183	0	0.00	0.00	0.00	0.00	0.00	n/a
	Loop	1590	8	7.30	2.92	3.63	3.25	3.99	-0.761
	Internal loop	390	1	3.72	3.79	0.183	1.26	0.234	-0.339
	Globular loop	1407	8	8.25	3.30	4.10	3.67	4.51	-0.761
	Extracellular	282	1	5.15	5.24	0.254	1.74	0.322	-0.339
	Cytoplasm	1308	7	7.77	3.27	4.35	3.92	4.79	-0.610

Population genetic statistics were calculated in different loops and in the transmembrane domain (TMD). The tail loop represents the N- and C-terminal tails; short loop represents loops < 50 amino acids in length; the Loop represents all segments not part of the transmembrane domain; the internal loop excludes the tail loops; the globular loop represents loops > 50 amino acids; Extracellular loops are found in the extracellular plane; Cytoplasmic loops are found in the cytoplasm.

Table 6.11 Nucleotide Diversity (π x 10^{-4}) in Structural Regions

	TMD			Tail Loops			Internal Loops		
Gene	**Total**	**NS**	**Syn**	**Total**	**NS**	**Syn**	**Total**	**NS**	**Syn**
ABCB1	0.053	0.071	0.00	6.50	2.42	20.49	4.76	3.22	10.2
ABCB4	5.70	0.071	22.6	0.578	0.051	2.37	3.78	2.59	7.96
ABCB11	7.08	0.000	28.8	1.77	0.294	6.93	5.16	4.06	9.08
ABCC1	3.45	0.050	14.0	4.58	0.000	19.66	2.76	0.242	11.2
ABCC2	0.639	0.496	1.09	11.31	3.05	39.06	2.19	2.55	0.952
ABCC3	0.135	0.091	0.265	5.23	1.30	17.20	1.86	0.877	4.95
ABCC4	4.33	0.324	15.7	4.85	0.643	20.36	10.1	3.25	33.7
ABCC5	1.96	0.00	7.31	2.82	0.034	11.98	6.17	0.043	27.8
ABCC6	4.44	1.88	11.2	4.69	5.76	1.50	7.12	3.93	16.9
ABCG2	0.381	0.372	0.410	4.74	5.23	3.12	0.183	0.234	0.00

Variability in three structural regions across ABC transporters measured by π. Listed are nucleotide diversities among non-synonymous and synonymous sites for comparison. TMD, transmembrane domain; NS, non-synonymous; Syn, synonymous.

Table 6.12 Selective Pressure in Structural Regions Measured by π_{NS} / π_{Syn}

Gene	TMD	Tail loops	Short loops	Loop	Internal loops	Globular loops	Extracellular	Cytoplasm
ABCB1	N/A	0.118	N/A	0.216	0.314	0.218	N/A	0.217
ABCB4	0.003	0.021	0.000	0.288	0.325	0.298	0.000	0.291
ABCB11	0.000	0.042	1.20	0.337	0.447	0.335	0.000	0.405
ABCC1	0.004	0.000	0.022	0.013	0.022	0.002	0.004	0.024
ABCC2	0.457	0.078	0.601	0.237	2.68	0.229	N/A	0.228
ABCC3	0.341	0.075	0.048	0.119	0.177	0.155	0.042	0.158
ABCC4	0.021	0.032	N/A	0.078	0.096	0.052	N/A	0.068
ABCC5	0.000	0.003	0.000	0.002	0.002	0.002	0.000	0.002
ABCC6	0.169	3.82	0.029	0.340	0.232	0.354	0.000	0.353
ABCG2	0.907	1.68	N/A	1.67	N/A	1.68	N/A	1.68

Selective pressure measured by π_{NS} / π_{Syn} in structural regions across ABC transporter genes. TMD, transmembrane domain; NS, non-synonymous; Syn, synonymous.

6.3.6 Predicting the Importance of Transporters in Tissues

ABC transporters are expressed in a myriad of tissues in the body. Nucleotide diversity in these transporters may help predict the importance of the roles these transporters play in protecting these tissues. ABCB11, ABCB1, ABCB4, ABCC1, ABCC2, ABCC3, ABCC6 and ABCG2 are all expressed in the liver, although ABCC1 and ABCC3 are at low levels (10, 11). Although the function of MRP6 is still unclear, the other transporters play a role in removing compounds from the hepatocyte into either the blood or bile. Transporters expressed in the liver had comparable π values, and only *ABCC1* had a π_{NS}/π_{Syn} ratio below .025 (Table 6.13). MDR3 and MRP2 have roles in the regulation of bile flow, and MRP3 is believed to play a compensatory role if and when MRP2 function is lost. MRP3 provides a backup for toxin elimination from the liver if bile flow is compromised. MRP1 was also shown to play a compensatory role, as mRNA expression was increased in cholestatic rat liver (22) and human liver diseases (23). It is interesting that BSEP, the main bile salt transporter, has a high π value compared to the other liver transporters. However, a majority of the variations in *ABCB11* occurred at synonymous sites.

P-glycoprotein, MRP1, MRP4, MRP5 and MXR are found in blood-tissue barriers where they are thought to protect such organs as the brain and testis, the placenta, and cerebrospinal fluid from toxins (14, 24). MDR1 has been extensively studied and is shown to be important in the protection of rodent brain from xenobiotics (25). MRP1 is found in the blood-CSF barrier (12), while MRP4 and MRP5 are found in capillary endothelium making up the blood-brain barrier (26). MXR expression has also been

Table 6.13 Nucleotide Diversity (π x 10^{-4}) in ABC Transporters in the Liver

	π_{Total}	π_{NS}	π_{Syn}	π_{NS} / π_{Syn}
ABCB1	5.63	2.41	10.7	0.226
ABCB4	3.55	1.43	9.60	0.149
ABCB11	6.99	2.30	12.6	0.182
ABCC1	4.07	0.149	13.6	0.011
ABCC2	4.28	2.18	8.81	0.248
ABCC3	4.16	0.783	6.37	0.123
ABCC6	7.39	4.24	13.3	0.318
ABCG2	7.70	3.30	1.99	1.66

Nucleotide diversity among transporters expressed in the liver. Listed are π values in for non-synonymous and synonymous sites. As a measure of function, ratios between the two are given.

detected in brain microvessel endothelial cells (27). *ABCC1* and *ABCC5* have lower average heterozygosity values than *ABCB1*, *ABCC4*, and *ABCG2*, and may be an indicator of the importance of their protective roles (Table 6.14). In fact their π_{NS}/π_{Syn} ratios in the coding sequence were 0.011 and 0.002, respectively, suggesting that the native protein is important in its function. Studies in triple knockout mice provided evidence that Mrp1 was important at the blood-CSF barrier (28). Mice lacking both *mrp1* and *mdr1a/b* that were administered an i.v. dose of eotoposide had a 10-fold increase in etoposide concentration in the CSF compared to the *mdr1a/b* double knockout. However, another study used an Mrp1 null mouse and found no difference in brain distribution of the typical MRP1 substrates $E_2$17βG and DNP-SG (29). Interestingly, MRP4, which transports similar compounds as MRP5, is found to be twice as variable as MRP5. MRP4 may be allowed to evolve, while MRP5 remains constant in

its structure and function. MDR1, although having higher π values, showed extremely low variability in the TMD. This suggests selection against amino acid changes that would affect substrate binding.

Table 6.14 Nucleotide Diversity (π x 10^{-4}) of ABC Transporters in the Brain

	π_{Total}	π_{NS}	π_{Syn}	π_{NS} / π_{Syn}
ABCB1	5.63	2.41	10.7	0.226
ABCC1	4.07	0.149	13.6	0.011
ABCC4	9.24	1.88	25.9	0.073
ABCC5	4.48	0.033	18.5	0.002
ABCG2	7.70	3.30	1.99	1.66

Comparison of nucleotide diversity between ABC genes expressed in the brain. Nucleotide diversity, as measured by π, is calculated for non-synonymous and synonymous sites for each gene. Their ratios are given as a measure of selection for function. NS, non-synonymous; Syn, synonymous.

6.3.7 Genetic Diversity in Disease Causing Transporters

Currently, there are several known diseases that are associated with ABC transporters. Genetic variations in *ABCB11*, *ABCB4*, *ABCC2* and *ABCC6* have been associated with progressive familial intrahepatic cholestasis type (PFIC) 2, PFIC3, Dubin-Johnson syndrome (DJS), and pseudoxanthoma elasticum (PXE), respectively. The large scale sequencing effort in this study was only able to identify one disease causing SNP that was reported in the literature for BSEP at nucleotide position 890A>G (30, 31). This was as a singleton in a Caucasian. One variant identified in PXE patients was found in our sample population as a singleton in a Caucasian as well. None of the identified variants in PFIC3 (32, 33) and DJS (34-40) were found in our sample set.

These disease-causing SNPs are rare, however, and many of the DJS SNPs were reported in Japanese patients, who comprised only a third of the Asian American population in this study. There was no correlation between the nucleotide diversity in a gene and its association with disease. Both *ABCB4* and *ABCC2* have one of the lower π_{Total} values of the ABC transporters, while *ABCB11* and *ABCC6* have one of the higher values (Figure 6.3).

6.4 PERSPECTIVES

The data presented in this study provide a unique perspective on the variability in genes that belong to the same superfamily and have similar function and structure. The data provided suggest that these transporters are under negative selection due to the high abundance of rare non-synonymous SNPs and the subsequent low values for π_{NS}/π_{Syn} ratios. The selection against protein changes is further evidence that ABC transporters have a crucial role in cellular processes. *ABCG2* had a π_{NS}/π_{Syn} ratio above one, indicating that it was undergoing positive selection.

Since greater than 50% of all disease-causing genetic variations are due to amino acid changes, understanding the amount of variation in genes and being able to predict their functional consequences are critical. For ABC transporters, two important regions in the protein are where substrate binding and ATP hydrolysis occur. Our data suggest that several of the transporters are under selective pressure and do not tolerate protein changes in these areas. *ABCC2* and *ABCC3*, however, show lower selective constraints and may be allowed to adapt to changing environmental conditions. These transporters have overlapping substrate specificities and MRP3 expression is upregulated when MRP2

function is diminished (41). Having a functional backup gene may allow variations to occur without suffering from harm.

The most polymorphic gene among the ABC transporters was *ABCC6*, a gene that hasn't been fully sequenced. Genetic variations in this gene are associated with pseudoxanthoma elasticum, a genetic disorder afflicting connective tissues (42, 43). It is interesting to note that *ABCC6* has several pseudogenes on chromosome 16 (44). These pseudogenes may provide a reservoir of genetic variation through gene conversion (4). The π_{NS}/π_{Syn} ratio in the coding sequence of *ABCC6* was higher than other ABC transporters, and this may be due to relaxed selective constraints due to duplicate copies.

The importance of ABC transporters is most notable in the treatment against cancer. MDR1, MRP1, and MXR overexpression have been implicated in cancer resistance (45). The other ABC transporters are able to transport cancer drugs as well, and may play a possible role in cancer resistance (46). Gene expression is regulated by cis-acting elements found in the 5'-, 3'-, and intronic regions (47). The sequencing efforts for the ABC transporters in this study covered only coding exons and flanking intronic boundaries. To better understand the role of genetic variation and cancer resistance, further analysis is required in these regulatory regions.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1 SUMMARY

ABC transporters are major players in the absorption, distribution and elimination of compounds from the body (1-3). Their ability to transport a wide array of chemical substrates marks them as important proteins that can affect the efficacy of drug treatment. With expression in major organs of elimination and protective barriers, these transporters limit the exposure of potentially toxic compounds by effluxing them into the lumen or blood vessels for removal (2-7). Any defect in this mechanism, either through changes in expression or protein function, can alter the cellular homeostasis and lead to disease or adverse reactions.

It has been established that the diverse group of individuals in the United States respond differently to drugs (8, 9) and that this phenomena may be due, at least in part, to differences in transporter function and/or expression (10, 11). The goals of the work presented here, therefore, were to determine the extent of variation in ABC transporters and to investigate the functional and clinical consequences of this variation.

There have been very few attempts to identify and characterize genetic variations in ABC transporters, with the exception of *ABCB1*. In Chapter 2, we sought to determine the extent of genetic variation in *ABCC2*. MRP2 is mainly expressed in the liver and plays a role in liver detoxification and bile flow (2, 12). Therefore any genetic variations that affect function of the protein may disrupt the body's ability to remove toxins through the liver. Sequence diversity in *ABCC2* was analyzed in the proximal promoter, the coding sequence, as well as flanking intronic regions. Using an ethnically diverse

collection of DNA samples, a total of 68 variants were identified. Of these, thirteen were found in the proximal promoter region and 34 were found in the coding region. Nineteen coding SNPs caused an amino acid change and 10 had an allele frequency > 1%.

Haplotypes and nucleotide diversity were also estimated from the SNPs identified. A total of 88 haplotypes were estimated through the use of PHASE (13). Nucleotide diversity estimates revealed that there was selective pressure against amino acid changes, especially at conserved sites. This was reflected in the low π values at evolutionarily conserved, non-synonymous sites compared to evolutionarily conserved, synonymous sites (0.60×10^{-4} vs. 12.58×10^{-4}).

Several assays were developed to characterize coding variants in *ABCC2*. MRP2 function could be analyzed using a flux assay in transfected MDCK II cells. Since these cells are polarizable, membrane trafficking can also be monitored. A similar assay for protein function was developed for fluorescence activated cell sorting (FACS) in which the accumulation of a fluorescent compound was inversely proportional to protein function. By far the best method to determine protein function was through the use of inside-out vesicles. This eliminated the need for Phase II enzymes, as many MRP2 substrates require conjugation before transport. Inside-out vesicles also allow for the determination of K_m and V_{max} parameters.

Proximal promoter SNPs were characterized using an *in vitro* reporter gene assay system (Chapter 3). The -1549G>A, -1292A>G, -1239G>A, and -1065C>A single variant constructs showed statistically significant decreases in promoter activity compared to a reference sequence. Haplotypes carrying combinations of variants also

showed decreased activity (-24C>T/-1019A>G/-1549G>A, -1292A>G/-1019A>G/-1549G>A, and -1065C>A/-1023G>A). Using EMSA, three variant sites showed differential binding of nuclear proteins. However, the identities of the proteins bound to the -1549A, -1292G, and -1065A probes have not been elucidated. It is feasible that these proteins interfere with the transcription of the downstream gene. Further analysis is required to determine the mechanism of this effect and the possible ramifications of having decreased expression of mRNA transcripts.

Recently, there were data suggesting that a synonymous SNP in *ABCC2* was associated with increased AUC of the anti-cancer drug irinotecan (14). Individuals carrying the *ABCC2* 3972TT showed higher AUC for irinotecan and its metabolite SN-38G, suggesting decreased function or expression of MRP2. Genetic analysis of *ABCC2* revealed that this SNP at 3972C>T was in linkage disequilibrium with the promoter haplotype carrying the -1549G>A, -1019A>G and -24C>T variants that showed decreased promoter activity *in vitro* (Chapter 3). Using allele-specific expression, we sought to determine whether there was an allelic imbalance in mRNA expression that could explain the *in vivo* association (Chapter 4). Forty-one Caucasian liver samples were analyzed for allelic expression at the 3972 locus. Overall there was a 10% increase in abundance for the C allele compared to the T allele at postion 3972 in *ABCC2*. The abundance increased to 14% when examining individuals carrying one copy of the promoter haplotypes #2 and #3. The difference between the two haplotypes was that haplotype #2 carried the -1549G>A, -1019A>G, -24C>T, and 3972C>T variants, while haplotype #3 carried only the 1249G>A variant (Chapter 4, Figure 4.3). Further analysis is needed to determine whether a 10% increase in abundance in the C allele translates

into decreased MRP2 expression in individuals carrying one or two copies of the T allele. This then may account for the increase in irinotecan AUC observed in patients carrying the TT genotype at 3972. There may also be other cis-acting elements tightly linked with the T allele further upstream in the 5'-region or in 3' or intronic sequences that may influence irinotecan pharmacokinetics.

In Chapter 5, we addressed the ability of genetic variations in transporters to influence substrate drug pharmacokinetics. We employed the use of twins to determine the heritability of drug pharmacokinetics. Digoxin was used as a probe drug, as it is minimally metabolized by Phase I enzymes and is known to be transported by P-glycoprotein in the gut (15, 16), and possibly by some uptake transporters (17). A one milligram oral dose was administered to ten monozygotic and ten dizygotic twin pairs. Pharmacokinetic parameters were calculated for each twin and results showed differences from data published in the literature. Oral clearance was higher in our population and the fraction of digoxin excreted in urine was lower (18, 19) using a mass spectrometric assay that only measures the parent unmetabolized drug. There were no significant differences in the pharmacokinetic means calculated between the two twin groups.

To determine whether the PK parameters were influenced by genetics, the heritability index was calculated using three methods. The first determined heritability by using the variance seen between twin pairs. The other two methods used the correlation coefficient for their calculations. For three PK estimates, $AUC_{0\text{-}3h}$, apparent nonrenal clearance and oral clearance, genetic factors were found to be involved in their variability. These parameters imply that bioavailability and elimination may be heritable, and that transporter genetics may be involved. Renal clearance was estimated to have no

genetic influence and this was similar to a measurement using the repeated dose application (20). This can be explained by the lack of active secretion of digoxin in the kidneys being only approximately 25% of total clearance.

Nonrenal clearance also has a significant genetic contribution and, with the fraction of digoxin dose excreted into urine being only 38%, it would be expected that biliary and intestinal elimination may play a prominent role in digoxin disposition. P-gp is expressed in the canalicular membrane in the hepatocyte and brush-border membrane of the intestine and is able to mediate the transport of compounds for elimination. Further analysis is needed to determine the effect of genetic variations in *ABCB1*, and possibly those of uptake transporters in the intestine and liver, on digoxin PK.

Lastly, in Chapter 6, we analyzed the genetic variation in ten ABC transporters. Overall genetic diversity in these transporters was similar to other genes when comparing total π and θ values (21-23). There was an overall bias towards variations at synonymous sites compared to non-synonymous sites in the coding region with *ABCC1* and *ABCC5* having the lowest ratios, possibly signifying their importance in cellular processes. However, a closer examination revealed that nine of the ten transporters did not tolerate variations at conserved nucleotides. Non-synonymous to synonymous ratios provide evidence of selection and function (24) and for the ABC transporters the ratios were low in the transmembrane domains, with the exception of *ABCG2*. The tail loops which contain the nucleotide binding folds also showed low ratios, however *ABCC6* and *ABCG2* had much higher ratios. The transmembrane domains (TMDs) and nuceleotide binding folds (NBFs) are important for protein function as they control substrate binding and ATP hydrolysis, respectively. Low ratios show that amino acid changes that could

affect substrate binding or ATP hydrolysis are selected against. *ABCG2* appeared to show positive selection in the TMD and the tail loop, while *ABCC6* only showed positive selection in the tail loop.

Nucleotide diversity also provided insight into the importance of transporters in the brain and liver. Many of the ABC transporters are found in the liver hepatocyte and mediate the efflux of chemical compounds either into the bile or blood for removal. A measure of selection for function, given by π_{NS}/π_{Syn}, showed that *ABCC1* had the lowest ratio of the liver transporters. However, ABCC1 mRNA expression is low in the liver. *ABCB1*, *ABCB4*, *ABCB11*, *ABCC2*, *ABCC3*, *ABCC6* and *ABCG2* had relatively higher ratios. The variability in these genes may be a result of functional redundancy as in the case of *ABCC2* and *ABCC3* or the presence of pseudogenes as in the case of *ABCC6*. It is also possible that since the liver is the major site of metabolism, these transporters are more variable to adapt to the ever changing barrage of chemical compounds. The π_{NS}/π_{Syn} ratios for transporters expressed in the brain found *ABCC1* and *ABCC5* to be under selective pressure as both had ratios under 0.025, whereas ratios for *ABCB1*, *ABCC4* and *ABCG2* had higher ratios. This may signify the importance of their protective function in the brain.

7.2 CONCLUSIONS

It is important to understand genetic variation in the ABC family of transporters. With many ABC transporters expressed in organs of elimination or at protective barriers, they serve to limit the exposure of drug compounds. Genetic variation in these transporters can thus lead to interindividual variability in drug response. ABC

transporters are also involved in the development of disease and have been implicated in drug resistance in tumor cells. Overexpression of MDR1, MRP1 and MXR (1, 25-28) plays a role in cancer drug resistance. Other members, although not implicated in multidrug resistance, are able to transport various anti-cancer drugs and thus may potentially play a role in resistant tumors (1, 29, 30). Four ABC transporters have been associated with PFIC2, PFIC3, Dubin-Johnson syndrome and pseudoxanthoma elasticum and genetic variation in *ABCB11*, *ABCB4*, *ABCC2* and *ABCC6*, respectively, are the genetic basis for these diseases (31-36).

It is interesting to note that these diseases are caused by rare variants. Nearly one-third of all variants identified in the ten ABC transporters were singletons and another 10% were found in only two chromosomes. Deleterious variants are under selection and through time remain low in frequency. It is reasonable to believe that a fair number of the rare variants in the ABC transporters may be non-functional.

The investigations presented in this dissertation have focused on coding sequence variants and have added significant information on the variability in ABC genes. For *ABCC2*, analysis was extended into the proximal promoter region. The variability in the ABC genes studied in this dissertation research is comparable to other genes studied (21, 22, 37). There was a bias towards synonymous SNPs compared to non-synonymous SNPs. Coding sequence variations that alter the amino acid may potentially affect protein function by altering substrate binding, protein trafficking or protein expression. ABC genes may possibly be under selective pressure to reduce the amount of variation due to their importance in transporting chemical compounds from various tissues. Currently, limited information is available on variations in cis-acting elements that

control gene expression (38, 39). Future work in regulatory regions would enhance our understanding of genetic variation in a gene. It is possible that genetic variation in regulatory regions is a mechanism for overexpression of efflux transporters in tumor cells.

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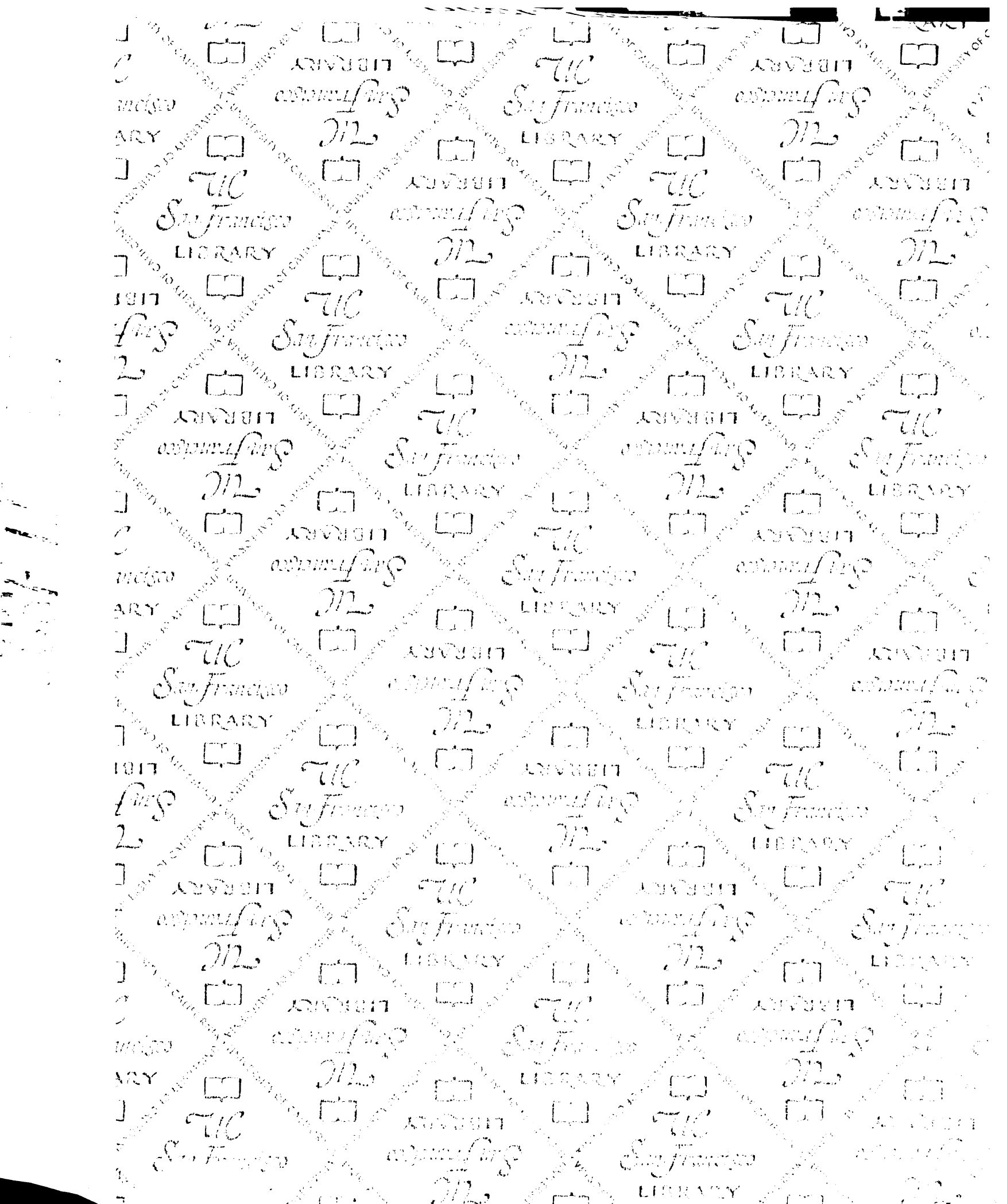
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