

mRNA EXPRESSION PROFILING OF THE ATP-BINDING CASSETTE TRANSPORTER  
FAMILY

by

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## ABSTRACT

Multidrug resistance (MDR) in cancer is characterized by cross-resistance to a variety of structurally unrelated chemotherapeutic compounds. Altered expression levels of P-glycoprotein (Pgp) and multidrug resistance-associated protein-1 (MRP1) are strongly associated with MDR. Pgp and MRP1 are members of the ATP-binding cassette (ABC) superfamily of membrane proteins. Members of this family transport a wide spectrum of substrates (e.g. peptides, metals, ions, toxins and chemotherapeutic drugs) across cell membranes. Completion of the human genome-sequencing project revealed there are close to 50 ABC genes (Dean et al., 2001); however, function or substrate specificity is known for only a few. It is anticipated that several of these proteins may also contribute to MDR and mutations or altered expression levels of a number of ABC proteins are causal of diseases such as cystic fibrosis and Tangier disease. Despite these associations, there has been no systematic attempt to profile expression of ABC transcripts.

We have developed a competitive-RT-PCR assay to examine RNA expression profiles of 35 human ABC transcripts. This assay uses RNA competitors for each transcript, and fluorescently labeled PCR products are resolved using capillary electrophoresis. The data is highly reproducible and accurate.

We have examined ABC transcript expression profiles in sensitive and vincristine-resistant cell lines, as well as cells exposed short-term (24 hours) to various chemotherapeutic drugs. Vincristine-selection demonstrated multiple, possibly sequential, expression changes may collectively contribute to MDR, whereas short-term drug exposure revealed few expression changes, suggesting that altered mRNA expression is not a common response to xenobiotic exposure. ABC expression profiles were also generated for 12 normal human tissues. Analysis of individual transcripts and the collective expression profiles provided insights into the relative contribution of ABC proteins to MDR, defense against xenobiotic toxins and phospholipid and cholesterol homeostasis. Another study examined the effect of enforced expression of the proapoptotic BAX cDNA on the collective ABC expression profile and relative drug-resistance of a cell line. Our results suggest Pgp-mediated MDR may be regulated by BAX protein in some cell lines.

The continued systematic evaluation of ABC transcript profiles under various experimental conditions should provide further insights into the normal physiological roles of ABC proteins.

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## ABBREVIATIONS

9-(2-phosphonylmethoxy-ethyl) adenine . . . . .	PMEA
12-O-tetradecanoylphorbol-13-acetate . . . . .	TPA
Acute myeloid leukemia . . . . .	AML
Adrenoleukodystrophy protein . . . . .	ALDP
All- <i>trans</i> retinoic acid . . . . .	ATRA
Alpha minimal essential medium . . . . .	$\alpha$ -MEM
Amino acid . . . . .	AA
ATP-binding cassette . . . . .	ABC
Bcl-2 homology domains . . . . .	BH1-BH4
Bicinchoninic acid . . . . .	BCA
Bovine serum albumin . . . . .	BSA
Breast cancer resistance protein . . . . .	BCRP
Chloramphenicol acetyl transferase . . . . .	CAT
Coefficient of variation . . . . .	COV
Complementary DNA . . . . .	cDNA
Concentration of chemotherapeutic drug that inhibits 3-day cell proliferation 50% . . .	IC <sub>50</sub>
Cystic fibrosis transmembrane conductance regulator . . . . .	CFTR
Deoxynucleotide-triphosphates . . . . .	dNTPs
Deoxyribonucleic acid . . . . .	DNA
Diethyl pyrocarbonate . . . . .	DEPC
Dimethylsulfoxide . . . . .	DMSO
Dithiothreitol . . . . .	DTT
Dulbecco's modified eagle's medium . . . . .	DMEM
Ethylenediaminetetraacetic acid . . . . .	EDTA
Expressed sequence tag . . . . .	EST
Expressed sequence tag database . . . . .	dbEST
Fetal bovine serum . . . . .	FBS
Glutathione-S-transferase $\pi$ . . . . .	GST $\pi$
Hemagglutinin . . . . .	HA
High pressure liquid chromatography . . . . .	HPLC
Human genome organization . . . . .	HUGO

Human ovarian carcinoma cell line . . . . .	SKOV3
Human ovarian carcinoma cell line . . . . .	SW626
Human promyelocytic leukemia cell line . . . . .	HL60
Human total genome sequence database . . . . .	HTGS
Human T-lymphoid cell line . . . . .	CEM
Human vinblastine-resistant SKOV3 subline . . . . .	SKVLB1.0
Human vincristine-resistant SKOV3 sublines . . . . .	SKVCR0.015, SKVCR0.1 and SKVCR0.25
Kilodalton . . . . .	kDa
Lung resistance protein . . . . .	LRP
Messenger RNA . . . . .	mRNA
Minutes . . . . .	min
Multidrug resistance . . . . .	MDR
Multidrug resistance-associated protein-1 . . . . .	MRP1
N-terminal transmembrane domain . . . . .	TMD <sub>0</sub>
Non-redundant database . . . . .	nr
Nucleotide-binding domain . . . . .	NBD
P-glycoprotein . . . . .	Pgp
Phosphate buffered saline . . . . .	PBS
Polymerase chain reaction . . . . .	PCR
Reduced glutathione . . . . .	GSH
Reverse transcription . . . . .	RT
Reverse-transcription-polymerase-chain-reaction . . . . .	RT-PCR
Ribonuclease protection assay . . . . .	RPA
Ribonucleic acid . . . . .	RNA
Revolutions per minute . . . . .	rpm
Room temperature . . . . .	rt
Roswell Park Memorial Institute 1640 medium. . . . .	RPMI 1640
Seconds . . . . .	sec
Serial analysis of gene expression . . . . .	SAGE
Sodium dodecyl sulphate . . . . .	SDS
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis . . . . .	SDS-PAGE
Standard deviation . . . . .	SD

Standard error of the mean .....	SEM
Tetrazolium salt (3-(4,5-dimethylthiazol-2-yl))-2, 5-diphenyl-tetrazolium bromide) .....	MTT assay
Transmembrane domain .....	TMD
Tris-buffered saline .....	TBS
Untranslated region .....	UTR

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## CHAPTER I

### INTRODUCTION TO THE ATP-BINDING CASSETTE (ABC) SUPERFAMILY

#### 1.1 Introduction to Transport Processes

All cells acquire a significant proportion of the molecules and ions they need from their surrounding extracellular fluid. Biological membranes serve as highly selective filters and as devices for active transport, control the entry of nutrients and the exit of waste-products, generate concentration gradients of ions crucial to many physiological processes and act as sensors of external signals. There is an unceasing traffic of compounds in and out of the cell through its plasma membrane. In eukaryotic cells, there is also transport in and out of membrane-bounded intracellular compartments such as the nucleus, endoplasmic reticulum and mitochondria. The lipid bilayer of the plasma membrane and intracellular membranes is permeable to water molecules and other small, uncharged molecules, but is impermeable to many essential compounds such as small hydrophilic molecules (glucose) and macromolecules [protein and ribonucleic acid (RNA)].

Molecules that are unable to freely diffuse across the lipid bilayer must be assisted by either facilitated diffusion or active transport. Facilitated diffusion is an energy-independent process that takes place through proteins, or assemblies of proteins, embedded in the plasma membrane that have access to both sides of the membrane. This transport can only equalize the concentration of the molecules. These transmembrane proteins form a water-filled channel through which ions can pass down a concentration gradient. These channels are selective such that the structure of the protein admits only certain types of molecules.

Active transport requires the expenditure of energy, usually in the form of ATP, to move molecules against their concentration gradient. Some transporters bind ATP directly and use the

energy of its hydrolysis to drive active transport (direct active transport), whereas other transporters use the energy already stored in the gradient of a directly pumped ion (indirect active transport). When these ions are transported back by facilitated diffusion (usually  $\text{Na}^+$ ), the energy released can be harnessed to transport another molecule against a concentration gradient. ABC proteins act as direct energy-dependent transporters.

## 1.2 Historical Review of ABC Transporters

The characterization of transmembrane transport proteins over the past 20 years has identified a myriad of membrane transport systems that can be grouped into a limited number of families, based upon sequence, functional similarity and mechanism of action (Higgins, 1992). The largest and most diverse of these families is the ABC superfamily whose members are associated with many important physiological processes in organisms ranging from bacteria to humans. The first ABC proteins to be characterized were bacterial uptake systems. The hemolysin protein complex, a pathogenicity factor of *Escherichia coli*, was the first bacterial ABC export protein to be discovered (Felmlee et al., 1985) and by 1992, 50 ABC proteins had been identified, the majority from prokaryotic species. Among the first eukaryotic ABC transporters to be characterized were: P-glycoprotein (Pgp), an ATP-dependent drug efflux pump over-expressed in some tumour cells (Gerlach et al., 1986); the *Saccharomyces cerevisiae* Ste6 protein, necessary for the secretion of the  $\alpha$ -mating pheromone (Kuchler et al., 1989); the *white* and *brown* gene products of *Drosophila melanogaster*, involved in the transport of pigment precursors (Dreesen et al., 1988); and the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a chloride ion channel (Riordan et al., 1989). Comparison of amino acid (AA) sequences revealed significant similarity between the prokaryote, yeast and higher eukaryote open reading frames, suggesting these proteins belonged to a single family, now known as the ABC family. This observation and the large number of prokaryotic ABC genes

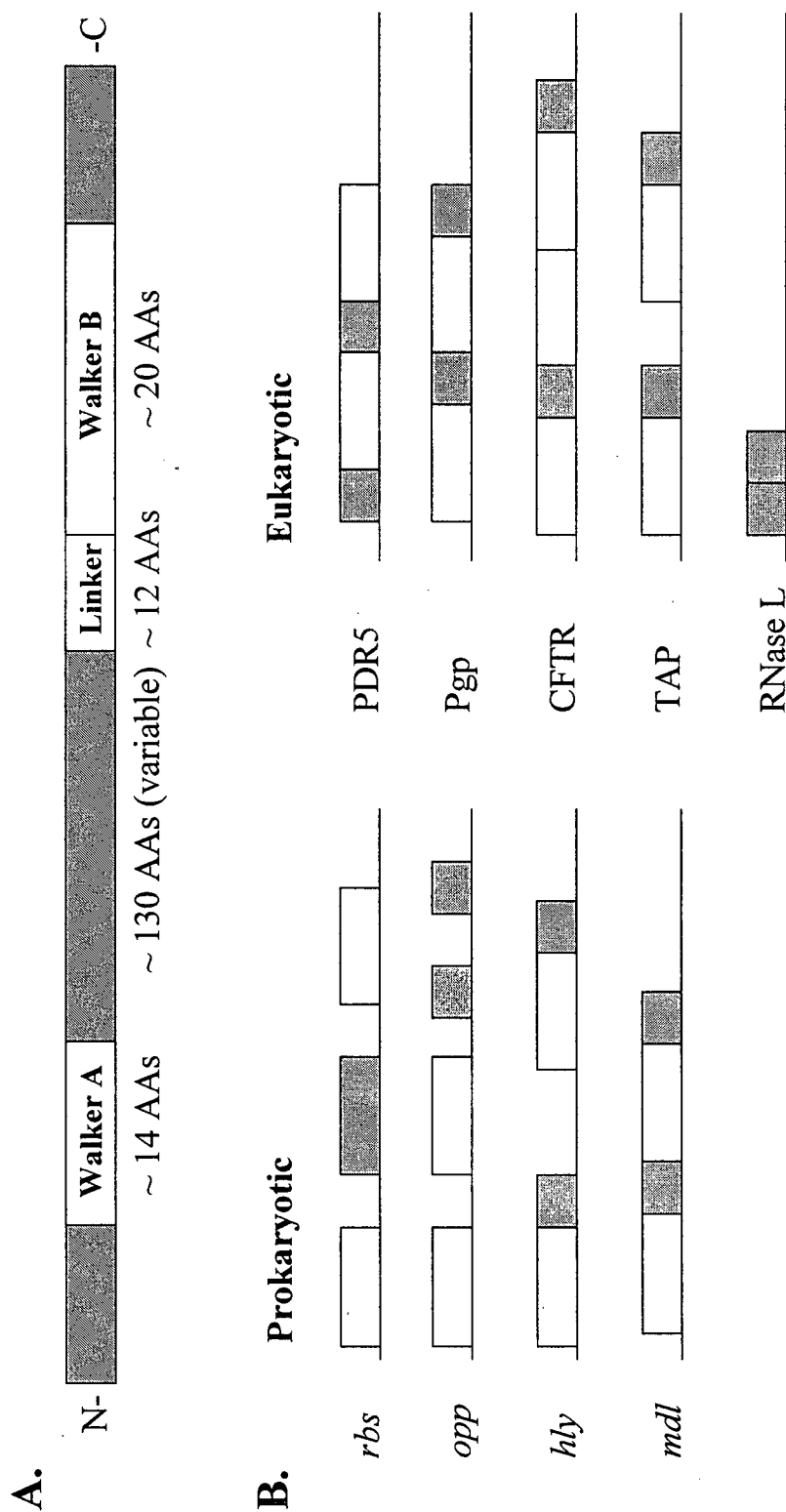
that had been identified by the early 1990s, prompted an intensive search for novel ABC proteins in humans and other organisms. With the recent completion of the human genome sequencing project, 48 ABC genes have been identified in the human genome (Dean et al., 2001).

The majority of ABC proteins identified act as transmembrane pumps; however, other roles have also been described, such as in deoxyribonucleic acid (DNA) repair in *E. coli* (*UvrA*) (Husain et al., 1986), protein synthesis in *S. cerevisiae* (EF-3) (Qin et al., 1990) and as part of the mammalian RNase L complex (OABP) (Bisbal et al., 1995). All eukaryotic ABC transporters with known functions are either responsible for cellular export of compounds (across the plasma membrane) or sequestration of substrates into intracellular compartments (across organelle membranes). In contrast, some bacterial ABC proteins mediate either the import or export of substrates at the bacterial plasma membrane, including sugars, AAs, vitamins, metals and organic ions, while others mediate pathogenicity via the secretion of protein toxins such as hemolysins and proteases (Higgins, 1992). A high degree of substrate diversity is also found among eukaryotic ABC proteins. Substrates transported by eukaryotic ABC proteins include ions, metals, steroid hormones, sugars, peptides and drugs. Despite the large range of substrate diversity across the entire superfamily, most individual ABC proteins exhibit limited substrate specificity. Notable exceptions are Pgp and the multidrug resistance-associated protein-1 (MRP1), each of which transports a variety of structurally unrelated compounds. It is poorly understood how such substrate diversity is achieved among the superfamily as a whole, while each specific transporter retains a high degree of selectivity for its own particular substrate. It is also this great diversity that makes it virtually impossible to predict the function or substrate specificity of a particular ABC transporter based on sequence information alone.

### 1.3 Structural Characteristics of ABC Transporters

ABC proteins are defined by the presence of at least one nucleotide-binding domain (NBD). Within the NBD are three consensus sequences: the first two are the ABC Walker A (G-X<sub>2</sub>-G-X-G-K-S/T-T/S-X<sub>4</sub>-hydrophobic) and ABC Walker B (R-X-hydrophobic<sub>2</sub>-X<sub>2</sub>-P/T/S/A-X-hydrophobic<sub>4</sub>-D-E-A/P/C-T-S/T/A-A/G-hydrophobic-D) motifs, similar but not identical to the consensus sequences first described by Walker et al. (1982) for a variety of ATP-requiring enzymes (Figure 1.1). The third motif, called the “Linker” or “ABC Signature Motif” (hydrophobic-S-X-G-Q-R/K-Q-R-hydrophobic-X-hydrophobic-A), is found only in proteins of the ABC superfamily (Figure 1.1) (Shyamala et al., 1991). These conserved regions are always found in the order (from N-terminus to C-terminus): ABC Walker A, followed by a 100-150 AA non-conserved stretch, Linker and then Walker B, with the Linker immediately preceding the Walker B consensus sequence.

It is believed that the binding and hydrolysis of ATP by the NBD is coupled to, and provides energy for, substrate transport. The minimal structural requirement for an active ABC transporter appears to be two transmembrane domains (TMDs) and two NBDs. These may be present within one polypeptide chain (‘full-transporters’) or homo- or hetero-dimers may form between two ‘half-transporters’, each consisting of a single TMD and NBD. The majority of TMDs possess four, six or eight predicted membrane spanning  $\alpha$ -helices that are divergent among ABC proteins, consistent with the notion that TMDs are important determinants of substrate specificity. The NBDs are located in the cytoplasm and transfer the energy to transport the substrate across the membrane. Of the dozen or so ABC proteins characterized thus far, full-transporters predominantly appear to localize to the plasma membrane, whereas most half-transporters are localized to membranes of intracellular organelles. An example of an early hypothetical structural model of Pgp embedded in the plasma membrane is shown in Figure 1.2.

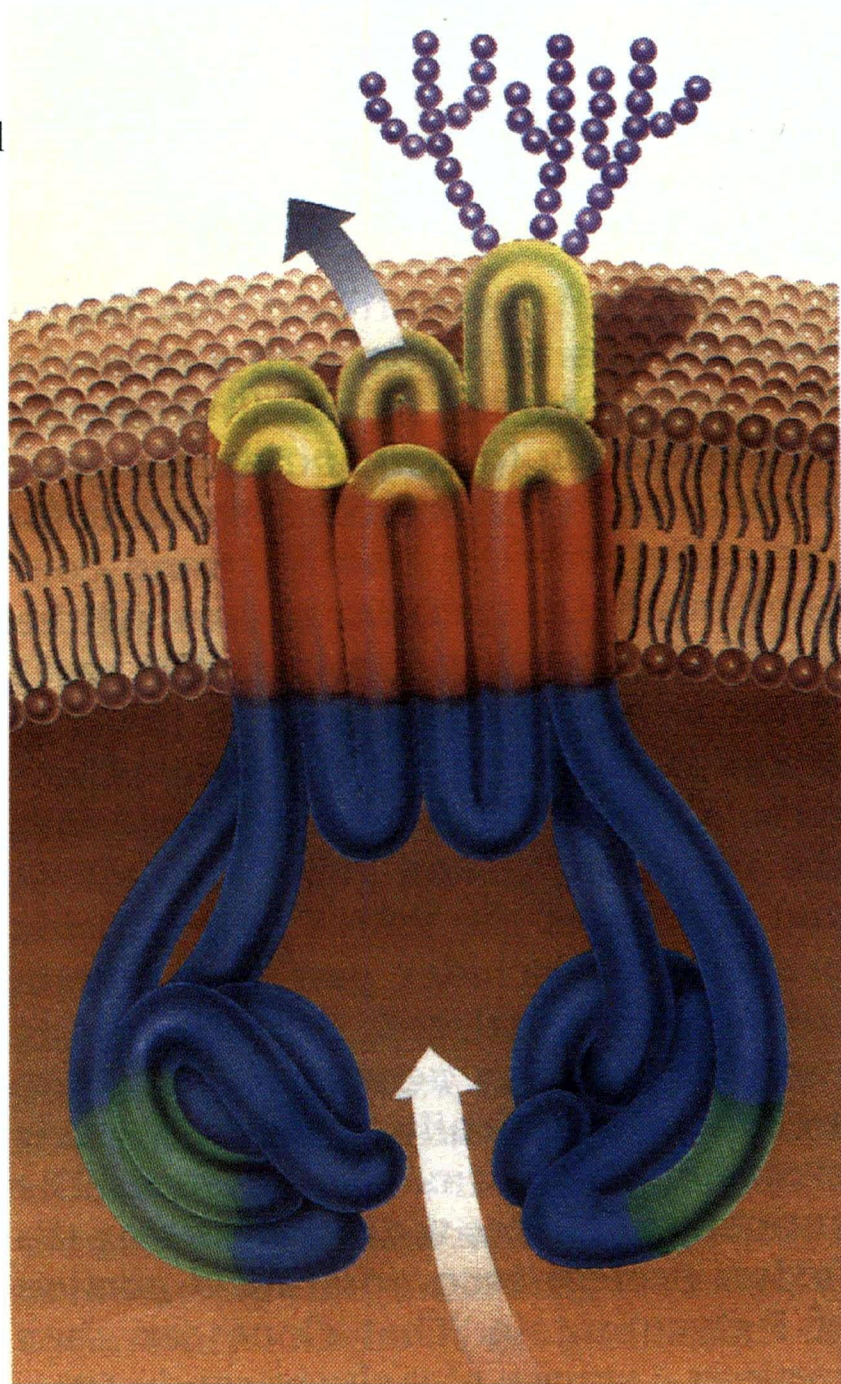


**Figure 1.1 Conserved Amino Acid Sequences and Structural Domain Arrangements of ABC Genes.** (A) The NBD of an ABC gene contains the Walker A and B motifs found in all ATP-binding proteins, as well as a Linker motif, that is unique to ABC proteins (open boxes). Hatched boxes represent non-conserved regions of the NBD. The approximate number of AAs within each motif are indicated below. See main text for complete consensus sequences. (B) The organization of prokaryotic ABC genes into operons is indicated with the NBDs displayed as gray shaded boxes and the TMDs as open boxes. Similarly for eukaryotic genes, the arrangement of domains in the RNA transcript is shown. See main text for references.

Outside of cell

Plasma  
membrane

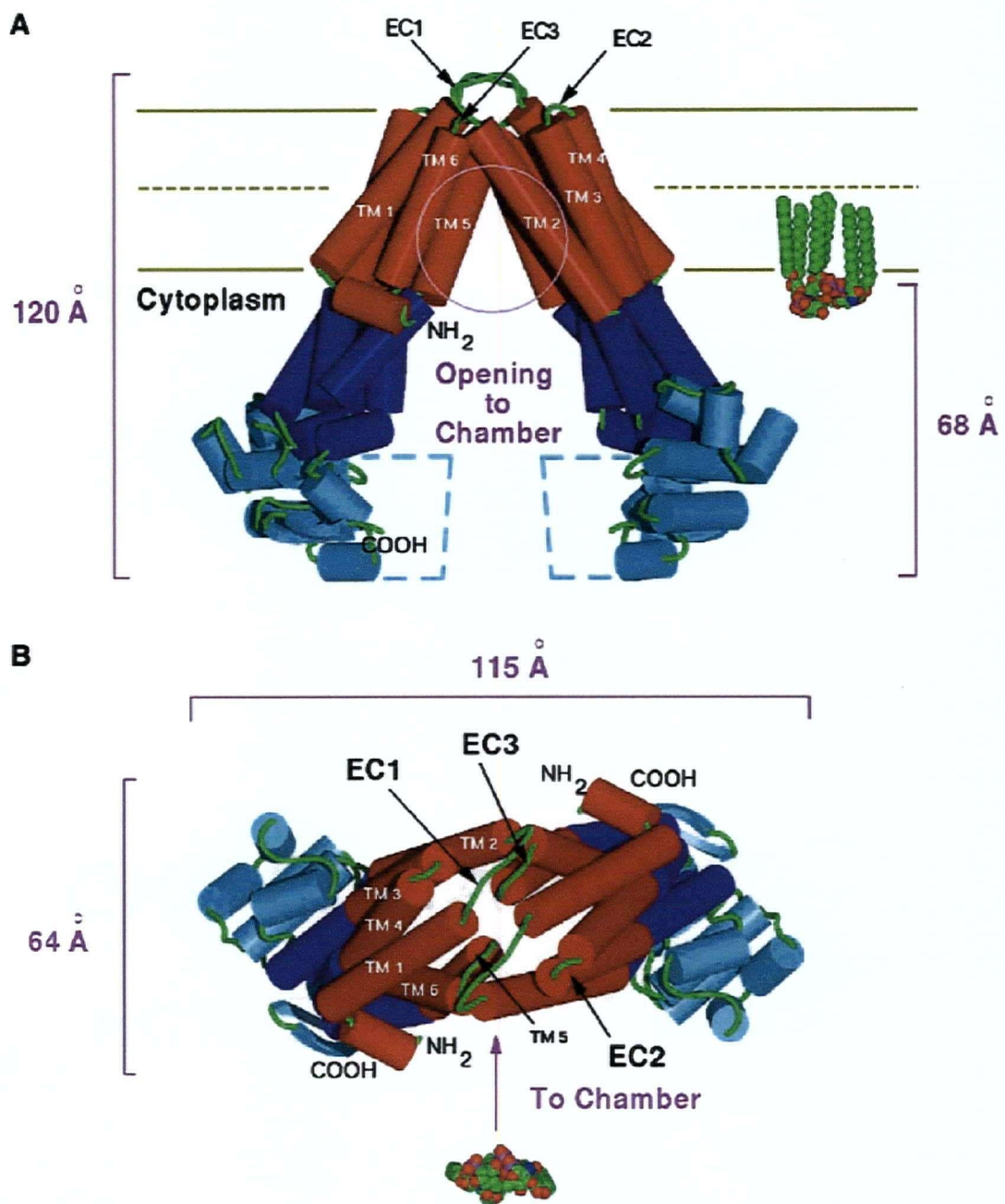
Inside of cell



**Figure 1.2 Hypothetical Structure of Pgp Embedded in the Plasma membrane.** An early hypothetical model of Pgp based on hydropathy plot analysis of the AA sequence. The protein chain was proposed to cross the lipid bilayer 12 times to form a 12-sided pore (red). The two ATP-binding domains were suggested to be positioned inside the cell (green). The sugar chain was predicted to be attached to the protein outside of the cell (purple). Taken from: Kartner, N. and Ling, V. (1989). Multidrug resistance in cancer. *Scientific American*, **260**(3), pg 50.

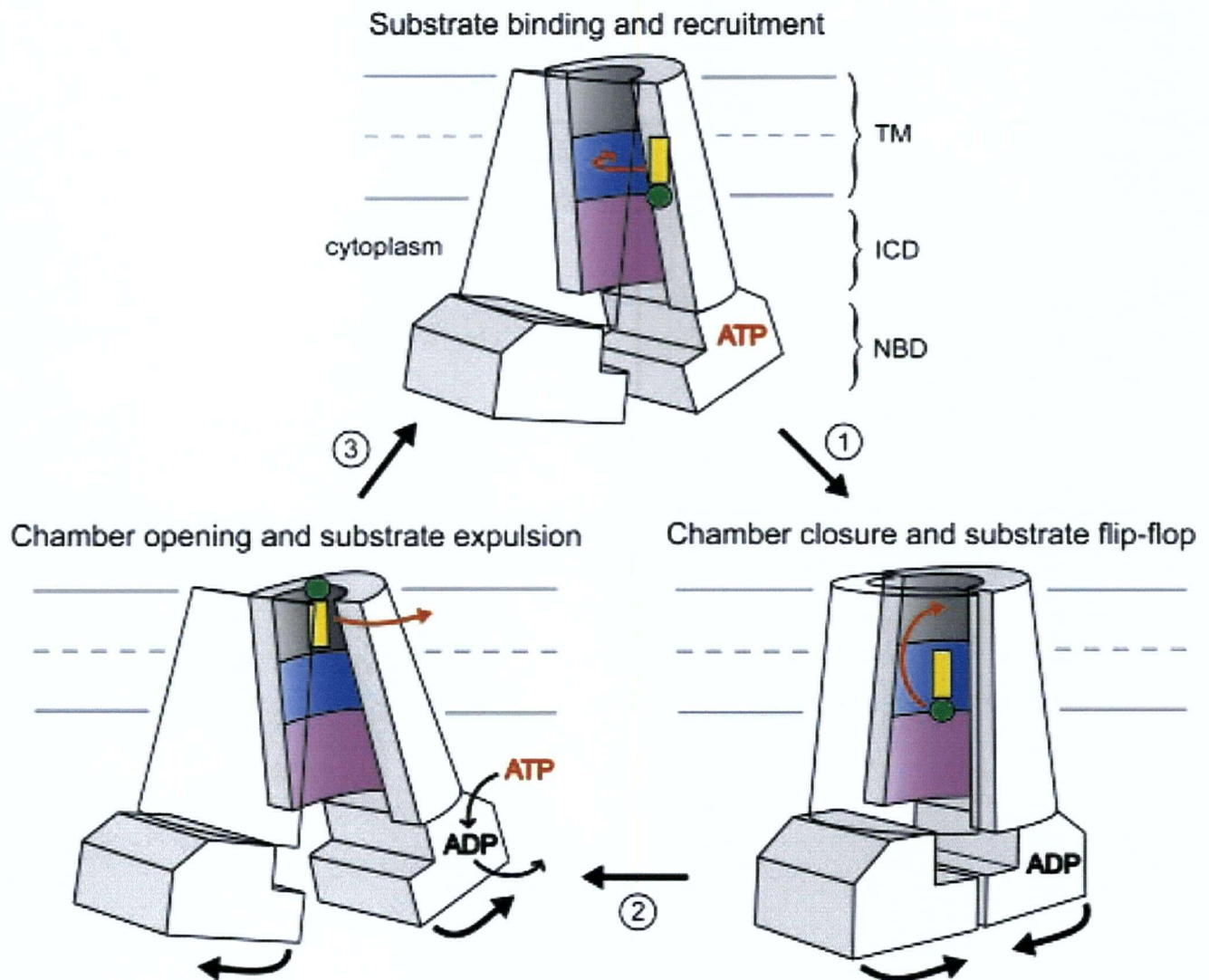
With respect to gene organization of ABC transporters, it is thought that the functional ABC transporter uses two TMDs and two NBDs, even though these domains may be encoded by one to four genes. For example, in the *Salmonella typhimurium* Opp oligopeptide importer system, the genes *OppB*, *OppC*, *OppD* and *OppF*, code for two separate transmembrane proteins and two separate nucleotide binding proteins, respectively (Figure 1.1) (Hiles et al., 1987). Similarly, in the Rbs operon, the *RbsA* gene encodes two nucleotide binding proteins side by side, whereas two genes, *RbsC* and *RbsD* each encode a single transmembrane protein (Bell et al., 1986). In most prokaryotic ABC transporters, the TMDs and NBDs are encoded by separate genes, though always within the same operon. However, for several genes the domains are fused into either half- (e.g. HlyB, Felmlee et al., 1985) or full-transporters (e.g. Mdl, Allikmets et al., 1993). A similar degree of diversity is observed among eukaryotic gene arrangements, although without the use of operons (Figure 1.1). The most common arrangement consists of all four domains present in a single gene, as in the case of the eukaryotic Pgp multigene family (Ng et al., 1989); and in reverse order, the yeast PDR5 pleiotropic drug resistance gene (Balzi et al., 1994). In another less common arrangement, one TMD may be joined to one NBD within the same gene, as found in the two mammalian TAP genes (Monaco, 1992); and in reverse order, the *D. melanogaster* brown eye pigment precursor importer gene (Dreesen et al., 1988). Another 'full-transporter', CFTR, contains an additional large hydrophilic domain in between the two halves of the molecule, known as the R domain (Riordan et al., 1989). This domain contains multiple sites for phosphorylation and is believed to be involved in the regulation of channel activity. Several mammalian ABC genes (e.g. MRPs 1, 2, 3, 6 and 7, SUR1 and SUR2) also contain an additional TMD at the N-terminus (TMD<sub>0</sub>); however, the function of this domain is unknown. Finally, some eukaryotic genes lack TMDs altogether, as in the case of RNase L, which consists of two side by side NBDs and no TMDs (Bisbal et al., 1995).

Despite years of work on both bacterial and mammalian ABC transporters, particularly Pgp, it has not been clear exactly how these proteins work. A crystal structure for an ABC transporter has been difficult to achieve due to the inherent difficulties of working with membrane proteins. Finally, some insight has been provided by the recent publication by Chang and Roth (2001) of the first high-resolution structure of a complete ABC transporter, the MsbA lipid A transporter of *E. coli* (Figure 1.3). The structure of MsbA suggests a general mechanism for hydrophobic substrate translocation by ABC transporters consisting of: substrate binding, triggering of ATP hydrolysis, and recruitment of the substrate to the chamber; closure of the chamber and translocation of the substrate, with potential coordinated interaction between the two NBDs; and finally, opening of the chamber, possibly via movement of transmembrane  $\alpha$ -helices 2 and 5, release of the substrate to the outer bilayer leaflet, and nucleotide exchange (Figure 1.4) (Chang and Roth, 2001).



**Figure 1.3 X-ray Crystallography Structure of *Escherichia coli* MsbA.**

**(A)** View of dimer looking into the chamber opening. The TMD, NBD and intracellular domain are coloured red, cyan and dark blue, respectively. Transmembrane  $\alpha$ -helices are marked and the connecting loops are shown in green. A model of lipid A (not in the crystal structure) is shown to the right, embedded in the lower bilayer leaflet. Solid and dotted green lines represent the boundaries of the membrane bilayer leaflets. **(B)** View of MsbA from extracellular side, perpendicular to the membrane model of lipid A. Transporter dimensions are labeled and images were rendered using BOBSCRIPT and RASTER 3D. Taken from: Chang, G. and Roth, C.B. (2001). Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science*, **293**, 1793-1800.



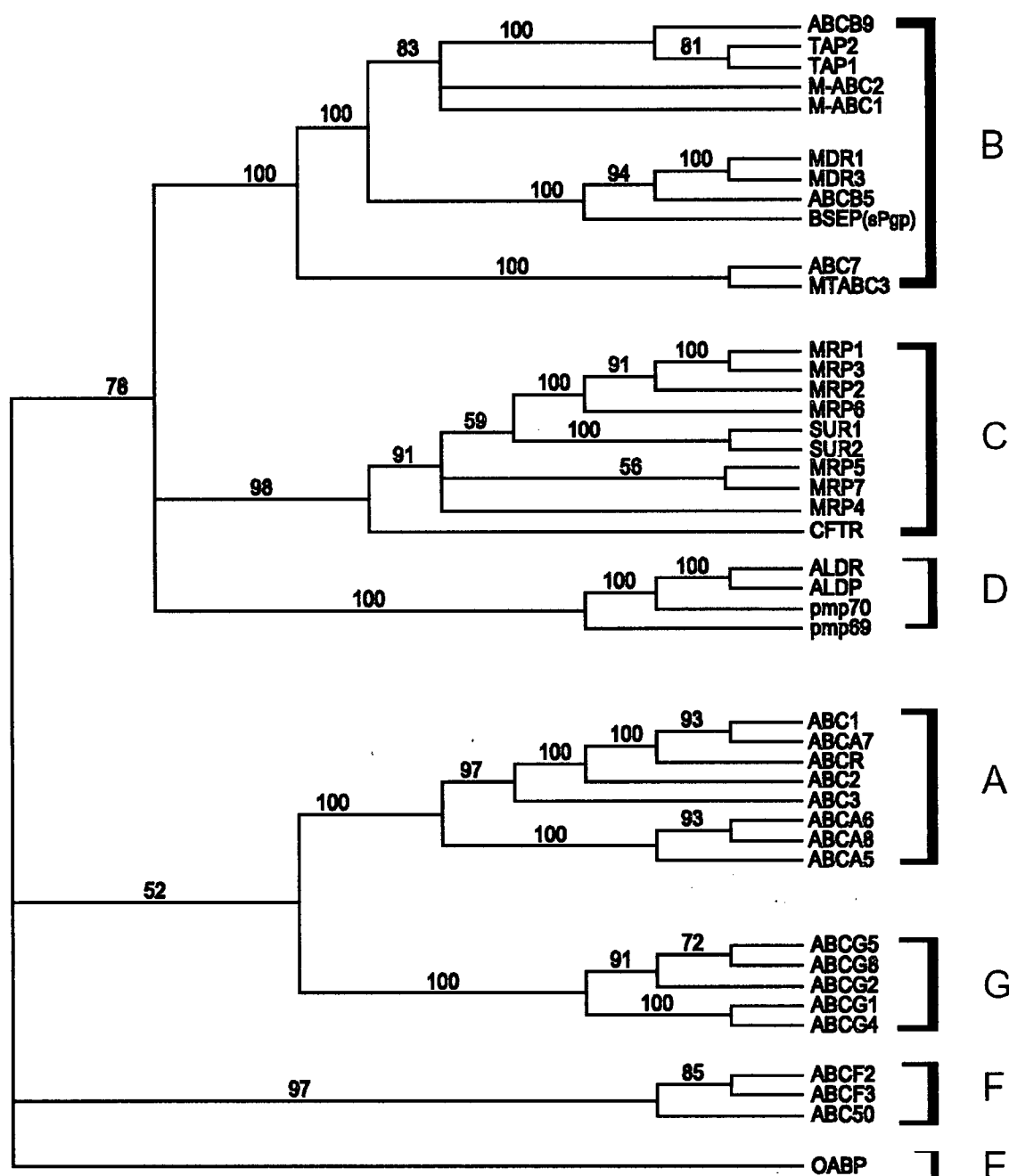
**Figure 1.4 Potential Model for Lipid A Transport by MsbA.**

Stages 1 to 3 begin at top and proceed clockwise. (1) Lipid A binding, triggering ATP hydrolysis, and recruitment of substrate to chamber. (2) Closure of the chamber and translocation of lipid A. Interaction between the two NBDs is possible. (3) Opening of the chamber, movement of TM2/TM5, release of lipid A to the outer bilayer leaflet, and nucleotide exchange. A small yellow rectangle and a green circle denote the hydrophobic tails and sugar head groups of lipid A, respectively. The transmembrane domain (TM), intracellular domain (ICD) and nucleotide-binding domain (NBD) are labeled. The cell membrane is represented as a set of two horizontal lines separated by a dash to indicate the separation of bilayer leaflets. Blue regions indicate positive charge lining the chamber, and purple regions represent the intracellular domain. The gray region in the outer membrane side of the chamber is hydrophobic. Taken from: Chang, G. and Roth, C.B. (2001). Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science*, **293**, 1793-1800.

## 1.4 Human ABC Subfamilies

An analysis of the phylogenetic relationships amongst human ABC proteins using all available full-length sequences, as well as partial sequences of sufficient length, indicate the presence of 7 distinct subfamilies (Figure 1.5). The existence of several ABC proteins has been inferred from database searches and/or early work describing human ABC proteins using expressed sequence tags (ESTs) (Allikmets et al., 1996) and will be mentioned only briefly. An analysis of the recently published human genome sequence indicates as many as 48 human ABC proteins may exist (Dean et al., 2001). A summary of biological functions and disease associations for each gene is presented in Table I.I.

Subfamily A consists of 6 known members, all of which are full-transporters, and 6 other members (ABCA5, ABCA8, ABCA9, ABCA10, ABCA12 and ABCA13) are predicted based on available EST and genomic sequence information, and in some cases, full-length complementary DNA (cDNA) sequences available in the nucleotide databases (Broccardo et al., 1999; Dean et al., 2001). ABC1 and ABCR are associated with inherited genetic disorders of lipid export processes involving vesicular budding between the Golgi and plasma membrane (Orso et al., 2000) and transport of protonated N-retinylidene-phosphatidylethanolamine out of retinal disk cells (Weng et al., 1999), respectively. The function of ABC1 may also be required for the engulfment of apoptotic bodies (Hamon et al., 2000; Luciani and Chimini, 1996). ABC2 has been shown to be expressed predominantly in brain tissue and to localize to the lysosomal/endosomal compartment in cell lines transfected with the cDNA (Vulevic et al., 2001). Amplification of the ABC2 gene in an ovarian carcinoma cell line has also been associated with resistance to the chemotherapeutic agent estramustine (Laing et al., 1998). Gene expression analysis in human macrophages showed that ABC2 messenger RNA (mRNA) is induced during cholesterol import indicating that ABC2 may be a cholesterol-responsive gene (Kaminski et al., 2001a).



**Figure 1.5. Phylogenetic Tree of ABC proteins in *Homo sapiens*.**

Predicted AA sequences were aligned using ClustalX. The cladogram is derived from a neighbour-joining analysis using PAUP\*4.03a of the resulting alignment. Bootstrapping was used to determine the relative support for the various branches of the tree (1000 replicates), and nodes with less than 50% support were collapsed to form polytomies. In order to avoid problems aligning proteins with different domain organisations the phylogeny was determined according to a two step protocol. Relationships between ABC transporter subfamilies was determined using only sequences from the conserved ABC domains, followed by separate re-analyses using complete sequences for groups (A & G), B and C, rooted according to the Illustrator v8.0.1. Letters to the right of the tree indicate membership in the corresponding subfamilies according to the Human Genome Organisation (HUGO) nomenclature. Contributed by Jonathan A. Sheps.

**Table I.I Functions and/or Diseases Associated with Human ABC Proteins<sup>a</sup>.**

Sub-family	ABC protein nomenclature		Function or association <sup>c</sup>	Disease or clinical phenotype <sup>c</sup>
	HUGO <sup>b</sup>	Common		
<b>A</b>	ABCA1	ABC1	<ul style="list-style-type: none"> <li>Export of cholesterol and phospholipids from cells</li> <li>Phagocytosis of apoptotic cells</li> </ul>	<ul style="list-style-type: none"> <li>Tangier disease</li> </ul>
	ABCA2	ABC2	<ul style="list-style-type: none"> <li>Overexpression confers resistance to estramustine</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCA3	ABC3, ABC-C	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCA4	ABCR	<ul style="list-style-type: none"> <li>Transport of N-retinylidene-phosphatidylethanolamine</li> </ul>	<ul style="list-style-type: none"> <li>Stargardt's disease</li> <li>Retinitis pigmentosa</li> </ul>
	ABCA5	ABCA5	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCA6	ABCA6	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCA7	ABCA7	<ul style="list-style-type: none"> <li>Upregulated in the presence of low density lipoprotein</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCA8	ABCA8	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
<b>B</b>	ABCB1	MDR1, Pgp	<ul style="list-style-type: none"> <li>Broad specificity amphipathic drug transporter</li> <li>Overexpression confers resistance to many natural product drugs</li> </ul>	<ul style="list-style-type: none"> <li>MDR in cancer</li> </ul>
	ABCB2/3	TAP1/2	<ul style="list-style-type: none"> <li>HLA Class I antigen transporter</li> </ul>	<ul style="list-style-type: none"> <li>Herpes simplex virus infection</li> <li>Immune surveillance</li> </ul>
	ABCB4	MDR3	<ul style="list-style-type: none"> <li>Phosphatidylcholine secretion into bile</li> </ul>	<ul style="list-style-type: none"> <li>Progressive familial intrahepatic cholestasis</li> </ul>
	ABCB6	MTABC3	<ul style="list-style-type: none"> <li>Mitochondrial iron homeostasis</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCB7	ABC7	<ul style="list-style-type: none"> <li>Mitochondrial iron homeostasis</li> </ul>	<ul style="list-style-type: none"> <li>X-linked sideroblastic anemia and ataxia</li> </ul>
	ABCB8	M-ABC1	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCB9	TAP-L, ABCB9	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCB10	M-ABC2, ABC-me	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	<ul style="list-style-type: none"> <li>N.D.</li> </ul>
	ABCB11	BSEP, SPgp	<ul style="list-style-type: none"> <li>Bile acid transporter</li> </ul>	<ul style="list-style-type: none"> <li>Progressive familial intrahepatic cholestasis</li> </ul>
<b>C</b>	ABCC1	MRP1	<ul style="list-style-type: none"> <li>Organic anion transporter</li> <li>Transports cysteinyl leukotriene LTC<sub>4</sub>, 17<math>\beta</math>-estradiol 17-(<math>\beta</math>-D- glucuronide), aflatoxin B<sub>1</sub>, hydrophobic anticancer agents, and anionic drug conjugates</li> <li>Over-expression confers resistance to many natural product drugs</li> </ul>	<ul style="list-style-type: none"> <li>MDR in cancer</li> </ul>
	ABCC2	MRP2, cMOAT	<ul style="list-style-type: none"> <li>Organic anion transporter with substrate specificity similar to MRP1</li> <li>Over-expression confers resistance to cisplatin, methotrexate, etoposide, vincristine and doxorubicin</li> </ul>	<ul style="list-style-type: none"> <li>Dubin-Johnson syndrome</li> </ul>

<b>C</b>	ABCC3	MRP3, MOAT-D	<ul style="list-style-type: none"> <li>Organic anion transporter with substrate specificity similar to MRP1 with a preference for glucuronide-conjugates and also transports bile salts</li> <li>Over-expression confers resistance to etoposide and methotrexate</li> </ul>	• N.D.
	ABCC4	MRP4, MOAT-B	<ul style="list-style-type: none"> <li>Organic anion transporter</li> <li>Over-expression confers resistance to antiviral nucleoside analogs</li> </ul>	• N.D.
	ABCC5	MRP5, MOAT-C	<ul style="list-style-type: none"> <li>Organic anion transporter</li> <li>Over-expression confers resistance to thiopurines and antiviral nucleoside analogues</li> </ul>	• N.D.
	ABCC6	MRP6, MOAT-E	<ul style="list-style-type: none"> <li>Unknown</li> </ul>	• Pseudoxanthoma elasticum
	ABCC7	CFTR	<ul style="list-style-type: none"> <li>Chloride ion channel</li> </ul>	• Cystic fibrosis
	ABCC8	SUR1	<ul style="list-style-type: none"> <li>Neuronal/pancreatic sulfonylurea receptor subunit (K<sub>ATP</sub> channel)</li> </ul>	• Familial persistent hypoglycemia of infancy
	ABCC9	SUR2	<ul style="list-style-type: none"> <li>Cardiac/vascular smooth muscle sulfonylurea receptor subunit (K<sub>ATP</sub> channel)</li> </ul>	• Familial persistent hypoglycemia of infancy
	ABCC10	MRP7	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• N.D.
	ABCC11	ABCC11	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• N.D.
	ABCC12	ABCC12	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• N.D.
<b>D</b>	ABCD1	ALDP	<ul style="list-style-type: none"> <li>Regulates very long chain fatty acid <math>\beta</math>-oxidation (?)</li> </ul>	• Adrenoleukodystrophy
	ABCD2	ALDR	<ul style="list-style-type: none"> <li>Regulates very long chain fatty acid <math>\beta</math>-oxidation (?)</li> </ul>	• Adrenoleukodystrophy
	ABCD3	PMP70, PXMP1	<ul style="list-style-type: none"> <li>Long chain acyl-CoA transporter (?)</li> </ul>	• Zellweger's syndrome
	ABCD4	PMP69, PXMP1L, P70R	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• N.D.
<b>E</b>	ABCE1	RNase LI, OABP	<ul style="list-style-type: none"> <li>Endoribonuclease inhibitor</li> </ul>	• N.D.
<b>F</b>	ABCF1	ABC50	<ul style="list-style-type: none"> <li>Homology to yeast ribosomal proteins involved in translation</li> </ul>	• N.D.
	ABCF2	ABCF2	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• N.D.
	ABCF3	ABCF3	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• N.D.
<b>G</b>	ABCG1	WHITE1, ABC8	<ul style="list-style-type: none"> <li>Regulator of macrophage cholesterol and phospholipid transport</li> </ul>	• N.D.
	ABCG2	BCRP, ABCP, MXR1	<ul style="list-style-type: none"> <li>Over-expression confers resistance to mitoxantrone, daunorubicin, doxorubicin, and topotecan</li> </ul>	• N.D.
	ABCG4	WHITE2, ABCG4	<ul style="list-style-type: none"> <li>Expression is regulated by oxysterols and retinoids</li> </ul>	• N.D.
	ABCG5	WHITE3, ABCG5	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• Sitosterolemia
	ABCG8	WHITE4, ABCG8	<ul style="list-style-type: none"> <li>N.D.</li> </ul>	• Sitosterolemia

<sup>a</sup>Only genes for which we have constructed a competitor or for which there is published data have been included in this table.

<sup>b</sup>HUGO refers to the nomenclature for the ABC gene superfamily assigned by the Human Genome Organization.

<sup>c</sup>N.D. - not determined.

The function of ABC3, which is highly expressed in normal lung, is unknown (Klugbauer and Hofmann, 1996; Connors et al., 1997). Yamano et al. (2001) localized ABC3 protein expression to the limiting membrane of the lamellar bodies in alveolar type II cells, and based on this localization, they propose ABC3 may be important in the formation of pulmonary surfactant. ABCA7 was recently discovered as a sterol-sensitive protein (Kaminski et al., 2000). The cDNA sequence of ABCA6 has recently been published; however, the function of this gene is unknown (Kaminski et al., 2001b). The cDNA sequences of ABCA5, ABCA8, ABCA9, ABCA10 and ABCA12 have recently been deposited in the nucleotide databases; however, there is no additional published information.

There are 4 MDR1-like full-transporters and 7 half-transporters in subfamily B. The MDR1 transporter (Pgp) can extrude a wide range of structurally unrelated natural product drugs (e.g. vincristine, doxorubicin, mitoxantrone, paclitaxel and many others) from the cell (Germann, 1996). Pgp is localized to apical membranes of endothelial cells at the blood-brain barrier, the biliary canaliculi of hepatocytes and the proximal tubules of kidney suggesting the physiological function may be protection against xenobiotic compounds. Other full-transporters include the phospholipid transporter MDR3 (De Vree et al., 1998) and the bile acid transporter BSEP (Strautnieks et al., 1998), originally discovered in rat (SPgp)(Childs et al., 1995), both of which are expressed predominantly in the plasma membrane of liver hepatocytes. Mutations within the coding regions of these genes have been found in patients with progressive familial intrahepatic cholestasis. Among the half-transporters, TAP1 and TAP2 associate to form a heterodimer that has been localized to the endoplasmic reticulum. This complex actively transports peptides from the cytosol into the lumen of the endoplasmic reticulum where the peptides associate with class I molecules for presentation and immunorecognition by cytotoxic lymphocytes (Abele and Tampe, 1999). ABCB9 localizes to lysosomes in transfected human ovarian carcinoma (SKOV3) cells but its function is unknown (Zhang et al., 2000a). The other 4 half-transporters (M-ABC1 –

Hogue et al., 1999; M-ABC2 – Zhang et al., 2000b; ABC7, and MTABC3) are localized to the mitochondria and although their specific functions are unknown, at least two are likely to play a role in iron homeostasis (ABC7 - Allikmets et al., 1999; MTABC3 - Mitsuhashi et al., 2000). Linkage analysis and the identification of mutations in the coding sequence of ABC7 in affected patients suggests it is the causal gene in X-linked sideroblastic ataxia and anemia (Allikmets et al., 1999). The cDNA sequence of ABCB5 has recently been deposited in the nucleotide databases; however, there is no additional published information.

Subfamily C consists of 12 full-transporters including CFTR, SUR1, SUR2, MRPs 1-7 and ABCC11-12. Seven proteins of this subfamily (e.g. MRPs 1, 2, 3, 6 and 7, SUR1 and SUR2) form a subcluster within which each member possesses an additional N-terminal transmembrane domain (TMD<sub>0</sub>) of unknown function. CFTR is a cAMP activated chloride channel that when mutated causes the inherited disease cystic fibrosis (Riordan et al., 1989). SUR1 and its isoform, SUR2, are regulatory components of the potassium channels responsible for glucose-related insulin release that when mutated can cause the rare genetic disease, persistent hyperinsulinemic hypoglycemia of infancy (Bryan and Aguilar-Bryan, 1999). MRPs 1, 2, 3, 4 and 5 are organic anion transporters that differ in their tissue distribution and substrate preference (Borst et al., 1999a; Konig et al., 1999). It remains to be determined whether MRP6 and 7 are also organic anion transporters. The discovery of MRP1 expanded our understanding of multidrug resistance (MDR) since it is now established that non-Pgp mediated drug resistance is frequently due to over-expression of this protein (Hipfner et al., 1999). Mutations in MRP2 cause Dubin-Johnson syndrome, a defect in the secretion of amphiphilic anionic conjugates from hepatocytes into bile (Wada et al., 1998). Mutations in MRP6 have recently been associated with the inheritable connective tissue disorder pseudoxanthoma elasticum but its substrate(s) is unknown (Bergen et al., 2000). Very little is known about MRP7 except that the transcript is ubiquitously expressed in human tissues (see appendix I) (Hopper et al., 2001). The substrate

specificity of MRPs 1-5 is discussed in greater detail in section 1.6. The cDNA sequences of ABCC11 and ABCC12 have recently been published; however, the function of these genes is unknown (Tammur et al., 2001).

The four half-transporters of subfamily D have been localized to the peroxisome and mutant forms of two, and possibly three, of these proteins cause peroxisomal disorders (Shani and Valle, 1998). Mutations in the adrenoleukodystrophy protein (ALDP) cause this neurodegenerative disorder characterized by accumulation of unbranched saturated fatty acids likely due to impairment of degradation of very long chain fatty acids in peroxisomes. Transient and stable over-expression of ALDR has been shown to restore the impaired peroxisomal beta-oxidation in fibroblasts of adrenoleukodystrophy patients (Netik et al., 1999). This defect could also be restored in ALDP-deficient mice after stimulation with the peroxisome proliferator fenofibrate, which increases ALDR expression. These data suggest that ALDR may also be important in adrenoleukodystrophy (Netik et al., 1999). Mutations of PMP70 have been found in some patients with Zellweger's disease, a disorder of peroxisome biogenesis. However, the association of PMP70 with disease and the identity of the substrate for this protein are unclear. The function of the other peroxisomal ABC, PMP69, is unknown (Holzinger et al., 1997a).

RNase L inhibitor (subfamily E) and ABC50 (subfamily F) possess two ATP-binding cassettes without any TMDs, suggesting they are not actual transporters. RNase L inhibitor is a regulatory protein whose expression inhibits the 2-5A-dependent activation of RNase L, an interferon-inducible pathway that may regulate mRNA stability (Bisbal et al., 1995). ABC50 was originally predicted to be a ribosomal protein involved in translation based on homology to the yeast GCN20 (Richard et al., 1998). More recently, ABC50 has been shown to be associated with eukaryotic initiation factor 2 (eIF2) and ribosomal subunits, providing additional support for a likely function in mRNA translation (Tyzack et al., 2000). The cDNA sequences for

ABCF2 and ABCF3 (subfamily F) have been deposited in the nucleotide databases; however, no additional published information is available.

Members of subfamily G are orthologous to the putative pigment transporters in *D. melanogaster* (*white*, *scarlet*, and *brown*). Endogenous human WHITE1 is expressed at the plasma membrane and in intracellular compartments of cholesterol-laden macrophages and is likely one of several ABC proteins that regulate cholesterol and phospholipid transport (Klucken et al., 2000). The breast cancer resistance protein (BCRP) has been shown to be over-expressed in several mitoxantrone-selected cancer cell lines and may contribute to transport of this drug (Doyle et al., 1998). Recently, it has been shown that BCRP mRNA is expressed at high levels in primitive hematopoietic stem cells and enforced expression confers a "side-population" phenotype (immature stem cells that actively efflux the fluorescent dye Hoechst 33342) (Zhou et al., 2001 and Scharenberg et al., 2002). This data suggests BCRP may be important for the defense of pluripotent stem cells against xenobiotics. ABCG5 and ABCG8 are located adjacent to each other on chromosome 2p21, in opposing orientations, and mutations have been found in the coding regions of these genes in patients with sitosterolemia, a disease characterized by the accumulation of dietary sterols and atherosclerosis (Berge et al., 2000). The cDNA sequence for ABCG4 was recently published, and mRNA expression levels of this gene were reported to be regulated by oxysterols and retinoids (Engel et al., 2001). For a complete up-to-date overview of ABC transporters, please see the homepage of Michael Muller <http://www.med.rug.nl/mdl/humanabc.htm>

## 1.5 Multidrug Resistance

Inherent or acquired resistance to chemotherapeutic agents often precludes the successful treatment of cancer. Cancerous cells are frequently refractory to a variety of drugs with different structure and function, despite exposure to only a single chemotherapeutic agent in some cases.

The phenomenon of simultaneous cross-resistance to multiple unrelated drugs is referred to as MDR. Some mechanisms that contribute to MDR include: enhanced drug efflux, mediated by members of the ABC protein family such as Pgp and MRP1 (reviewed by Germann, 1996 and Hipfner et al., 1999, respectively); drug detoxification, involving glutathione-S-transferase and related enzymes (O'Brien and Tew, 1996); altered drug targets, involving DNA topoisomerase II (Nitiss and Beck, 1996); and alteration in drug-induced apoptosis, involving genes in the Bcl-2 pathway (Reed, 1995). Other molecular markers are also associated with MDR, such as the lung resistance protein (LRP) (Izquierdo et al., 1996), but their contribution to MDR is poorly understood. The multifactorial nature of MDR has made it difficult to determine the contribution of specific ABC proteins to drug efflux.

## **1.6 Contribution of ABC Transporters to MDR**

Decreased drug accumulation due to increased expulsion of drug from cancer cells is a significant contributing mechanism to the MDR phenotype. "Classical" MDR is usually characterized by cross-resistance to four classes of chemotherapeutic drugs: the anthracyclines, vinca alkaloids, taxanes and epipodophyllotoxins. In 1976, Juliano and Ling noted a correlation between decreased drug accumulation and the expression of a 170 kilodalton (kDa) cell surface glycoprotein in cells that displayed this classical MDR phenotype. This protein was later designated Pgp. Subsequent gene transfection studies demonstrated that Pgp could confer a classical MDR phenotype. Drug accumulation experiments using membrane vesicles containing high concentrations of Pgp elucidated the wide array of drugs transported by Pgp. For many years it was widely believed that Pgp was the exclusive vehicle for drug transport, until the discovery by Cole et al. (1992) of a small cell lung carcinoma cell line that displayed resistance to a multitude of drugs but did not express MDR1 mRNA or Pgp protein. MRP1 was later identified as the causal factor of this non-Pgp-mediated drug resistance in studies using these

cells that over-expressed MRP1 (Cole et al., 1994; Grant et al., 1994). Pgp transports hydrophobic natural product drugs in unmodified form [e.g. vinblastine, vincristine, doxorubicin, daunorubicin, etoposide, teniposide, mitoxantrone, topotecan, colchicine and paclitaxel (Germann, 1996)], whereas MRP1 can transport a similar spectrum of drugs either conjugated to anionic ligands such as reduced glutathione (GSH), glucuronide, or sulfate, or in unmodified form, possibly together with GSH (Leier et al., 1994; Jedlitschky et al., 1996; Loe et al., 1998; Loe et al., 1996). MRP1 also confers resistance to heavy metal oxyanions that are not within the spectrum of compounds transported by Pgp (Cole et al., 1994). In contrast to Pgp-mediated MDR, MRP1 over-expressing tumour cells often possess only modest levels of resistance to paclitaxel and colchicine.

In light of the established links between Pgp/MRP1 expression and chemotherapeutic drug efflux, many studies have attempted to correlate expression of these genes with clinical prognosis. A number of studies support the notion that high Pgp/MDR1 expression is associated with an increased risk of treatment failure in several types of cancer. The most supportive evidence has been found in lymphoblastic and myeloid leukemias (Campos et al., 1992; Goasguen et al., 1993), and soft tissue sarcomas (Chan et al., 1990). However, the role of Pgp in mediating MDR in other cancers such as neuroblastoma, osteosarcoma and cancers of the lung and breast remains controversial. High levels of MRP1 have been noted in some tumour cell lines derived from cancers that typically respond poorly to chemotherapeutic treatment, for example, lung cancer and melanoma. Patients with tumour cells expressing high levels of MRP1 in these types of cancer have significantly worse survival following chemotherapy (Ota et al., 1995; Berger et al., 1997). High MRP1 expression is also associated with increased risk for treatment failure in breast cancer (Nooter et al., 1997) and neuroblastoma (Norris et al., 1996). The associations of these proteins with resistance in at least some forms of cancer has prompted intensive efforts to identify ways to reverse the detrimental effects of MRP1 and/or Pgp. Current

pharmacological approaches to this problem include agents that enhance the cytotoxicity of known chemotherapeutic drugs and the use of novel drugs and analogues. Chemosensitizers such as cyclosporine A, verapamil and trifluoperazine are among the most effective modulators of Pgp-mediated MDR; however, these agents are generally much less effective at reversing MRP-associated drug resistance (Loe et al., 1996; Germann, 1996). Additional well designed studies with large patient cohorts will be required to elucidate the contribution of Pgp and MRP1 to clinical outcome and to develop strategies to circumvent their effects.

Numerous laboratory studies have demonstrated that ABC proteins, other than Pgp and MRP1, contribute to MDR, although there is only limited preliminary evidence that any of these proteins is relevant to clinical MDR in cancer. The most extensively studied member is MRP2. The substrate specificity of this transporter was originally defined by an inborn error in rats lacking this protein and their inability to secrete bilirubin glucuronides into their bile (Paulusma et al., 1996). Absence of MRP2 protein in humans results in an analogous inborn error of metabolism, the Dubin-Johnson syndrome (Wada et al., 1998). MRP2 mRNA has been shown to be moderately over-expressed in some cisplatin-resistant cell lines (Taniguchi et al., 1996) and transfection with an MRP2 antisense construct into the human hepatic cancer cell line HepG2 reduced MRP2 protein levels and increased the sensitivity of these cells to cisplatin (Koike et al., 1997). Additionally, transfection of MRP2 cDNA into human ovarian carcinoma 2008 cells confers short-term resistance to several antifolates including methotrexate (Hooijberg et al., 1999).

Another member of the MRP subfamily, MRP3, has been shown to be an organic anion transporter, which when transfected into 2008 cells confers resistance to etoposide, teniposide and methotrexate (Kool et al., 1999a). Membrane vesicles isolated from LLC-PK1 and HeLa cells transfected with the rat MRP3 cDNA demonstrated that several kinds of organic anions are

transported via MRP3, with a substrate specificity distinct from that of MRP1 and MRP2 in that glutathione conjugates are poor substrates for MRP3 (Hirohashi et al., 1999).

The recently identified MRP4 protein (Lee et al., 1998) was demonstrated to be over-expressed with gene amplification in a human T-lymphoid cell line (CEM) selected in the presence of the acyclic nucleoside phosphonate PMEA (9-(2-phosphonylmethoxy-ethyl)adenine) (Schuetz et al., 1999). Another T-lymphoid cell variant (CEM-r1) was shown to be resistant to PMEA, as well as several other nucleoside reverse transcriptase inhibitors, and at least in the case of PMEA, this was the result of enhanced energy-dependent drug efflux (Schuetz et al., 1999). These results suggest that MRP4 may contribute to the efflux of nucleoside monophosphate analogs from mammalian cells. In a recent study, membrane vesicles prepared from insect cells infected with MRP4 baculovirus were capable of energy-dependent transport of estradiol 17-beta-D-glucuronide and several cyclic nucleotides (Chen et al., 2001).

There is also emerging evidence that the recently identified MRP5 protein (McAleer et al., 1999) can confer drug resistance. Wijnholds et al. (2000a) demonstrated that MRP5-transfected cells were resistant to the thiopurine anticancer drugs, 6-mercaptopurine and thioguanine, and the anti-HIV drug PMEA. This resistance was due to increased extrusion of these compounds.

The last member of this subfamily, MRP6, has only recently been identified (Kool et al., 1999b) and its ability to confer resistance to chemotherapeutic drugs is unknown. To assess the possible role of these MRP1 homologues in MDR, Kool et al. (1997) examined a large set of resistant cell lines for mRNA expression of MRPs 1-5. MRPs 3-5 were found not to be over-expressed significantly in the cell lines analyzed. In contrast, MRP2 was substantially over-expressed in some of the cisplatin resistant cell lines examined, although there was no clear quantitative correlation between degree of over-expression and resistance level.

Human ABC transporters outside of the MRP subfamily have also recently been shown to confer drug resistance. Transfection of the cDNA for BCRP into MCF-7 breast cancer cells confers resistance to mitoxantrone, doxorubicin and daunorubicin (Doyle et al., 1998) and BCRP mRNA is over-expressed in drug-resistant human cancer cell lines derived by selection with mitoxantrone (Ross et al., 1999). Maliepaard et al. (1999) have shown BCRP mRNA to be over-expressed in ovarian carcinoma cell lines selected with either topotecan or mitoxantrone. Another ABC protein, ABC2, has been shown to be genomically amplified and over-expressed at the RNA level in the estramustine-resistant human ovarian carcinoma cell line SKEM (Laing et al., 1998). In addition, ABC2-specific antisense RNA sensitized these resistant cells to estramustine. Finally, an ABC protein closely related to Pgp, called BSEP, is mutated in individuals with an inherited disorder with severe cholestatic liver disease that is consistent with defective bile transport (Strautnieks et al., 1998). Transfection of the rat homologue, SPgp, into SKOV3 confers low-level resistance to paclitaxel that is verapamil reversible (Childs et al., 1998). However, no resistance was observed when other chemotherapeutic drugs were tested.

The complexity of MDR, with respect to the multitude of proteins contributing to drug efflux, is only now beginning to be investigated. Several studies involving the step-wise selection of a series of drug-resistant cells have demonstrated over-expression of MRP1 in low-level resistant cells, followed by Pgp/MDR1 over-expression at higher levels of resistance (Jensen et al., 1995; Hasegawa et al., 1995), suggesting that multiple drug efflux mechanisms may simultaneously contribute to MDR. Furthermore, the contribution of known ABC proteins to drug efflux is unclear since it has been difficult to generate transfected cell lines that over-express these proteins with proper cellular localization (Borst et al., 1999a). Previous studies indicate that an MDR phenotype is often expressed in tumours derived from tissues with high Pgp/MDR1 expression and that this may also occur with other MDR-associated proteins (MRPs 2-5 and BCRP) (Germann, 1996). It remains to be determined if other MDR-associated ABC

proteins contribute to a multi-component drug efflux phenotype or if they act alone. A comprehensive understanding of the relationship between expression levels of individual ABC proteins, the collective expression profile of the entire ABC gene superfamily and drug resistance is required to define their roles in the MDR phenomenon.

## **1.7 Historical Perspective and Thesis Overview**

When this thesis research was initiated in the fall of 1996, cDNA sequences had been identified and published for a total of 15 human ABC genes. The list included members of what is now subfamily A (ABC1 and ABC2), subfamily B (MDR1, MDR3, TAP1, TAP2), subfamily C (CFTR, SUR1, SUR2, MRP1 and MRP2), subfamily D (PMP70, ALDR, ALDP) and subfamily G (WHITE1). No members of subfamilies E or F had yet been identified. Beyond cDNA sequence, genomic localization and cursory mRNA expression data, there was limited information concerning the biological function of most of these proteins. With the discovery of first the TAP proteins and later the WHITE1 protein, the significance of half-ABC proteins began to be recognized. Altered expression and/or mutations of several genes had been linked to genetic diseases including cystic fibrosis, hyperglycemia of infancy, adrenoleukodystrophy, Dubin-Johnson syndrome and MDR. At the time, only Pgp and MRP1 were considered relevant to MDR, in contrast to the 7 or so ABC proteins considered important today.

The focus of this thesis research has been to enhance our understanding of the mechanisms that contribute to MDR in cancer, with an emphasis on the contribution of ABC transporters. My earliest research, which is not described in this thesis, investigated methods to down-regulate MDR1 mRNA expression in order to reverse MDR in cancer cell lines. The selection of this research topic was determined in part by Dr. Ling's experience studying the contribution of Pgp to MDR in cancer. Plasmids were created that contained inserts encoding hammerhead ribozymes designed to bind to the initiation codon of the MDR1 mRNA sequence

and thus incapacitate translation. Preliminary experiments using cell lines transfected with these constructs suggested we could partially reverse the MDR phenotype using this approach. As the number of newly identified ABC cDNAs continued to grow and the complexity of MDR became apparent, we decided to abandon this research and instead pursue approaches that would permit analysis of mRNA expression patterns of the entire human ABC superfamily.

During the early stages of developing an assay for RNA expression profiling, I also worked on other research, which is not described in this thesis, involving the cloning and partial characterization of the ABC cDNA MRP5. In the fall of 1997, a publication reported the cloning of a new human ABC cDNA, SMRP, from a cisplatin-resistant lung adenocarcinoma cell line (Suzuki et al., 1997). This cDNA was 4.8 kb in length, considerably shorter than other MRP subfamily cDNAs, and lacked most of the N-terminal TMD present in all other full-transporters. Previously in our laboratory, in an attempt to identify new human ABC cDNAs, a technician (Lin Liu) performed hybridization screening of cDNA libraries using conserved regions of ABC cDNAs as probes. Any positive clones of significant length were end-sequenced. Among this population of clones, approximately 30 had 3' ends that matched that of SMRP and several were greater than 6 kb in length, suggesting that the SMRP cDNA was not full-length. In the summer of 1998, a collaborative effort was initiated between myself and a post-doctoral researcher in the Ling laboratory (Dr. Douglas Hogue) to sequence, with overlapping coverage in both directions, the longest cDNA clone matching SMRP (clone 12-27, 6.3 kb). The DNA sequence of clone 12-27 matched SMRP and contained an additional 1.5 kb of sequence at the 5' end that, according to hydropathy plot analysis, encoded the expected TMD. However, clone 12-27 contained three mutations that disrupted the open reading frame. Two other shorter cDNAs that matched SMRP (clone 12-12 and 12-24) were subsequently partially sequenced (~3 kb each), with overlapping coverage in both directions, in the regions of the mutations present in clone 12-27. Although other mutations were present in clones 12-12 and 12-24, they did possess an open reading frame

in the region of the three mutations in clone 12-27. To create a continuous open reading frame, fragments from both clones 12-12 and 12-24 were inserted into clone 12-27. After removing the 5' and 3' untranslated regions (UTRs) of this cDNA by restriction digests and polymerase chain reaction (PCR), a VSV epitope tag was added to the 3' end to permit detection by antibody. Transient transfections with this cDNA, followed by preliminary fluorescent confocal microscopy, suggested this protein was localized at the plasma membrane. This cDNA was then used to initiate the generation of stable transfected cell lines. In February of 1999, we attended a conference on ABC transporters in Austria where several research groups presented posters on the characterization of cells transfected with cDNAs matching our clone 12-27. This cDNA was later named MRP5. Since these research groups had progressed farther on the characterization of MRP5 than we had and since both Dr. Hogue and I had other research projects ongoing, we decided to terminate our MRP5 project.

Methods for the quantitative detection of mRNA species have improved considerably during the past 5 years. Previously, available methods included labour-intensive Northern blots and the ribonuclease protection assay (RPA), both of which permit simultaneous analysis of only a few transcripts, as well as PCR approaches, which were difficult to apply in a quantitative manner. By the late 1990s a number of other methods such as real-time PCR, serial analysis of gene expression (SAGE) and cDNA microarrays were developed which provided advantages over traditional methods.

Before developing a method for comprehensive mRNA expression analysis of ABC genes we first needed to identify as many human ABC cDNAs as possible by screening the EST and genomic nucleotide databases. The strategy for this approach and the resulting set of ABC cDNAs that was identified are described in chapter II.

The primary criteria for a method for mRNA quantification were as follows: 1) quantitative (detect < 2-fold differences in mRNA copy number); 2) sensitive (detect less than

1000 copies of a mRNA species; and 3) specific (able to distinguish and quantitate up to 50 mRNA species). After investigating several possibilities, it was decided that only a PCR-based approach could satisfy these criteria. At the time, we had recently acquired an automated DNA sequencer and opted to take advantage of the high throughput and fluorescent detection that this instrument provided. In the fall of 1997, the best available PCR-based approach for quantitative detection of mRNA transcripts was competitive-reverse-transcription-polymerase-chain-reaction (competitive-RT-PCR). Although this method was considered laborious, we felt that the use of capillary electrophoresis, rather than standard agarose slab gel electrophoresis, would significantly reduce labour and time requirements. However, the construction of competitor molecules specific for each ABC cDNA would still prove to be a difficult and time-consuming process. The development and validation of this fluorescent competitive-RT-PCR assay is described in detail in chapter II.

Considering that Dr. Ling's research group has historically been one of the pioneering laboratories investigating MDR, it was decided to first apply our competitive-RT-PCR assay to MDR cell lines. When this study was initiated, emerging evidence suggested that multiple ABC transporters could confer an MDR phenotype and that these transporters may collectively contribute to MDR. In addition, we also had a large collection of cancer cell lines that had been characterized with respect to Pgp/MDR1 expression and resistance to various chemotherapeutic drugs. We chose a series of vincristine-selected SKOV3 cell lines that had been previously characterized to over-express MDR1 and Pgp with high selection pressure. In conducting this study, we hoped to determine if other ABC mRNA species were also up- or down-regulated with vincristine-selection, especially in cell lines with low levels of resistance that do not show a change in MDR1 or Pgp compared to the parental cell line. These low-level resistant cell lines are considered more clinically relevant than those with high levels of resistance to a drug. The

mRNA expression profiles from this experiment and a discussion of their significance are presented in chapter III.

As part of a joint project with Allison Pahl in Dr. Ling's laboratory (MSc. graduate, Department of Biochemistry & Molecular Biology, UBC), we next began to examine the effect of short-term exposure of the human leukemia cell line HL60 to chemotherapeutic drugs. It has been speculated that chemotherapy treatment itself may alter the expression of MDR-associated proteins, resulting in the gradual development of an MDR phenotype as treatment progresses. The objective of these experiments was to identify individual ABC genes that were responsive to short-term chemotherapeutic drug exposure, as well as groups of genes that were potentially co-regulated. These short-term exposures more closely resemble the dosing strategy of chemotherapy regimens, compared to stable drug-resistant cell lines, and therefore may provide more relevant data than the experiments using the SKOV3 cell lines. Understanding the conditions (e.g. type of chemotherapeutic drug, dosage and duration of exposure) that lead to up- or down-regulation of MDR-associated transcripts may assist refinement of treatment protocols to minimize the development of drug-resistant tumours during chemotherapy. The mRNA expression profiles from these experiments and a discussion of their significance are presented in chapter III. We also examined the effect of short-term exposure to biological compounds such as cholesterol and dihydrotestosterone. Since the focus of this thesis is MDR, the data from these experiments has not been included in this chapter. The transcript that underwent the largest change in expression levels upon exposure to chemotherapeutic drugs was MRP7. At the time these studies were conducted there was no full-length cDNA sequence available in any of the nucleotide databases. Our attempts to clone and characterize this gene are described in appendix I.

In addition to investigating the effect of chemotherapeutic drug exposure on ABC transcript expression, we also examined ABC transcript expression profiles in normal tissues.

The expression pattern of an ABC transcript in normal tissues can provide clues to the biological function of the gene, particularly if the transcript is present in only a limited number of tissues. In addition, comprehensive expression profiles of the ABC family in normal tissues can provide insights into the relative contribution of each protein to the efflux of natural substrates that are recognized by several ABC transporters. When this project was initiated in the fall of 1998, there was only a limited amount of mRNA expression data in the literature for most ABC genes. The available expression data was limited to only a few tissues and no attempts had been made to provide comprehensive expression profiles allowing comparisons among the various ABC mRNAs. Our competitive-RT-PCR approach allowed us to examine more than 35 ABC transcripts in a dozen tissues and permitted quantitative comparisons among mRNA levels of each transcript. The mRNA expression profiles from these experiments and a discussion of their significance are presented in chapter IV.

Another application of this method is to screen ABC transcripts for changes in expression that correspond to gain or loss of a particular phenotype. In the fall of 1998 we acquired ovarian carcinoma cell lines that had been transfected with the pro-apoptotic BAX cDNA or a control vector. It had been reported in the literature that these cell lines, which over-express BAX protein, were chemosensitive relative to the control transfectants and that this was due to an energy-dependent transport mechanism. However, it was reported that no differences in Pgp or MRP1 expression levels were observed between the cell lines. This suggested that another ABC transporter might be down-regulated as a result of BAX protein over-expression and thus responsible for transport of chemotherapeutic drugs to which the cells were sensitive. An analysis of expression profiles for each of these cell lines indicated that there was a 20-fold reduction in MDR1 mRNA in the BAX transfectants. Luciferase reporter assays were subsequently conducted using different regions of the MDR1 promoter, to determine if luciferase

activity was dependent on the level of BAX protein. These experiments are described in detail in chapter V.

## **CHAPTER II**

### **DEVELOPMENT OF A COMPETITIVE-RT-PCR ASSAY FOR RNA PROFILING OF ABC TRANSPORTERS**

#### **INTRODUCTION**

##### **2.1.1 Approaches for Expression Profiling of the ABC Protein Family**

A number of new technologies have been developed in recent years to perform large-scale expression analysis. SAGE and cDNA microarrays provide genome-scale expression profiles, but have shortcomings for some applications. These methodologies require substantial amounts of starting RNA and, while SAGE generates comprehensive and sensitive expression profiles, its prohibitive costs limit its applicability. Microarrays provide a quality screen for expression changes but their sensitivity and reproducibility are limited by the difficulties of applying a single set of hybridization conditions to thousands of cDNAs. Traditional labour-intensive methods such as Northern blots and RPA are limited by the inability to compare more than a few transcripts due to the amount of RNA required and the limited resolution of separating gels. These methods are also hampered by the considerable sequence similarity among ABC transcripts, particularly within the NBDs, that can result in cross-reactivity among subfamily members if hybridization conditions are not properly optimized.

Conversely, PCR-based approaches are relatively simple to apply and provide excellent sensitivity with limited amounts of RNA. Moreover, this approach can be conducted on a relatively large number of samples and/or many different genes in the same experiment. This provides the investigator a measure of flexibility unavailable with more traditional approaches (e.g. Northern blots and RPA). Along with the power and relative ease of PCR come a number of technical hurdles to utilizing this approach in a reliable manner. Specifically, as the PCR

process exponentially amplifies the target, errors are also amplified. As a result, variability can be very large and preclude accurate and reliable quantification. Inherent factors that will lead to tube-to-tube or sample-to-sample variability are, for example, thermocycler-dependent temperature deviations, the presence of individual DNA polymerase inhibitors in samples, pipeting variations, or the abundance of the target sequence in the specimen of interest (Reischl and Kochanowski, 1995).

There are many approaches that have been used as controls in RT-PCR, both internal and external; however, few of these are suitable to control for the many sources of variation. Although external control genes such as  $\beta$ -actin,  $\beta_2$ microglobulin and GAPDH are frequently used to normalize for the amount of input RNA or cDNA, they can only provide a relative indication of PCR product quantity in different reactions. This type of semi-quantitative RT-PCR method can partially compensate for tube-to-tube and sample-to-sample variation. Quantification is limited, however, since these housekeeping transcripts differ in abundance among RNA sources, their expression is sensitive to some experimental conditions and the efficiency of the reverse transcription (RT) step for the transcript(s) of interest and external control mRNA may vary. In addition, it is not possible to compare quantitatively transcripts that have been amplified with different primer pairs and it is necessary to identify the initiation of the plateau phase of PCR (Cross, 1995). Another semi-quantitative RT-PCR approach uses serial dilution of a known amount of standard, often a plasmid containing the target DNA sequence, that can be amplified in parallel (external control) with the samples of interest to generate a standard curve. Potential drawbacks to this procedure are the sensitivity of PCR for small variations in the setup (tube-to-tube) and the need to identify the initiation of the plateau phase of PCR. If quantification with this type of external control is established, precision (replicates in the same PCR run) and reproducibility (replicates in separate PCR runs) must be analyzed to understand the limitations.

The limitations associated with semi-quantitative RT-PCR and external controls are overcome through the use of competitive-RT-PCR, which employs co-amplification of native mRNA with an RNA or DNA internal standard (Wang et al., 1989; Becker-Andre and Hahlbrock, 1989; Gilliland et al., 1990). Initial mRNA copy number is determined from the ratio of the endpoint native mRNA and internal standard PCR products. The principle of an internal standard is that by co-amplifying a second PCR product in the same tube, differences in PCR setup among tubes, presence of contaminants and quality of starting material can be controlled for. An internal competitive standard is constructed that competes with the native transcript for enzyme, nucleotides and primer molecules. The competitive standard bears the same primer binding region as the native mRNA, but the sequence in between is modified in such a way that amplification products derived from the competitor and the native mRNA can be differentiated. Both DNA and RNA standards have been reported in the literature; however, it is generally accepted that DNA standards are not an optimal choice because they do not compensate for the inherent variability of the RT step. Competitive standards are created by inserting a portion of the native transcript into a plasmid containing an RNA polymerase promoter suitable for *in vitro* transcription. Heterologous competitive RNA standards possess the same primer binding sites as the native mRNA, but the intervening region differs. Several studies have demonstrated that the greater the differences in size or internal sequence composition between the competitor and native mRNA, the more likely their amplification efficiencies will differ (Hayward et al., 1998; McCulloch et al., 1995). It is for this reason that most investigators chose to use homologous competitors.

The PCR products of homologous competitors are differentiated from native transcript PCR products by introducing into the competitor a very small deletion, insertion or a mutation that alters a restriction site. As mentioned, the smaller the alteration, the more similar the amplification characteristics of the competitor and native transcript. This can be explained by

the fact that amplification efficiency is related to the length and secondary structure of the RNA transcript and ensuing product. Sequences immediately upstream or downstream of the primer sites may also influence RT and PCR efficiencies. The use of mutagenesis to create an altered restriction site in the competitor has the advantage that the sequences of competitor and native transcript are virtually identical; however, the extra restriction digestion step required to distinguish the PCR products does introduce another undesired source of variability. It is for this reason that most investigators choose to minimize the size of the deletion or insertion rather than engineer a unique restriction site.

One difficulty with homologous standards is that as the competitor and native mRNA are amplified by PCR, single strands of competitor and native mRNA amplification products can re-anneal to form heteroduplexes, which cannot be distinguished from homoduplexes with non-denaturing electrophoresis (Henley et al., 1996). The likelihood of heteroduplex formation is dependent on the degree of sequence similarity between competitor and native mRNA sequence, making point mutants and constructs with small deletions/insertions the most problematic. Greater quantities of total PCR product and an excess of competitor relative to native mRNA will both exacerbate this heteroduplex formation. Solutions to this problem are to use denaturing conditions to prevent the formation of DNA dimers such as with denaturing gel capillary electrophoresis (Pannetier et al., 1993) or to use high pressure liquid chromatography (HPLC) that permits reliable identification of heteroduplex products (Hayward-Lester et al., 1996) (see below).

Several methods for detection of competitive-RT-PCR products have been employed, with the choice of assay determined by competitor design and availability of specialized equipment. The most common method for detection of end-point competitive-RT-PCR products is through the use of intercalating dyes (e.g. ethidium bromide or SYBR Green) and separation of PCR products by agarose gel electrophoresis (Lyttelton et al., 1994). This method requires

equipment available in any molecular biology laboratory and is inexpensive. However, the limited resolving capacity of agarose gel separation requires that the competitor and target PCR products differ in size by at least 50 nucleotides, which precludes the use of homologous competitors. Another common approach involves measurement of incorporated radioactivity by autoradiography or phosphor imaging (Wang et al., 1989). In this case, the use of polyacrylamide gels permits higher resolution but, requires the additional labour of using radioactive isotopes. A third type of end-point product measurement uses solid-state approaches in which a bound enzyme produces fluorescence or luminescence (Martin et al., 1995). This method requires multiple incubation and wash steps, with somewhat limited sensitivity, and is therefore most suitable for repeatedly measuring a few transcript species in numerous specimens as is done in clinical laboratories. Finally, several laboratories measure the production of amplification products following resolution by HPLC (Hayward-Lester et al., 1996) or capillary electrophoresis (Borson et al., 1998). These approaches can provide up to single nucleotide resolution and can be semi-automated, but do require specialized equipment.

Competitive-RT-PCR is inherently an indirect method of measurement. For reliable quantitative information to be extracted from this variable system, an understanding of the mathematical models of RT-PCR is necessary (Hayward et al., 1998). In this regard, the RT and PCR phases are entirely different types of enzymatic reactions and thus should be considered separately. The RT phase is simple in that there is no amplification and the sole variable is the percentage of mRNA converted into cDNA. This can be stated as Equation 1:

$$[\text{cDNA}] = [\text{RNA}] \times \text{Efficiency} \quad [\text{Eq. 1}]$$

Efficiency is measured as the percentage of RNA transcribed into cDNA. This reaction is extremely susceptible to contaminants such that efficiency can fluctuate from 5-90% (Ferre et al., 1994). If two separate reactions have equal amounts of starting RNA, but their RT efficiency is unequal, the final amounts of the amplification products will be dissimilar following PCR.

The mathematical description of PCR is more complex. The amplification of cDNA by PCR is not constant in that it differs for each transcript and primer pair and also as the reaction progresses. Under ideal amplification conditions, with each cycle of temperatures (denature, anneal and extend) the amount of DNA is theoretically doubled. This can be expressed as Equation 2:

$$P = T (1+E)^n \quad 0 \leq E \leq 1 \quad [\text{Eq. 2}]$$

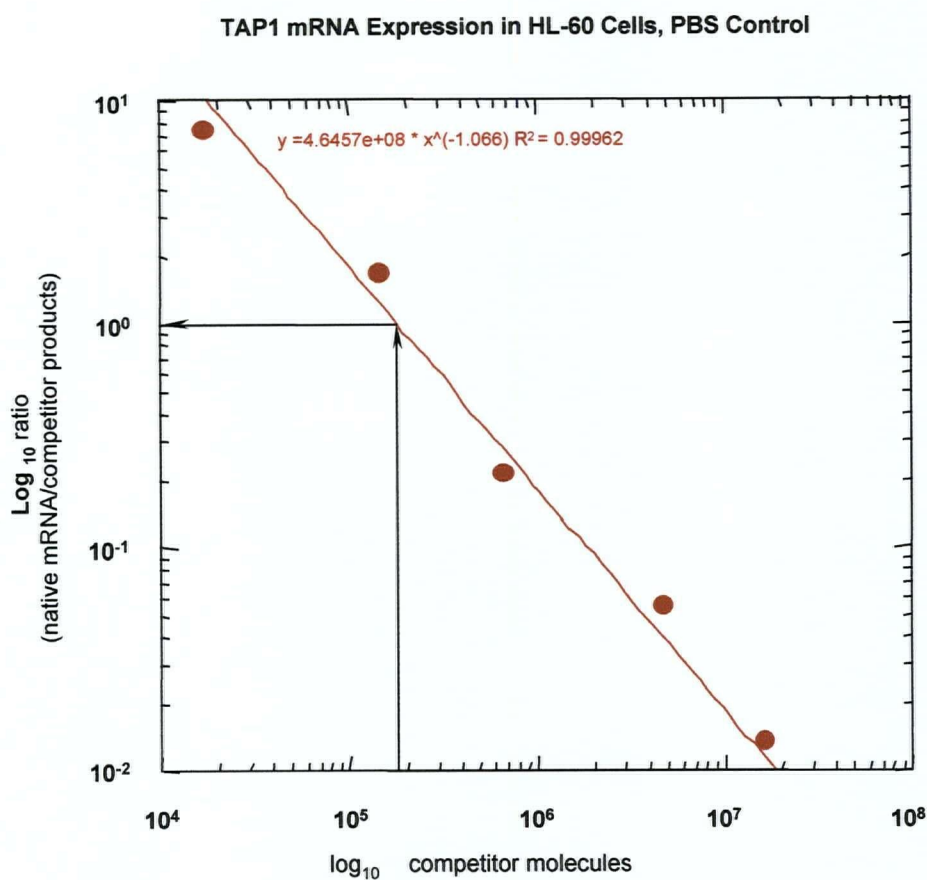
where P is product (amount measured after n cycles); T is template (amount of cDNA from RT reaction); E is efficiency (percentage of cDNA copied in a PCR cycle); and n is cycle number. From Equation 2 it can be seen that small differences in amplification efficiency are compounded exponentially. Also from Equation 2 it can be seen that a 5% difference in amplification efficiency between two initially equal targets can result in an end-point difference of 2-fold after 26 cycles. The overall efficiency of the amplification process is dependent on the primer/target hybridization, the relative amounts of each reagent [deoxynucleotide triphosphates (dNTPs), DNA polymerase, primers, template and  $\text{MgCl}_2$ ] and the presence of DNA polymerase inhibitors. From these equations, it would seem that RT-PCR inherently lacks the consistency necessary for accurate quantification; however, the use of co-amplified internal competitors can control for this potential variability.

In competitive-RT-PCR, the amount of initial native transcript can be determined from a graph of  $\log_{10}$  ratio (native mRNA/competitor end-point PCR products) versus  $\log_{10}$  competitor molecules added to the reaction (Figure 2.1). The initial number of native mRNA copies can be determined by locating the point at which the ratio of native mRNA/competitor end-point PCR products is 1 and determining the corresponding number of input competitor molecules from the equation of the line (x-intercept), which is equal to the number of initial native mRNA molecules. However, for competitive-RT-PCR results to be reliable, several conditions must be met. As with any standard curve, there must be data points above and below the equivalence

point. The equation of the line for this plot should have a slope close to 1 and the  $r^2$  value should also be close to 1. Mathematically, this slope is the ratio of the native mRNA and competitor amplification efficiencies. If the slope is  $> 1$ , the native mRNA has a higher efficiency, whereas if the slope is  $< 1$ , the competitor has a higher efficiency.

The sensitivity of competitive-RT-PCR, combined with its relatively inexpensive application, has made this method a popular choice for numerous applications. Examples of use of competitive-RT-PCR include the detection of viruses in clinical specimens (Kohsaka et al., 1993); quantification of minimal residual disease in patients after therapy by amplification of leukemia-specific fusion genes or translocations, for example BCR-ABL in chronic myeloid leukemia (Cross et al., 1993) or t(14:18) breakpoints in follicular lymphoma (Meijerink et al., 1993); measuring the degree of oncogene amplification in tumours (Sestini et al., 1994); and determining differences in patterns of gene expression after various stimuli (Fandrey and Bunn, 1993).

In light of these advantages, we have modified a competitive-RT-PCR method coupled to capillary electrophoresis and fluorescent detection (Borson et al., 1998; Thiery et al., 1996; Pannetier et al., 1993; Porcher et al., 1992) for use in moderate-scale expression analysis of the human ABC protein superfamily. In this chapter we will describe the development and validation of a competitive-RT-PCR assay for the quantification of 35 human ABC genes, several MDR-associated genes and the housekeeping gene  $\beta_2$ microglobulin.



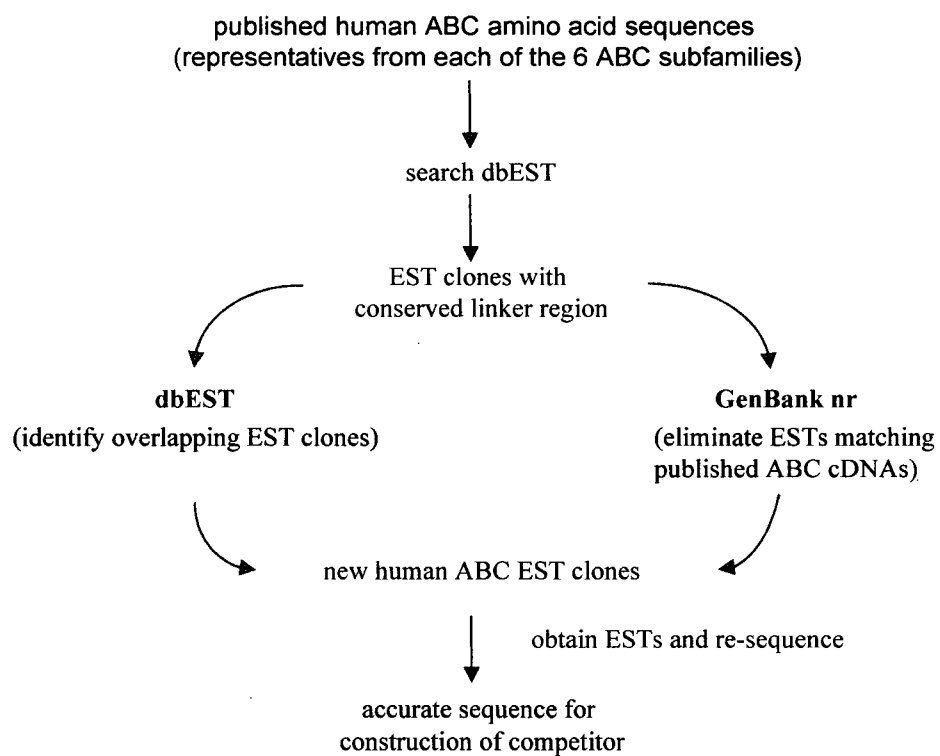
**Figure 2.1 Determination of Transcript Copy Number.**

Competition plot for TAP1 mRNA based on peak areas obtained using GeneScan2.1 software. Each data point represents the ratio of the area under the curve for native mRNA and competitor PCR products from a single reaction tube with a known quantity of competitor molecules added. The estimated number of initial native mRNA copies in the PCR reaction is determined from the equation of the line. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

## MATERIALS AND METHODS

### 2.2.1 Identification of Human ABC cDNAs Using DNA Sequence Databases

We have searched for new members of the human ABC protein family by utilizing the expressed sequence tag database (dbEST). Specifically, we searched dbEST using the conserved Walker A (G-X<sub>2</sub>-G-X-G-K-S/T-T/S-X<sub>4</sub>-hydrophobic), Walker B (R-X-hydrophobic<sub>2</sub>-X<sub>2</sub>-P/T/S/A-X-hydrophobic<sub>4</sub>-D-E-A/P/C-T-S/T/A-A/G-hydrophobic-D) and ABC Linker (hydrophobic-S-X-G-Q-R/K-Q-R-hydrophobic-X-hydrophobic-A) motifs of published ABC AA sequences and the tBLASTn program (Altschul et al., 1990) (Figure 2.2). At least one member from each of the six human ABC subfamilies was used as bait in this search. This search identified several hundred ESTs containing conserved ABC motifs. These EST nucleotide sequences were used to search the non-redundant database (nr) with the tBLASTn program to remove ESTs matching published ABC cDNAs. Finally, the remaining EST nucleotide sequences were used to search dbEST with the program tBLASTn to identify overlapping ESTs from the same ABC cDNA. This approach produced a list of 40 previously unidentified “human” ABC cDNAs. Due to the sequence inaccuracies inherent to entries in dbEST (single-pass sequencing), we decided to sequence representative EST clones to obtain more accurate sequence data. This information along with the publication of a comprehensive list of ABC genes in the *S. cerevisiae* (Decottignies and Goffeau, 1997) and *E. coli* (Linton and Higgins, 1998) genomes, allowed us to develop a confirmed list of 20 novel human ABC cDNAs (based on published sequence information available in 1997). A similar approach was used to search the human total genome sequence database (HTGS); however, this effort did not identify any additional novel human ABC cDNAs that were not represented in dbEST. This information, along with more recently submitted sequence data, has been summarized in the dendrogram in Figure 1.5 (chapter I).



**Figure 2.2 Identification of New Human ABC cDNAs Using Nucleotide Database Searches.**  
See Materials and Methods section 2.2.1 for details.

## 2.2.2 Preparation of Total RNA

Total RNA from cell lines was isolated using a modified guanidine isothiocyanate/cesium chloride method (Chirgwin et al., 1979). The extracted RNA was cleared of genomic DNA by a 15 minute (min) incubation at room temperature (rt) in RNase-free DNase I along with RNase Inhibitor (Gibco BRL) according to manufacturer's instructions, followed by phenol-chloroform extraction, precipitation with isopropanol and a wash with 70% ethanol. Pelleted RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated H<sub>2</sub>O with 10 mM Tris-Cl and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, pipetted up and down 50 times and quantified in triplicate by absorbance at 260 nm and stored at -80°C.

Integrity of RNA was assessed by formaldehyde-agarose gel electrophoresis and the ability to serve as a template for production of full-length  $\beta_2$ microglobulin mRNA by RT-PCR. To determine the optimal number of PCR cycles, so as to avoid amplification in the plateau phase and to obtain enough PCR product to assess RNA integrity, we used a single RNA source with a varying number of cycles ranging from 20-32 cycles. From this we determined the optimal number of PCR cycles to be 24 (data not shown).

Reverse transcription was performed, using 250 ng of total RNA, under the following conditions: 50°C (50 min), then 70°C (10 min) in a 9600 thermal cycler (Perkin-Elmer). RT buffer consisted of: 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 500  $\mu$ M dNTPs, 2.5 mM MgCl<sub>2</sub>, 500 ng oligo (dT) primer and 10 mM dithiothreitol (DTT). To denature RNA secondary structure, RT reactions were heated at 70°C (10 min), and then chilled to 4°C (5 min). The reaction was then incubated at rt (10 min) to allow primers to anneal. 180 U of Superscript II reverse transcriptase and 2 U of RNase Inhibitor (Gibco BRL) were then added to a final volume of 20  $\mu$ l. PCR was performed using the following primers for  $\beta_2$ microglobulin: 5'-ATTCGGGCCGAGATGTCTCG-3' and 5'-CCAGATTAACCACAACCATGC-3'. Cycling parameters were as follows: 95°C (2 min), then 24 cycles of 95°C [15 seconds (sec)], 64°C (15 sec), 72°C (90

sec) and a final extension period of 72°C (5 min). PCR buffer consisted of: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100 µM dNTPs, 2 mM MgCl<sub>2</sub>, 20 µg/ml bovine serum albumin (BSA), 0.1% (v/v) Triton X-100, 2.5 U of *Taq* enzyme (laboratory stock) and 1 µM of forward and reverse primers. 5 µl of cDNA was added to 20 µl of PCR mix. PCR products were separated electrophoretically on a 1% agarose gel, stained with ethidium bromide and visualized on a Eagle Eye II (Stratagene) imaging system.

### **2.2.3 Construction of ABC RNA Competitors**

Competitor ABC cDNAs were generated by PCR amplification using forward and mutagenizing primers (Table II.I) to create deletions ranging from 4-8 bp. DNA templates used included commercially available EST cDNAs (Research Genetics), a human kidney cDNA library (kind gift of Dr. Douglas Hogue, Dalhousie University, Halifax) and full-length cDNAs (ABCD2 cDNA kindly provided by Dr. Andreas Holzinger, Ludwig-Maximilian University, Munich; ABCG2, ABCG4 and ABCG5 cDNAs generously provided by Dr. Rando Allikmets, Columbia University, New York). The PCR protocol used to introduce the deletion varied depending on the template used and predicted annealing temperature of the primers.

PCR products were blunt-ended and phosphorylated using T4 DNA polymerase and T4 polynucleotide kinase (Gibco BRL) according to manufacturer's instructions. The modified DNA was then purified using phenol/chloroform, precipitated with isopropanol, washed with 70% ethanol and resuspended in 10 µL H<sub>2</sub>O. pBluescript II (SK+ or KS-) plasmid (ampicillin-resistant) was digested with *EcoRV* (Gibco BRL) and dephosphorylated with Calf Intestinal Alkaline Phosphatase (Gibco BRL) according to manufacturer's instructions. The digested plasmid was then separated electrophoretically on a 0.7% gel, along with non-digested plasmid, and the band containing the linearized form of the plasmid was cut out and purified with the QIAEXII Gel Extraction kit according to manufacturer's instructions (Qiagen). The modified

PCR product and linearized plasmid were ligated overnight at 14°C with DNA ligase enzyme and buffer (Gibco BRL). Ligation products were transformed, using the heat-shock method, into calcium chloride-competent DH5 $\alpha$  *E. coli* cells, plated on ampicillin-resistant plates containing X-galactosidase (blue/white selection) and grown overnight at 37°C. Several white colonies were selected and suspended in 10  $\mu$ L H<sub>2</sub>O and 2  $\mu$ L of this mixture was used immediately as template for colony PCR.

Colony PCR was performed to confirm the presence, size and orientation of the insert. PCR was performed using primers T7 universal (5'-CCCTATAGTGAGTCGTATT-3') and M13 reverse (5'-CAGGAAACAGCTATGA-3'). To determine orientation of the insert, a second PCR was performed using T7 or M13 and an appropriate internal primer matching the expected insert sequence (Table II.I). PCR buffer consisted of: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100  $\mu$ M dNTPs, 2 mM MgCl<sub>2</sub>, 20  $\mu$ g/mL BSA, 0.1% (v/v) Triton X-100, 4 U of *Taq* polymerase (laboratory stock), 0.5  $\mu$ M of T7 and M13 primers and 2.0  $\mu$ L of the colony mixture in a 15  $\mu$ L volume. PCR cycling parameters were 95° (5 min) and 30 cycles at 95° (30 sec), 56° (15 sec), 72° (45 sec). PCR Products were separated on a 1% agarose gel and visualized after ethidium bromide staining.

Colonies containing plasmids with the appropriate insert size and orientation, as determined by PCR, were re-plated on ampicillin-resistant plates containing X-galactosidase and grown overnight at 37°C. The next day, a single white colony was selected and used to inoculate an overnight culture of LB media containing ampicillin. Plasmids were then extracted using the Quantum Prep Plasmid Miniprep Kit (Biorad) according to manufacturer's instructions. cDNA identity and orientation was confirmed by DNA sequencing on an ABI 310 automated DNA sequencer. Plasmids were sequenced using 4  $\mu$ L of 0.8  $\mu$ M T7 universal or M13 reverse primers, 4  $\mu$ L of Big Dye Terminator Mix (Applied Biosystems) and 2  $\mu$ L of plasmid template in a total volume of 10  $\mu$ L. Sequencing PCR conditions were: 95° (2 min) and 28 cycles of 95° (10

sec), 47° (5 sec), 60° (3 min). PCR products were precipitated with isopropanol, washed with 70% ethanol, resuspended in 13 µL Template Suppression Reagent (Applied Biosystems) and processed in the 310 DNA sequencer.

After verifying competitor constructs by DNA sequencing, these plasmids were re-transformed into calcium chloride-competent DH5α *E. coli* cells, plated on ampicillin-resistant plates containing X-galactosidase and grown overnight at 37°C. The next day, a single white colony was selected and used to inoculate an overnight culture of LB media containing ampicillin. The bacterial cells were then pelleted and resuspended in 5 mL of a solution containing 50 mM glucose, 25 mM Tris (pH 8.0) and 10 mM EDTA (pH 8.0) (TE buffer) and left to stand for 5 min at rt. Cells were then lysed with the addition of 10 mL of a solution of 0.2 M NaOH and 1% sodium dodecyl sulphate (SDS) for 5 min at 4°C. Next, 7.5 mL of a chilled (4°C) solution of 3 M potassium acetate and 11.5% (v/v) glacial acetic acid was added and incubated at 4°C for 5 min. The plasmid DNA was then purified using phenol/chloroform extraction, precipitated with isopropanol, washed with 70% ethanol and resuspended in 250 µL of TE buffer. To remove any contaminating RNA, 1.25 µL of a 2 mg/mL solution of RNase A was added and incubated for 30 min at 37°C. To precipitate the plasmid DNA, 200 µL of a solution of 20% polyethylene glycol and 2 M NaCl was added and this was placed at 4°C for 1 hour. After precipitation, pellets were washed with 70% ethanol and resuspended in 250 µL H<sub>2</sub>O.

Plasmids were linearized by restriction endonuclease digestion with *ScaI* (Gibco BRL). A portion of the *ScaI* restriction digest was then separated electrophoretically, along with non-digested plasmid, on a 0.7% agarose gel to confirm that digestion was complete. A single linearized band was consistently observed. The modified DNA was then purified using phenol/chloroform, precipitated with isopropanol, washed with 70% ethanol and resuspended in 10 µL DEPC-treated H<sub>2</sub>O. *In vitro* transcription was performed at 37°C (2 hours), then 70°C

(10 min) in a 9600 thermal cycler (Perkin-Elmer) using a Megascript T7 *in vitro* transcription kit (Ambion) and the resulting RNA transcript was purified according to the manufacturer's instructions. Competitor RNA was quantified and stored as described above for total RNA preparation. Transcript size and purity were verified by formaldehyde-agarose gel electrophoresis. We consistently observed a strong band of expected size (2.0-2.4 kb), as well as a second band approximately 500 nts shorter and 1/5 the intensity. This pattern was consistently observed for every RNA competitor, whereas transcription from the linearized control plasmid (TRIPLScript plasmid containing a 1.85 kb *Xenopus* elongation factor 1 $\alpha$  gene) provided with the kit produced a single band of expected size, suggesting the secondary band is plasmid-dependent. There may be strong secondary structure in the RNA competitor transcript that is only partially resolved using formaldehyde denaturing gels. RNA competitors run on regular agarose gels have a secondary band of 1/2 the intensity of the expected band, in comparison to a secondary band of 1/5 the intensity on formaldehyde denaturing gels, suggesting incomplete denaturation is likely the cause of this secondary band. This would not affect quantification of any transcript species using competitive-RT-PCR. Alternatively, the manufacturer does suggest that prematurely terminated products, manifested as smaller discrete bands, may occur with some templates (Ambion technical bulletin #182). Possible causes are sequences that resemble the phage termination signals, stretches of single nucleotides and GC-rich templates. Since the ABC competitor insert is located ~50 nts downstream of the T7 promoter it would be present in both bands. The presence of a truncated transcript ~500 nts shorter and 1/5 the intensity of the expected band will cause an approximate 5% overestimation of the number of RNA competitors present in an *in vitro* transcription reaction [ $\#$  of RNA competitors/ $\mu$ L = RNA yield determined by absorbance at 260 nM (ng/ $\mu$ L) / (transcript length (nts) x weight per ribonucleotide (ng))]. This would correspond to a 5% underestimation of the number of copies of a specific transcript species when using competitive-RT-PCR. Since all competitors were generated in the same

manner, with the same plasmid backbone, this potential underestimation would apply uniformly to all ABC transcripts. This potential underestimation has not been incorporated into the ABC transcript expression levels reported in this thesis.

#### **2.2.4 Reverse Transcription**

Each RT consisted of 6 competition reactions and an H<sub>2</sub>O-only negative control without competitor or RNA template added. Competitive-RT reactions contained 200 ng of total RNA and competitor RNAs of five different ABCs at varying concentrations. Competitor RNAs were added in 5-fold or 2-fold (increasing or decreasing) increments of concentration across the 6 competitive reactions to span a 3125-fold or 32-fold range, respectively. RT buffer consisted of: 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 500  $\mu$ M dNTPs, 2.5 mM MgCl<sub>2</sub>, 10 ng/ $\mu$ L random hexamers, 20  $\mu$ g/mL BSA and 10 mM DTT. To denature RNA secondary structure, RT reactions were heated at 70°C (10 min), and then chilled to 4°C (5 min). The reaction was then incubated at rt (10 min) to allow primers to anneal. 180 U of Superscript II reverse transcriptase and 2 U of RNase Inhibitor (Gibco BRL) were then added to a final volume of 40  $\mu$ L. Reverse transcription was performed at 50°C (50 min), then 70°C (10 min) in a 9600 thermal cycler (Perkin-Elmer).

#### **2.2.5 Amplification and Analysis of PCR Products**

PCR amplification for each ABC transcript consisted of six competition reactions, an H<sub>2</sub>O-only negative control and a non-reverse-transcribed total RNA control. Uniplex PCR buffer consisted of: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100  $\mu$ M dNTPs, 2 mM MgCl<sub>2</sub>, 20  $\mu$ g/ml BSA, 0.1% (v/v) Triton X-100, 2 U of Amplitaq Gold (Perkin-Elmer), 2  $\mu$ M of forward (5'-6FAM labeled) and reverse primers and 4  $\mu$ L of the appropriate cDNA mixture or 40 ng of non-reverse-transcribed total RNA in a 20  $\mu$ L volume. Multiplex PCR buffer was the same as

the uniplex PCR buffer except that it employed 3.5 U of Amplitaq Gold, scaling the amount of primer to product size (100-150 bp/0.8  $\mu$ M, 151-200/1.15  $\mu$ M...451-500 bp – 3.25  $\mu$ M) and adding 6  $\mu$ L of cDNA in a final volume of 30  $\mu$ L. Uniplex cycling parameters were: 95°C (7 min), 45 cycles at 95°C (15 sec), 64°C (15 sec) and 72°C (90 sec) and a final extension at 72°C (90 min). Multiplex cycling parameters were: 95°C (7 min), 45 cycles at 95°C (15 sec), 66°C (4 min) and a final extension at 72°C for 90 min.

PCR products from five different uniplex reactions or a single multiplex reaction (non-overlapping native mRNA and competitor products) were routinely pooled and diluted 2- to 50-fold in H<sub>2</sub>O. The PCR mixture (2  $\mu$ L) was added to 11  $\mu$ L of Template Suppression Reagent and 0.5  $\mu$ L of TAMRA-500 DNA size standard (Applied Biosystems). This mixture was heat denatured for 2.5 min at 95°C and immediately cooled to 4°C. PCR products were separated electrophoretically on a PE Biosystems 310 DNA sequencer and the resulting 6FAM labeled DNA peaks were quantified using GeneScan2.1 software. Conditions for the electrophoretic separation were as follows: module – GS STR POP4 (1 mL) C, gel temperature 60°C, sample injection voltage – 15 kV, sample injection time – 1-10 sec, run voltage – 15 kV and run time of 20-28 min. A commercially prepared liquid polyacrylamide formulation, POP4 – 4% polyacrylamide, was used as a matrix along with a 47 cm uncoated capillary (Applied Biosystems).

#### **2.2.6. Generation of ABC7 RNA Transcript**

A pcDNA3 vector containing full-length human ABC7 cDNA was kindly provided by Dr. Rando Allikmets, Columbia University, New York. The plasmid was transformed into calcium chloride-competent DH5 $\alpha$  *E. coli* cells, plated on ampicillin-resistant plates containing X-galactosidase and grown overnight at 37°C. The next day, a single colony was selected and used to inoculate an overnight culture of LB media containing ampicillin. Plasmid DNA was

then prepared as described in section 2.2.3. Plasmid linearization and *in vitro* transcription were also performed as described in section 2.2.3. Formaldehyde-agarose gel electrophoresis revealed a single band of expected size (data not shown). The RNA yield was quantified in triplicate by absorbance at 260 nm.

**Table II.I Oligonucleotides for Competitive Standard Generation and Competitive-RT-PCR.**

ABC nomenclature		Genbank accession number	Forward primer (5'to 3')	Reverse primer (5' to 3')	Mutagenizing primer (5' to 3')
<b>HUGO<sup>a</sup></b>	<b>Common</b>				
ABCA1	ABCI	NM 005502	GGCAATCATGGTCAATGGAAAG	GGAGAGGATGCTGAATATATCTG	GGAGAGGATGCTGAATATCTCTGAGAAAGATAATG
ABCA2	ABCI	AB028985	GTGGTGTGACATCACACAGC	ACCGTGATCATGTAGCCATCTCCCGGTTCTTC	ACCGTGATCATGTAGCCATCTCCCGGTTCTTC
ABCA3	ABCI	NM 001089	CATCCAGTACAGGAACTTC	CTGCTGTACACCTTGGAGATAATCAG	CTGCTGTACACCTTGGAGATAATCAG
ABCA4	ABCI	NM 000350	CATGGCATCAACAGCATGC	ATCTGTACAGCTGGCTGGCTCAG	ATCTGTACAGCTGGCTGGCTCAG
ABCA5	ABCI	U66672	GTGTTGTGAGGAGAAACCATCC	AGGACAGTAAACCCATACACTTC	AGGACAGTAAACCCATACACTTC
ABCA6	ABCI	U66680	CTCAAGAGAACGTGCTGTGGC	TTCCGCTCAGCCAGGTTATG	TTCCGCTCAGCCAGGTTATGCTCAGGAGG
ABCA8	ABCI	NM 007168	GAGTTGAGATGTATCGGTTCC	TTGCACATCTTCCACTGGCAAC	TTGCACATCTTCCACTGGCAAC
ABCB1	MDRI	NM 000927	TCCTATGCTGGATGTTCCGG	AATGAACCTGACTTGCCTCCAG	AATGAACCTGACTTGCCTCCAG
ABCB2	TAPI	NM 000593	TGCCCTACAGTTCGAAGCTTTGC	TGTAAGGAGTCAACAGACCCAC	TGTAAGGAGTCAACAGACCCACTGGGACGC
ABCB3	TAP2	NM 000544	GATGCTGATGCTGAGCTGTGG	CAGTGTTCATCTGTGAGATGG	CAGTGTTCATCTGTGAGATGGTTCATCCAGC
ABCB4	MDR3	NM 000443	GGTGCAATATCTCATTTGTGAATGG	CTGAAGCACTGGCAGCTTTGC	CTGAAGCACTGGCAGCTTTGCGATAGTTGAACAG
ABCB6	MTABC3	AF070598	TCACAGCTGGGAATGATGAGG	GGTTCTTCTCTGCTCCCTGCTG	GGTTCTTCTCTGCTCCCTGCTGCTGCCACATGTC
ABCB7	ABCI	NM 004299	GAAGTGATGCTGTGATGAGTGG	TAGATCTCCAACAGTAAGGTTAC	TAGATCTCCAACAGTAAGGTTACAAATCCC
ABCB8	M-ABCI	NM 007188	CCAAGGCTTTCCACATCCG	GGTGAAGTCTTTCAGCACTTC	GGTGAAGTCTTTCAGCACTTCGCGGCGCAGG
ABCB9	TAP-L	U66676	TCTACAGTGGCTGATGAG	ACAGGCTGAAGGAGACATCTCTG	ACAGGCTGAAGGAGACATCTCTGCTGGTGTG
ABCB10	M-ABCI	AA31819	CTCTCCGGAACCTGATCGTG	CTCCAAAGCACTTGGGAAAGC	CTCCAAAGCACTTGGGAAAGCATTAAAGATG
ABCB11	BSEP	NM 003742	TCAGTAACATCCGCACTGTGC	TCTTCCAAGAGCTGTGCACTC	TCTTCCAAGAGCTGTGCACTCCTGCGAGAG
ABCC1	MRP1	NM 004996	CTTCGTCAGAGGTTCTACG	CCACCTGTGGCCACGATGTAATAGGCC	CCACCTGTGGCCACGATGTAATAGGCC
ABCC2	MRP2	NM 000392	GGCTGGTGAGGATGACATCAG	CCAGATCCAGCTCAGGTCGG	CCAGATCCAGCTCAGGTCGGCACTTGGTAG
ABCC3	MRP3	NM 003786	GAGACCTGAGTCTGATGTGC	CCTCTGAGCACTGGAAATGCC	CCTCTGAGCACTGGAAATGCCGCTGGGAG
ABCC4	MRP4	NM 005845	GAAACCTTGAAGATCTCTCTGC	TCAATCTGTGCAATGTTAGTTC	TCAATCTGTGCAATGTTAGTTCAGTGGGC
ABCC5	MRP5	NM 005688	GGTCTCGCATCTCTTATGC	CTTAATCTGGCAGGTGCTTCC	CTTAATCTGGCAGGTGCTTCCAGTCTTAATG
ABCC6	MRP6	NM 001171	GGCAGATCGAGTTCGGGAC	AGATAGCCTCGTCCGAGTGC	AGATAGCCTCGTCCGAGTGCAGGTCGAGG
ABCC7	CFTR	NM 000492	TCCAGATCGATGGTGTCTTG	CTGGATGGAATCGTACTGCTCCG	CTGGATGGAATCGTACTGCTCCGCTTCTTTC
ABCC10	MRP7	AL133613	CCTGAGTGAAGTATTACATCC	TCAGGATCGTGTGAGCCTATG	TCAGGATCGTGTGAGCCTATGATGTCAGC
ABCD1	ALDP	NM 000033	ATCAACCTCATCTCTCTGGAAC	CTTGAAGTGACAGCGCTGAAC	CTTGAAGTGACAGCGCTGAACAAATACC
ABCD2	ALDR	AF119822	TTTGACAGTGGTGAAGATGGC	TCTCTGCTGGTGAATTAATG	TCTCTGCTGGTGAATTAATGGAATTTTAC
ABCD3	PMP70	NM 002858	GGTCTGGAGAAATCAATTATGC	ATCACTTGAATCTGAAAGTGTCC	ATCACTTGAATCTGAAAGTGTCCGTCATGTAAG
ABCD4	PMP69	NM 005050	AGATTGGGTGAATGCGGAGC	GAGGTAGATGCACACAAAGGC	GAGGTAGATGCACACAAAGGCTTGTGACC
ABCF1	ABCI	AF027302	TGAAGAGCTGAAGGACGCG	CATATCTGCTGGTGAAGAACGC	CATATCTGCTGGTGAAGAACGCTCAGCCGG
ABCF2	ABCI	NM005692	CACATGAAGAACTACATTCGAG	AGCTGCTTGTAAATGCTGATG	AGCTGCTTGTAAATGCTGATGCGCCCTATC
ABCF3	ABCI	AK002060	TCTGTCTGCTGATCTCGAG	TAGGTCAGCTGCTCCACATG	TAGGTCAGCTGCTCCACATGCTGGTGAATAG
ABCG1	WHITE1	NM 004915	TCTATTCGGTTCCTGAAGGAC	TCTGCTTCACTCTCTCTCTG	TCTGCTTCACTCTCTCTCTCTCTGATGTC
ABCG2	BCRP	NM 004827	CACAGAGATCATAGAGCCTTCC	CACCTGTAGTATCGGCAATATG	CACCTGTAGTATCGGCAATATGGAAGGAGG
ABCG4	WHITE2	NM 022169	CTGTCGCTATGGCTGAGAG	TGCTGGCATCTGCGCAATATG	TGCTGGCATCTGCGCAATATGGAAGGAGG
ABCG5	WHITE3	NM 022436	TACGCGTCTCTCAGAACTCG	GCCAGCATCATCTGCCACTTC	GCCAGCATCATCTGCCACTTCGCGCGTC
	$\beta_2$ microglobulin	XM032402	CCGACATTGAAGTTGACTTACTG	CATCTCAAACCTCCATGATG	CATCTCAAACCTCCATGATGCTGCTGATC
	LRP	X79882	CACAACCTACTGGTGAATTCG	CTCCGACGACATACATC	CTCCGACGACATACATCCTGCTGTTTC
	TopoisomeraseII $\alpha$	J04088	TCTGTGCTGAAGATGATGCTG	GTATCTGGACTAGCAATCC	GTATCTGGACTAGCAATCCACCATGTAG
	GST $\pi$	X15480	CTGTACCAGTCCAAATACCATCC	GTAGTCAGCGAAGGAGATCTG	GTAGTCAGCGAAGGAGATCTGCCACAATG

<sup>a</sup>HUGO refers to the nomenclature for the ABC gene superfamily assigned by the Human Genome Organization.

## RESULTS

### 2.3.1 Construction of ABC RNA Competitors

We have designed RNA competitors, for each of 36 human ABC transcripts, that possess primer binding sites identical to the native mRNA sequence and an intervening sequence that differs from the native mRNA by only a 4-8 nucleotide deletion (Table II.II). In addition to ABC genes, we have also generated competitors for  $\beta_2$ microglobulin, topoisomerase II $\alpha$ , glutathione-S-transferase  $\pi$  (GST $\pi$ ) and LRP. Our method of competitor construction requires single mutagenizing PCR and cloning steps suited to generating dozens of different competitors (Figure 2.3).

### 2.3.2 Optimization of a Competitive-RT-PCR Protocol

The process of optimizing a competitive-RT-PCR protocol is fraught with significantly more difficulties than standard RT-PCR. Firstly, to evaluate the effect of altering a single assay parameter (e.g. [dNTPs], [primer], annealing temperature etc.), the investigator must evaluate the effectiveness of the competition between the internal competitor and native mRNA, as well as changes in PCR product yield and presence of non-specific PCR products. This requires multiple competitive-RT-PCR reactions (at least 6 – negative and positive controls plus 4 reactions spanning a range of competitor concentrations) to assess each incremental change of a single parameter, which precludes assessing a range of conditions for a parameter in a single experiment. In comparison to standard RT-PCR, this increases, by several fold, the time and labour required for optimization. Secondly, the relatively narrow spectrum of reaction conditions, in comparison to standard RT-PCR, over which effective competitive can be achieved complicates the determination of optimal conditions. Thirdly, the added difficulty of resolving competitor and native mRNA PCR products can complicate the interpretation of

results, as happened in our experiments using agarose and polyacrylamide gels (see below). Finally, due to the high concentration of competitor molecules that are employed in competitive-RT-PCR, contaminating competitor PCR products in negative control reactions can be a frequent problem. Taken together, these difficulties proved a considerable obstacle to overcome. Our case was particularly challenging since we needed to design an assay for 40 unique transcripts with PCR amplicons ranging in size from 100 to 500 nucleotides.

The first phase of optimization utilized 2% agarose gels to separate electrophoretically PCR products with a resolution of ~20 bp over the size range of interest. To increase the size difference between competitor and native mRNA PCR products, the first three competitive standards ( $\beta_2$ microglobulin, MRP1 and MDR1) were designed such that the engineered 4-8 nucleotide deletion generated a new restriction site that was not present in the native mRNA amplicon sequence. After the completion of competitive-RT-PCR, products were digested with *Bgl*II (MDR1-native mRNA amplicon of 128 nucleotides versus 100 nucleotides for competitor), *Nsi*I ( $\beta_2$ microglobulin-native mRNA amplicon of 227 nucleotides versus 200 nucleotides for competitor) or *Bst*XI (MRP1-native mRNA amplicon of 214 nucleotides versus 190 nucleotides for competitor). Typical competitive-RT-PCR reactions consisted of a range of competitor concentrations mixed with a constant amount of SKOV3 or SKVLB1.0 (human vinblastine-resistant SKOV3-subline) total RNA, competitor-only positive control, total RNA-only positive control and a H<sub>2</sub>O-only negative control.

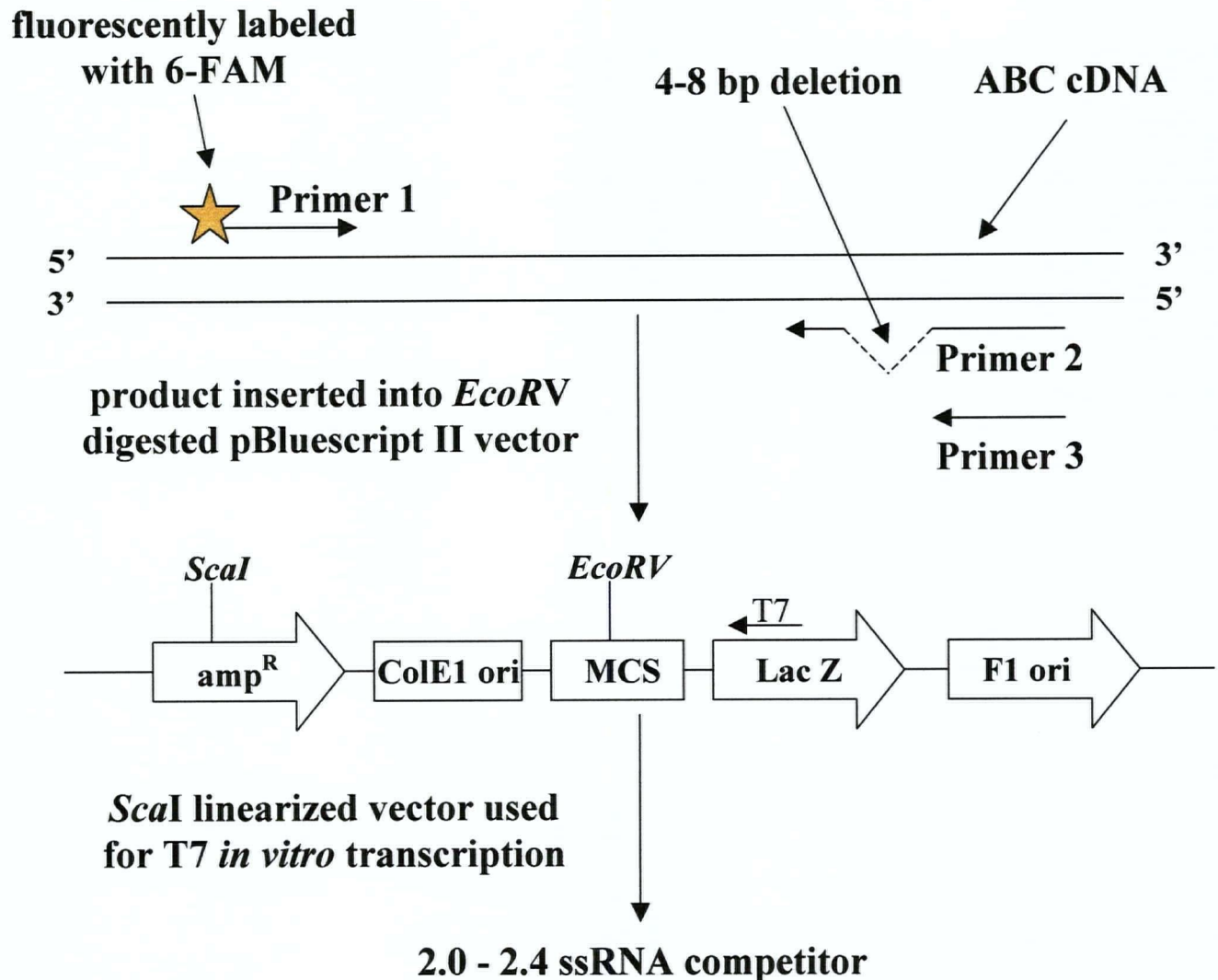
Using this approach, we compared the standard Gibco BRL RT buffer [50 mM Tris-HCl (pH8.3), 18.75 mM KCl, 3 mM MgCl<sub>2</sub>] with a second buffer system [20 mM Tris-HCl (pH8.3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub> and 20  $\mu$ g/mL BSA - Patrik Medstrand, personal communication], and the latter was found to produce a slightly greater yield of desired PCR product without an increase in non-specific PCR products (Figure 2.4). Using this new buffer system we observed the first evidence of competition between a competitor and native mRNA ( $\beta_2$ microglobulin

**Table II.II Competitor Construct Versus Native mRNA Amplicon Sizes.**

ABC nomenclature		Product size (nts) <sup>a</sup>		Size difference in nts (%)
HUGO <sup>b</sup>	Common	Native mRNA	Competitor	
ABCA1	ABC1	246	241	5 (2.03)
ABCA2	ABC2	141	137	4 (2.84)
ABCA3	ABC3	313	307	6 (1.92)
ABCA4	ABCR	166	162	4 (2.41)
ABCA5	ABCA5	295	291	4 (1.36)
ABCA6	ABCA6	353	349	4 (1.13)
ABCA8	ABCA8	193	187	6 (3.10)
ABCB1	MDR1	128	123	5 (3.91)
ABCB2	TAP1	373	369	4 (1.07)
ABCB3	TAP2	456	452	4 (0.88)
ABCB4	MDR3	285	277	8 (2.81)
ABCB6	MTABC3	428	424	4 (0.93)
ABCB7	ABC7	402	397	5 (1.26)
ABCB8	M-ABC1	349	341	8 (2.29)
ABCB9	TAP-L	195	190	5 (2.56)
ABCB10	M-ABC2	270	264	6 (2.22)
ABCB11	BSEP	268	262	5 (2.24)
ABCC1	MRP1	214	208	6 (2.80)
ABCC2	MRP2	186	181	5 (2.69)
ABCC3	MRP3	345	337	8 (2.32)
ABCC4	MRP4	238	234	4 (1.68)
ABCC5	MRP5	150	142	8 (5.33)
ABCC6	MRP6	326	318	8 (2.45)
ABCC7	CFTR	485	481	4 (0.82)
ABCC10	MRP7	257	253	4 (1.56)
ABCD1	ALDP	357	353	4 (1.12)
ABCD2	ALDR	365	361	4 (1.10)
ABCD3	PMP70	287	283	4 (1.39)
ABCD4	PMP69	278	274	4 (1.44)
ABCF1	ABC50	420	415	4 (0.95)
ABCF2	ABCF2	410	406	4 (0.98)
ABCF3	ABCF3	177	173	4 (2.26)
ABCG1	WHITE1	333	329	4 (1.20)
ABCG2	BCRP	454	447	7 (1.54)
ABCG4	WHITE2	261	255	6 (2.29)
ABCG5	WHITE3	216	209	6 (3.24)
	$\beta_2$ microglobulin	227	220	7 (3.08)
	LRP	159	155	4 (2.52)
	TopoisomeraseII $\alpha$	494	489	5 (1.01)
	GST $\pi$	275	270	5 (1.82)

<sup>a</sup>Size of PCR products based upon capillary electrophoresis may differ slightly from actual size.

<sup>b</sup>HUGO refers to the nomenclature for the ABC gene superfamily assigned by the Human Genome Organization.



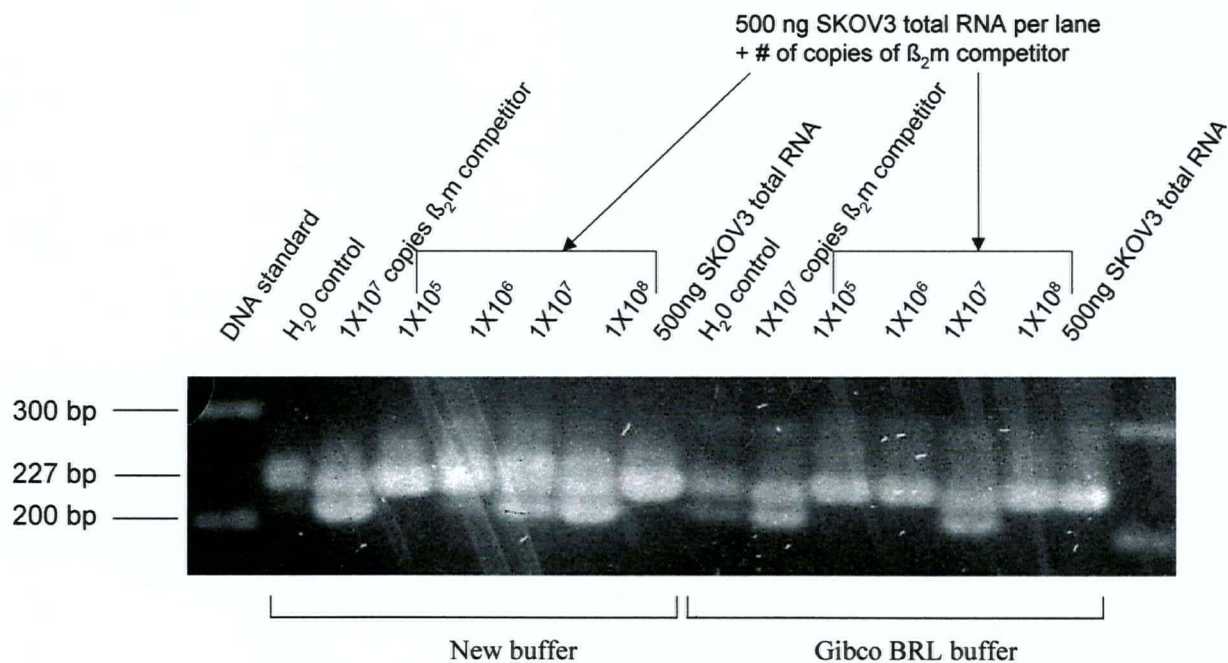
**Figure 2.3 Schematic of RNA competitor generation.**

An unlabeled forward primer (#1) and a mutagenizing reverse primer (#2), the latter of which matches the native transcript except for a gap of 4-8 bp, are used to PCR amplify each ABC competitor cDNA. These mutagenized PCR products are then directionally cloned into the pBluescript II plasmid. For competitive-RT-PCR, a 6FAM labeled forward primer (#1) and a reverse primer (#3) are used. See Materials and Methods section 2.2.3 for details.

competitor and native mRNA bands at 200 and 227 nts, respectively, in lanes 4,5 and 6). Contaminating PCR products were present in the H<sub>2</sub>O-only negative control and competitor-only positive control lanes. The DNA smearing is likely a result of the high concentration of restriction enzyme used to digest the PCR products.

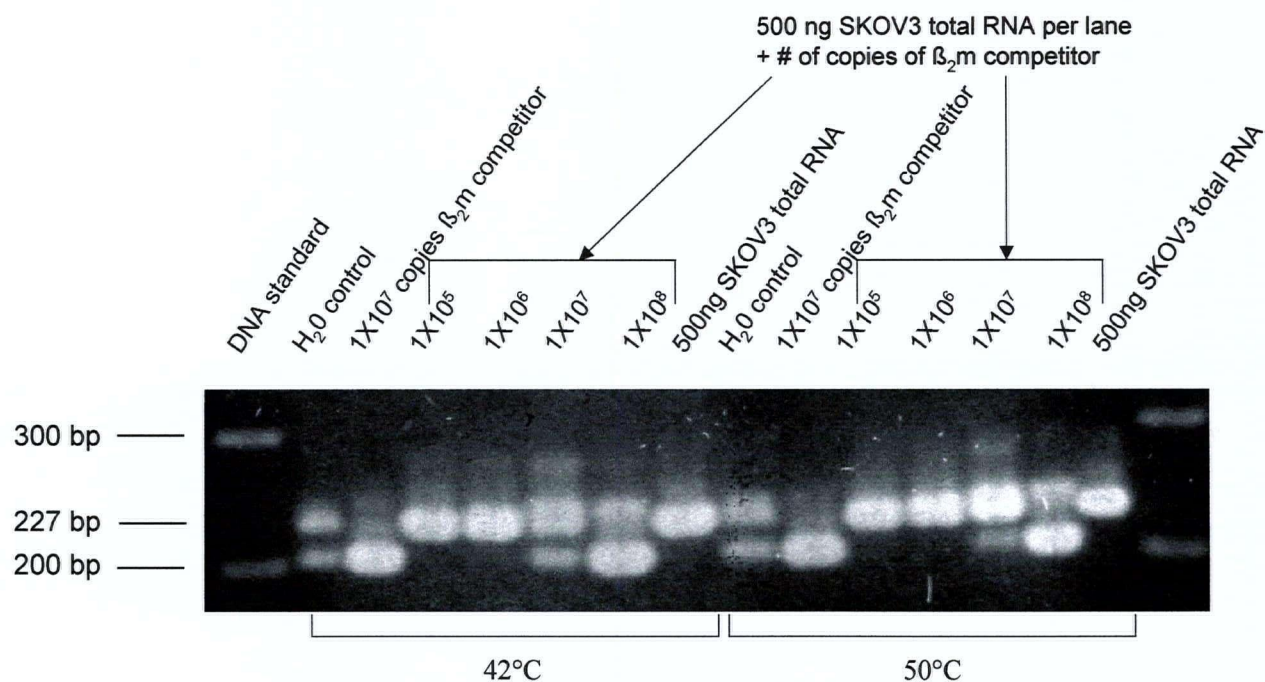
We next examined the effect of RT reaction temperature. The manufacturer's protocol recommends 37°C (Superscript II reverse transcriptase), but also states that the enzyme is still fully active at 50°C. We gradually increased the RT temperature over a series of experiments (37°C, 42°, 46°, 50° and 54°) and observed no difference in RT-PCR product yield or presence of non-specific PCR products and therefore chose to use 50°C to minimize RNA secondary structure. An example of one such experiment is shown in Figure 2.5.

During the initial phase of competitive-RT-PCR optimization, we consistently observed competitor PCR products in our H<sub>2</sub>O-only negative control reactions (Figures 2.4 and 2.5). This is a frequent problem in competitive-RT-PCR due to the high concentrations of competitor molecules employed. To minimize the occurrence of these undesired PCR products, we switched to aerosol-resistant pipette tips, set aside a dedicated set of micro-pipettors for PCR and thoroughly cleaned the inside and outside of these micro-pipettors prior to each use. We also constructed a self-contained PCR hood for PCR set-up that had no air circulation and had an ultraviolet light source to decontaminate the work surface between uses. This PCR hood was kept on a bench separate from that used for the handling of post-PCR products. Finally, all RT and PCR reagents were divided into small aliquots (< 5 reactions each) to minimize the number of times each tube was accessed. These measures significantly reduced the occurrence of contamination in H<sub>2</sub>O-only controls, although this problem did still occur infrequently. For quantification, any competitive-RT-PCR reactions with contaminating PCR products in the control reactions were disregarded.



**Figure 2.4 Comparison of Buffers for Reverse Transcription.**

The effect of different reverse transcription buffer systems was compared using competitive-RT-PCR amplification of  $\beta_2$ microglobulin present in 500 ng of SKOV3 total RNA. PCR products were digested with *Nsi*I prior to separation by electrophoresis on a 2% agarose gel followed by staining with ethidium bromide. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



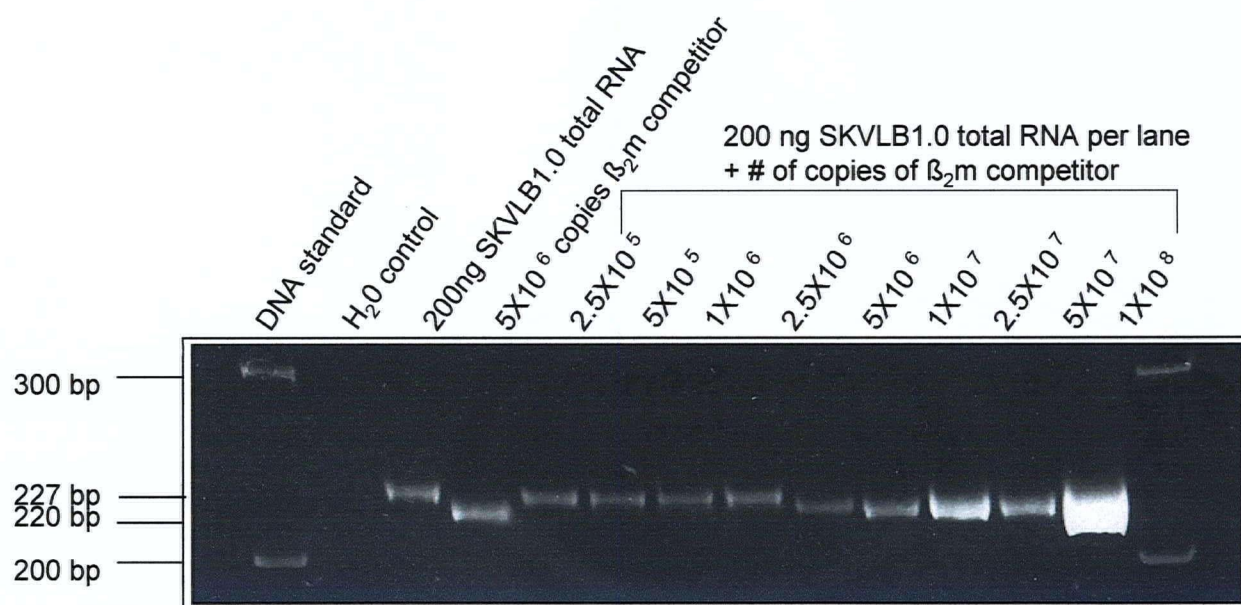
**Figure 2.5 Comparison of Reverse Transcription Reaction Temperatures.**

The effect of different reverse transcription temperatures (42°C or 50°C) was examined using competitive-RT-PCR amplification of  $\beta_2$ microglobulin present in 500 ng of SKOV3 total RNA. PCR products were digested with *NsiI* prior to separation by electrophoresis on a 2% agarose gel followed by staining with ethidium bromide. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

To simplify the visualization of competitor and native mRNA RT-PCR products, we switched to 11% non-denaturing polyacrylamide gels with a resolution of  $\sim 5$  bp over the size range of interest. Using this approach, a range of primer concentrations in the PCR reaction was compared (0.5  $\mu$ M, 1.0  $\mu$ M and 2.0  $\mu$ M) and it was determined that 2  $\mu$ M primer produced the greatest yield of PCR product without an increase in non-specific PCR products (data not shown). We also gradually increased the annealing temperature (57°, 59°, 61° and 63°C) of the PCR reaction over a series of experiments and observed no change in PCR product yield with a decrease in non-specific PCR products (data not shown). A typical image of competitor and native mRNA PCR products separated by electrophoresis on an polyacrylamide gel is shown in Figure 2.6. Using polyacrylamide gels we were able to demonstrate effective competition for several competitors ( $\beta_2$ microglobulin, MRP1 and MDR1), but we still observed significant contaminating PCR products in negative control reactions. The efficiency of PCR amplification was inconsistent, apparently due to the non-optimal protocol being employed.

With sufficient evidence that competition was occurring between competitor and native mRNA transcripts, we proceeded to analyze the competitive-RT-PCR products using capillary electrophoresis on an ABI 310 model automated DNA sequencer. To optimize capillary electrophoresis and fluorescent detection, we adjusted a number of parameters (e.g. run time and voltage, sample injection voltage, gel temperature, % polyacrylamide and sample dilution prior to loading) over a period of several weeks. In addition, we determined the number of samples that could be processed through a single capillary without reduction in fluorescent signal strength and resolution, and the effective life span of liquid polyacrylamide at room temperature in the DNA sequencer. Samples were heat-denatured at 95°C prior to loading and capillary electrophoresis was carried out at 60°C to maintain the denatured state of the PCR products.

One complication of using denaturing capillary electrophoresis with single nucleotide resolution is the detection of PCR doublet peaks. These doublets are due to the tendency of *Taq*



**Figure 2.6 Optimization of Competitive-RT-PCR Using Non-Denaturing Polyacrylamide Gels.**

The number of  $\beta_2$ microglobulin transcripts present in 200 ng of SKVLB1.0 total RNA was determined using competitive-RT-PCR. PCR products were separated by electrophoresis on an 11% non-denaturing polyacrylamide gel followed by staining with ethidium bromide. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

polymerase to add a non-templated nucleotide to the end of an extended cDNA during PCR (Smith et al., 1995). To correct this problem, we compared our laboratory prepared stock of *Taq*, with or without *Pfu* or T4 DNA polymerase, with Gibco BRL *Taq* polymerase. The use of the Gibco BRL *Taq* was problematic because of the increase in non-specific PCR products compared to our laboratory *Taq* stock, whereas the addition of *Pfu* (1 *Pfu*:8 *Taq* units) or T4 DNA polymerase (post-PCR) had no significant effect on the occurrence of doublet peaks (data not shown). Rather than trying to prevent the non-templated addition of nucleotides, we decided to modify PCR conditions, over a series of experiments, to promote the addition of an extra nucleotide to all PCR products. As shown in Figure 2.7, using a laboratory *Taq* polymerase stock, and without any 72°C extension after the completion of PCR cycling, there was a 50:50 ratio of *n* and *n*+1 PCR products (panel I). With a 15 min post-cycling 72°C extension (panel II), the ratio remained 50:50, whereas with a 30 min 72°C extension (panel III), the ratio was reduced to ~30:70. A further increase to a 40 min 72°C extension (panel IV) shifted the ratio of *n* and *n*+1 products to ~10:90. We later increased the extension times within each PCR cycle (4 min at 66°C – multiplex PCR) and post-cycling (90 min at 72°C) and the percentage of *n* + 1 peaks was maximized, although some PCR products still tended to form doublets (data not shown). Similar results were observed for MDR1 PCR products.

We next examined the effect of varying MgCl<sub>2</sub> concentrations (25–0.19 mM) in the PCR buffer (Figure 2.8). In standard PCR reactions, MgCl<sub>2</sub> ranges from 1 to 5 mM; however, we decided to test a broad range of concentrations to determine how this would influence the effectiveness of competition and the formation of doublet peaks. At high MgCl<sub>2</sub> concentrations (25 mM-panel I and 12.5 mM-panel II), there was a significant reduction in PCR product yield. At moderate MgCl<sub>2</sub> concentrations (6.25 mM-panel III, 3.12 mM-panel IV and 1.56 mM-panel V), PCR product yield was maximized. At 1.56 mM MgCl<sub>2</sub>, approximately 20% of the PCR products lacked the addition of a non-templated nucleotide. As MgCl<sub>2</sub> concentrations were

further reduced (0.78 mM-panel VI, 0.39 mM-panel VII and 0.19 mM-panel VIII), the percentage of PCR products without a non-templated nucleotide increased to a maximum of 50% and the PCR product yield was significantly reduced. The ratio of MDR1 competitor:native mRNA PCR products was consistent over the range of  $\text{MgCl}_2$  concentrations tested. Similar results were observed for other MDR1 competitor concentrations and other transcripts (MRP1 and  $\beta_2$ microglobulin) (data not shown). Based on these data, an optimal concentration of 2.5 mM was selected for the PCR buffer, although a subsequent round of optimization lowered this to 2 mM.

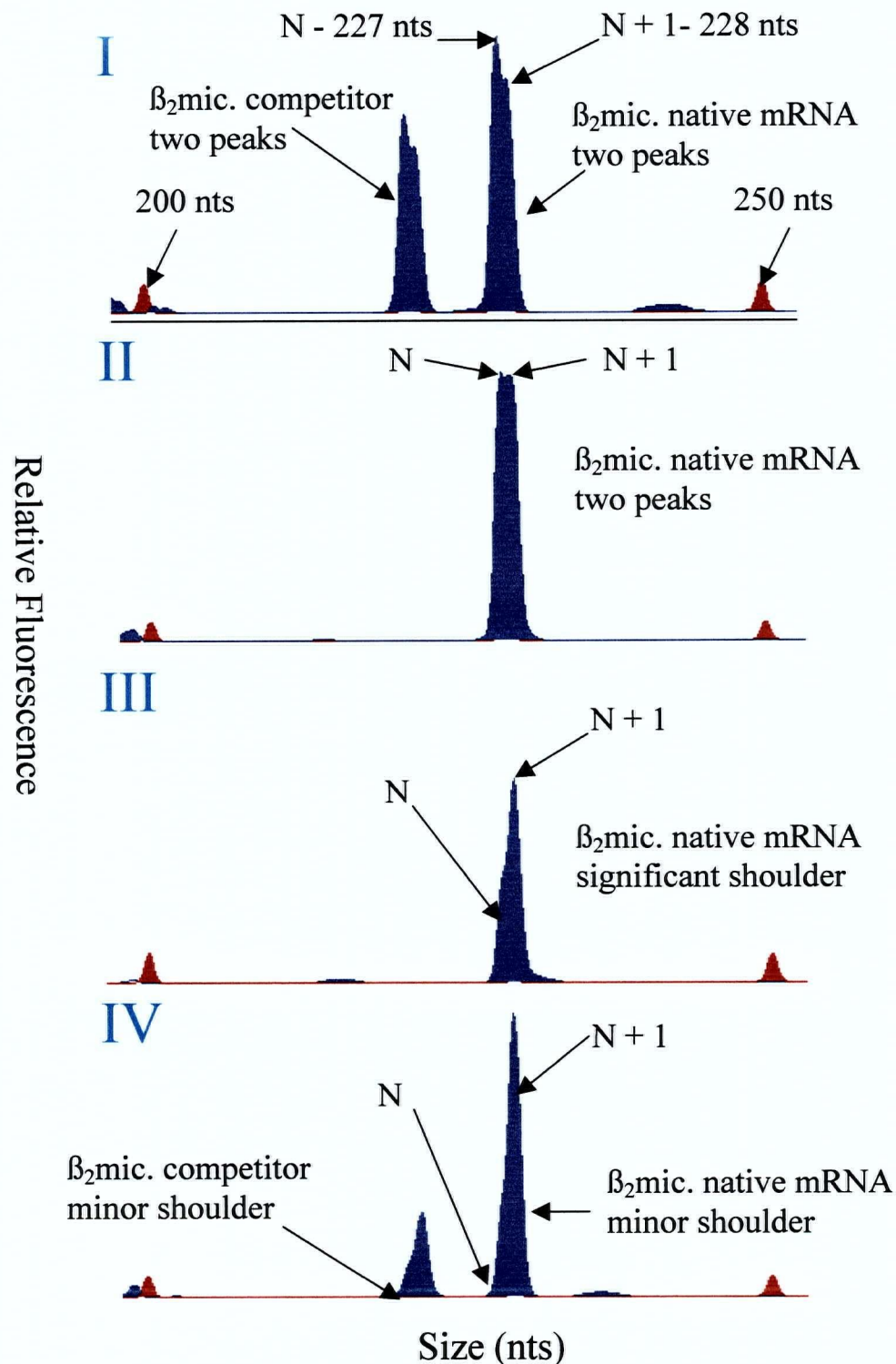
To further improve the PCR component of our protocol, we compared our standard PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH8.3), 1.5 mM  $\text{MgCl}_2$  and 20  $\mu\text{g/mL}$  BSA] with a modified PCR buffer with increased  $\text{MgCl}_2$  (2 mM) and the addition of dNTPs (100  $\mu\text{M}$  plus 100  $\mu\text{M}$  carry-over from the RT reaction) and Triton X-100 (0.1% v/v) (Figure 2.9). For each of the four transcripts examined, there was significantly greater PCR product yield without an increase in non-specific PCR products, using the modified PCR buffer. The trend was consistent across a range of competitor concentrations (reactions 1-4). The ratio of competitor:native mRNA PCR products did not differ between the two buffers. This modified PCR buffer was used for all subsequent experiments.

To further improve PCR product yield and reduce the number of non-specific noise peaks, we next tested the effect of increasing the annealing temperature (60°, 62°, 64° and 66°C) (Figure 2.10), as we had done previously with polyacrylamide gels. The increase in annealing temperature did not affect PCR product yield for the transcripts examined ( $\beta_2$ microglobulin, MRP1 and MDR1). Increasing annealing temperature to 62°C removed the relatively minor non-specific peaks seen at 60°C. A second round of annealing temperature optimization (66°, 68° and 70°C) revealed a marginal reduction of PCR product yield. Since all competitive-RT-PCR primers are designed to have a predicted annealing temperature of 64-66°C [ $2 \times (A+T)$

+4x(G+C)], an optimal annealing temperature of 66°C was chosen. Similar results were observed using different concentrations of competitors (data not shown).

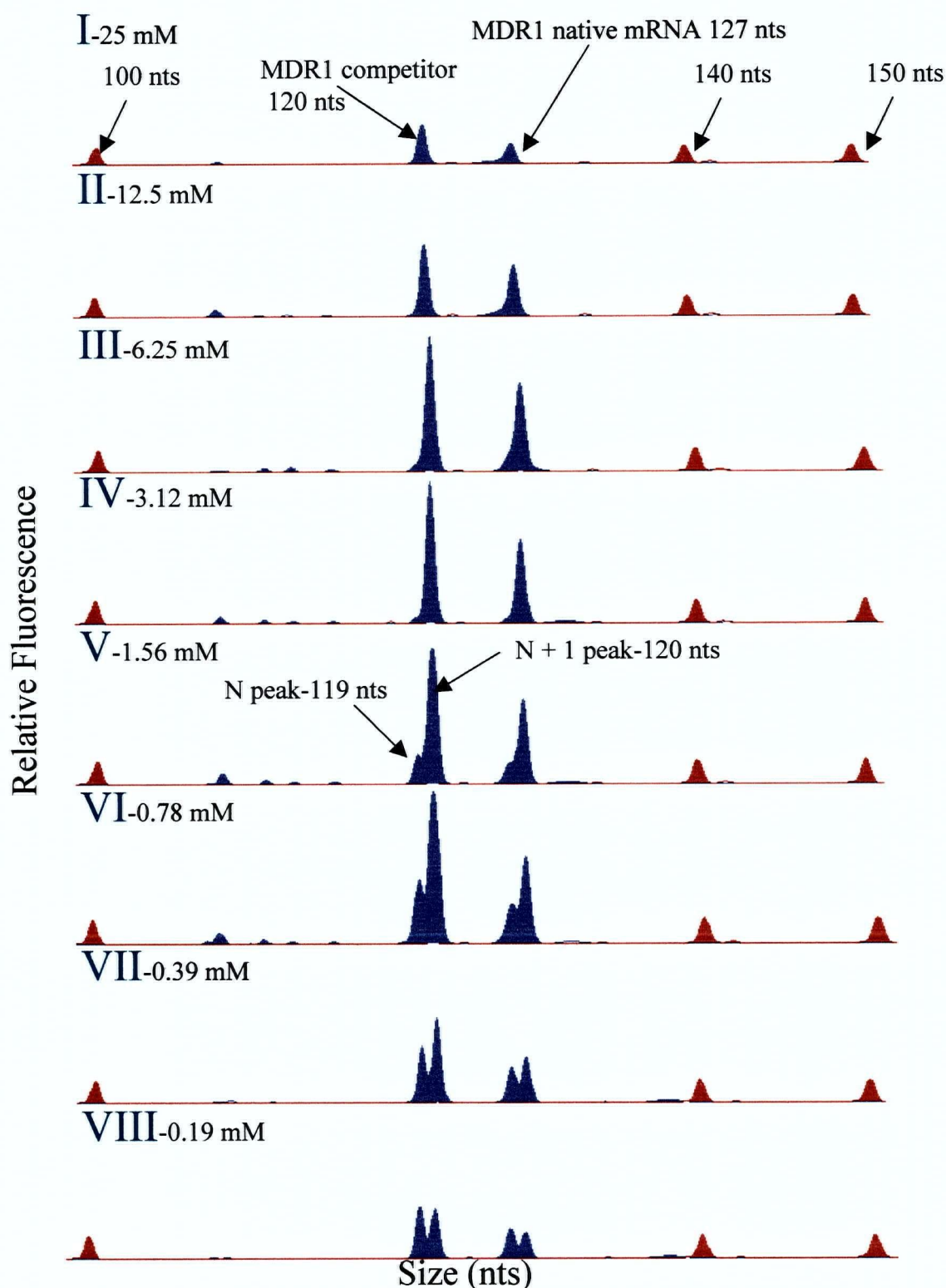
Other modifications to the competitive-RT-PCR protocol included scaling the amount of primer to PCR product size (100-150 bp/0.8  $\mu$ M, 151-200/1.15  $\mu$ M...451-500bp – 3.25  $\mu$ M), which produced a more evenly distributed yield of PCR products across the size range of 100 to 500 nucleotides (small PCR amplicons are preferentially amplified) (data not shown). Finally, to minimize the number of non-specific noise peaks we compared our laboratory *Taq* stock with the Perkin-Elmer Amplitaq Gold enzyme, which is a genetically engineered version of *Taq* that is not active until heated at 95°C for 12 minutes. The use of Amplitaq Gold marginally reduced the appearance of non-specific PCR products, in comparison to laboratory *Taq* stock, but did not enhance PCR product yield (data not shown). However, the addition of Amplitaq Gold was deemed necessary since the length of time required for the addition of the numerous reagents in a competitive-RT-PCR reaction is considerable, thus creating the potential for non-specific priming at low temperatures (Kebelmann-Betzing et al., 1998). This is a significant problem when using regular *Taq* polymerase, which is still partially active at room temperature.

Overall, the parameters that influenced competitive-RT-PCR performance the most were: RT and PCR buffer composition, MgCl<sub>2</sub> concentration and primer concentration. PCR annealing temperature, RT reaction temperature and the source of *Taq* polymerase were the least influential. A typical image of competitive-RT-PCR products generated using our optimized protocol is shown in Figure 2.11.



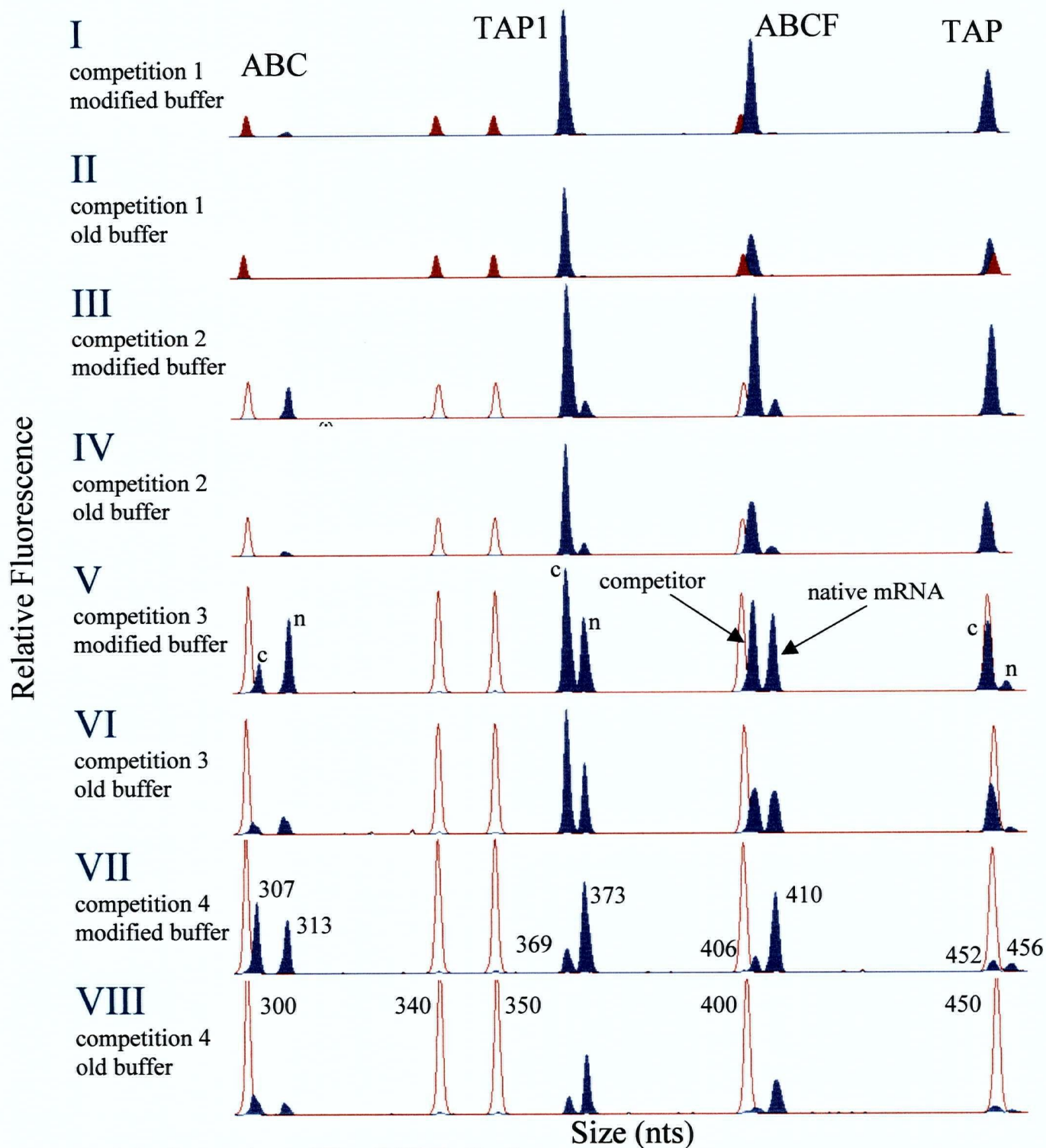
**Figure 2.7. Effect of Increased Post-cycling Extension Time in PCR.**

$\beta_2$ microglobulin PCR products were separated via capillary electrophoresis on an ABI 310 DNA sequencer and analysed using GeneScan2.1 software. Blue peaks represent PCR products fluorescently labeled with 6FAM and red peaks represent a size standard fluorescently labeled with TAMRA. Row I - competitor and native mRNA amplified with no extension at 72°C, row II - native mRNA amplified with 15 min. 72°C extension, row III - native mRNA amplified with 30 min. 72°C extension and row IV - competitor and native mRNA amplified with 40 min. 72°C extension. See Materials and Methods sections 2.2.4 and 2.2.5.



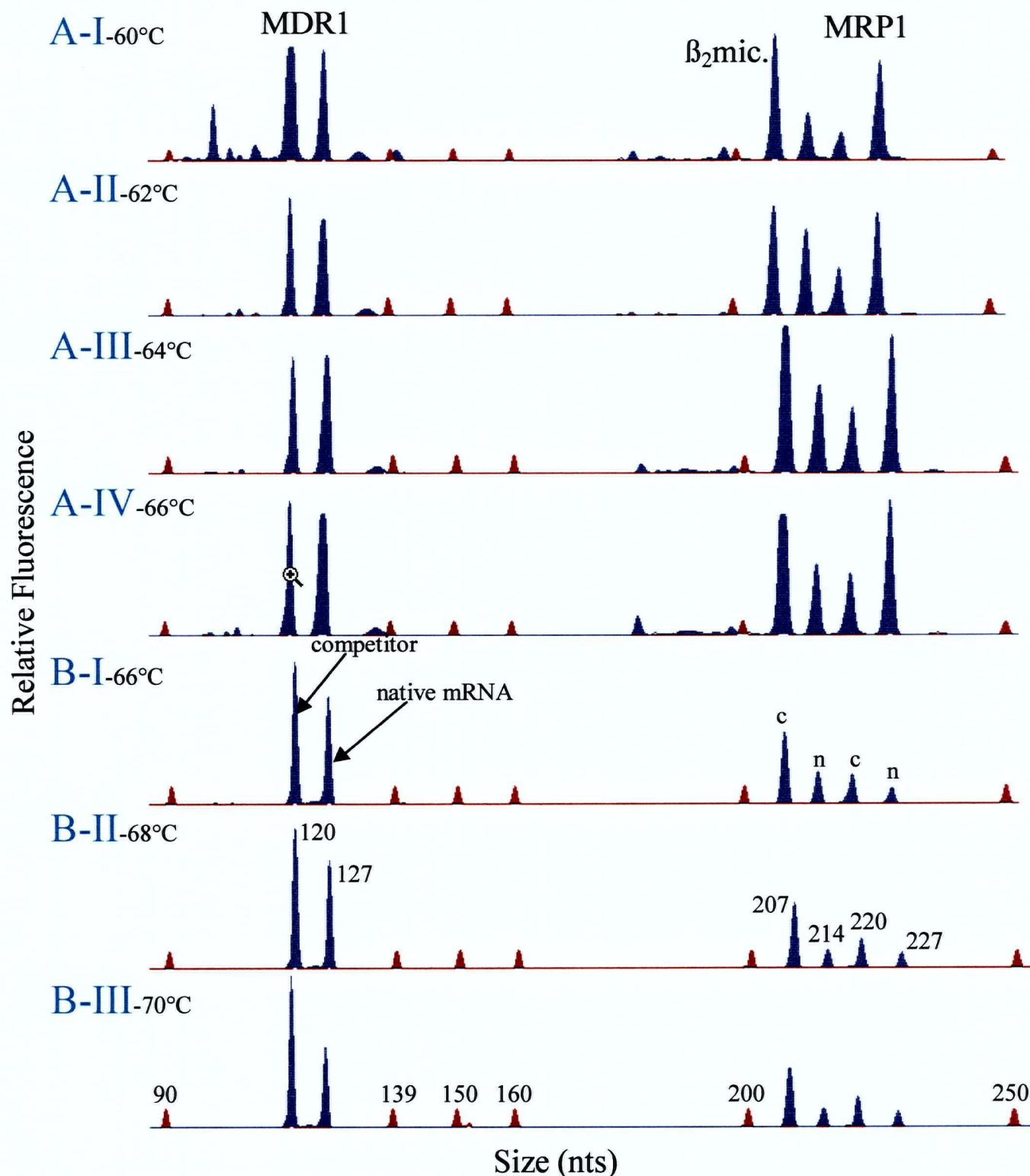
**Figure 2.8. Effect of Varying  $MgCl_2$  Concentration in PCR Buffer.**

MDR1 competitive-RT-PCR products were separated via capillary electrophoresis on an ABI 310 DNA sequencer and analysed using GeneScan2.1 software. Blue peaks represent PCR products fluorescently labeled with 6FAM and red peaks represent a size standard fluorescently labeled with TAMRA. The same RT reaction mixture with 100 ng SKVLB1.0 total RNA and  $2.5 \times 10^6$  copies of MDR1 competitor was used for each PCR reaction. Rows I - VIII have 2-fold decreasing amounts of  $MgCl_2$  from 25 mM to 0.19 mM. See Materials and Methods sections 2.2.4 and 2.2.5.



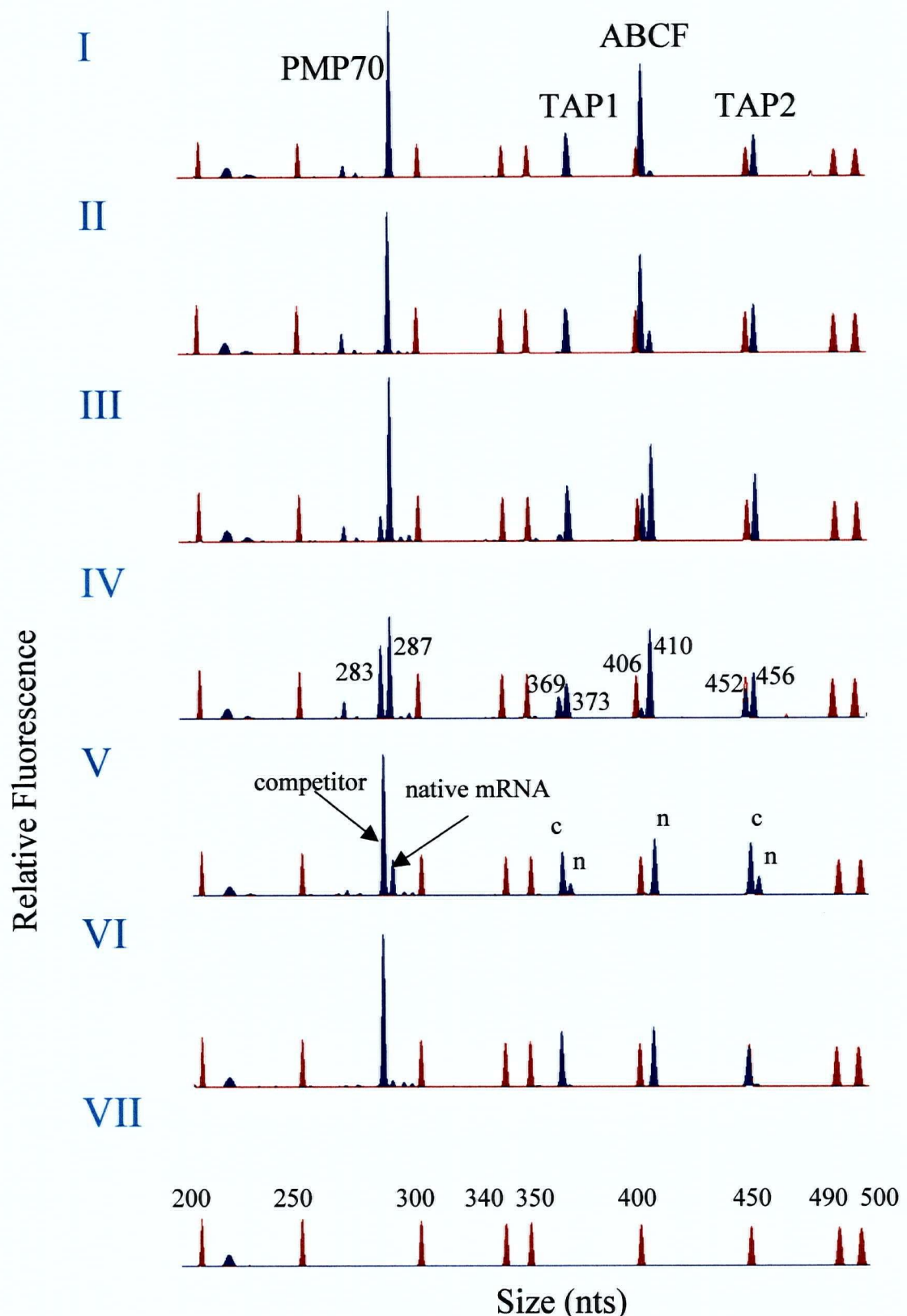
**Figure 2.9. Comparison of Two PCR Buffer Systems.**

ABC3, TAP1, TAP2 and ABCF2 competitive-RT-PCR products were separated via capillary electrophoresis on an ABI 310 DNA sequencer and analysed using GeneScan2.1 software. Blue peaks represent PCR products fluorescently labeled with 6FAM and red peaks represent a size standard fluorescently labeled with TAMRA. Rows I /II, III/IV, V/VI, and VII/VIII are paired competition reactions that are identical except for the PCR buffer used. Increasing or decreasing amounts of each competitor were added across the four competition reactions. The vertical scale is identical within each set of rows, but differs among competition reactions 1-4. Peak positions (nts) are given for PCR products and size standards in panels VII and VIII, respectively. c = competitor and n = native mRNA. See Materials and Methods sections 2.2.4 and 2.2.5.



**Figure 2.10. Effect of Increasing PCR Annealing Temperature.**

MDR1,  $\beta_2$ microglobulin, and MRP1 competitive-RT-PCR products were separated via capillary electrophoresis. Blue peaks represent PCR products fluorescently labeled with 6FAM and red peaks represent a size standard fluorescently labeled with TAMRA. Two different sets of RT reaction mixtures were used for PCR reactions in panels A and B, both with 100 ng SKVLB1.0 total RNA, but with different concentrations of MDR1,  $\beta_2$ microglobulin and MRP1 competitors added. Peak positions (nts) are given for PCR products and size standards in panels B-II and B-III, respectively. c = competitor and n = native mRNA. See Materials and Methods sections 2.2.4 and 2.2.5.



**Figure 2.11. Electrophoretically Resolved Competitive-RT-PCR Products.**

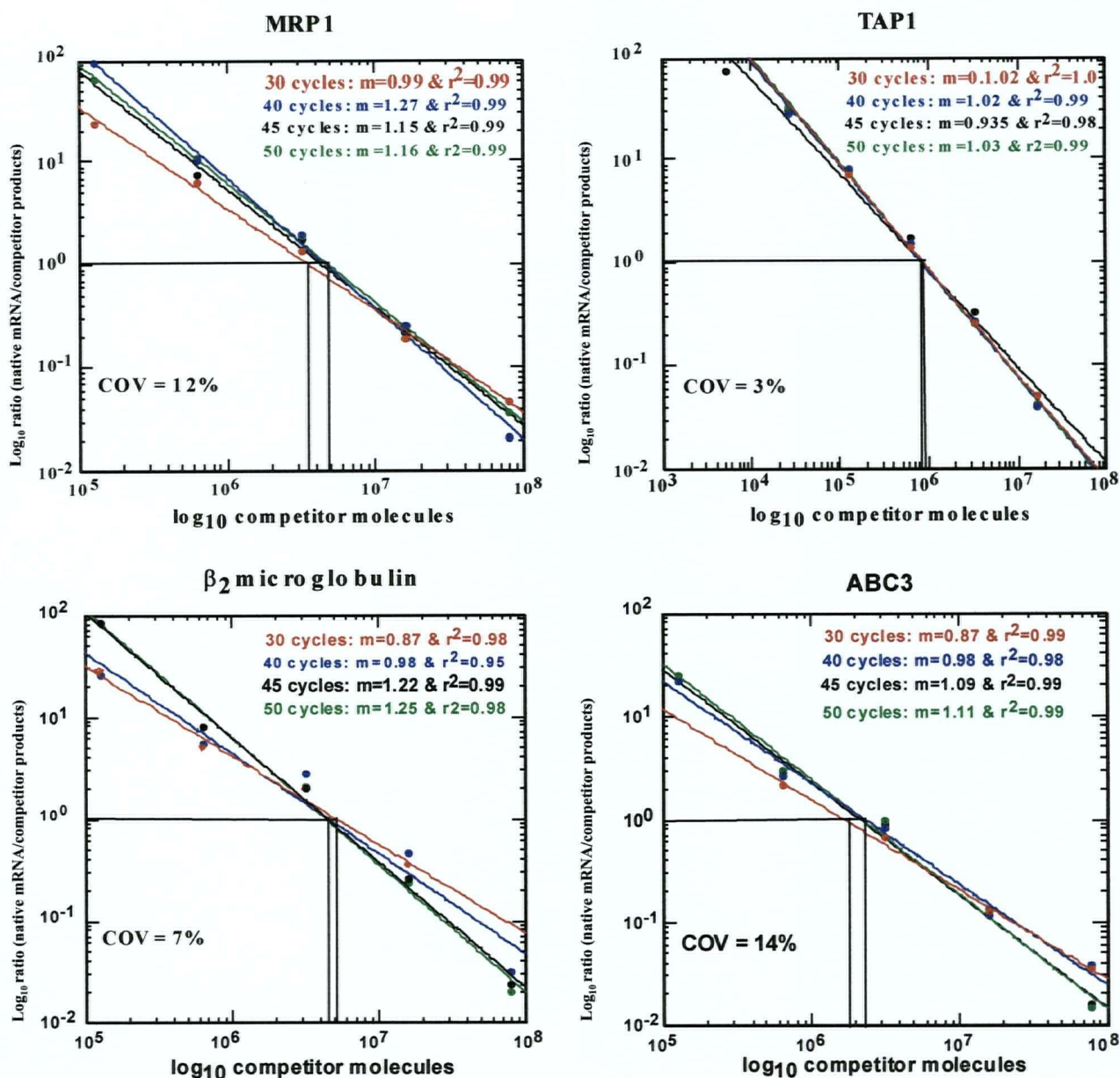
PMP70, TAP1, TAP2 and ABCF2 competitive-RT-PCR products were separated via capillary electrophoresis on an ABI 310 DNA sequencer and analysed using GeneScan2.1 software. Blue peaks represent PCR products fluorescently labeled with 6FAM and red peaks represent a size standard fluorescently labeled with TAMRA. Rows I -VI are competitive reactions with 5-fold increasing or decreasing [competitor] added. Row VII is an H<sub>2</sub>O control reaction. Peak positions (nts) are given for PCR products and size standards in panels IV and VII, respectively. c = competitor and n = native mRNA. See Materials and Methods sections 2.2.4 and 2.2.5.

### 2.3.3 Validation of Competitive-RT-PCR

To determine the optimal number of cycles for PCR, an RT was performed using SKOV3 total RNA and competitors for MRP1, TAP1, ABC3 and  $\beta_2$ microglobulin. This cDNA source was then used for a series of multiplex PCR reactions with 30, 40, 45 or 50 cycles (Figure 2.12). For all four transcripts, with an increasing number of PCR cycles, there was no significant change in the slope of the line. All but one  $r^2$  value fell between 0.98 and 1.0. At 50 cycles there was an increase in the number of non-specific PCR products, but these were still relatively minor and did not interfere with quantification of the desired peaks. The coefficients of variation (COV) for the estimated number of transcripts, among the varying number of PCR cycles, were 3% (TAP1), 7% ( $\beta_2$ microglobulin), 12% (MRP1) and 14% (ABC3). This data indicates that regardless of the number of PCR cycles used, the estimated number of transcripts in the SKOV3 RNA was consistent. Similar results were observed using 30, 38, 45 or 50 cycles of uniplex PCR (data not shown). Multiplex PCR was used for all subsequent experiments described in chapters III-V since it permits quantification of more transcripts per reaction than uniplex PCR. The number of PCR cycles during which the Amplitaq Gold enzyme is active is likely 5-10 cycles fewer than the total number of cycles used in a PCR reaction, since complete activation of the enzyme requires 12 minutes at 95°C and our protocol contains only a 7 minute 95°C step.

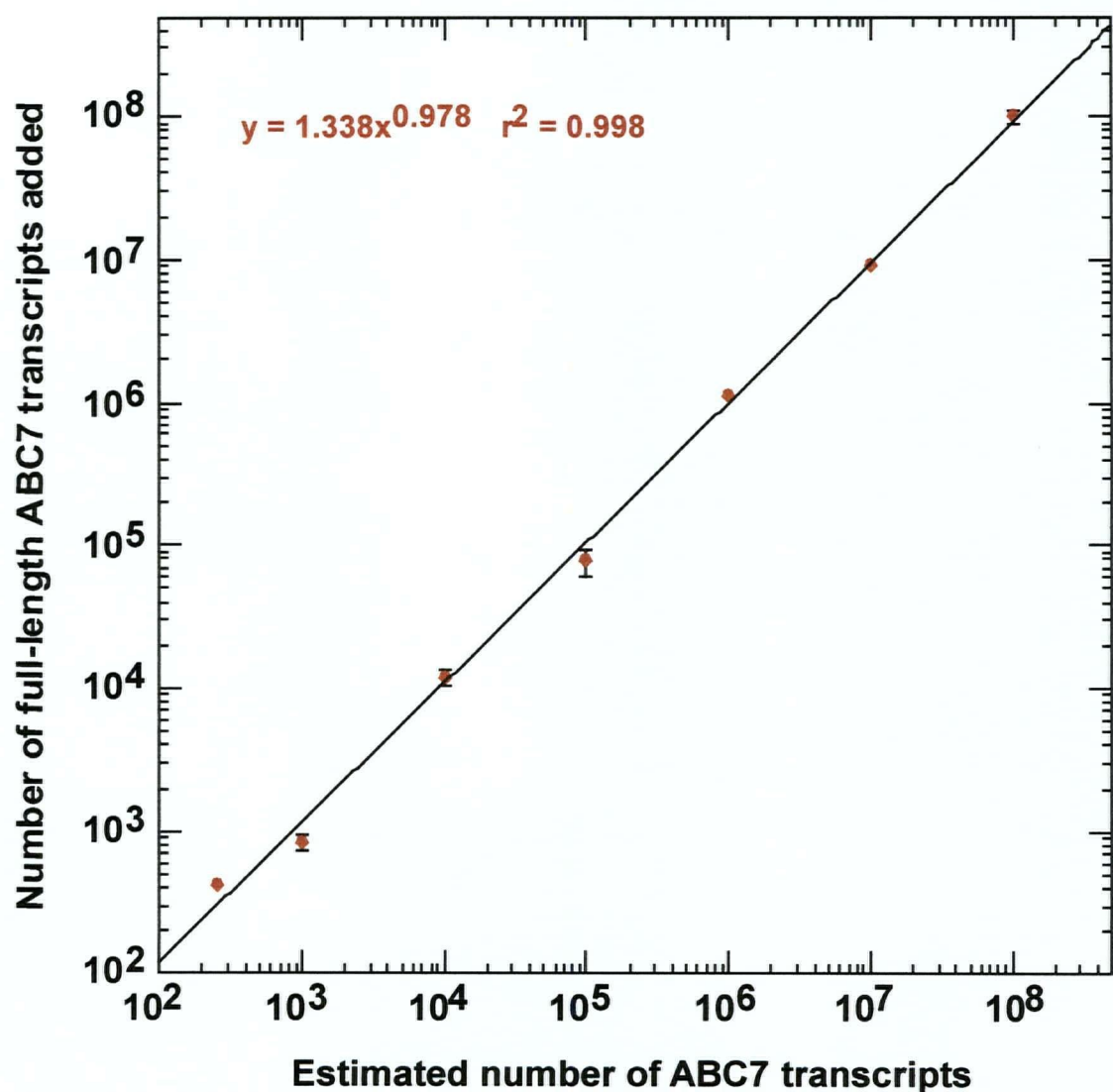
To determine the performance characteristics of our competitive-RT-PCR assay, full-length human ABC7 RNA transcript was added at various copy numbers to a constant background of *E. coli* total RNA. The primers used to amplify the human ABC7 transcript do not match any nucleotide sequences in the *E. coli* genome. Competitive-RT-PCR quantification of ABC7 copy number was performed in triplicate for each quantity of ABC7 transcript spiked into the reaction. An estimate of ABC7 copy number was obtained over a broad range from 500 to  $1 \times 10^8$  copies (Figure 2.13). Although as few as 50 transcripts could be quantified, there was significant variability at this low copy number. The slope of the line (0.978) and the  $r^2$  value

(0.998) were both close to 1. The average percent discrepancy between input and estimated amount of ABC7 transcript was ~15% and the average COV was ~20%.



**Figure 2.12. Effect of PCR Cycle Number.**

A single RT reaction was performed using SKOV3 total RNA and competitors for various transcripts. Multiplex PCR was then performed using 30, 40, 45 or 50 cycles. PCR products were analyzed by capillary electrophoresis on an ABI 310 automated DNA sequencer (GeneScan 2.1 software). The resulting competition plots for each transcript are shown above. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



**Figure 2.13. Validation of Competitive-RT-PCR Performance.** Full-length human ABC7 RNA transcript was added to 200 ng of *E.coli* DH5 $\alpha$  total RNA at varying copy number and competitive-RT-PCR was performed. Each data point represents the mean  $\pm$  standard error of the mean (SEM) of three independent experiments. See Materials and Methods sections 2.2.4, 2.2.5 and 2.2.6 for details.

## DISCUSSION

The present studies demonstrate that accurate quantification of gene expression can be accomplished using a competitive-RT-PCR assay coupled with capillary electrophoresis and fluorescent detection. The homologous RNA competitive standards for this assay are identical to the native mRNA amplicon except for a 4-8 nucleotide deletion that represents on average 2% of the total length. This size/sequence difference between native mRNA and competitor amplicon is smaller than for any other competitors in published competitive-RT-PCR studies and this minimizes differences in amplification efficiencies between competitor and native mRNA. We have chosen to use RNA standards to control for both variations in transcription (RT) and amplification (PCR) efficiencies among transcripts, the former of which has been shown to be more variable (Ferre et al., 1994; Hayward et al., 1998). This is contrary to real-time PCR methods (Heid et al., 1996; Gibson et al., 1996), which do not control for variability at the RT step and thus, underestimate variability and overestimate accuracy. To control for differences in RNA extraction efficiency and quality, we have designed a competitor for the housekeeping gene  $\beta_2$ microglobulin. The expression level of this gene is quantified in any RNA source being studied and is used to normalize ABC expression levels between RNA sources. All RNA competitors were stored at  $-80^\circ\text{C}$  and the RNA competitor transcript stocks were regenerated from the DNA plasmid stocks every 12-18 months. Using this approach we have not observed any appreciable degradation.

There are several advantages associated with using an automated DNA sequencer for resolving competitive-RT-PCR products. Unlike agarose or polyacrylamide gels, the use of potentially hazardous DNA intercalating dyes (e.g. ethidium bromide and SYBR Green) or incorporation of radioactivity is not required. In addition, the volume of polyacrylamide used in capillary electrophoresis is insignificant compared to that used in standard polyacrylamide gels.

The separation is rapid, usually 25 min/5 transcripts in a sample, easily automated and the reaction products need only to be diluted and heat-denatured before analysis. Separation of DNA fragments differing in size by only 1-2 nucleotides is readily accomplished, allowing competitors to be constructed that are virtually identical to the native mRNA amplicon. The precise sizing performed by capillary electrophoresis provides a double check of specificity since it is unlikely that contaminant DNA species will be of exactly the same size as the native mRNA or competitor amplicons. Separation is easily optimized to accommodate additional competitive-RT-PCR products ranging from 100 to 500 nucleotides in size. Samples can be repeatedly re-injected to increase or decrease PCR product signal strength since only  $\sim 1/100$  of the PCR product is required per injection. Over 1000 reactions can be run through a single capillary without noticeable reduction in separation efficiency or signal strength. The use of denaturing gel electrophoresis eliminates the formation of heteroduplexes that can complicate analysis. The cost of reagents (for electrophoretic separation) to quantify a single transcript in an RNA source is less than \$1.00.

Once an optimized competitive-RT-PCR protocol was developed, we proceeded to validate the assay by first examining the effect of PCR cycle number. Using a range of 30 to 50 PCR cycles for various transcripts, there was no significant difference in the estimated transcript copy number,  $r^2$  value or slope of the line. Similar results were obtained using either uniplex or multiplex PCR. These data confirm the results of other studies (Vu et al., 2000; Hayward et al., 1998; Pannetier et al., 1993) that have demonstrated that accurate quantification can be achieved with competitive-RT-PCR in the plateau phase of the PCR reaction. This permits the user to apply a standardized set of PCR conditions for multiple transcripts rather than identifying the initiation of the plateau phase for each transcript in each RNA source.

To validate that this assay system is producing accurate estimates of transcript copy number, we tested the system against a range of known input amounts of full-length native

transcript. Since all competitive standards are constructed and quantified in the same manner and contain the same vector backbone, it is expected that they will have very similar performance characteristics. Using ABC7 transcript, it was determined that this competitive-RT-PCR assay could quantify transcripts over a range of  $> 5$  orders of magnitude with an average percent discrepancy between input amount and estimated amount of only 15%. The COV for triplicate measurements of the input amount of each transcript was consistently close to 20%. This experiment further validates that accurate quantification is unaffected by entry of reactions into the plateau phase.

The ability to quantify mRNA species at lower levels of abundance with accuracy and precision is influenced by a number of factors. These phenomena have been modeled in the context of the sensitivity of competitive-RT-PCR using a computer simulation that incorporates the random nature of reactions involving very low abundance reagents (Peccoud and Jacob, 1996). Predictions from this model indicate that the threshold at which uncertainty begins to rise rapidly is between 20 and 40 copies. These values are comparable to the lower limits of sensitivity experimentally determined for the competitive-RT-PCR assay described here.

The completion of these studies demonstrates that when adequately designed, validated and applied, this competitive-RT-PCR assay is a versatile tool for quantitative measurements of gene expression. The application of this assay to generate RNA expression profiles of the ABC protein family in drug-sensitive and drug-resistant cell lines, as well as in cell lines exposed for a short-term to chemotherapeutic drugs, is presented in chapter III.

## **CHAPTER III**

### **EXPRESSION OF ABC TRANSCRIPTS IN VINCRISTINE-RESISTANT CELL LINES AND AFTER SHORT-TERM EXPOSURE TO CHEMOTHERAPEUTIC DRUGS**

#### **INTRODUCTION**

##### **3.1.1 Intrinsic versus Acquired Drug-Resistance in Clinical Disease**

Drug resistance in cancer cells has been defined as either intrinsic (inherent to the tissue of origin) or acquired (resistance that arises following chemotherapy or drug exposure). Numerous studies over the past 15 years have attempted to determine if Pgp/MDR1 expression in untreated cancer correlates with clinical outcome (intrinsic resistance) and/or if Pgp/MDR1 expression is induced during chemotherapy (acquired resistance). Expression of the MDR1 transcript in untreated cancers can be categorized as high (hepatoma, multiple myeloma, colon, renal, adrenocortical and pancreatic carcinoma), moderate or occasionally high [acute lymphocytic leukemia, acute myeloid leukemia (AML), neuroblastoma and non-Hodgkin's lymphoma] or low or absent (melanoma, breast, bladder, gastric, head and neck, ovarian, prostate and lung carcinoma) (Goldstein, 1996). Cancers of the kidney (Hartmann and Bokemeyer, 1999) and pancreas (Borst, 1999b) are typically intrinsically unresponsive to chemotherapy, and as such are among the most difficult cancers to treat successfully. Intrinsic resistance usually means that the tissue of origin is relatively well protected against drugs and that the tumour has not lost this protection during the multi-step process of carcinogenesis (Borst, 1999b). It has been postulated that intrinsically resistant tumours are often derived from normal tissues with high expression of Pgp, MRP1 or other ABC proteins that contribute to MDR. The steady-state level of MDR1 mRNA in normal kidney is among the highest of any

tissue (Fojo et al., 1987) and Pgp, as detected by immunohistochemistry of normal tissues, is highly expressed at the brush border of kidney proximal tubules and the apical surface of small ductules of the pancreas (Thiebaut et al., 1987). Not surprisingly, MDR1 mRNA is also highly expressed in kidney (Goldstein et al., 1990; Kakehi et al., 1988) and pancreatic (Suwa et al., 1996; Goldstein et al., 1990) tumours prior to treatment.

In contrast to kidney and pancreatic cancer, many hematological neoplasms are initially sensitive to chemotherapy, but the relapse rate is usually high. Expression of Pgp/MDR1 is usually low or undetectable prior to treatment, but it is frequently increased during disease progression and, most noticeably, after chemotherapy. Pgp is expressed in early hematopoietic progenitors (Chaudhary and Roninson, 1992), probably to protect this pool of stem cells, capable of self-renewal, by pumping out natural xenobiotics. The expression of MDR1 mRNA decreases gradually in more differentiated hematopoietic cells (Drach et al., 1992). The expression of Pgp/MDR1 in AML presents an interesting dichotomy. MDR1 mRNA is often expressed at diagnosis and this is correlated with decreased survival (Leith et al., 1999), suggesting that intrinsic drug resistance contributes to treatment failure in this disease. However, Pgp is more frequently encountered in AML after chemotherapy, mainly in samples from patients who are refractory to chemotherapy (Zhou et al., 1995), suggesting a role for acquired resistance in AML. In other tumours such as breast cancer, Pgp/MDR1 expression is usually absent in normal tissue or untreated tumours, but treatment with chemotherapeutic drugs, particularly substrates of Pgp, has been found in many studies to be associated with an increase in the proportion of tumours expressing Pgp (Trock et al., 1997). Researchers have been unable to determine conclusively the role of Pgp/MDR1 expression in intrinsic and acquired resistance due to the limited availability of sequential clinical samples from the same patient, the wide range and lack of standardization of techniques used for detection and limited statistical power owing to the small number of samples used in these studies.

### **3.1.2 Drug Resistance: A Result of Drug Selection, Induction, or Both?**

The effect of cytotoxic drug exposure on gene expression, and in turn, the MDR phenotype, is not well understood. Drug resistance arising via repeated exposure to a chemotherapeutic drug can be a result of the natural genetic heterogeneity present in a population of cells, whereby a small number of cells that inherently possess genetic differences that confer a survival advantage are selected.

A second mechanism for the development of drug resistance after exposure to chemotherapeutic drugs involves changes in gene expression in individual cells. It is well established that regulation at the level of gene transcription is the primary means by which cells respond rapidly to changes in their environment, and it has been shown that many genes can be induced in response to drug exposure. A 15 hour exposure of MCF7 breast cancer cells to the anthracycline doxorubicin resulted in approximately 10% of the ~5000 DNA elements on a cDNA microarray fluctuating by more than 2-fold (Kudoh et al., 2000). Transcripts with altered expression included transcription factors (e.g. RNA Polymerase II, AP-3 and AP-4), cell cycle regulators (e.g. CDC28 protein kinase I and cyclin D2), apoptotic and antiapoptotic genes (e.g. cytochrome C and Bcl-2) and genes of the ubiquitin-proteasome pathway. Repeated exposure to chemotherapeutic drugs can give rise to a subpopulation of cells that, as a result of their altered gene expression profile, are resistant to the selecting agent. Eventually this genetically altered cell population predominates and the result is a drug-resistant tumour. Development of drug resistance via this route requires repeated drug exposures over a prolonged period of time.

A third mechanism of drug resistance can arise due to the action of the drug itself. Exposure to some chemotherapeutic drugs may cause point mutations (anthracyclines) (Gerson, 1998) or genomic alterations, such as gene amplification or deletions (DNA strand breaks caused by mitoxantrone) (Blum, 1998), in cancer cells that are already genetically unstable. If these genetic alterations are not lethal to the cell, they may confer a survival advantage.

The concepts of selection and induction are not mutually exclusive and it is probable that both mechanisms contribute to the development of clinical drug resistance. Treatment protocols typically consist of several rounds of chemotherapy with intervening recovery periods. The initial rounds of chemotherapy might induce expression changes in multiple genes, conferring some short-term survival advantage on a small population of cells that could persist through to subsequent rounds of chemotherapy. Although these expression changes are usually only transient, they may last anywhere from a few hours (Manzano et al., 1996) to several weeks (Chaudhary and Roninson, 1993). If these genes are induced repeatedly, this population of cells becomes “selected” for survival in the presence of drug. In addition to induced gene expression, treatment with chemotherapy drugs would also eliminate the most inherently drug-sensitive cells from the population by selection. Cells selected for their intrinsic resistance along with cells that have been induced to alter their expression profile will both eventually predominate during the later stages of treatment, resulting in drug-resistant cancer.

### **3.1.3 Pgp/MDR1 Gene Expression is Inducible by Chemotherapeutic Drugs**

It has been proposed that the major cause of Pgp-mediated drug resistance during chemotherapy is due to the selection of cells that inherently express high levels of Pgp, rather than via induction of MDR1 gene expression. It has also been suggested that during the development of a tumour, a small number of untreated cancer cells accumulate mutations that result in a high constitutive level of Pgp, conferring a preferential growth advantage in the presence of MDR-associated drugs (Chen et al., 2000; Manzano et al., 1996; Chen et al., 1994; Gekeler et al., 1994). However, it has proven difficult to investigate this phenomenon in clinical samples for reasons already mentioned. Numerous investigators have attempted to replicate the conditions of chemotherapy treatment in a cancer patient by exposing cancer cells in *in vitro* culture systems to chemotherapeutic drugs. Under these conditions, the increased Pgp/MDR1

expression is frequently a result of gene amplification after exposing cells to drug concentrations hundreds of times higher than would be used in the clinic. Furthermore, the degree of genomic amplification of the MDR1 gene does not always adequately explain the increased Pgp/MDR1 expression and drug resistance (Kohno et al., 1994) and genomic amplification is rarely observed in clinical samples.

Several investigators have reported that Pgp/MDR1 expression is rapidly inducible in response to a variety of stresses, including heat shock, arsenite (Chin et al., 1989), differentiating agents (Mickley et al., 1989) and ultraviolet irradiation (Uchiumi et al., 1993). Using an MDR1 promoter/reporter gene construct, Kohno et al. (1989) demonstrated that the transfected human MDR1 promoter could be activated by anticancer agents. In addition, Chin et al. (1990), Manzano et al. (1996), Gekeler et al. (1994) and Chaudhary and Roninson (1993) demonstrated that brief exposure to clinically-relevant concentrations of a number of chemotherapeutic drugs, including Pgp and non-Pgp substrates, resulted in an up-regulation of MDR1 mRNA expression in several cell lines.

More recent studies using a human multidrug-resistant leukemia cell line showed rapid induction of MDR1 mRNA after as few as 4-24 hours exposure to anthracyclines (Hu et al., 1995) and anthracycline analogues (Hu et al., 1999a). Up-regulation of MDR1 expression has been observed with *ex vivo* exposure (16 hour) of blasts from AML patients to anthracyclines and the nucleoside analogue ara-C (Hu et al., 1999b). Induction of MDR1 has also been shown to occur *in vivo* following short term exposure (50 min) of human metastatic sarcomas to doxorubicin using an isolated chemotherapy perfusion circuit, followed by MDR1 mRNA analysis of tumour tissues obtained by biopsy (Abolhoda et al., 1999). From these studies it is clear, at least in cultured cells, that induction of Pgp/MDR1 expression can occur relatively quickly with clinically relevant doses of numerous Pgp substrates and in response to a variety of stresses.

### 3.1.4 Induction of Other ABC Genes

Although our understanding of the contributions of ABC proteins to MDR has expanded in recent years, very little is known about the potential induction of these genes by chemotherapeutic drugs or other agents. As described in chapter I, over-expression of several ABC proteins can confer a drug-resistant phenotype (e.g. MDR1, MRPs 1-5, MDR3 and BCRP) and it is therefore important to determine the conditions under which these genes may be up- or down-regulated.

There are only a few examples of induction of ABC genes, other than MDR1, by chemotherapeutic drugs. The canalicular conjugate transporter MRP2 is inducible by treatment with the chemotherapeutic drug cisplatin in rat kidney (Kaufmann et al., 1997; Demeule et al., 1999). MRP1 expression can be induced by heavy metals and cisplatin in a cisplatin-resistant HL60 leukemic cell line (Ishikawa et al., 1996; Akimaru et al., 1996) and also by doxorubicin and vincristine in a doxorubicin-resistant lung cancer cell line (Manzano et al., 1996). Some ABC genes are also induced by exposure to biological compounds. For example, the human WHITE1 and ABC1 genes are transcriptionally activated when macrophages accumulate excess lipids (Kluchen et al., 2000; Venkateswaran et al., 2000) and ABCA7 expression is up-regulated in the presence of low density lipoprotein and down-regulated by high density lipoprotein (Kaminski, et al. 2000). Finally, the promoter activity of the adrenoleukodystrophy-related gene ALDR is up-regulated by 9-cis-retinoic acid and forskolin (Pujol et al., 2000).

Given the evidence of MDR1 induction by chemotherapeutic drugs and the number of ABC genes potentially contributing to MDR, it is reasonable to propose that exposure of cancer cells to chemotherapeutic drugs might result in the simultaneous induction of several ABC genes. Based on the recent work of Kudoh et al. (2000) using cDNA microarray technology, it appears that there may be a coordinated transcriptional response after exposure to a chemotherapeutic drug. However, it is not known if this response is unique for each type of

chemotherapeutic drug or other stress and if the response differs based on the basal expression profile of the tissue exposed. There is also no evidence as to whether or not multiple ABC genes are part of this transcriptional response. To begin to address these questions, we have generated expression profiles of 25 ABC genes, using competitive-RT-PCR, in HL60 leukemia cells following 24 hour exposure to individual chemotherapeutic drugs. HL60 was chosen because it grows in cell suspension and is, therefore, easy to manipulate. It is well characterized with respect to drug resistance and it is of promyelocytic origin that provides a useful comparison to other AML research being done in our laboratory. However, there is only limited information concerning an inducible transcriptional response following exposure of HL60 cells to chemotherapeutic drugs. A brief exposure time was chosen to minimize cell division and expansion of any subpopulation of cells that might be inherently resistant. This enables us to focus on expression changes that are a result of transcriptional activity rather than changes due to drug selection. This work represents the first attempt to study, in a comprehensive manner, the effects of short-term chemotherapeutic drug exposure on ABC gene expression.

The chemotherapeutic drugs chosen for these experiments act by a variety of different mechanisms. Mitoxantrone is a substituted anthracenedione, which exerts a cell-cycle-specific cytotoxic effect on cells, by binding to guanine-cytosine base-pair rich regions and causing DNA strand breaks. It also induces cell membrane and mitochondrial lipid peroxidation and inhibition of glutathione synthesis (Blum, 1998). The anthracycline daunorubicin interferes with the action of DNA topoisomerase II in regions of transcriptionally active DNA, specifically by inhibiting the reannealing of the DNA strand after cleavage by topoisomerase II. Secondary mechanisms of action include free-radical formation, direct membrane interactions and DNA/RNA intercalation (Gerson, 1998). Ara-C is an antimetabolite that interferes with DNA synthesis. Cellular kinases convert ara-C to active nucleotide metabolites (e.g. ara-cytosine triphosphate), which inhibits enzymes of DNA synthesis and repair. Other antimetabolic and biologic effects

of ara-C include inhibition of ribonucleotide reductase and promotion of differentiation of leukemic cells *in vitro* (Chiao, et al., 1998). All-*trans*-retinoic acid (ATRA) is a member of the retinoid family of molecules that exert a profound effect on cell growth and differentiation and has been shown to induce differentiation of blasts from patients with acute promyelocytic leukemia (Baar et al., 1998). Vincristine is discussed in detail in the following section.

### 3.1.5 Drug Resistance After Multiple Serial Selections with *Vinca* Alkaloids

In contrast to the effect of short-term chemotherapeutic drug exposure, we have also chosen to use the SKOV3 cell line to examine the effect of multiple serial selections with the chemotherapeutic drug vincristine. Previously in our laboratory, SKOV3-derivatives spanning a wide range of resistance to vincristine (2- to 1000-fold) were obtained by serial selection and analyzed for Pgp expression (Bradley et al., 1989). At the time this research was conducted only a few human ABC proteins had been discovered. It was anticipated that the magnitude of vincristine-resistance of these SKOV3-derivatives would be associated with correspondingly significant changes in the expression of ABC mRNA species, in comparison to the expected relatively moderate mRNA changes associated with short-term drug exposure. In addition to MDR1 mRNA, we have examined mRNA expression of 34 ABC transcripts, LRP, topoisomerase II $\alpha$ , GST $\pi$  and the housekeeping gene  $\beta_2$ microglobulin.

The *Vinca* alkaloids vincristine and vinblastine are widely used chemotherapeutic drugs that were developed in the 1950s after being isolated from *Cantharanthus roseus*, the common periwinkle. Vincristine and vinblastine are unusual among chemotherapeutic drugs in that they do not target DNA; rather they selectively bind to tubulin. In doing so, these compounds inhibit microtubule assembly and are, therefore, cell-cycle dependent, causing arrest in the M phase of the cell-cycle (Agarwala, 1998). The *Vinca* alkaloids may also disrupt lipid membranes by virtue of their hydrophobic properties and lead to cellular disruption. Vincristine is used in the

treatment of leukemias, Hodgkin's and non-Hodgkin's lymphoma and the childhood tumours neuroblastoma, rhabdomyosarcoma and Wilm's tumour. Vinblastine is used against lymphomas, testicular cancer, renal cell carcinoma and Kaposi's sarcoma (Rowinsky and Donehower, 1991).

Drug resistance frequently limits the effectiveness of these compounds and can result in cross-resistance to other naturally derived chemotherapeutic drugs including the podophyllotoxins, the taxanes and the DNA intercalators (daunorubicin, doxorubicin, mitoxantrone), but not the antimetabolites or alkylating agents (Agarwala, 1998). The primary mechanism of resistance to the *Vinca* alkaloids is via increased extrusion of drug from the interior of cancer cells due to over-expression of members of the ABC protein family (e.g. Pgp and MRP1). A less frequently described mechanism involves the development of mutations in the  $\alpha$  or  $\beta$  subunits of tubulin which reduces the binding of the alkaloids to the tubulin substrate (Cabral et al., 1986).

The primary mechanism of extrusion of *Vinca* alkaloids from cancer cells is via Pgp. Plasma membrane vesicles partially purified from MDR cell lines that over-express Pgp, but not from drug-sensitive cell lines, have been shown to accumulate vinblastine and vincristine in an ATP-dependent manner (Horio et al., 1988). Similar results have been obtained using cell lines transfected with an MDR1 cDNA compared to non-transfected control cell lines with low levels of MDR1 expression (Hammond et al., 1989). More recently, proteoliposomes containing purified Pgp have also been demonstrated to transport vinblastine (Dong et al., 1996). Over-expression of Pgp is a frequent consequence of long-term selection with either vincristine or vinblastine. *Vinca* alkaloid-resistant cell lines that over-express Pgp have been derived from various tissue sources including myelogenous leukemia K562 cells (Sugimoto et al., 1987), colon carcinoma KB cells (Kohn et al., 1988), breast cancer MCF-7 cells (Whelan et al., 1992) and SKOV3 cells (Bradley et al., 1989) to name a few.

MRP1 transport of *Vinca* alkaloids is another mechanism of resistance, although it appears to be less common than Pgp-mediated transport. Proteoliposomes containing purified MRP1 co-transport vincristine and reduced glutathione (Mao et al., 2000), as do MRP1-enriched plasma membrane vesicles from MDR cell lines that over-express MRP1 (Loe et al., 1998). Unlike for Pgp, *in vitro* transport studies using MRP1-enriched vesicles, under conditions similar to those used to study drug transport by Pgp, have failed to demonstrate that MRP1 directly transports natural product chemotherapeutic agents (Loe et al., 1996; Loe et al., 1998). Among cell lines generated by selection with *Vinca* alkaloids, few have been described without over-expression of Pgp as a primary mechanism of resistance. In fact, although there are several cell lines selected in anthracyclines that over-express MRP1 (Kool et al., 1997), there is only one published example of a cell line selected in either vincristine or vinblastine that over-expresses MRP1 (murine erythroleukemia PC4 cells; Slapak et al., 1994a).

A few other ABC proteins have also been implicated in resistance to *Vinca* alkaloids or have been demonstrated to be capable of mediating their transport, although often with conflicting reports. Transfection experiments indicate that MRP2 confers resistance to *Vinca* alkaloids, anthracyclines, cisplatin and possibly etoposide (Chen et al., 1999; Cui et al., 1999; Koike et al., 1997). Over-expression of MRP2 has been detected in cisplatin-resistant cell lines (Kool et al., 1997), but has not been reported for any cell lines selected with *Vinca* alkaloids. MRP3 transfected cells confer low-level resistance to etoposide, tenoposide, methotrexate (Kool et al., 1999a) and possibly vincristine (Zeng et al., 1999) and MRP3 antisense transfectants show increased drug sensitivity to etoposide and cisplatin, but not vincristine (Haga et al., 2001). Transfection of the TAP1 and TAP2 cDNAs into a sensitive cell line conferred low-level resistance to vincristine, etoposide and doxorubicin and increased expression of TAP1 was observed in several drug selected cell lines, although *Vinca* alkaloid-resistant cell lines were not used (Izquierdo et al., 1996b). There is also data suggesting that MDR3 transfected cell lines are

capable of vincristine transport (Smith et al., 2000). However, there is no evidence that increased MDR3 expression contributes to *Vinca* alkaloid resistance and several other studies have failed to find evidence of MDR3-mediated drug transport or resistance (Ruetz and Gros, 1994; Schinkel et al., 1991). Other ABC proteins have been shown to contribute to MDR (MRP4, MRP5 and BCRP – see chapter I), but there is no evidence for a role in *Vinca* alkaloid-resistance.

## **MATERIALS AND METHODS**

### **3.2.1 Cell Lines**

SKOV3-derivatives were originally generated in this laboratory using serial selection for growth in the presence of increasing concentrations of vincristine (Bradley et al., 1989). SKOV3 and vincristine-selected SKVCR0.015, SKVCR0.1 and SKVCR0.25 cells were maintained in alpha minimal essential medium ( $\alpha$ -MEM) plus 15% fetal calf serum and 1/100 streptomycin/penicillin (Gibco BRL) at 37° with 5% CO<sub>2</sub> in a humidified incubator. 24 hours after cells were passaged, vincristine was added to the resistant clones as follows: SKVCR0.015 – 50  $\mu$ L of 10  $\mu$ g/mL media; SKVCR0.1 – 10  $\mu$ L of 100  $\mu$ g/mL media; and SKVCR0.25 – 25  $\mu$ L of 100  $\mu$ g/mL media, in a total volume of 10 mL. Drug-resistant cell lines were selected for at least two weeks prior to their use in experiments.

The promyelocytic HL60 leukemia cell line was a kind gift from Dr. Connie Eaves' laboratory at the British Columbia Cancer Research Centre, and was maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Gibco BRL) without phenol red [inhibitory for the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl))-2, 5-diphenyl-tetrazolium bromide) (MTT) assay], 10% charcoal/dextran-stripped fetal bovine serum (FBS) (Hyclone) and 1/100 streptomycin/penicillin. Stripped serum was used to minimize the potential masking effects of growth factors and cytokines present in normal FBS, to permit detection of subtle changes in expression after exposure to chemotherapeutic drugs. Cells were grown at 37°C with 5% CO<sub>2</sub> in a humidified incubator.

### **3.2.2 Cell Viability Determination**

Cell viability was determined by diluting cells 1/20 in Trypan Blue Exclusion Dye (Sigma), incubating for 5 minutes at room temperature, plating 10  $\mu$ L of cells on a

hemacytometer and performing two counts of 100 cells. Only cells with normal morphology were counted and those that completely excluded the dye were considered viable.

### **3.2.3 Preparation of Total RNA**

Total RNA was prepared as described in section 2.2.2 of this thesis.

### **3.2.4 Reverse Transcription**

Reverse transcription was performed as described in section 2.2.4 of this thesis.

### **3.2.5 Amplification and Analysis of PCR Products**

Amplification and analysis of PCR products was performed as described in section 2.2.5 of this thesis.

### **3.2.6 Chemotherapeutic Drug Inhibition of Cell Proliferation**

The concentration of chemotherapeutic drug that inhibited 3-day cell proliferation 50% ( $IC_{50}$ ) was determined using the MTT assay (Mosmann, 1983). The assay utilizes a tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl))-2, 5-diphenyl-tetrazolium bromide) (ICN Biomedical), which is converted to a blue precipitate in living cells. The measured absorbance is proportional to the number of live cells. To first determine the optimal number of cells per well (in a 150  $\mu$ L volume), we generated standard curves relating the number of cells plated to the absorbance value of solubilized MTT for the HL60 cell line (Figure 3.4). HL60 cells in the exponential growth phase were plated in duplicate wells in a 1/3 dilution series over a 130-fold range of cell concentrations in 96-well flat bottom plates and were grown for 3 days in 100  $\mu$ L of normal media as described in section 3.2.1. After 3 days, 20  $\mu$ L of 2 mg/mL sterile-filtered MTT solution was added to each well and plates were incubated for a further 18 hours at 37°C. The

resulting blue precipitate was solubilized using 75  $\mu$ L of 1:24 HCl/isopropanol solution, pipetted up and down 50 times and incubated on a plate shaker at 37°C for 1 hour. Plates were read on an ELISA Microplate Autoreader EL309 (Bio-Tek Instruments) at a wavelength of 570 nm. Experiments were repeated in triplicate.

To determine IC<sub>50</sub> values for HL60 cells with various drugs, cells were plated at  $4.5 \times 10^5$  cells/ml in 100  $\mu$ L of appropriate media in 96-well flat bottom plates. Treatment conditions included media only, media+drug only, cell+medium, cell+drug+medium and cell+medium+solvent [either phosphate buffered saline (PBS)(Sigma) or dimethylsulfoxide (DMSO)(Sigma)]. 50  $\mu$ L of drug dissolved in PBS or DMSO solvent was added in a 1/3-dilution series, spanning an approximate 200- to 2000-fold range of drug concentrations. The plates were then incubated for 3 days at 37°C. IC<sub>50</sub> values were determined using the MTT assay as described above and three independent experiments were conducted for each drug examined.

### **3.2.7 Chemotherapy Drugs (Short-term Drug Exposure)**

Etoposide (Sigma) and mitoxantrone (Sigma) were dissolved in DMSO at stock concentrations of 16 mM and 2.3 mM, respectively, then diluted in PBS to the appropriate concentration. Vincristine (Sigma), cisplatin (Sigma), ara-C (ICN Biomedical), 5-Fluorouracil (Sigma) and daunorubicin (Sigma) were dissolved in PBS at concentrations of 5.4 mM, 1.4 mM, 36 mM, 80 mM and 3.7 mM respectively, and diluted with PBS to the appropriate concentrations. ATRA (Sigma) was dissolved in ethanol at a concentration of 1.3 mM and diluted with PBS to the appropriate concentration. The concentration of ATRA used was based upon the minimum level required to induce promoter transcriptional activity in HL60 cells, as detected by luciferase reporter gene assays, for a family of lysosome-associated transport proteins (Douglas Hogue, personal communication).

### **3.2.8 24 hour Exposure of HL60 Cells to Chemotherapeutic Drugs**

HL60 cells in the exponential growth phase were plated at a density of  $2 \times 10^5$  cells/ml (50 mL volume) in cell culture flasks (T125) and incubated at 37°C for 24 hours. A single dose of a chemotherapeutic drug or ATRA was then added and cells were incubated for a further 24 hours. Controls included cells without drug and cells treated with the highest concentration of carrier (PBS, ethanol or DMSO) used in drug treatments. Since only modest changes in ABC mRNA expression occurred after treatment with chemotherapeutic drugs, the expression profile of carrier treated HL60 cells was not determined, although RNA was extracted and stored.

### **3.2.9 Statistical Analysis**

Statistical analysis of the competitive-RT-PCR expression data was performed with two-tailed unpaired t-tests using the statistical analysis software of the GraphPad Prism 3.00 program.

## RESULTS

The experiments on the effects of short-term chemotherapeutic drug exposure were conducted in collaboration with Allison Pahl (MSc. student, Dr. Victor Ling's Laboratory).

### 3.3.1 Drug Resistance Profile of SKOV3-Derivatives

Table III.I reports the levels of resistance, to a panel of cytotoxic drugs, of SKOV3 cell lines selected with increasing concentrations of vincristine. There is a consistent rank-order correlation between resistance to the selecting drug and cross-resistance to other drugs.

**Table III.I Levels of Drug Resistance in SKOV3 Cells Selected With Increasing Concentrations of Vincristine<sup>a</sup>.**

<u>Cell Line</u>	<u>Relative Resistance<sup>b</sup></u>				
	<u>Vincristine</u>	<u>Vinblastine</u>	<u>Adriamycin</u>	<u>Colchicine</u>	<u>Gramicidin D</u>
SKVCR0.015 <sup>c</sup>	8	4	16	4	2
SKVCR0.1	64	260	32	8	130
SKVCR0.25	510	1000	260	64	4100

<sup>a</sup>Data taken from Bradley et al., 1989.

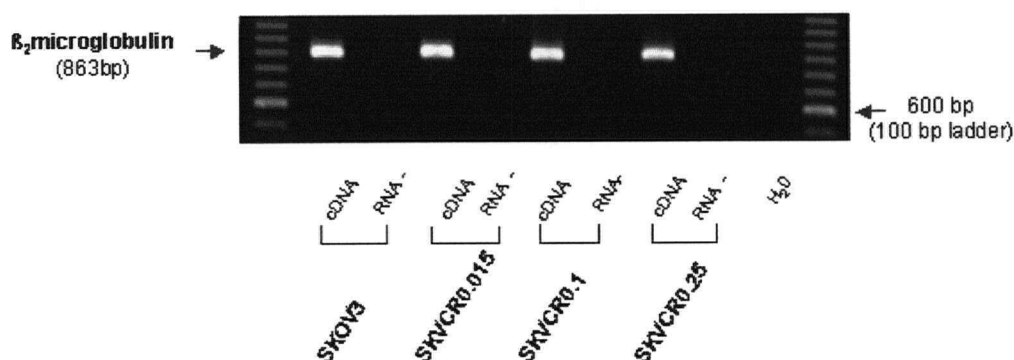
<sup>b</sup>Relative resistance was calculated as the ratio of drug concentrations that inhibited colony growth of each drug-resistant cell line and the drug-sensitive cell line.

<sup>c</sup>Numbers refer to selecting drug concentration in µg/mL.

### 3.3.2 Assessment of RNA Integrity (SKOV3 and Derivatives)

The quality of total RNA isolated from SKOV3 and SKOV3-derivative cell lines was assessed by RT-PCR amplification of full-length  $\beta_2$ microglobulin (Figure 3.1). There was no PCR product in the control lanes containing non-reverse-transcribed RNA or H<sub>2</sub>O as template and the PCR product representing full-length  $\beta_2$ microglobulin was present at approximately

equal levels in all four cell lines. Cell viability was determined to be greater than 95% for each of the four cell lines.



**Figure 3.1 RT-PCR of Full-Length  $\beta_2$ microglobulin in Total RNA From SKOV3 Cells and Vincristine-selected Sublines.**

See Materials and Methods section 2.2.2 for details.

### 3.3.3 Competitive-RT-PCR Expression Profile of SKOV3 Cells

We have applied competitive-RT-PCR to examine the expression profile of 35 ABC transcripts, LRP, GST $\pi$ , topoisomerase II $\alpha$  and  $\beta_2$ microglobulin in the SKOV3 drug-sensitive cell line (Figure 3.2). The most striking feature observed was that the majority of transcripts examined, approximately 75%, was expressed at detectable levels. More than one half of these were below the detection limits of Northern blot or cDNA microarray analyses (1000 copies/ng) (Kane et al., 2000). Based on the amount of RNA recovered and the approximate number of cells harvested, we estimate a yield of approximately 10 pg total RNA per cell. A transcript expressed at 1000 copies/ng of total RNA would correspond to 10 mRNA copies per cell. The

amounts of all transcripts were determined in triplicate with an average COV of 31%. This is similar to the COV for the assay (20%) that was determined using full-length ABC7 transcript spiked into an *E. coli* background (chapter II).

The expression levels of several transcripts in SKOV3 cells are noteworthy. A number of transcripts, including ALDR, MDR3, BSEP, MRP6 and CFTR, have expression patterns known to be limited to one or only a few tissues and are, therefore, not surprisingly absent in SKOV3 cells. Among ABC transcripts associated with MDR, MDR1 and BCRP are both expressed at low levels, whereas MRP1 is the most highly expressed ABC transcript in SKOV3 cells. Other ABC proteins associated with MDR such as MRPs 2,4 and 5 are expressed at moderate levels. Among the transcripts associated with drug resistance, topoisomerase II $\alpha$  and GST $\pi$  are moderately expressed and LRP is weakly expressed.

#### **3.3.4 Competitive-RT-PCR Expression Profile of Vincristine-Selected SKOV3 Sublines**

Expression changes associated with MDR were examined by comparing the expression profiles of SKOV3 cells and the vincristine-selected SKVCR0.015, SKVCR0.1 and SKVCR0.25 cell lines (Figure 3.3). Vincristine-selection conferred a  $\geq 5x$  differential expression, in at least one cell line, for 7 of 39 transcripts and had no significant effect on the expression of the MDR-associated transcripts BCRP, MRPs 3-5, LRP, or topoisomerase II $\alpha$ . MDR1 mRNA expression was unchanged in either SKVCR0.015 or SKVCR0.1 cells and was  $\sim 600x$  higher in SKVCR0.25 cells, in accordance with the genomic amplification of the MDR1 gene observed in these cells (Bradley et al., 1989). Expression of MRP1 mRNA increased 15x and 7x in SKVCR0.015 and SKVCR0.1 cells, respectively, but was unchanged relative to SKOV3 in SKVCR0.25 cells.



**Figure 3.2 Competitive-RT-PCR Expression Profile of SKOV3 Cells.**

Expression profile of 35 ABC transcripts, LRP, GST $\pi$  and topoisomeraseII $\alpha$ . All data are normalized to  $\beta_2$ microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable. ABC transcript labels (x-axis) highlighted with a red asterisk are associated with MDR. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

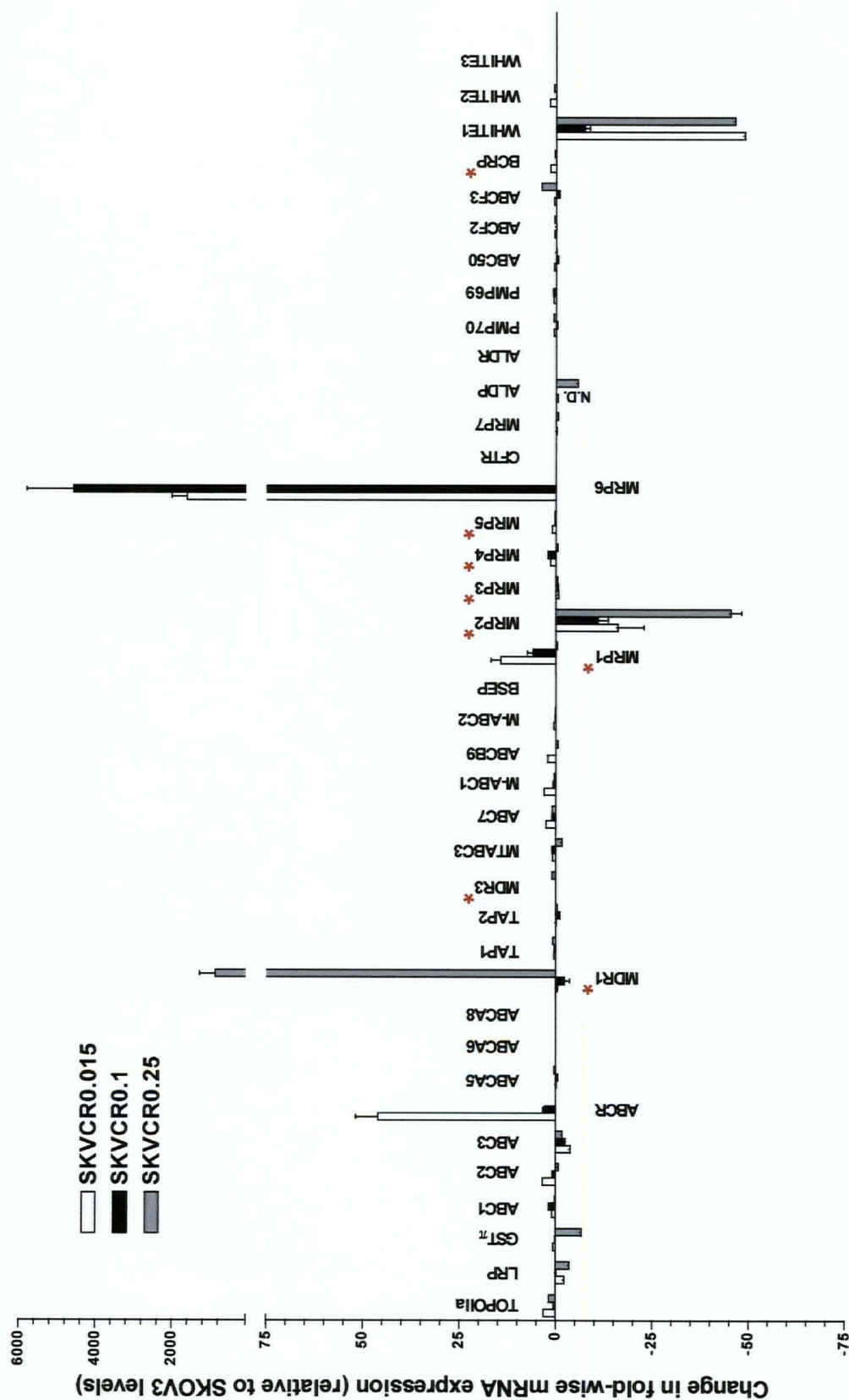
Increased MRP1 levels were significant considering it is the mostly highly expressed transcript in SKOV3 cells and can transport vincristine. MRP6 mRNA was undetectable in SKOV3 and SKVCR0.25 cells, but like MRP1, there was a large increase in MRP6 mRNA in SKVCR0.015 and SKVCR0.1 cells. Expression of ABCR mRNA in SKVCR0.015 cells was surprising given its absence of expression in SKOV3 and SKVCR0.25 cells, but is likely not relevant considering its low expression. Interestingly, the levels of MRP2 and WHITE1 mRNAs were significantly reduced in all 3 vincristine-selected cell lines.

### **3.3.5 MTT Standard Curve for the HL60 Cell Line**

From the MTT standard curve generated for the HL60 cell line (Figure 3.4), as well as from MTT standard curves for other cell lines (Allison Pahl, Master's thesis), we determined the minimum starting number of cells to be approximately  $1 \times 10^4$  cells/well (linear range of absorbance). Based on this data and on similar studies done by other groups (Campling et al., 1988; Twentyman et al., 1989), we chose a value of  $4.5 \times 10^4$  cells/well as the optimal number of cells to be used in our MTT assay.

### **3.3.6 Chemotherapeutic Drug Inhibition of Cell Proliferation**

IC<sub>50</sub> values were determined for the parental HL60 cell line for comparison with any drug-resistant HL60 sublines generated in our laboratory. This data was also used for comparison with other leukemia cell lines under study. IC<sub>50</sub> values for the HL60 cell line for multiple chemotherapeutic drugs are shown in Table III.II and representative dose-response curves are given for each drug in Figure 3.5a and 3.5b.



**Figure 3.3 Competitive-RT-PCR Expression Profile of Vincristine-selected SKOV3 Cell Lines.**

Expression profile of 35 ABC transcripts, LRP, GST $\pi$  and topoisomerase II $\alpha$ . All data are normalized to  $\beta_2$ microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detected. ND = not determined. ABC transcript labels (x-axis) highlighted with a red asterisk are associated with MDR. To calculate the change in fold-wise mRNA expression for transcripts that were not detectable in SKOV3 cells, a baseline of 5 transcripts/ng of total RNA was used. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

### **3.3.7 Cell Viability Measurements**

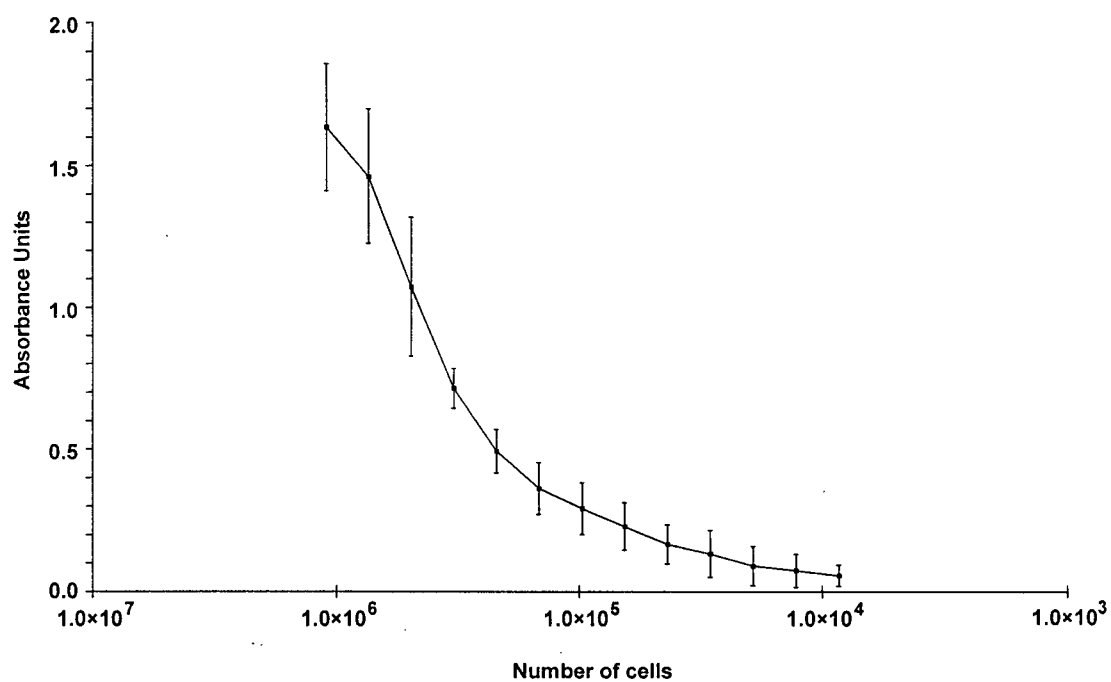
The drug concentrations used in the 24 hour drug exposures were determined based on  $IC_{50}$  values for the HL60 cell line, as well as trial and error based on cell viability measurements. We initially decided that a minimum viability of 70% was required to obtain sufficient quantities of high quality total RNA; however, we later discovered that viability was not necessarily a reliable indicator of RNA quality, depending on the drug in question. RNA yields, as a percentage of the PBS control, ranged from 100.3% to below 20% and the higher the dosage of drug, the lower the RNA yield (data not shown). Viability, as measured using trypan blue dye exclusion, was at least 70% for all drug-treated cells, and greater than 95% in most cases (data not shown). Although cells treated with daunorubicin appeared to be viable ( $> 80\%$ ), RNA yields were low ( $\sim 20\%$  of the PBS control). Moreover, these cells were morphologically distinct compared to the control cells or other drug-treated cells and significant cell debris was usually present. A possible explanation for the poor RNA yield is that daunorubicin may cause cross-linking between RNA strands, impeding the RNA isolation procedure and inhibiting RT-PCR.

To determine the highest concentrations of drug that could be used in the induction experiments, exposures at multiples of the  $IC_{50}$  values were performed. In the event that these concentrations killed the majority of cells, or failed to yield quality RNA, the concentrations were reduced accordingly.

### **3.3.8 Assessment of RNA Integrity of Drug-treated HL60 Cells**

The quality of total RNA isolated from HL60 and drug-treated HL60 cells was assessed by RT-PCR amplification of full-length  $\beta_2$ microglobulin (Figure 3.6). The control lanes containing non-reverse-transcribed RNA were blank, as were the  $H_2O$  control lanes. PCR product was present for all RNA samples examined, with weaker signals for daunorubicin- and mitoxantrone-treated cells. The band intensity of the  $\beta_2$ microglobulin PCR product was

proportional to the level of  $\beta_2$ microglobulin transcript determined by competitive-RT-PCR (data not shown).



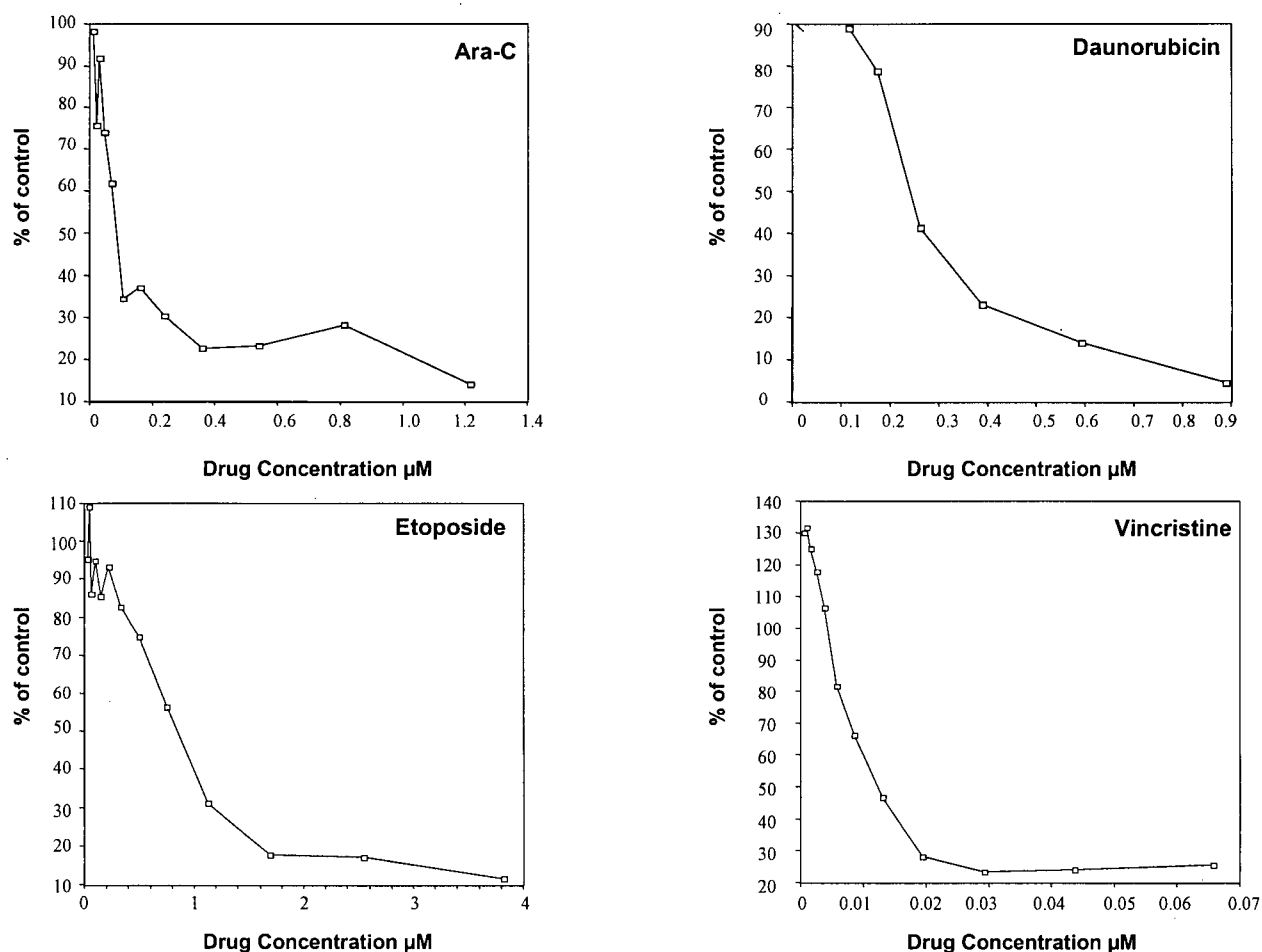
**Figure 3.4 MTT Standard Curve for the HL60 Cell Line.**

A 3-day MTT assay was used to determine the degree of cell proliferation in HL60 cells. Absorbance values were obtained using a range of initial cell concentrations in the absence of any chemotherapeutic drug. Error bars represent the standard deviation of three independent experiments. See Materials and Methods section 3.2.6 for details.

**Table III.II Drug Resistance Profile of the HL60 Cell Line Determined Using the MTT Assay<sup>a</sup>**

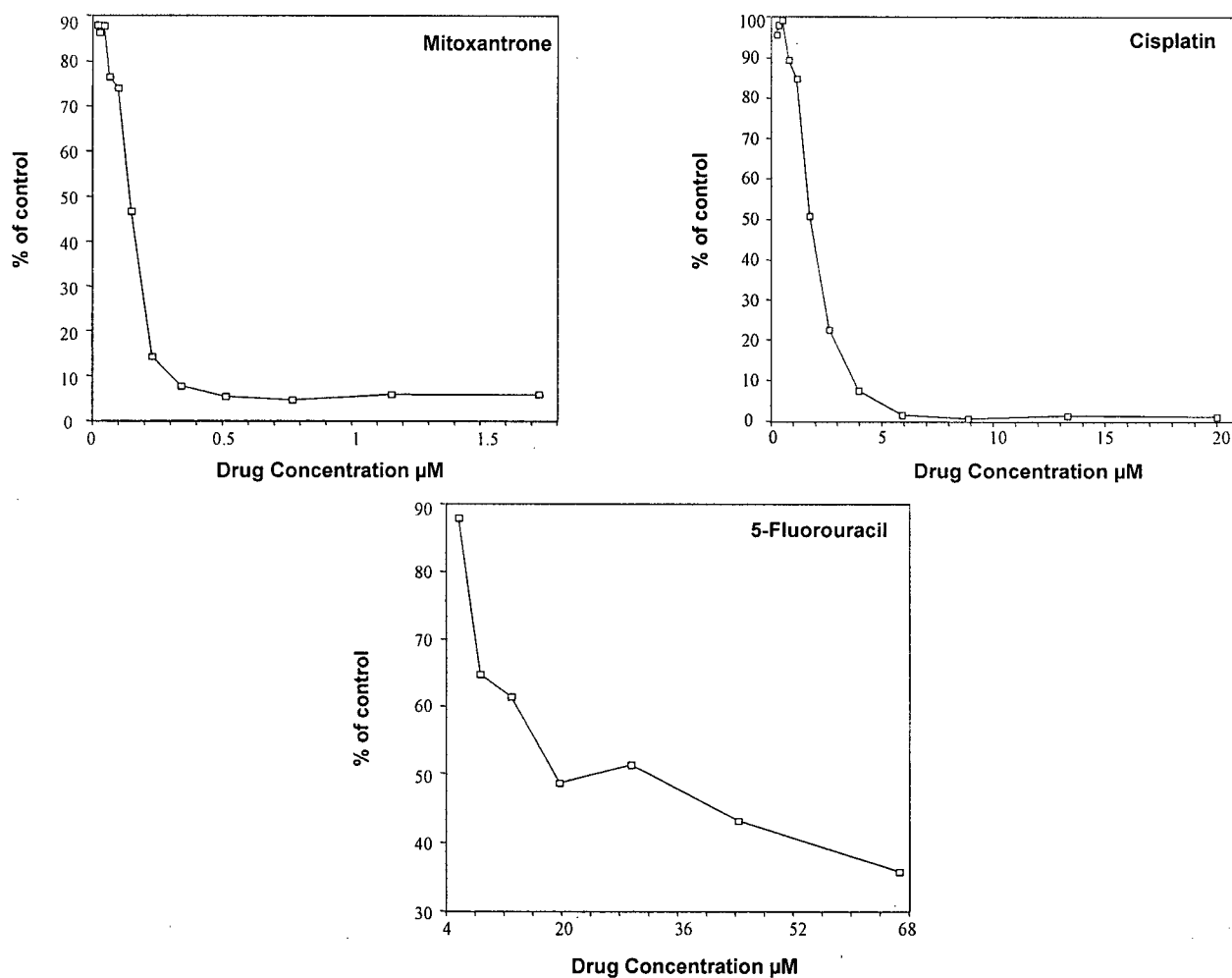
Drug	Drug Concentration ( $\mu$ M)		
	IC <sub>10</sub> Mean $\pm$ SD	IC <sub>50</sub> Mean $\pm$ SD	IC <sub>90</sub> Mean $\pm$ SD
<b>Ara C</b>	0.025 $\pm$ 0.0097	0.2 $\pm$ 0.079	0.95 $\pm$ 0.21
<b>Etoposide</b>	0.25	0.72 $\pm$ 0.1	2.4 $\pm$ 0.89
<b>Daunorubicin</b>	0.068	0.18 $\pm$ 0.05	0.45 $\pm$ 0.17
<b>Vincristine</b>	0.002 $\pm$ 0.0016	0.0082 $\pm$ 0.0024	0.04 $\pm$ 0.02
<b>Mitoxantrone</b>	0.053	0.14 $\pm$ 0.01	0.33
<b>Cisplatin</b>	0.93 $\pm$ 0.14	1.8 $\pm$ 0.25	7.9 $\pm$ 3.8
<b>5-FU</b>	5.0	37 $\pm$ 18	800

<sup>a</sup>A 3-day MTT assay was used to determine the concentration of chemotherapeutic drug that inhibited HL60 cell proliferation by 10% (IC<sub>10</sub>), 50% (IC<sub>50</sub>) or 90% (IC<sub>90</sub>). Data is expressed as mean  $\pm$  standard deviation (SD) of three independent experiments. See Materials and Methods section 3.2.6 for details.



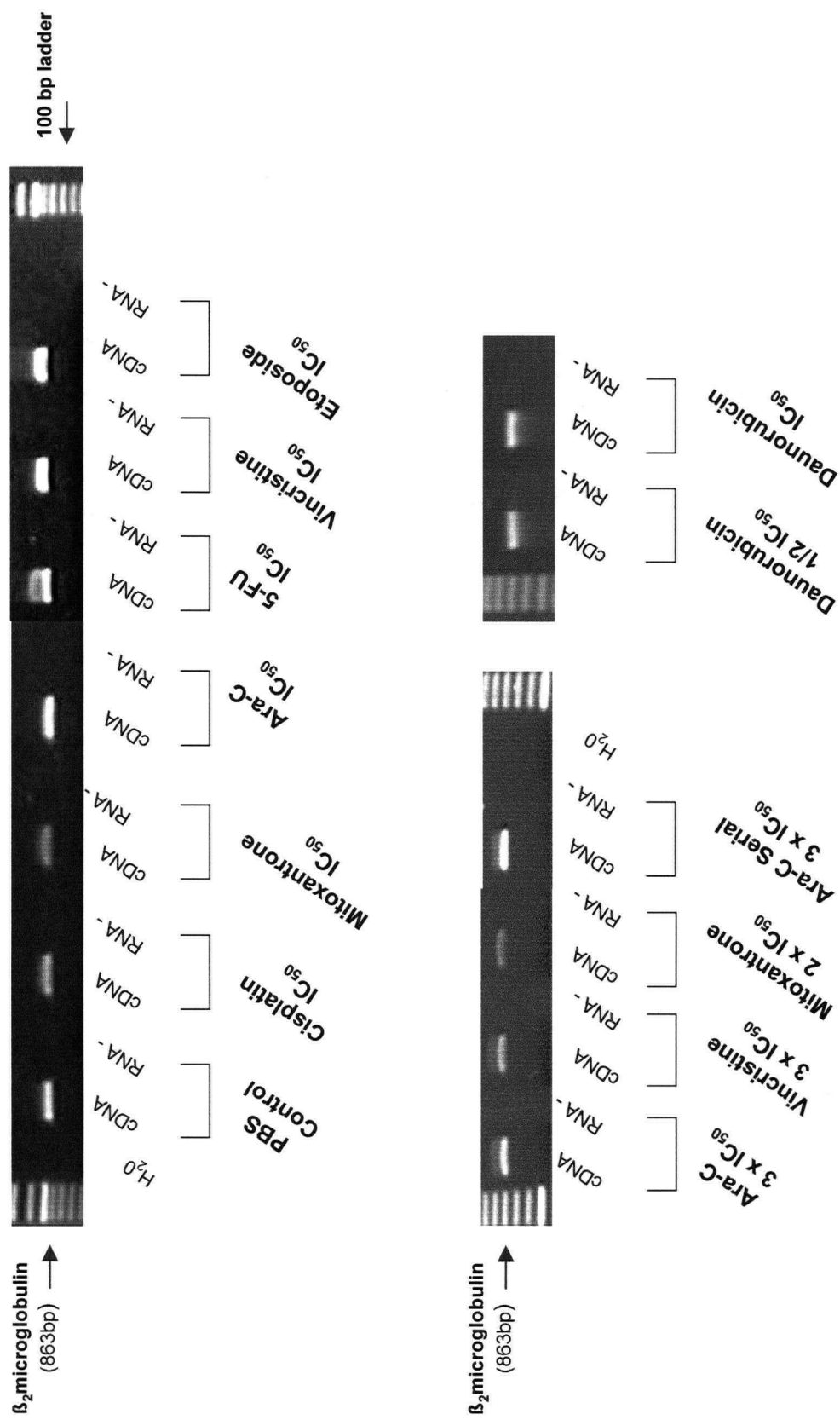
**Figure 3.5a. Dose-response Curves for HL60 Cells Determined for Chemotherapeutic Drugs Using the MTT Assay.**

HL60 cells were exposed for 3 days to a range of concentrations of a chemotherapeutic drug and the degree of cell proliferation was measured using the MTT assay. Representative plots were selected for each drug from experiments performed in triplicate. The vertical axis expresses the degree of HL60 cell proliferation as a percentage of the media+solvent+cells control. See Materials and Methods section 3.2.6 for details.



**Figure 3.5b. Dose-response Curves for HL60 Cells Determined for Chemotherapeutic Drugs Using the MTT Assay.**

HL60 cells were exposed for 3 days to a range of concentrations of a chemotherapeutic drug and the degree of cell proliferation was measured using the MTT assay. Representative plots were selected for each drug from experiments performed in triplicate. The vertical axis expresses the degree of HL60 cell proliferation as a percentage of the media+solvent+cells control. See Materials and Methods section 3.2.6 for details.



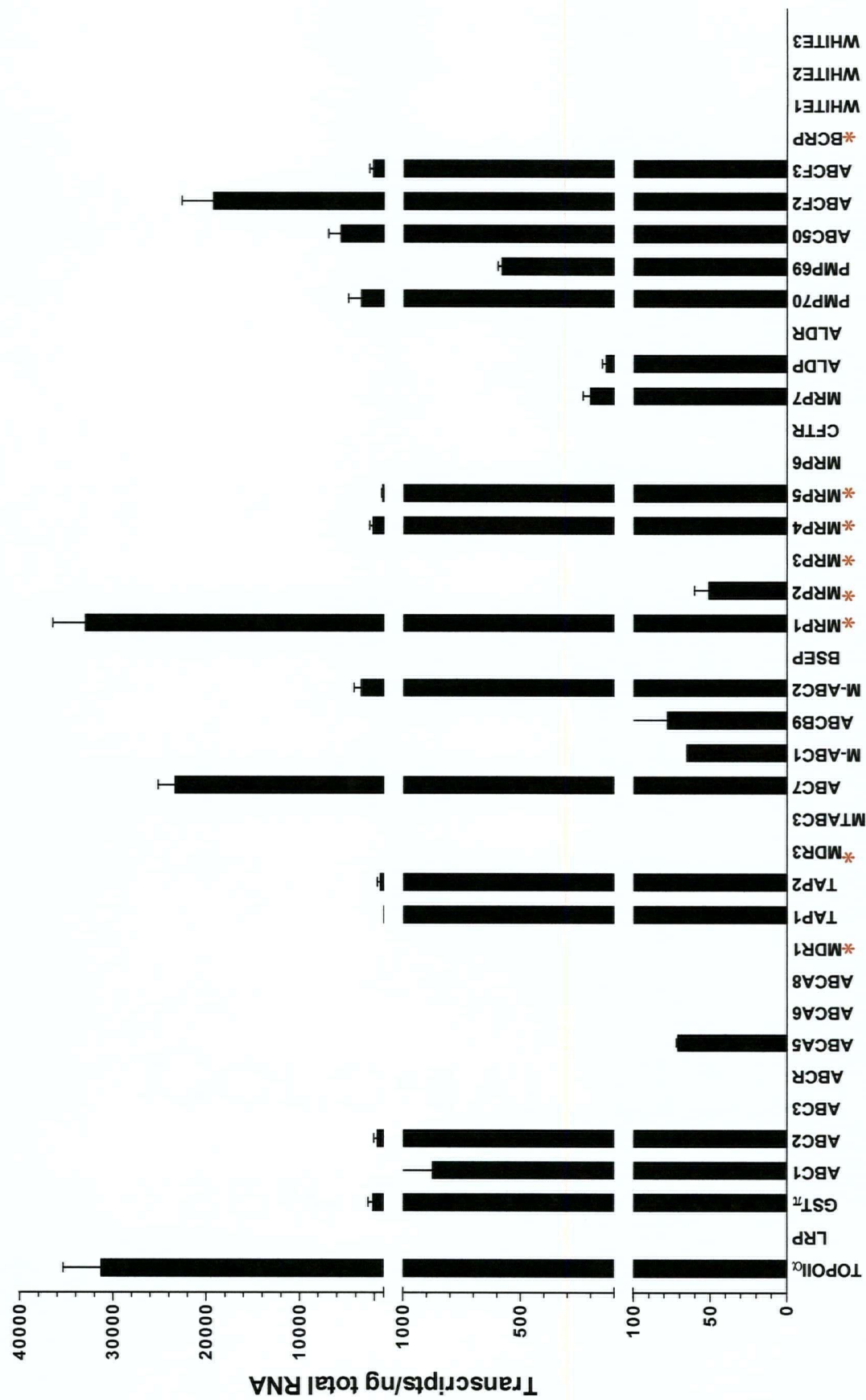
**Figure 3.6. RT-PCR of Full-Length β<sub>2</sub>-microglobulin in Total RNA from HL60 Cells Exposed 24-Hours to Various Concentrations of Chemotherapeutic Drugs.**

See Materials and Methods section 2.2.2 and 3.2.8 for details.

### 3.3.9 Competitive-RT-PCR Expression Profile of HL60 Cells

The competitive-RT-PCR expression profile of 36 ABC transcripts, LRP, GST $\pi$  and topoisomerase II $\alpha$  in untreated HL60 cells is reported in Figure 3.7. Slightly less than 60% of transcripts examined were expressed at detectable levels compared to 75% reported for SKOV3 cells (Figure 3.2). Among the transcripts detected by competitive-RT-PCR, 40% were below the detection limits of Northern blot or cDNA microarray analyses (1000 copies/ng). The expression levels of all transcripts were determined in triplicate with an average COV of 27%, which is similar to the 31% reported for SKOV3 cells and that determined for the assay (20%) using full-length ABC7 transcript spiked into an *E. coli* background (chapter II).

A significant number of transcripts, including ALDR, ABCR, WHITE2, WHITE3, MDR3, BSEP, MRP3, MRP6 and CFTR were not detectable in HL60 cells. This is not surprising since these transcripts are normally expressed in a very limited number of tissues and are not typically expressed in hematopoietic cells. M-ABC2 mRNA has been reported to be predominantly expressed in hematopoietic cells (Shirihai et al., 2000) and was expressed at relatively high levels in HL60 cells. Among ABC transcripts associated with drug resistance, MDR1 and BCRP were not detectable, MRP2 was weakly expressed and MRPs 4 and 5 were expressed at moderate levels. MRP1 was one of the most highly expressed transcripts in HL60 cells, at levels comparable to the drug-sensitive SKOV3 cell line (Figure 3.2), but still far less than that observed in SKVCR0.015 cells which overexpress MRP1. Among transcripts associated with drug resistance, topoisomerase II $\alpha$  was highly expressed, GST $\pi$  was moderately expressed and LRP was weakly expressed.



**Figure 3.7. Competitive-RT-PCR Expression Profile of HL60 Cells.**

Expression profile of 35 ABC transporters, LRP, GSTπ and topoisomeraseIIα.. All data are normalized to β<sub>2</sub>microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable. ABC transporter labels (x-axis) highlighted with a red asterisk are associated with MDR. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

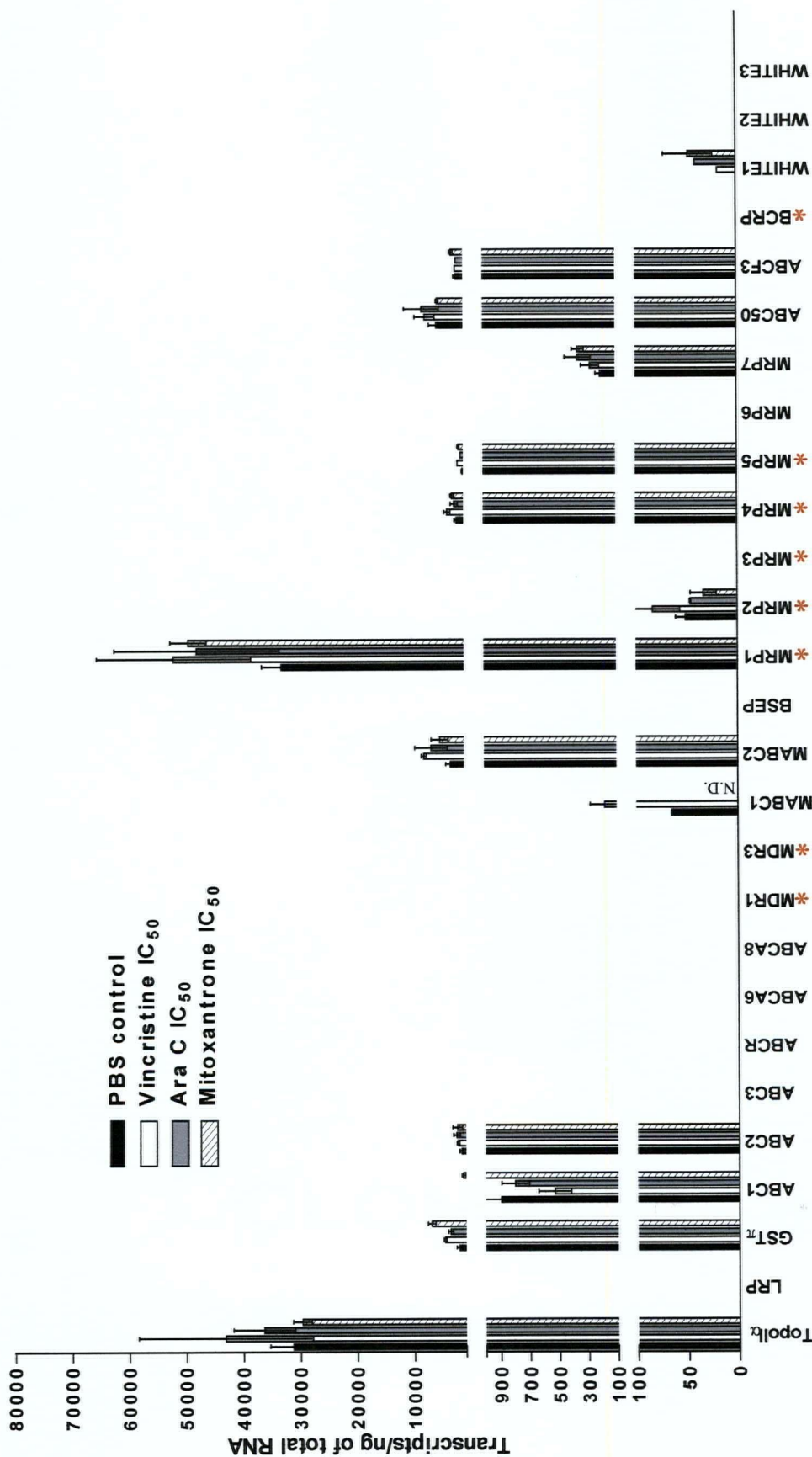
### 3.3.10 Competitive-RT-PCR Expression Profile of Drug-treated HL60 Cells

We have applied competitive-RT-PCR to examine expression profiles of 26 ABC transcripts, LRP, GST $\pi$ , topoisomerase II $\alpha$  and  $\beta_2$ microglobulin in HL60 cells exposed to IC<sub>50</sub> (Figure 3.8) and higher concentrations (Figure 3.9) of chemotherapeutic drugs. We have also examined expression of 33 ABC transcripts after treatment of HL60 cells with 100 nM ATRA (Figure 3.10). Each of the compounds used in these experiments (e.g. daunorubicin, mitoxantrone, ara-C, vincristine and ATRA) have different mechanisms of action and most are used in the treatment of leukemia.

Among the 28 transcripts examined after treatment with one of four chemotherapeutic drugs at IC<sub>50</sub> concentrations (Figure 3.8), only 7 statistically significant changes (at the  $P < 0.05$  level) in expression were observed. Furthermore, only two of these changes were greater than two-fold in magnitude: a  $2.3 \pm 0.1$ -fold increase ( $p < 0.01$ ) (mean  $\pm$  SEM) in the expression of the mitochondrial half-transporter M-ABC2 after exposure to vincristine; and a  $3.2 \pm 0.4$ -fold increase ( $p < 0.01$ ) in GST $\pi$  expression after exposure to mitoxantrone. No alteration of mRNA expression occurred after exposure to ara-C. The experiment was repeated using higher drug concentrations (Figure 3.9), based on the hypothesis that the IC<sub>50</sub> concentrations were insufficient to induce large changes in gene expression. The concentrations of chemotherapeutic drugs used in these treatments were increased to 3x IC<sub>50</sub> for vincristine and ara-C, and daunorubicin at  $\frac{1}{2}$  IC<sub>50</sub> was also included in this analysis. Even at these higher drug doses, there were only a few expression changes observed. The most notable expression changes were: a  $3.6 \pm 0.7$  fold reduction ( $p < 0.05$ ) in ABC1 mRNA with ara-C treatment (3x IC<sub>50</sub>); a  $4.8 \pm 1.1$  fold increase ( $p < 0.01$ ) in MRP2 mRNA and a  $6.2 \pm 1.1$  fold increase ( $p < 0.01$ ) in MRP7 mRNA with daunorubicin ( $\frac{1}{2}$  IC<sub>50</sub>) treatment. No alterations in expression occurred with exposure to higher concentrations of vincristine. Overall, only 2% of the transcripts examined after treatment with low or high drug concentrations showed expression changes greater than 2-fold in

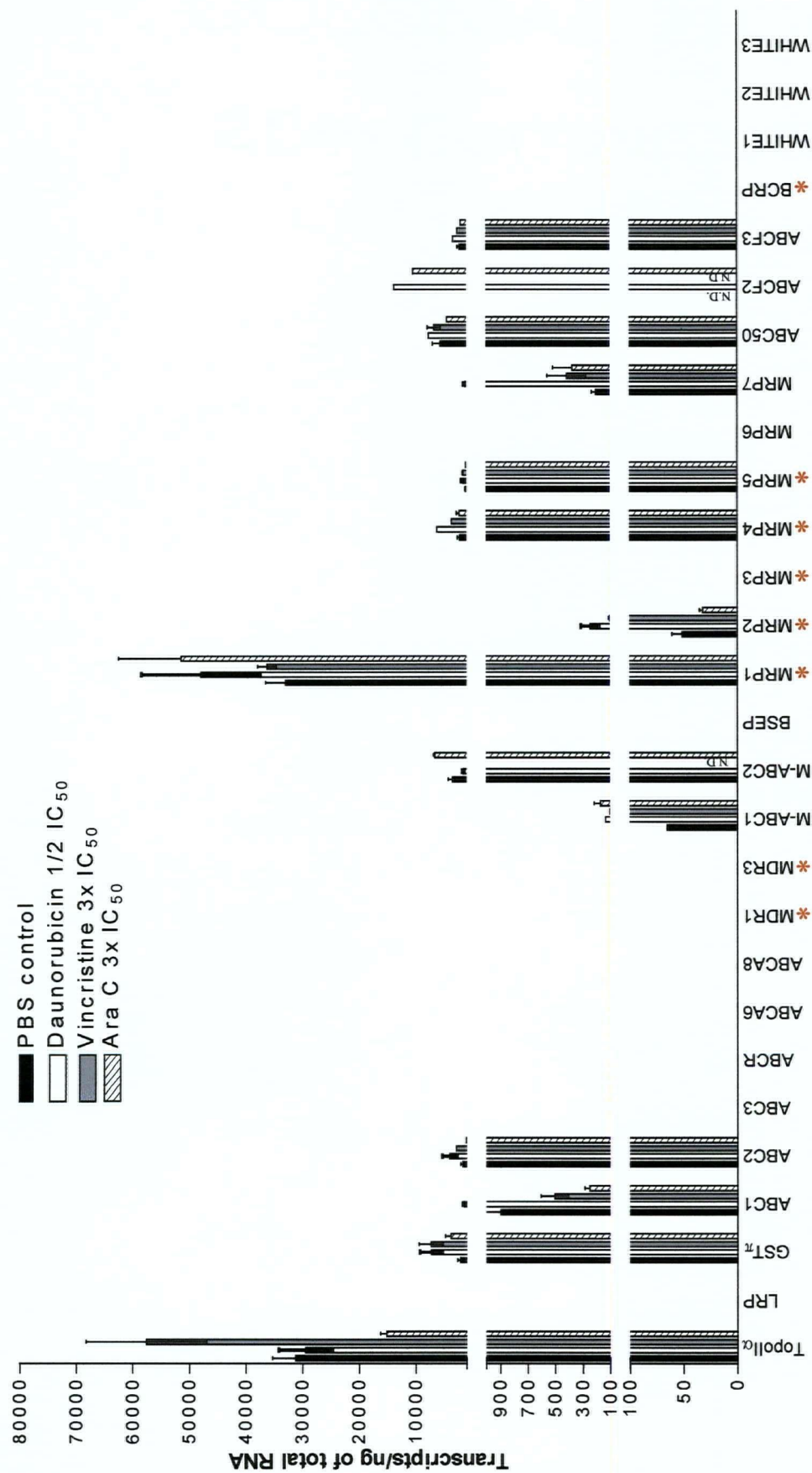
magnitude. Higher doses of each drug were also used, but cell viability fell below 70% and in most cases we were unable to isolate sufficient quantities of intact RNA.

Only two statistically significant changes were observed with ATRA exposure: a  $3.2 \pm 0.9$ -fold increase ( $p < 0.05$ ) in MRP7 mRNA expression and a  $1.8 \pm 0.1$  fold increase ( $p < 0.05$ ) in ABC1 mRNA expression (Figure 3.10).



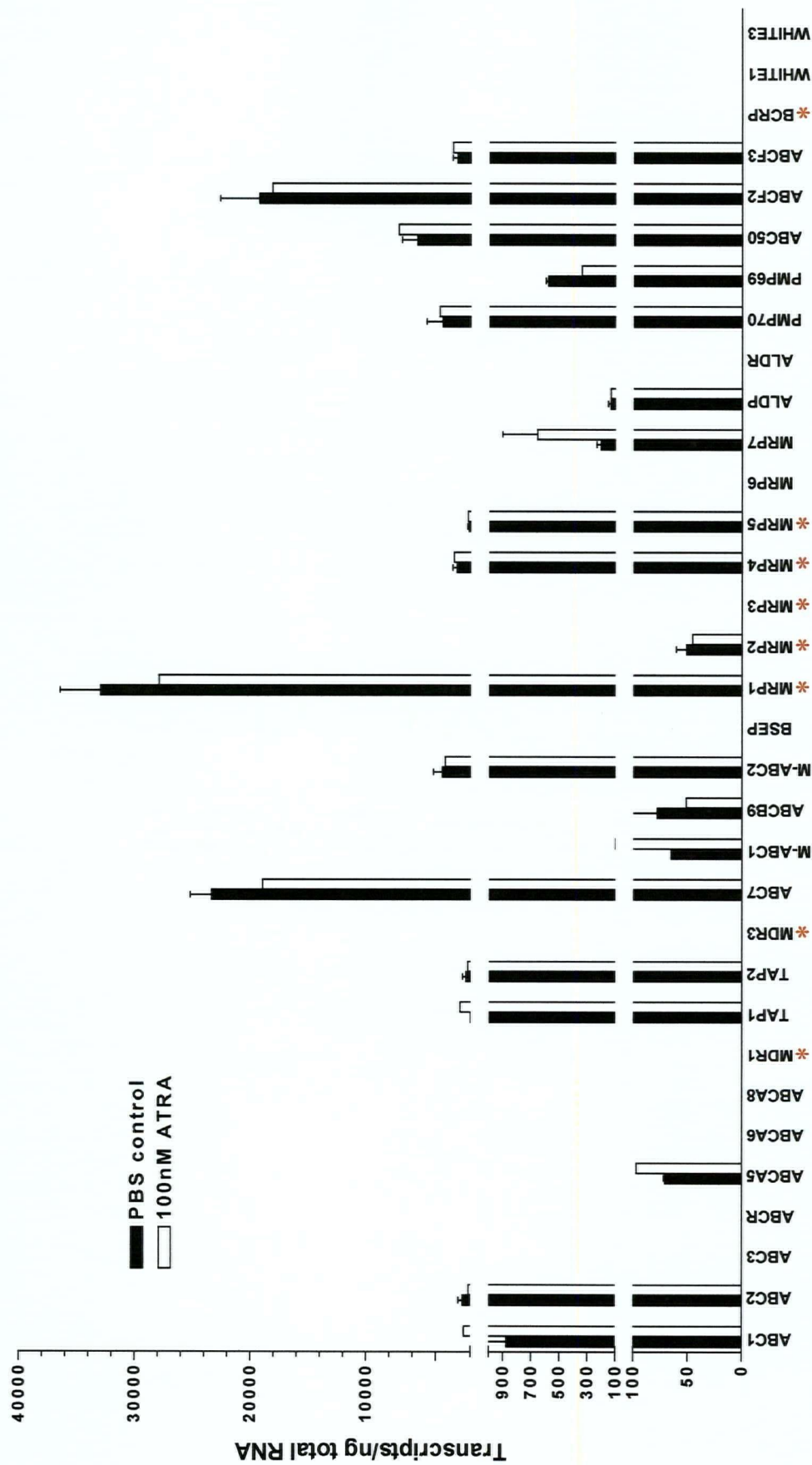
**Figure 3.8. Competitive-RT-PCR Expression Profile of HL60 Cells After 24-hour Treatment with Chemotherapeutic Drugs (IC<sub>50</sub>-low dose).**

Profile of 24 ABC transporters, LRP, GST $\pi$  and topoisomerasell $\alpha$ . All data are normalized relative to  $\beta_2$ microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable. ND = not determinable. ABC transporter labels (x-axis) highlighted with a red asterisk are associated with MDR. See Materials and Methods sections 2.2.4, 2.2.5 and 3.2.8 for details.



**Figure 3.9. Competitive-RT-PCR Expression Profile of HL60 Cells After 24-hour Treatment with Increased Concentrations of Chemotherapeutic Drugs (high dose) and Daunorubicin 1/2@IC<sub>50</sub>.**

Profile of 25 ABC transcripts, LRP, GSTπ and topoisoerasellα. All data are normalized relative to β<sub>2</sub>microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable. ND = not determined. ABC transcript labels (x-axis) with a red asterisk are associated with MDR. See Materials and Methods sections 2.2.4, 2.2.5 and 3.2.8 for details.



**Figure 3.10. Competitive-RT-PCR Expression Profile of HL60 Cells After 24-hour Treatment with 100 nM ATRA.**

Profile of 32 ABC transcripts. All data are normalized relative to  $\beta_2$ microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable. ABC transcript labels (x-axis) with a red asterisk are associated with MDR. See Materials and Methods sections 2.2.4, 2.2.5 and 3.2.8 for details.

## DISCUSSION

The mRNA expression profiles of SKOV3 and HL60 cells provide, for the first time, a comprehensive analysis of ABC transcript expression in a cell line. Analysis of a series of vincristine-selected SKOV3 cell lines revealed both changes in expression levels of individual ABC mRNAs, as well as changes to the collective expression profiles. These data demonstrate that multiple expression changes may collectively contribute to MDR and that altered expression of a single ABC transporter cannot be assumed to be causative of drug resistance.

We observed a very low level of expression of MDR1 mRNA in SKOV3 cells which is in agreement with other reports (Bradley et al., 1989; Speicher et al., 1994). The expression of other ABC proteins was not examined in those studies. It has been reported previously that in western blots of plasma membrane preparations of the SKOV3 cell line and SKOV3-derivatives, over-expressed Pgp could be detected in SKVCR0.25 and SKVCR0.1, but not in SKVCR0.015 and SKOV3 (Bradley et al., 1989). In Northern blot analyses, each of the multidrug-resistant SKOV3-derivatives showed over-expression of MDR1 mRNA, but in SKOV3 it was not detectable. The relative amounts of Pgp protein and mRNA in different SKOV3-derivatives generally correlated with the observed MDR, but with some exceptions. Using competitive-RT-PCR we did not observe any significant MDR1 expression changes except the dramatic increase (~600-fold) in MDR1 in SKVCR0.25. There is no comparable numerical data available from the work of Bradley et al. since they were unable to detect MDR1 mRNA in SKOV3; however, we have observed a similar range of values for MDR1 in other drug-resistant cell lines and in normal tissues (chapters IV and V). The large increase in MDR1 mRNA in SKVCR0.25 cells is likely due to genomic amplification of the Pgp gene. In the work of Bradley et al., initial steps of selection with vincristine did not result in amplification of the Pgp gene (SKVCR0.015 and SKVCR0.1), as assessed by a Southern blot. However, further steps of selection with vincristine

resulted in the emergence of a cell population (SKVCR0.25) with the Pgp gene amplified 8-fold. The reasons for the discrepancy in MDR1 mRNA expression between the earlier work of Bradley et al. and our most recent work (SKVCR0.015 and SKVCR0.1 cell lines) are unclear. It is possible that with the repeated passaging and drug selection of these cell lines over the past decade new cell populations have emerged.

Over-expression of MRP1 and Pgp are not mutually exclusive, and as we have observed in the SKOV3-derivatives, both mechanisms of resistance can be selected using the same chemotherapeutic drug (Brock et al., 1995; Slapak et al., 1994b; Izquierdo et al., 1996c; Hasegawa et al., 1995; Zhou et al., 1996). It is not clear what factors determine which mechanism(s) of resistance will predominate after selection. However, analysis of several sets of related cell lines selected for sequentially higher levels of drug resistance suggest that MRP1 may be involved at lower drug concentrations, whereas Pgp over-expression often emerges at higher levels of resistance. On the other hand, resistant variants of the same parental cell line exclusively over-expressing MRP1 (HL60/AR) or Pgp (HL60/Tax) (Gollapudi et al. 1997), have been isolated using similar selection protocols, indicating that this model may be overly simple. The mechanism of resistance that is activated is likely the result of a combination of factors including the selecting drug, selection protocol, cell type and basal expression profile.

The altered expression of ABCR, WHITE1 and MRP6 is intriguing since these genes have not previously been associated with drug resistance. However, these changes may indicate that a large number of genetic alterations occur upon long-term drug-selection, many of which are not associated with altered drug sensitivity. The increase in MRP6 mRNA parallels an increase in MRP1 and is likely due to its genomic localization adjacent to MRP1 on chromosome 16p13; although the extent of MRP1 genomic amplification, if any, was not examined. A large panel of resistant cell lines has been examined for over-expression of MRP1 and MPR6 (Kool et al., 1999b). This study demonstrated that whenever over-expression of MRP6 occurred this was

the result of genomic amplification and always coincided with genomic amplification of MRP1. Although the expression levels of ABCR and WHITE1 are low, the changes in expression are large and may alter drug sensitivity by an unknown mechanism.

The altered expression of MRP2 in vincristine-selected cell lines is not surprising since this transcript has previously been associated with drug resistance and also undergoes significant changes in expression levels. Vincristine is a substrate for both Pgp and MRP1 and possibly MRP2, but the relative contribution of each of these proteins to the observed drug resistance in SKOV3-derivatives has not been assessed.

The competitive-RT-PCR expression profiles of HL60 leukemia cells and SKOV3 ovarian carcinoma cells are surprisingly similar. The only significant mRNA expression differences were the lower levels of M-ABC2 and ABC7 in SKOV3, the much greater levels of MRP2 in SKOV3 and the low-level expression of MDR1, BCRP, LRP and MRP3 in SKOV3, all of which were not detectable in HL60 cells.

A comparison of the competitive-RT-PCR expression profiles of the promyelocytic HL60 cell line and a series of myeloid-derived AML cell lines (AML1, AML2 and AML3 - derived from blasts recovered from AML patients prior to chemotherapy - Allison Pahl, Master's thesis) reveals a high degree of similarity (data not shown). Among ABC transcripts associated with MDR, MRPs 1-7 were expressed at very comparable levels among the four hematopoietic cell lines (MRP1 – high expression, MRP4 and MRP5 – moderate, MRP2 and MRP7 – low and MRP3 and MRP6 – not detectable), MDR1 was only detectable in AML1 (~10 copies/ng of total RNA) and BCRP was not detectable in any of the cell lines. Among other transcripts associated with MDR, GST $\pi$  was moderately expressed in all the hematopoietic cell lines and LRP was very lowly expressed in AML1, 2 and 3 (~10 copies/ng of total RNA), but not detectable in HL60. Topoisomerase II $\alpha$  mRNA expression was not examined in the AML cell lines. Other transcripts of interest include M-ABC2, which was relatively highly expressed in all the

hematopoietic cell lines and the ABC3 and ABC7 transcripts which were weakly expressed in the AML cell lines (~25-400 copies/ng total RNA and ~200-600 copies/ng of total RNA, respectively), but absent and highly expressed, respectively, in HL60. The significance of these limited expression differences among these myeloid cell lines is unknown.

In general, there appear to be fewer differences in the expression profiles among cancer cell lines derived from different tissues than among the profiles of a panel of normal tissues (chapter IV). This may reflect the common selection pressure inherent in cell culture systems or expression changes common to the development of cancer. Further study is needed to address this observation.

We observed no detectable MDR1 mRNA and high levels of MRP1 mRNA and topoisomerase II $\alpha$  in HL60 cells using competitive-RT-PCR. Using a similar competitive-RT-PCR technique, Wada et al., (1999) reported extremely low expression of MDR1 (no numerical values available), whereas another study was unable to detect MDR1 mRNA or Pgp (Ogretmen and Safa, 2000). MRP1 and topoisomerase II $\alpha$  transcripts have also been reported to be expressed in HL60 cells (Wada et al., 1999). We were unable to find any other expression data in the literature concerning HL60 cells and the transcripts we examined using competitive-RT-PCR.

The IC<sub>50</sub> values we have obtained for HL60 cells are similar to those reported by other groups, although IC<sub>50</sub> values differ between groups by as large as 10-fold for some drugs (Wada et al., 1999; Jonsson et al., 1995). Genetic differences between HL60 clones in different laboratories, varying experimental procedures and different drug preparations may account for the disparity of IC<sub>50</sub> values for some drugs.

The decision to pursue further study of any gene that underwent expression changes greater than 2-fold was based upon the function of the gene, reported associations with drug resistance and the magnitude of the expression change. The increase in M-ABC2 expression

after vincristine treatment is unlikely to be associated with resistance to the drug since this protein is localized in mitochondrial membranes and not the plasma membrane (Zhang et al., 2000b). Furthermore, there is no previous evidence for its role in drug resistance. Despite the 2.3-fold increase in M-ABC2 expression at the  $IC_{50}$  dose of vincristine, M-ABC2 expression actually decreased to a level below that of the PBS control at the  $3 \times IC_{50}$  dose of vincristine. The lack of a dose-dependent response suggests this alteration could be a random expression change due to cellular stress rather than part of a common defense mechanism to xenobiotics. There was also increased ABC1 expression after exposure to ara-C, but no evidence exists to support a role for ABC1 in drug transport and its proposed function is the export of cholesterol and phospholipids from the cell (Orso et al., 2000). MRP2, an anion transporter expressed at the canalicular membrane of hepatocytes, confers resistance to a variety of chemotherapeutic drugs in transfected cells including doxorubicin (a structural relative of daunorubicin) and mitoxantrone (Borst et al., 1999a), and its role in drug resistance has already been well characterized (see chapter I). The 4.8-fold increase in MRP2 mRNA after daunorubicin treatment is intriguing, but even at these elevated levels the transcript is still very weakly expressed in these cells and may, therefore, be of no consequence with respect to drug resistance. The role of GST $\pi$  in drug detoxification is well established; hence, the increase in GST $\pi$  expression with mitoxantrone treatment was not considered for further analysis. The most intriguing alteration in expression was that of MRP7 after daunorubicin treatment. This 6.2-fold increase was the largest we observed and at the time of this observation, no full-length MRP7 cDNA had been reported in the literature. Appendix I describes the rationale behind the decision to further characterize the induction of MRP7 mRNA after daunorubicin exposure and the results of this work.

The selection of a model cell line was a crucial aspect of these studies. Several reports have described the selection of stable drug-resistant variants of the HL60 cell line and the

resulting mechanisms of resistance. In each case, drug-resistant HL60 sublines were generated by exposure to stepwise increasing concentrations of the drug for several months. In almost every resistant subline, enhanced transport of the xenobiotic out of the cell was a major mechanism of resistance. In addition to resistance to the selecting agent, cross-resistance to structurally unrelated chemotherapeutic agents was always observed.

Anthracycline-resistant HL60 sublines have been generated independently by at least six different laboratories and at least two different resistance mechanisms have been reported. Bhalla et al. (1985) generated an adriamycin-resistant HL60 subline (HL60/AR) that was 111-fold resistant to the selecting drug and it was later shown that these HL60/AR cells over-express MRP1 protein, but not Pgp (Huang et al., 1997). Another adriamycin-resistant HL60 subline (HL60/ADR) (Marsh et al., 1986) was determined to be 10-fold resistant to the selecting drug and like the HL60/AR subline, HL60/ADR cells were later shown to over-express MRP1 protein, but did not express Pgp (Krishnamachary and Center, 1993). The HL-60-R<sub>0.5</sub>, HL-60-R<sub>5</sub> and HL-60-R<sub>10</sub> sublines generated by Jonsson et al. (1995) after exposure to doxorubicin were resistant to the selecting agent (14-, 142- and 328-fold, respectively) and displayed elevated MDR1 mRNA and Pgp compared to the parental cells. Southern blot analysis indicated amplification of the MDR1 gene in all 3 resistant sublines, suggesting that the increased Pgp/MDR1 levels were the result of genomic amplification. Marks et al. (1996) generated a 4-fold epirubicin-resistant subline (HL60/E2) which displayed over-expression of both Pgp and MRP1 mRNA, suggesting that multiple resistance mechanisms can simultaneously contribute to drug resistance in HL60 cells. The authors also note that although treatment with low levels of epirubicin produced an HL60 subline with increased Pgp expression, parallel treatment with only a 2.5-fold higher drug concentration, which resulted in approximately 10% cell death in a 4-day cell viability assay, gave rise to a resistant HL60 subline without increased Pgp or MRP1 expression. This suggests that the selection of this phenotype is associated with minimal cellular

damage and sufficient time for the cells to respond and possibly repair the damaged DNA. The HL60/DOX subline generated by Wada et al. (1999) after co-exposure to doxorubicin and the Pgp inhibitor cepharanthine, was 13-fold resistant to doxorubicin and over-expressed MRP1 mRNA 8-fold, as might be expected. Southern blot analysis also revealed amplification of the MRP1 gene. Finally, Takemura et al. (2001) isolated the HL60/AD subline which was 10-fold resistant to ara-C and doxorubicin, both of which were used simultaneously as selecting agents. Pgp was not detected in either HL60/AD or HL60 cells, but MRP1 protein was over-expressed in the former.

Drug-resistant HL60 sublines have also been generated using other structural classes of chemotherapeutic agents. McGrath and Center (1987) generated two vincristine resistant HL60 sublines that were 25- and 140-fold resistant to the selecting agent (HL60/vincC2 and HL60/vincC1, respectively) and unlike HL60 cells, these sublines over-expressed Pgp. Harker et al. (1989) generated 10- and 35-fold mitoxantrone-resistant (HL60/MX1 and HL60/MX2, respectively) HL60 sublines that did not over-express Pgp and the authors did not examine MRP1 expression. It was later shown that the levels of nuclear topoisomerase II catalytic activity and enzyme sensitivity to mitoxantrone-induced DNA cleavage were both reduced ~3-4 fold in HL-60/MX2 cells in association with a marked reduction in nuclear topoisomerase II  $\beta$  protein levels (Harker et al., 1991). Bhalla et al. (1994) generated taxol-resistant HL60 subclones that were 110- and 790-fold resistant to the drug (HL-60/TAX100 and HL-60/TAX/1000, respectively). Both cell lines over-expressed MDR1 mRNA and Pgp. Ishikawa et al. (1994) generated a cisplatin-resistant HL60 subclone named HL-60/R-CP that was 10-fold resistant to the selecting drug. It was later shown that MRP1 mRNA was 5-fold higher in HL-60/R-CP cells compared to the parental HL60 cells, but there was no amplification of the MRP1 gene as assessed by Southern blotting (Ishikawa et al., 1996). Culturing HL-60/R-CP cells in cisplatin-free medium for a month resulted in reduced MRP1 mRNA levels, but these levels

could be induced to rise within 30-hours by treatment with cisplatin and heavy metals such as arsenic, cadmium and zinc.

From these studies it is clear that the generation of stable drug-resistant HL60 sublines is frequently accompanied by increased expression of Pgp, MRP1 or both proteins. Increased expression often coincided with genomic amplification of the gene, but not always. This suggests that both increased transcription or mRNA/protein stability and greater gene copy numbers contribute to higher expression. The magnitude of increased expression was relatively small for MRP1 and Pgp, although no quantitative assessment was performed for Pgp/MDR1. In the cases where MRP1 mRNA was measured quantitatively, the increase ranged from 2.5- to 8-fold with corresponding levels of resistance ranging from 4- to 13-fold. This suggests that if any expression changes were to occur via induction following short-term drug exposure, these would likely be small changes. Our experience with vincristine-resistant SKOV3 sublines suggests that multiple changes in ABC transcripts may occur with long-term drug selection, but ABC transcripts other than MDR1 and MRP1 were not examined in any of these drug-resistant HL60 sublines.

ATRA has been demonstrated to induce the *in vitro* differentiation of the HL60 cell line (Breitman et al., 1980). In addition, ATRA has been reported to induce complete remission in patients with acute promyelocytic leukemia. However, the majority of patients relapse, likely the result of the development of drug resistance. An ATRA-resistant HL60 cell line has been generated (HL-60/RA), but there was no evidence for cross-resistance to other chemotherapeutic drugs or defective transport of the compound (Dore et al., 1994). There is no previous evidence for drug resistance to ATRA via a drug transport mechanism, nor is there evidence for induction of ABC genes by treatment with this compound. Hence it was not surprising to find so few significant changes in expression with ATRA treatment.

In retrospect, the surprisingly few expression changes might have been unique to the HL60 cell line; perhaps the use of a different leukemia cell line would have yielded more significant changes. The work of Ishikawa et al. (1996) is the only example of induction of an ABC transcript in HL60 cells, albeit using a cisplatin-resistant subline. It recently came to our attention that Chaudhary and Roninson (1993) were unable to detect any Pgp induction in HL60 cells using a range of drugs (e.g. doxorubicin, vinblastine, methotrexate and ara-C), although they did have success using the K562 and H9 leukemia cell lines under the same experimental conditions. Induction of MDR1 by anthracyclines has also been documented in another leukemia cell line (CEM/A7R) (Hu et al., 1995). All of these leukemia cell lines, unlike HL60, express a basal level of MDR1 mRNA, which appears to be a necessary prerequisite for up-regulation of MDR1 expression (Kantharidis et al., 2000). In support of this view, Hu et al. (1995) observed increased MDR1 mRNA after exposure of CEM/A7R cells to several chemotherapeutic drugs, but there were no increases in MDR1 mRNA under the same conditions in the parental CCRF-CEM cells. CEM/A7R is a variant of the doxorubicin resistant CEM/A7 cell line (20-fold doxorubicin resistant) that is only 5-fold resistant and, unlike CCRF-CEM, these cells express MDR1 mRNA.

It is also possible that induction of mRNA expression after short-term exposure to drugs may not be a common mechanism of resistance to xenobiotics. There is a surprisingly small number of publications on induction after drug exposure in comparison to the number of publications reporting altered expression after long-term drug selection. This may be the result of the inherent bias against publishing negative data. The majority of papers reporting altered expression after short-term drug exposure have used leukemia-derived cell lines, suggesting that this may not be a common occurrence in other cell lines such as those derived from solid tumours.

It is possible that 24 hours was an insufficient time period for induction of genes in HL60 cells. However, most studies examining induction employ exposure times ranging from less than 1 hour up to 24 hours. It is believed that longer exposures might allow sufficient time for the death of drug-sensitive cells, or over a period of several days, cell division and expansion of an inherently resistant subpopulation of cells. In this case, it would be difficult to determine whether the observed expression changes were a result of drug selection or induction. Manzano et al. (1996) also reported that depending on the specific drug and dosage used, changes in mRNA expression (MRP1 and MDR1) may be observed after as little as 1 day or as long as 6 days, or even not at all.

In conclusion, we have demonstrated that the expression levels of multiple ABC transcripts are altered with drug selection suggesting that altered levels of a single ABC transcript cannot be assumed to be causative of resistance. Given the number of expression changes observed for ABC transcripts in vincristine-resistant SKOV3-derivatives, it is reasonable to predict that multiple changes in expression levels also occur under long-term selection with other classes of anti-cancer drugs (e.g. daunomycin, mitoxantrone, cisplatin). After short-term exposure of HL60 cells to various chemotherapeutic drugs, we have detected a few changes in ABC mRNA expression levels. The number of changes is surprisingly small and may reflect the fact that altered mRNA expression is not a common response to xenobiotic exposure. Alternatively, the limited alteration of the HL60 expression profile may reflect the general insensitivity or unresponsiveness of the transcriptional machinery in HL60 cells to these stresses or the shortcomings of the experimental design (e.g. length of exposure and drug concentration). Future experiments should investigate the use of other leukemic cell lines, possibly even drug-resistant sublines. Identification of ABC proteins that are up- or down-regulated in response to chemotherapeutic drug exposure, both long-term and short-term, will

contribute to our understanding of MDR and may ultimately influence the design of future treatment protocols.

## CHAPTER IV

### EXPRESSION OF ABC TRANSCRIPTS IN NORMAL HUMAN TISSUES

#### INTRODUCTION

##### 4.1.1 Benefits of RNA Expression Profiling of ABC Transcripts in Normal Tissues

It is generally difficult to predict the function of an ABC protein based upon sequence similarity with other subfamily members for which a function is known. For example, there is only limited evidence that either MDR3 or BSEP can transport chemotherapeutic drugs, although they share 71% and 51% AA identity, respectively, with the multidrug transporter Pgp. This challenge can be overcome, at least in part, through detailed knowledge of the RNA expression profile of each gene. For example, in the case of BSEP and ABCR, which are expressed predominantly in bile canaliculi and retina, respectively, the search for potential functions and/or substrates can be simplified due to their specific expression in tissues with particular functions. Unfortunately, the expression patterns of most ABC proteins are complex, with only subtle differences amongst tissues. Therefore, knowledge of the expression levels of individual ABC mRNA species in normal tissues, as well as the collective profiles of the entire gene superfamily, are required to gain insight into the functions of these proteins.

The currently available expression data for individual ABC transcripts is limited and typically consists of a single Northern blot analysis using RNA from a small number of tissues. Since most RNA expression data is based on Northern blot analysis, which lacks the sensitivity of RT-PCR, low-level expression, which can be of functional significance, is often overlooked. Another difficulty with traditional methods such as Northern blots and RPA is that they do not permit quantitative comparisons among transcripts due to their labour-intensive application, the amount of RNA required and differences in hybridization efficiencies among transcripts.

Finally, due to the high sequence similarity among ABC proteins, especially within the NBDs, cross-hybridization can occur among subfamily members (e.g. MDR1 and MDR3).

A review of available expression data for individual ABC transcripts also highlights the variability of results among investigators. For example, Allikmets et al. (1996), using Northern blot analysis, observed one EST (accession number R00050) matching MRP4 to be expressed predominantly in pancreas and skeletal muscle, with low or absence of expression in other tissues (8 tissues examined). However, as part of the same study, another EST (accession number R35797) matching MRP4 was expressed ubiquitously, with high expression in pancreas. In contrast, Kool et al. (1997) examined expression of MRP4 mRNA in 20 tissues using RPA and observed low expression in lung, kidney, bladder, gall bladder and tonsil and no detectable expression in the remaining 15 tissues examined, including pancreas and skeletal muscle. Adding further confusion, Lee et al. (1998) examined MRP4 mRNA expression in 16 tissues using Northern blot analysis and reported ubiquitous expression, with by far the greatest expression in prostate and lowest in liver and peripheral blood leukocytes, for which prolonged exposure of film was required. Intermediate levels of expression were observed in other tissues. Clearly, standardized, quantitative assays are required to clarify these situations.

In many organisms, multiple ABC proteins contribute to defense against xenobiotic toxic compounds. Knowledge of the expression levels of each of these transcripts in individual tissues can help discern the relative contribution of each ABC protein to this defense. Examples include the *C. elegans* orthologs of MRP1 and Pgp1 which contribute to heavy metal resistance (Broeks et al., 1996) and Pgp3 which contributes to colchicine and chloroquine resistance (Broeks et al., 1995). The functions of the other 7 MRPs and 13 Pgp-like proteins in *C. elegans* are unknown, but they may also contribute to host defense (J. Sheps et al., unpublished data). In the parasitic protozoa *Leishmania*, PgpA, an MRP ortholog, contributes to defense of this unicellular organism against arsenite and antimonite, whereas Mdr1, a Pgp-like ortholog, provides resistance

to hydrophobic drugs (Ouellette et al., 1994). In mice, the disruption of the *mdr1a* gene leads to elevated drug levels in tissues, especially the brain, demonstrating the importance of Pgp at the blood-brain barrier (Schinkel et al., 1994). These mice also demonstrate that Pgp serves to protect the body against a broad range of orally ingested xenobiotics (Sparreboom et al., 1997). Doubly homozygous *mdr1a*<sup>-/-</sup>, *mdr1b*<sup>-/-</sup> mice also demonstrated the importance of these Pgp-like proteins in protecting bone marrow cells against cytotoxic anticancer drugs (Schinkel et al., 1997). The disruption of the *mrp1* gene in mice leads to impaired response to inflammatory stimuli, likely due to decreased secretion of the glutathione-S-conjugate leukotriene C<sub>4</sub> (Wijnholds et al., 1997). Moreover, such mice were hypersensitive to the anticancer drug etoposide. Subsequent studies with *mrp1*-deficient mice demonstrated that the location of *mrp1* at the basolateral side of polarized epithelial cells contributes to protection for: (1) basal stem cells in the oral mucosa (Wijnholds et al., 1998); (2) testicular tubules where the male germ cells are surrounded by Sertoli cells that express high levels of *mrp1* (Wijnholds et al., 1998); and (3) the choroid plexus which forms part of the blood-cerebrospinal fluid barrier (Wijnholds et al., 2000b). In humans, in addition to Pgp and MRP1, several other MRP subfamily members contribute to the protection of their host against toxic compounds. MRP2 is localized at the apical membrane of epithelial cells and provides a major route for secretion of organic anions from the liver (Konig et al., 1999). MRP3 is localized to the basolateral membrane of epithelial cells and plays a metabolic role in the intestinal uptake of organic anions and removal of organic acids from bile and liver cells under conditions of cholestasis (Kool et al., 1999a). MRP6 is localized at the lateral and canalicular plasma membrane of hepatocytes and may also contribute to organic anion secretion out of the liver (Hagenbuch et al., 2000).

Previous studies indicate that an MDR phenotype is often expressed in tumours derived from tissues with high Pgp/MDR1 expression (Germann, 1996). This may also be true for other MDR-associated ABC proteins such as MRPs 1-5 and BCRP. The relative mRNA levels of each

of these transcripts may determine the spectrum of drugs to which a tumour is resistant and the extent of the resistance. A lack of awareness of the presence of multiple efflux systems in cells may potentially result in the erroneous classification of drugs as being, for example, solely Pgp substrates, since the drug could also be transported by other drug efflux proteins with overlapping substrate specificity. By examining the collective ABC expression profiles of drug-resistant/sensitive cell lines and normal/tumour biopsies, we will gain insight into the nature of these multi-component drug efflux systems.

A number of ABC proteins have been shown to be involved in cellular homeostasis of phospholipids and cholesterol. This list includes: MDR3, a phosphatidylcholine translocator (van Helvoort et al., 1996); ABC1, a regulator of high-density lipoprotein metabolism (Orso et al., 2000); BSEP, a bile acid transporter (Gerloff et al., 1998); MRP3, possibly a bile acid transporter (Hirohashi et al., 2000); ABCG5 and ABCG8, which have been implicated in the genesis of sitosterolemia, a genetic disorder of lipid metabolism (Berge et al., 2000); WHITE1, a regulator of cholesterol and phospholipid transport in macrophages (Klucken et al., 2000); and ABCR, a transporter of protonated N-retinylidene-phosphatidylethanolamine (Weng et al., 1999). In addition, the expression of MRP5, BSEP, ABCA5, ABCA7 and several other ABC transcripts is regulated by lipid loading and deloading in macrophages (Klucken et al., 2000). Based on published expression data, it appears that there are both relatively tissue-specific lipid transporters (e.g. MDR3, BSEP and ABCR) that likely translocate a limited number of substrates, and ubiquitously expressed proteins (e.g. ABC1, WHITE1 and possibly other ABCA subfamily members) involved in lipid processes common to most cell types. However, comprehensive RNA expression profiles of these transcripts are currently lacking.

As functions are assigned to more ABC proteins, other multi-component efflux systems may become apparent. Currently, there is only limited information regarding physical interaction (homo- and hetero-dimerization) or coordinated transport among mitochondrial

(subfamily B) and peroxisomal (subfamily D) proteins. A detailed understanding of expression differences among tissues and cell types may provide insights in the future.

In this chapter, we provide competitive-RT-PCR analysis of 34 ABC mRNA species and the housekeeping gene  $\beta_2$ microglobulin in 12 normal human tissues, as well as a discussion of the significance of these results.

## **MATERIALS AND METHODS**

### **4.2.1 Human Total RNA Sources**

Human Total RNA Panels I, II and III were purchased from Clontech. These RNA sources were obtained from either a single individual or from multiple individuals to create a pooled sample. The quality of total RNA was verified by the manufacturer using denaturing gel electrophoresis. We independently confirmed RNA quality using RT-PCR amplification of  $\beta_2$ microglobulin (data not shown), as described in section 2.2.2 of this thesis. RNA sources used from Panel I included: brain (Caucasian male), kidney (pooled from 8 male/female Caucasians), liver (Asian male), lung (Asian male), and trachea (pooled from 84 male/female Caucasians). RNA sources used from Panel II included: heart (pooled from 12 male/female Caucasians), mammary gland (pooled from 8 female Caucasians), prostate (pooled from 23 male Caucasians), skeletal muscle (pooled from 10 male/female Caucasians), testis (pooled from 25 male Caucasians) and uterus (pooled from 10 female Caucasians). Bone marrow RNA (pooled from 24 male/female Caucasians) was obtained from Panel III.

### **4.2.2 Preparation of Total RNA**

Total RNA was prepared as described in section 2.2.2 of this thesis.

### **4.2.3 Reverse Transcription**

Reverse transcription was performed as described in section 2.2.4 of this thesis.

### **4.2.4 Amplification and Analysis of PCR Products**

Amplification and analysis of PCR products was performed as described in section 2.2.5 of this thesis.

## RESULTS

### 4.3.1 Competitive-RT-PCR Expression Profile of Normal Human Tissues

We have applied competitive-RT-PCR to examine the expression profile of 34 ABC transcripts and the housekeeping gene  $\beta_2$ microglobulin in total RNA from 12 normal human tissues (Figures 4.1-4.6). The percentage of ABC transcripts expressed at detectable levels averaged 90% and ranged from a high of 100% in prostate to a low of 75% in bone marrow (Table IV.I). This value is considerably higher than the average observed for SKOV3 (75%) and HL60 (60%) cells (chapter III). A potential reason for this difference may be that some transcripts present at low levels in normal tissues are transcriptionally inactive under cell culture conditions, since the functions of cultured cells are often distinct from the same cell type in normal tissues. The percentage of ABC transcripts expressed at less than 1000 copies per ng of total RNA, the expected detection limit of Northern blot or cDNA microarray analyses, averaged 81% and ranged from a high of 97% in trachea to a low of 58% in testis (Table IV.I). This value is similar to the average observed for SKOV3 (77%) and HL60 (69%) cells (chapter III). The expression levels of  $\beta_2$ microglobulin and TAP1 transcripts were determined in triplicate with an average COV of 19% and 27%, respectively. This is similar to the COV for the assay (20%) that was determined using full-length ABC7 transcript spiked into an *E. coli* background (chapter II), as well as the average COV for 39 transcripts in SKOV3 cells (31%) and HL60 cells (27%) (Chapter III).

The most striking feature observed was the unique expression profile of each tissue. In Table IV.I, the average number of mRNA copies per cell for all 34 ABC transcripts, excluding  $\beta_2$ microglobulin, has been estimated for each tissue type. These values were determined based upon the data presented in Figures 4.1-4.6 without normalization to  $\beta_2$ microglobulin levels, and an estimated yield of 10 pg total RNA per cell. In order to approximate absolute expression

levels, we then normalized the data to 500 copies of  $\beta_2$ microglobulin per cell, which is the average  $\beta_2$ microglobulin mRNA expression among a collection of SAGE libraries (Velculescu et al., 1999). The same study reported that  $\beta_2$ microglobulin mRNA was among the top 1000 (~ top 3% of genes in the human genome) ubiquitously expressed genes, based on range/average and an expression level of at least 35 copies/cell in all tissues analyzed (Velculescu et al., 1999). The average number of copies per cell among the 12 tissues was 24.0 and ranged from a high of 57.5 in testis to a low of 3.5 in trachea (Table IV.I). In general, the majority of ABC transcripts were weakly expressed in trachea, mammary gland, prostate, uterus, lung and heart, whereas most transcripts were highly expressed in testis, brain and skeletal muscle. These differences may relate to the high-energy demands of some tissues, as well as their relative structural and cellular complexity. High expression of multiple ABC transcripts may be required in cell types actively transporting many biological molecules among organelle compartments, as well as for vectoral transport across polarized cells. The profiles of liver, bone marrow and kidney were distinct in that they displayed a limited number of highly or uniquely expressed transcripts (i.e. MRP2, MRP6, BSEP, MDR3, M-ABC2 and MDR1) that were generally absent or at low levels in other tissues.

When describing competitive-RT-PCR data, ABC transcript expression has been categorized as very high (> 10,000 copies per ng of total RNA), high (2,000 – 10,000), moderate (500 – 2,000), low (100 – 500) or marginally detectable (< 100), rather than providing numerical values available in Figures 4.1-4.6. When possible, we have compared the competitive-RT-PCR expression pattern of each ABC transcript with published data (see below). Published expression data from tissues other than the 12 examined in this study has not been included for the sake of space and clarity. Unless otherwise noted, all comparisons are between our competitive-RT-PCR data and Northern blot data published in other mRNA expression studies. These comparisons are only general in nature and are not intended to be quantitative.

### 4.3.2 Subfamily A

Using competitive-RT-PCR, we examined the expression patterns of 7 members of subfamily A (ABC1, ABC2, ABC3, ABCR, ABCA5, ABCA6 and ABCA8). ABC1 is associated with an inherited genetic disorder of lipid export involving vesicular budding between the Golgi and plasma membrane (Orso et al., 2000). We observed ABC1 mRNA to be ubiquitously expressed at low or marginally detectable levels in all tissues examined (Figures 4.1-4.6). As shown in Table IV.II, the range/average (range of transcript levels in the 12 tissues examined divided by the average mRNA expression in copies per cell) for ABC1 mRNA was 2.06, which was the lowest of all ABC transcripts. This parameter is a measure of the ubiquity of transcript expression that has been used previously in SAGE analysis (Velculescu et al., 1999). In partial contrast to our results, Langmann et al. (1999) observed using dot blot analysis that the highest ABC1 mRNA expression was in liver and lung, with generally low expression in all other tissues examined. Broccardo et al. (1999) reported ABC1 mRNA to be strongly expressed in liver and uterus, but was not detectable in brain, heart, lung, skeletal muscle and kidney. Considering the function of ABC1 as a regulator of high-density lipoprotein metabolism, which is essential in all cell types, it is anticipated that the ABC1 transcript would be ubiquitously distributed.

The function of ABC2 is unclear; however, the transcript is induced during cholesterol import indicating that ABC2 may be a cholesterol-responsive gene (Kaminski et al., 2001a). ABC2 mRNA was ubiquitously expressed at low or marginally detectable levels, with the exception of brain, where it was moderately expressed (Figures 4.1-4.6). The ABC2 mRNA level in brain was 10x greater than the next most prominent tissue (Figure 4.1). The range/average was high at 7.86 (Table IV.II), making ABC2 a relatively tissue-specific transcript. Vulevic et al. (2001) demonstrated that ABC2 mRNA was predominantly expressed in the brain, with lower levels in kidney, liver and skeletal muscle, and no detectable expression

in heart and lung. Broccardo et al. (1999) also observed ABC2 mRNA was predominantly expressed in brain, with lower levels in heart and liver, but no detectable expression in kidney, lung, skeletal muscle or uterus. These data are in general agreement with our results.

Although the function of ABC3 is unknown, this protein has been localized to the limiting membrane of the lamellar bodies in alveolar type II cells in the lung, suggesting ABC3 may be important in the formation of pulmonary surfactant (Yamano et al., 2001). The expression of ABC3 mRNA was variable (range/average of 3.93, Table IV.II), with moderate levels in lung, brain and testis, low levels in skeletal muscle, heart, prostate, kidney and uterus, and marginally detectable expression in most other tissues (Figures 4.1-4.6). In contrast to the relatively low expression of most ABC transcripts in lung (average of 13.6 copies per cell, Table IV.I), ABC3 mRNA, along with TAP1 and MRP1, was expressed at a level 6x greater than the next most prominent transcript (Figure 4.3). ABC3 mRNA was also among the 3 most highly expressed transcripts in brain (Figure 4.1). In agreement with our data, Connors et al. (1997) and Klugbauer and Hofmann (1996) both reported ABC3 mRNA was most highly expressed in lung, with lower levels in brain, weak expression in heart and skeletal muscle, and no detectable expression in liver or kidney (testis not examined). Broccardo et al. (1999) reported ABC3 mRNA was most highly expressed in lung, with strong expression in liver and kidney, in contrast to Connors et al. and Klugbauer and Hofmann, as well as lower levels in brain and uterus, and no detectable expression in heart and skeletal muscle.

ABCR is associated with an inherited genetic disorder of lipid export involving transport of protonated N-retinylidene-phosphatidylethanolamine out of retinal disk cells (Weng et al., 1999). Using competitive-RT-PCR, we observed ABCR mRNA levels were variable, with low expression in kidney and testis, and marginally detectable or undetectable expression in other tissues (Figures 4.1-4.6). The range/average for ABCR was the highest of all transcripts at 12.01 (Table IV.II). Allikmets et al. (1997) reported using Northern blot analysis that ABCR mRNA

was detectable only in retina RNA; however no published analysis has been conducted using RT-PCR, which has far greater sensitivity. Our surprising observation of ABCR mRNA in other tissues, albeit at low levels (average of 1.56 copies per cell compared to the ABC average of 24.0), raises the possibility that this gene may have functions outside the retina.

ABCA5 mRNA was expressed at variable levels (range/average of 4.42, Table IV.II), with moderate expression in skeletal muscle, brain and testis, low expression in prostate, trachea, kidney, mammary gland and uterus, and marginally detectable or undetectable expression in other tissues (Figures 4.1-4.6). ABCA5 mRNA was among the three most highly expressed ABC transcripts in skeletal muscle (Figure 4.2). In an early study examining expression of ABC mRNA species using EST probes, Allikmets et al. (1996) observed an EST matching ABCA5 to be expressed in skeletal muscle and heart, and was not detected in brain, lung, liver and kidney; however, a Northern blot image was not provided. The function of ABCA5 is unknown.

ABCA6 mRNA was ubiquitously expressed (range/average of 2.36, Table IV.II), with moderate expression in testis, mammary gland, liver and skeletal muscle, low expression in uterus, bone marrow, kidney, brain and trachea, and marginally detectable expression in other tissues (Figures 4.1-4.6). ABCA6 was among the 3 most highly expressed transcripts in mammary gland (Figure 4.3). In partial contrast to our data, Kaminski et al. (2001b) reported using dot blot analysis that ABCA6 mRNA was variably expressed with the highest levels in liver, with lower levels in lung, heart and uterus, and even lower levels in prostate, kidney and bone marrow. ABCA6 mRNA was not detectable in skeletal muscle, mammary gland or testis. The function of ABCA6 is unknown.

ABCA8 mRNA was ubiquitously expressed at relatively low levels (range/average 2.65, Table IV.II), with moderate expression in testis and low or marginally detectable expression in all other tissues examined (Figures 4.1-4.6). No published ABCA8 expression data is available for comparison. The function of ABCA8 is unknown.

### 4.3.3 Subfamily B

Using competitive-RT-PCR, we examined the expression patterns of 10 members of subfamily B (MDR1, TAP1/2, MDR3, MTABC3, ABC7, M-ABC1, ABCB9, M-ABC2 and BSEP). In agreement with the published literature, we observed MDR1 mRNA (multidrug transporter) to be expressed at variable levels (range/average of 5.74, Table IV.II), with moderate expression in kidney, brain and uterus, low expression in liver and testis and marginally detectable expression in most other tissues (Figures 4.1-4.6). MDR1 mRNA was among the 3 most highly expressed ABC transcripts in kidney (Figure 4.6). Noonan et al. (1990) demonstrated using RT-PCR that MDR1 mRNA was most highly expressed in kidney, with lower expression in liver and lung, and even lower expression in skeletal muscle, mammary gland and bone marrow. Fojo et al. (1987) used slot blot analysis to demonstrate MDR1 mRNA was most highly expressed in kidney, with lower levels in liver, lung and brain, and even lower levels in prostate, skeletal muscle, heart and bone marrow. Pgp/MDR1 is also expressed within endothelial cells of the central nervous system and testis (Cordon-Cardo et al., 1989) and epithelium of the uterine cervix (Riou et al., 1990).

Both TAP1 and TAP2 (transporters of antigenic peptides associated with major histocompatibility complex function) mRNA species were ubiquitously expressed (Figures 4.1-4.6), with range/average values of 2.25 and 2.45, respectively (Table IV.II). This ubiquitous pattern of expression would be expected considering the important role of these proteins in immune recognition, which is essential for all cell types. TAP1 mRNA was expressed at higher levels than TAP2 mRNA in all tissues examined, with an average differential of 14-fold. TAP1 mRNA was also higher than TAP2 in SKOV3 cells (chapter III) and SW620 cells (chapter V); however, these transcripts were at roughly equivalent levels in HL60 cells (chapter III) and the AML cell lines AML1, AML2 and AML3 (data not shown). Unequal mRNA expression is surprising considering these "half-transporters" form heterodimers. However, the corresponding

protein levels in these tissues may be similar to each other. TAP1 mRNA was highly expressed in testis and moderately expressed in all other tissues. TAP2 mRNA was moderately expressed in bone marrow and weakly expressed in most other tissues. TAP1 mRNA was the most highly expressed ABC transcript in heart (Figure 4.2) and among the three most highly expressed ABC transcripts in testis, prostate, trachea, uterus, lung, mammary gland and bone marrow (Figures 4.1, 4.3-4.6). Not surprisingly, numerous publications have demonstrated both TAP1 and TAP2 mRNA species are expressed in every cell type.

In agreement with data reported by other researchers, we observed MDR3 mRNA (phospholipid transporter) to be expressed at variable levels (range/average of 5.01, Table IV.II), with low expression in liver, testis and skeletal muscle, and marginally detectable or undetectable expression in other tissues (Figures 4.1-4.6). Chin et al. (1989) demonstrated using RT-PCR that MDR3 mRNA was highest in liver, with lower levels in kidney, and no detectable expression in skeletal muscle, lung or mammary gland (testis not examined). Smit et al. (1994) demonstrated using RPA that MDR3 mRNA was highest in liver, with lower levels in skeletal muscle and heart and no detectable expression in brain, lung, kidney and testis.

MTABC3 mRNA (mitochondrial protein) was ubiquitously expressed at variable levels (range/average of 3.13, Table IV.II). The transcript was moderately expressed in skeletal muscle, testis and liver, lowly expressed in uterus, prostate, brain, kidney and heart and marginally detectable in other tissues examined (Figures 4.1-4.6). In agreement with our results, Mitsuhashi et al. (2000) examined a limited number of tissues and reported MTABC3 mRNA was most highly expressed in skeletal muscle and heart, with lower levels in brain, liver and kidney, and no detectable expression in lung.

We determined ABC7 mRNA (mitochondrial protein) was ubiquitously expressed at variable levels (range/average of 4.48, Table IV.II). The transcript was moderately expressed in liver, skeletal muscle, heart, bone marrow, kidney, brain and testis, lowly expressed in prostate,

mammary gland and uterus, and marginally detectable in other tissues examined (Figures 4.1-4.6). ABC7 was among the 3 most highly expressed ABC transcripts in brain and heart (Figures 4.1 and 4.2). In partial agreement with our results, Allikmets et al. (1999) reported ABC7 mRNA was most highly expressed in heart and skeletal muscle, with lower levels in liver and kidney and no detectable expression in brain or lung.

We observed M-ABC1 mRNA (mitochondrial protein) to be expressed at variable levels in the tissues examined (range/average of 5.20, Table IV.II), with moderate expression in testis and marginally detectable or undetectable expression in other tissues examined (Figures 4.2-4.7). Hogue et al. (1999) reported M-ABC1 mRNA to be ubiquitously expressed at low levels in all tissues examined.

We determined ABCB9 mRNA expression to be variable (range/average of 5.20, Table IV.II), with moderate expression levels in brain and testis, low expression in bone marrow, and marginally detectable expression in most other tissues examined (Figures 4.1-4.6). The levels of ABCB9 expression in testis and brain were 9x greater than that of the next most prominent tissue source. In agreement with our observations, Zhang et al. (2000a) reported ABCB9 mRNA was most highly expressed in testis and brain, with much lower levels in bone marrow, trachea and kidney and no detectable expression in other tissues. ABCB9 is most closely related to the TAP proteins, with AA identity of 38% and 40%, respectively, with TAP1 and TAP2, whereas the identity between the two TAP proteins is 39% (Zhang et al., 2000a). Although this suggests ABCB9 may also be involved in peptide transport, the function of ABCB9 is unknown.

We observed M-ABC2 expression (mitochondrial protein) to be relatively tissue-specific (range/average of 8.07, Table IV.II). There was moderate expression in bone marrow, low expression in kidney, liver, skeletal muscle, heart, brain and testis, and marginally detectable expression in other tissues examined (Figures 4.1-4.6). M-ABC2 mRNA was expressed at a level 5.5x higher in bone marrow than the next most prominent tissue and M-ABC2 mRNA was

the most highly expressed ABC transcript in bone marrow (Figure 4.6). In agreement with our results, Zhang et al. (2000b) reported M-ABC2 mRNA was highly expressed in bone marrow, with much lower expression in all other tissues examined.

We observed BSEP mRNA (bile acid transporter) to be expressed in a relatively tissue-specific pattern (range/average of 6.10, Table IV.II), with moderate expression in liver and testis and marginally detectable or undetectable expression in all other tissues examined (Figures 4.1-4.6). In agreement with our data, Strautnieks et al. (1998) reported BSEP mRNA to be expressed in liver, and not detectable in brain, heart, skeletal muscle, kidney and lung (testis not examined). The surprising expression of BSEP mRNA in testis suggests it may have secondary functions other than at the canalicular membrane.

#### **4.3.4 Subfamily C**

Using competitive-RT-PCR, we examined the expression patterns of 8 members of subfamily C (MRPs 1-7 and CFTR). We observed MRP1 mRNA (multidrug transporter) to be ubiquitously expressed (range/average of 3.02, Table IV.II), and at a very high level in testis, high levels in lung, brain, skeletal muscle and prostate, and moderate levels in other tissues, except liver, where expression was low (Figures 4.1-4.6). MRP1 was the most highly expressed ABC transcript in lung, testis, brain, prostate, trachea, skeletal muscle, mammary gland and uterus, and among the 3 most highly expressed ABC transcripts in heart. However, MRP1 mRNA levels were lower than  $\beta_2$ microglobulin mRNA in every tissue examined by an average of 7.5-fold. In agreement with our results, Kool et al. (1997) and Zaman et al. (1993) compared RNA expression levels of MRPs 1-5 in 22 tissues and observed MRP1 mRNA to be expressed in 95% of tissues examined and to be the most highly expressed of the five transcripts, followed by MRP5. In contrast, MRPs 2,3 and 4 were detectable in only a few tissues. In general agreement with our results, the authors found MRP1 mRNA at the highest levels in lung and testis, with

slightly lower levels in kidney and skeletal muscle, lower levels in heart and brain and no detectable expression in liver. Nooter et al. (1995) examined a limited set of tissues and reported using RPA that MRP1 mRNA was at the highest levels in testis and lung, with slightly lower levels in kidney and in contrast to our results and those of Kool et al. (1997), liver. Legrand et al. (1996) also demonstrated using RT-PCR that MRP1 mRNA was ubiquitously expressed in all normal hematopoietic lineages isolated from bone marrow.

Mutations in MRP2 cause Dubin-Johnson syndrome, a defect in the secretion of amphiphilic anionic conjugates from hepatocytes into bile (Wada et al., 1998). We observed the expression of MRP2 mRNA to be relatively tissue-specific (range/average of 8.22, Table IV.II), with very high and high levels in liver and kidney, respectively, moderate levels in testis and low or marginally detectable levels in other tissues (Figures 4.1-4.6). MRP2 was the most highly expressed ABC transcript in liver and kidney (Figures 4.5 and 4.6). In agreement with our results, Kool et al. (1997) observed MRP2 to be predominantly expressed in liver, with lower expression in kidney, and no detectable expression in lung, mammary gland, testis, skeletal muscle, heart or brain. Taniguchi et al. (1996) observed expression in liver, but not kidney, heart, brain, lung and skeletal muscle. Schaub et al. (1999) also demonstrated using RT-PCR that MRP2 mRNA was expressed in normal kidney.

We observed MRP3 mRNA (organic anion transporter) was relatively tissue-specific (range/average of 8.78, Table IV.II), with moderate levels in liver and kidney, and marginally detectable or undetectable expression in other tissues (Figures 4.1-4.6). MRP3 mRNA was among the 3 most highly expressed ABC transcripts in liver (Figure 4.5). MRP3 mRNA levels in liver and kidney were 9x greater than the next most prominent tissues (Figures 4.5 and 4.6). Our data is similar to that reported by several other groups. Kool et al. (1997) observed MRP3 mRNA to be predominantly expressed in liver, with lower expression in kidney and lung, and no detectable expression in mammary gland, testis, skeletal muscle, heart and brain. Belinsky et al.

(1998) reported highest expression of MRP3 mRNA in liver, with lower levels in kidney and with prolonged exposure expression could be detected in prostate, testis and lung, but not heart, brain or skeletal muscle. Other groups have reported similar data (Kiuchi et al., 1998 and Uchiumi et al., 1998).

We observed MRP4 mRNA (organic anion transporter) to be ubiquitously expressed at variable levels (range/average of 3.40, Table IV.II). The transcript was moderately expressed in prostate, kidney, brain, testis, liver and skeletal muscle, and lowly expressed or marginally detectable in other tissues (Figures 4.1-4.6). In contrast to the relatively low expression of most ABC transcripts in prostate (average of 16.3 copies per cell, Table IV.I), MRP4 mRNA, along with MRP1 and TAP1, was expressed at a level 5x greater than the next most prominent transcript (Figure 4.4). In agreement with our results, Lee et al. (1998) observed MRP4 mRNA to be most highly expressed in prostate, with lower levels in testis, skeletal muscle and lung, and very low levels in heart, brain, liver and kidney. In contrast to our results and those of Lee et al., Kool et al., (1997) observed MRP4 to be expressed at low levels in lung and kidney and absent in testis, liver, skeletal muscle, heart, and brain (prostate not examined).

We observed MRP5 mRNA (organic anion transporter) to be ubiquitously expressed (range/average of 3.25, Table IV.II), with moderate expression in skeletal muscle, bone marrow, kidney, brain and testis, and low expression in most other tissues (Figures 4.1-4.6). MRP5 mRNA was among the 3 most highly expressed ABC transcripts in bone marrow (Figure 4.5). Kool et al. (1997) observed MRP5 mRNA to be most highly expressed in skeletal muscle and brain, with lower levels in lung, kidney, testis, and heart, and even lower levels in liver (bone marrow not examined). Belinsky et al. (1998) reported the highest expression of MRP5 mRNA in skeletal muscle, with intermediate levels in kidney, testis, heart and brain, lower levels in prostate, and with prolonged film exposure, lung and liver (bone marrow not examined). McAleer et al. (1999) observed the highest MRP5 mRNA levels in skeletal muscle, heart, kidney

and brain, with an intermediate level in bone marrow, lower expression in lung, and no detectable expression in liver. Our own Northern blot analysis demonstrated highest MRP5 mRNA levels in kidney and trachea, with intermediate levels in bone marrow, testis and prostate, lower levels in heart, brain and liver, and no detectable expression in lung and skeletal muscle (data not shown). Although there are significant differences among investigators, collectively, these results are similar to our competitive-RT-PCR data.

Mutations in MRP6 have recently been associated with the inheritable connective tissue disorder pseudoxanthoma elasticum but its substrate(s) is unknown (Bergen et al., 2000). We observed the expression of MRP6 mRNA to be relatively tissue-specific and detectable in only 3 of 11 tissues examined (range/average of 8.62, Table IV.II). The transcript was very highly expressed in liver, moderately expressed in kidney, marginally detectable in lung and absent in other tissues examined (Figures 4.1-4.6). MRP6 mRNA was among the three most highly expressed ABC transcripts in liver and kidney (Figures 4.5 and 4.6). In agreement with our results, Kool et al. (1999b) also observed MRP6 mRNA to be most highly expressed in liver and kidney, detectable at much lower levels in lung, and not detectable in testis, skeletal muscle, heart and brain.

We observed CFTR mRNA (cAMP activated chloride channel) to be marginally detectable or absent in all tissues examined (range/average of 5.01, Table IV.II) (Figures 4.1-4.6). However, using competitive-RT-PCR we observed CFTR mRNA was expressed at a very high level in pancreas (data not shown). Riordan et al. (1989) reported similar data and demonstrated that CFTR mRNA was most highly expressed in pancreas, with much lower levels in lung and liver, and no detectable expression in brain, testis or kidney.

We observed MRP7 mRNA expression to be variable (range/average of 7.42, Table IV.II), with low expression in heart and marginally detectable expression in most other tissues (Figures 4.1-4.6). Using Northern blot analysis, Hopper et al. (2001) reported that MRP7 mRNA

was undetectable (16 tissues); however, when using RT-PCR they observed highest expression in testis, and lower levels in heart, liver, kidney and brain. Our own dot blot analysis demonstrated ubiquitously low expression in the 76 tissues examined (appendix I). The function of MRP7 is unknown.

#### **4.3.5 Subfamily D**

Using competitive-RT-PCR, we examined the expression patterns of 4 members of subfamily D (ALDP, ALDR, PMP70 and PMP69). Mutations in the ALDP gene cause adrenoleukodystrophy, a neurodegenerative disorder characterized by accumulation of unbranched saturated fatty acids likely due to impairment of degradation of very long chain fatty acids in peroxisomes. We observed ALDP mRNA to be ubiquitously expressed at variable levels (range/average of 6.93, Table IV.II). ALDP mRNA was present at moderate levels in testis and skeletal muscle, low levels in uterus, heart, prostate, trachea and bone marrow, and at marginally detectable levels in other tissues (Figures 4.1-4.6). In agreement with our results, Mosser et al. (1993) reported that ALDP mRNA was most highly expressed in skeletal muscle, with lower levels in heart, lung and liver, and no detectable expression in brain and kidney (testis not examined).

We observed ALDR mRNA (peroxisomal protein) expression to be relatively tissue-specific (range/average of 8.79, Table IV.II), with moderate levels in brain, low levels in bone marrow, mammary gland, skeletal muscle and uterus, and marginally detectable expression in most other tissues (Figures 4.1-4.6). ALDR mRNA expression in brain was 8.5x greater than that of the next most prominent tissue (Figure 4.1). Similar data has been reported by Holzinger et al. (1997b), using Northern blot analysis, who observed ALDR mRNA to be predominantly expressed in brain, with much lower levels in heart, and no detectable expression in lung, liver,

skeletal muscle and kidney. Using RT-PCR, Holzinger et al. could also detect ALDR mRNA in liver, lung and uterus.

Mutations of PMP70 have been found in some patients with Zellweger's disease, a disorder of peroxisome biogenesis. We observed PMP70 mRNA to be ubiquitously expressed (range/average of 2.72, Table IV.II), with moderate expression in testis, brain, skeletal muscle, kidney and uterus, and low expression in most other tissues (Figures 4.2-4.7). PMP70 mRNA was among the 3 most highly expressed ABC transcripts in trachea (Figure 4.4). No published PMP70 expression data is available for comparison.

We observed PMP69 mRNA (peroxisomal protein) to be expressed ubiquitously at low levels in most tissues (range/average of 2.71, Table IV.II) (Figures 4.1-4.6). In agreement with our data, Shani et al. (1997) also reported PMP69 mRNA to be ubiquitously expressed at similar levels in most tissues.

#### **4.3.6 Subfamily F**

Using competitive-RT-PCR we examined the expression patterns of 3 of the members of subfamily E (ABC50, ABCF2 and ABCF3). We observed ABC50 mRNA (involved in mRNA translation) to be ubiquitously expressed at variable levels (range/average of 3.49, Table IV.II), with high expression in testis, moderate expression in brain, bone marrow, kidney, liver, uterus, mammary gland, skeletal muscle and heart, and low expression in other tissues examined (Figures 4.1-4.6). ABC50 mRNA was among the 3 most highly expressed ABC transcripts in testis, skeletal muscle and uterus (Figures 4.1, 4.2, and 4.5). In agreement with our data, Richard et al. (1998) demonstrated that ABC50 mRNA was ubiquitously expressed, with greatest abundance in skeletal muscle, testis and heart. The high and ubiquitous expression of ABC50 is expected since this protein is likely involved in the translation of mRNA.

We observed ABCF2 mRNA was ubiquitously expressed (range/average of 3.69, Table IV.II), with moderate levels in bone marrow, kidney, brain, testis, skeletal muscle and heart, and low levels in other tissues (Figures 4.1-4.6). In an early study examining expression of ABC mRNA species using EST probes, Allikmets et al. (1996) observed an EST matching ABCF2 to be expressed in lung, liver and skeletal muscle, and was not detected in heart, brain and kidney; however, a Northern blot image was not provided. The function of ABCF2 is unknown.

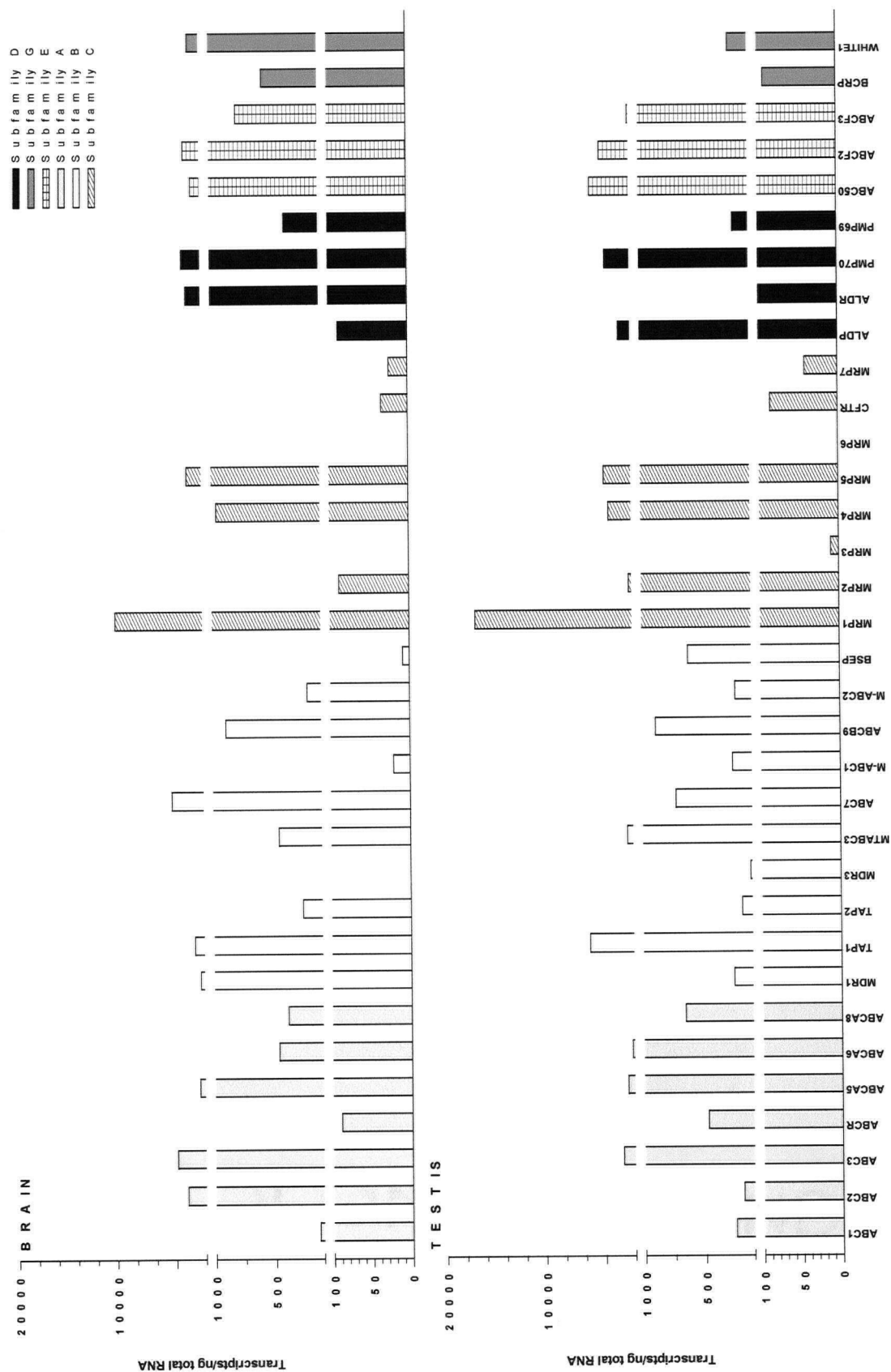
We observed ABCF3 mRNA was ubiquitously expressed (range/average of 3.03, Table IV.II), with moderate expression in brain, testis and skeletal muscle, and low expression in most other tissues (Figures 4.1-4.6). In agreement with our results, Allikmets et al. (1996) also observed an EST matching ABCF3 to be ubiquitously expressed, however a Northern blot was not provided. The function of ABCF3 is unknown.

#### **4.3.7 Subfamily G**

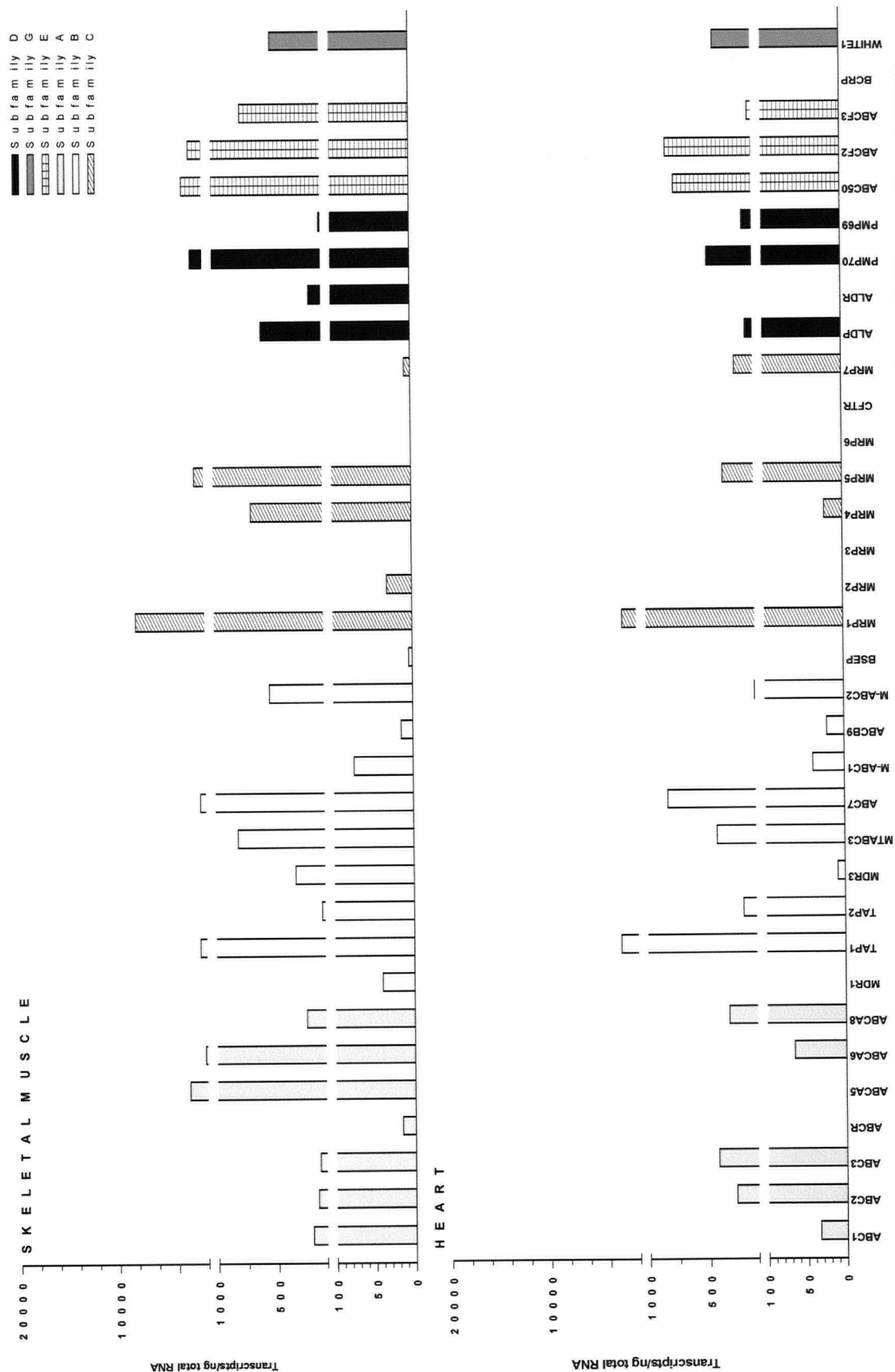
Using competitive-RT-PCR, we examined the expression patterns of 2 members of subfamily G (BCRP and WHITE1). We observed BCRP mRNA (multidrug transporter) to be expressed at variable levels (range/average of 7.66, Table IV.II), with moderate expression in brain, low expression in testis and uterus, and marginally detectable or undetectable expression in other tissues examined (Figures 4.1-4.6). Allikmets et al. (1998) demonstrated that BCRP mRNA was expressed predominantly in placenta, with very low expression in heart, and no detectable expression in the other 43 tissues examined. Doyle et al. (1998) also reported BCRP mRNA to be predominantly expressed in placenta, however, similar to our results, they detected lower levels in brain, prostate, testis and liver, but were unable to detect expression in heart, lung, skeletal muscle and kidney (uterus not examined).

WHITE1 is likely one of several ABC proteins that regulate cholesterol and phospholipid transport (Klucken et al., 2000). We observed WHITE1 mRNA to be expressed ubiquitously

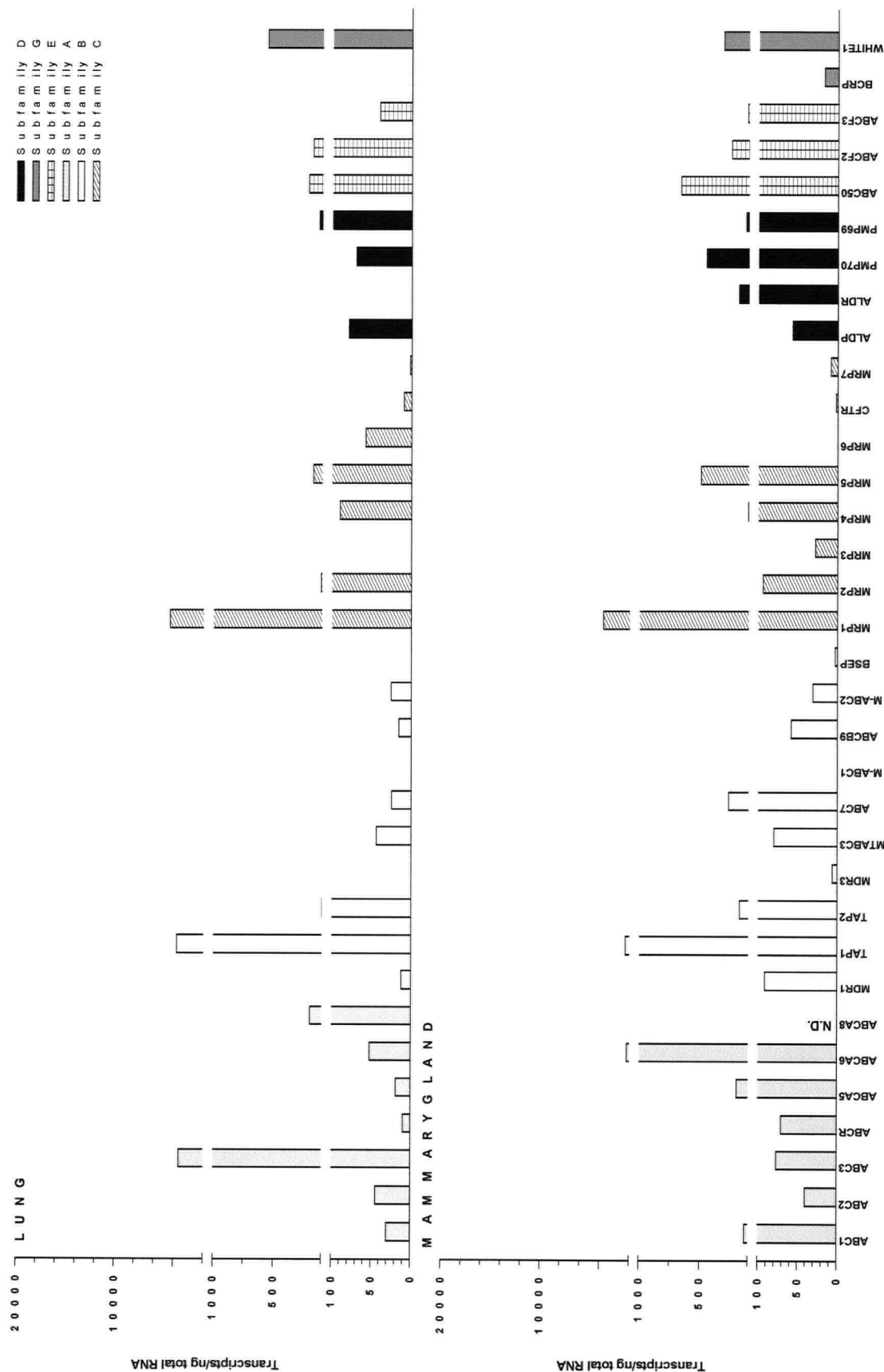
(range/average of 3.77, Table IV.II), with moderate expression in skeletal muscle, brain, lung, bone marrow, kidney, and low expression in all other tissues examined (Figures 4.1-4.6). Chen et al. (1996) demonstrated that WHITE1 mRNA was expressed strongly in skeletal muscle, with lower expression in heart, lung, kidney and prostate, and no detectable expression in brain, liver and testis. Croop et al. (1997) examined a limited number of tissues and reported strong expression in brain and lung, with lower levels in heart and skeletal muscle, and no detectable expression in liver. Collectively, these data resemble our results.



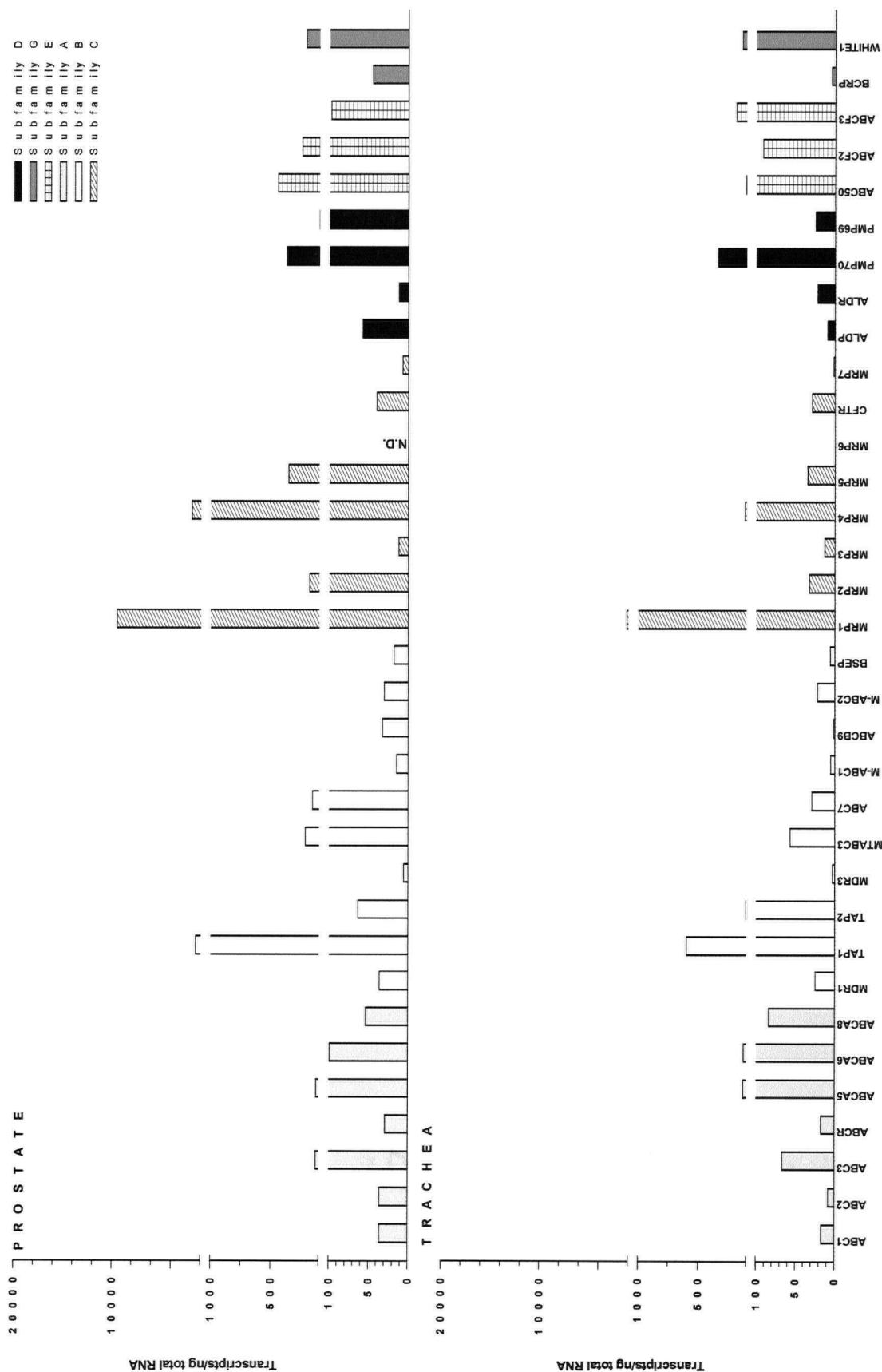
**Figure 4.1 Competitive-RT-PCR Expression Profile of Normal Human Brain and Testis Total RNA.** Profile of 34 ABC transporters normalized relative to  $\beta_2$ microglobulin levels. The absence of a bar denotes the transcript was not detectable. ND = not determined. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



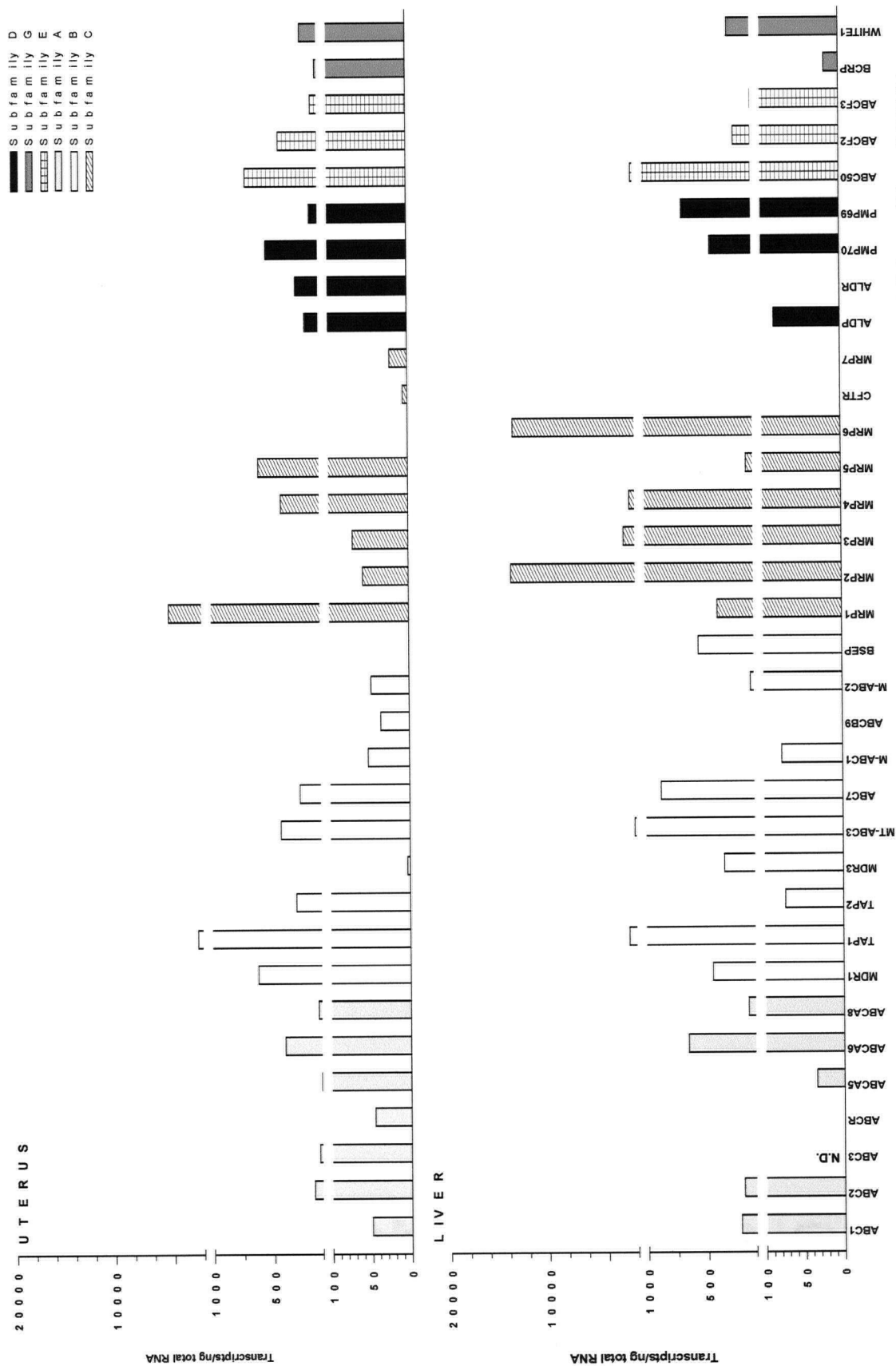
**Figure 4.2 Competitive-RT-PCR Expression Profile of Normal Human Muscle and Heart Total RNA.** Profile of 34 ABC transcripts normalized relative to  $\beta_2$ microglobulin levels. The absence of a bar denotes the transcript was not detectable. ND = not determined. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



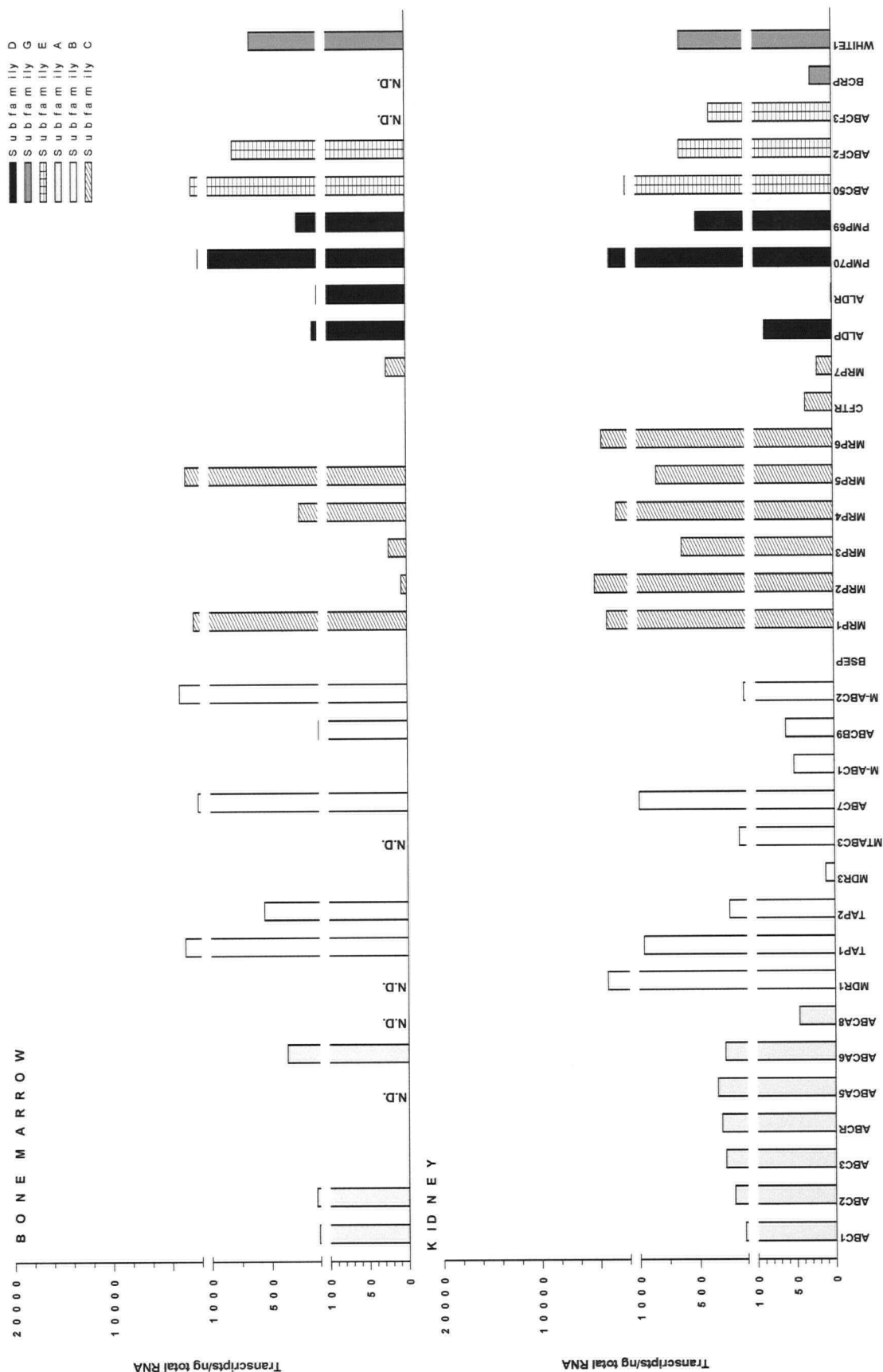
**Figure 4.3 Competitive-RT-PCR Expression Profile of Normal Human Lung and Mammary Gland Total RNA.** Profile of 34 ABC transcripts normalized relative to  $\beta_2$ microglobulin levels. The absence of a bar denotes the transcript was not detectable. ND = not determined. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



**Figure 4.4 Competitive-RT-PCR Expression Profile of Normal Human Prostate and Trachea Total RNA.** Profile of 34 ABC transporters normalized relative to  $\beta_2$ microglobulin levels. The absence of a bar denotes the transcript was not detectable. ND = not determined. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



**Figure 4.5 Competitive-RT-PCR Expression Profile of Normal Human Uterus and Liver Total RNA.** Profile of 34 ABC transporters normalized relative to  $\beta_2$ microglobulin levels. The absence of a bar denotes the transcript was not detectable. ND = not determined. See Materials and Methods sections 2.2.4 and 2.2.5 for details.



**Figure 4.6. Competitive-RT-PCR Expression Profile of Normal Human Bone Marrow and Kidney Total RNA.** Profile of 34 ABC transporters normalized relative to  $\beta_2$ microglobulin levels. The absence of a bar denotes the transcript was not detectable. ND = not determined. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

**TABLE IV.I ABC Transcript Expression in Normal Human Tissues<sup>a</sup>.**

<b>Tissue</b>	<b>Category of expression profile</b>	<b>% of detectable transcripts</b>	<b>% of transcripts below 1000 copies/ng of total RNA</b>	<b>Average copies/cell</b>
Testis	high	97	58	57.5
Brain	high	91	61	45.1
Liver	unique	84	79	41.4
Skeletal Muscle	high	91	73	29.6
Kidney	unique	97	79	27.6
Bone Marrow	unique	75	75	19.5
Prostate	low	100	90	16.3
Lung	low	85	91	13.6
Uterus	low	94	94	12.3
Heart	low	76	94	11.2
Mammary Gland	low	94	90	10.5
Trachea	low	97	97	3.5
<b>AVERAGE</b>		<b>90</b>	<b>81</b>	<b>24.0</b>

<sup>a</sup>Average copies per cell based upon an estimated yield of 10 pg total RNA per cell. Data is normalized to 500 copies of  $\beta_2$ microglobulin per cell.

**TABLE IV.II. Ubiquity of ABC Transcript Expression Among 12 Normal Human Tissues<sup>a</sup>.**

ABC gene	Average # of copies/cell	# of tissues with detectable transcript	Range		Range/average
			Low	High	
ABC1	3.76	12/12	0.57	8.34	2.06
TAP1	71.67	12/12	19.95	181.39	2.25
ABCA6	17.07	12/12	1.73	42.16	2.36
TAP2	6.67	12/12	2.12	18.49	2.45
ABCA8	7.68	11/11	1.53	21.95	2.65
PMP69	8.05	12/12	0.82	22.64	2.71
PMP70	41.88	12/12	2.34	116.54	2.72
MRP1	181.30	12/12	13.35	561.19	3.02
ABCF3	11.64	11/11	1.35	36.71	3.03
MTABC3	16.52	11/11	1.46	53.33	3.13
MRP5	37.87	12/12	1.17	124.48	3.25
MRP4	32.06	12/12	0.77	109.8	3.40
ABC50	46.52	12/12	3.46	166.00	3.49
ABCF2	35.00	12/12	3.06	132.24	3.69
WHITE1	17.58	12/12	4.49	70.78	3.77
ABC3	32.62	10/11	0	128.49	3.93
ABCA5	21.01	10/11	0	92.92	4.42
ABC7	31.01	12/12	0.83	139.97	4.48
CFTR	0.57	7/12	0	2.86	5.01
MDR3	2.35	9/12	0	11.79	5.01
ABCB9	5.91	11/12	0	29.74	5.02
M-ABC1	1.65	9/12	0	8.58	5.20
MDR1	18.42	10/11	0	105.91	5.74
BSEP	3.39	7/12	0	20.71	6.10
ALDP	10.46	12/12	0.31	72.8	6.93
MRP7	1.15	11/12	0	8.54	7.42
BCRP	2.44	8/11	0	18.7	7.66
ABC2	11.89	12/12	0.27	93.80	7.86
M-ABC2	12.75	12/12	0.74	102.93	8.07
MRP2	55.13	11/12	0	453.49	8.22
MRP6	51.62	3/11	0	445.06	8.62
MRP3	8.17	8/12	0	71.81	8.78
ALDR	9.40	9/12	0	82.66	8.79
ABCR	1.56	9/12	0	18.75	12.01

<sup>a</sup> All data is normalized to 500 copies per cell of  $\beta_2$ microglobulin transcript.

## DISCUSSION

For the majority of transcripts examined, our competitive-RT-PCR data was in agreement with the published literature. In some cases, for example MRP4, this comparison was not straightforward because of inconsistencies in the expression patterns reported by different investigators. For a very limited number of ABC transcripts there were notable differences between our data and those reported in the literature. These exceptions included: ABC1, low expression in liver and lung; ABCR, low expression in most tissues; BSEP, low expression in testis; and ABCA6, moderate expression in mammary gland, skeletal muscle and testis. The few differences between our results and the published literature may, in part, be due to the RNA sources used. Genetic heterogeneity among individuals can be a significant problem when analyzing transcript expression. Therefore, it is preferable to use RNA sources pooled from multiple individuals. In our case, the liver, lung and brain total RNA samples were obtained from single individuals and this may account for some of the observed variability, particularly in the case of ABC1. Other factors influencing the quality of the RNA source could include: the care taken when isolating RNA from different tissue sources (commercial source); the age, sex and race of the donor; the degree of contamination from blood and other bodily fluids; and the source of the tissue from which RNA was isolated (e.g. surgery or accidental death). As mentioned previously, cross-hybridization among members of the same ABC subfamily may also provide misleading results when using dot blot, Northern blot or RPA analysis. Inaccurate aliquoting of competitor RNA species may also contribute to over- or under-estimation of transcript copy number; however, we have rarely observed such differences between aliquots (data not shown). Regarding, ABCR and BSEP mRNA species, the lack of reported detection of these transcripts in tissues other than retina and liver, respectively, may be the result of the sensitivity of the assay used (Northern blot analysis). The generally low expression of ABC1

mRNA in most tissues is surprising considering the important role of this protein in the transport of cholesterol from extrahepatic tissues to the liver (reverse cholesterol transport). It is possible that the ABC1 mRNA is translated very efficiently or that the transporter exhibits a very high rate of substrate turnover.

Examination of the complete expression profile of each tissue reveals several trends regarding defense against xenobiotic toxins, MDR and potential dimeric interactions. The moderate mRNA expression levels of MRPs 1,4 and 5 in brain suggest they, in conjunction with MDR1, may contribute to transport across the blood-brain barrier. Other transcripts moderately expressed in brain and potentially localized at the plasma membrane could also be involved (e.g. WHITE1, ABC3 and ABCA5); however, none of these proteins have previously been associated with defense against toxins. MRPs 2, 4 and 5 were highly expressed in testis RNA and, along with MRP1, may serve a protective function for male germ cells. Additional candidates moderately expressed in testis could include ABC3, ABCA5 and ABCA6. The high expression of several MRPs in the excretory organs liver and kidney is compatible with a role in cellular detoxification by secretion of GSH S-conjugates or organic anions. Further study using RNA isolated from subsections of these organs and pure cell populations is obviously required.

As mentioned, it has been postulated that drug-resistant tumours are frequently derived from normal tissues with high expression of Pgp/MDR1. This may also be the case for other ABC proteins. At one end of the spectrum, MRP1 mRNA is consistently among the most highly expressed ABC transcripts in each tissue; hence, it is not surprising that increased MRP1 mRNA is frequently observed in drug-resistant tumours derived from many different tissue types. Although at somewhat lower levels, both MRP4 and MRP5 transcripts are also broadly expressed. Therefore, these transporters may also contribute to drug-resistance in tumours derived from a wide range of tissue types. MRP2 and MRP3 mRNA species have relatively tissue-specific expression patterns and based on this hypothesis, would only be relevant to liver

and kidney cancers. In contrast, BCRP mRNA is lowly expressed or absent in most tissues, rendering this protein less likely to be causative of drug-resistant tumours.

Mitochondrial proteins MTABC3, ABC7, M-ABC1 and M-ABC2 have been recently identified but their functions are unknown. We have shown that MTABC3 and ABC7 are consistently expressed at higher levels than M-ABC1 and M-ABC2 and this may have implications for the dimeric interaction of these half ABC proteins. In the case of mitochondrial and peroxisomal ABC proteins, some predictions can be made regarding protein associations between half-transporters for transcripts with relatively tissue-specific expression patterns. For example, ALDR is predominantly expressed in brain and is, therefore, most likely to interact with itself or another mitochondrial half-transporter highly expressed in this tissue (e.g. PMP70). In other tissues where ALDR is weakly expressed, it is unlikely to interact with other mitochondrial proteins and may form homo-dimers or be functionally irrelevant. A similar argument can be made for an association between the relatively tissue-specific peroxisomal protein M-ABC2 (predominantly in bone marrow) and the broadly expressed ABC7. However, for most mitochondrial and peroxisomal proteins it is difficult to predict protein associations based on expression data because these transcripts are ubiquitously expressed at variable levels (e.g. PMP70, PMP69 and ALDP).

In general, the mitochondrial proteins were most highly expressed in metabolically active tissues that contain greater numbers of mitochondria (e.g. brain, skeletal muscle and testis). The subtle differences in expression levels among these proteins may be especially important if they act coordinately in the translocation of substrates.

The 4 human peroxisomal transcripts were ubiquitously expressed, albeit at markedly different levels in each tissue. As with the mitochondrial proteins, subtle differences in expression levels among the peroxisomal proteins may be especially important if these proteins act coordinately to translocate substrates.

It is predicted that the three members of subfamily F are involved in protein translation based on their homology to members of the yeast subfamily IV. Members of subfamily F are ubiquitously expressed with the highest levels in brain, skeletal muscle and testis, tissues that exhibit high metabolic activity. ABCF2 is consistently expressed at lower levels than ABCF3 and ABC50. These differences in expression levels among the tissues examined may reflect differences in demand for both the number and types of proteins undergoing translation.

Analysis of the expression pattern of individual ABC transcripts in normal tissues has revealed that these genes can be classified into one of three groups based on their tissue distribution and level of expression. Firstly, a number of transcripts have a relatively tissue-specific pattern of expression such as ALDR (brain), ABCR (retina – not examined), MRP3 (liver and kidney), MRP6 (liver and kidney), MRP2 (liver, testis and kidney), M-ABC2 (bone marrow), ABC2 (brain), CFTR (pancreas), ABCB9 (brain and testis) and BSEP (liver and testis). These transcripts are localized predominantly to one or two tissues, with weak or non-detectable expression in other tissues. Several of these genes are known to have specialized functions related to the tissue in which they are expressed. Examples include ABCR, which transports protonated N-retinylidene-phosphatidylethanolamine out of retinal disk cells, and BSEP, a bile acid transporter. However, the narrow tissue distribution of other genes such as ABCB9 and ABC2 has not provided clear insight as to their normal function. A second class encompasses those transcripts expressed at variable levels in many tissues such as MDR1, ABC3, ABCA5, ALDP, and WHITE1. These transcripts are typically expressed at high levels in one or a few tissues (e.g. MDR – brain and kidney, ABC3 – lung and WHITE1 – brain), but are also expressed at significant levels in many other tissues. It is difficult to speculate on the normal function of these proteins based on expression pattern alone. Finally, the last category consists of transcripts that are ubiquitously expressed and often at high levels such as MRP1, MRP5, TAP1, ABC50 and ABCF2. The fact that these transcripts are present in most cell types and are often expressed

at significant levels implies that these are the “house-keeping” class of ABC genes. These genes are likely to either perform important functions required in most cell types (e.g. TAP1 – immune surveillance, MRP1 – protection against xenobiotics) or are highly active (e.g. ABC50 – translation of mRNA).

Competitive-RT-PCR has been used previously to quantitate ABC transcript species; however, its application has been limited primarily to MDR1 (Hashida et al., 2001; Masuda et al., 2000; Kobayashi, et al., 2000; Illmer, et al., 1999; El-Osta et al., 1999; Al-Katib et al., 1998; Norgaard et al., 1998; Kobayashi et al., 1997; Debuire et al., 1995; Lyttelton et al., 1994; de Kant et al., 1994; and Futscher et al., 1993). A few studies have also examined mRNA expression of other ABC transcripts including MRP1 (Kohler, et al., 1997, MRP1 and MDR1 (Wada et al., 1999; Xu et al., 1999; Xu et al., 1996) and MDR1, MDR3, MRP2, MRP3 and BSEP (Zollner et al., 2001). Published data that can be compared in a quantitative manner to our own results are described in greater detail below.

In the most straightforward application of competitive-RT-PCR, several investigators have applied this assay to study MDR1 mRNA expression in drug-sensitive and drug-resistant cell lines (Table IV.III). These data demonstrate that MDR1 mRNA is not detected in most drug-sensitive cell lines, but is frequently over-expressed at variable levels in drug-resistant cell lines. In comparison, we observed MDR1 mRNA levels ranging from 12 to 11,556 copies per ng of total RNA (0.4 - 910 copies per cell) in the parental SKOV3 cells and vincristine-resistant SKVCR0.25 cells, respectively (chapter III). MRP1 mRNA levels ranged from 17,572 to 269,037 copies per ng of total RNA (585 – 8,967 copies per cell) in SKOV3 and SKVCR0.015 cells, respectively (chapter III). In general, the range of expression of MDR1 mRNA in drug-sensitive and drug-resistant cell lines, as determined using competitive-RT-PCR, is similar to our observations for the SKOV3 series.

**Table IV.III MDR1 Expression in Drug-sensitive and Drug-resistant Cancer Cell Lines**  
**Determined Using Competitive-RT-PCR.**

Cell line (s)	MDR1 expression (# of copies) <sup>a</sup>	Reference
MCF7, ZR-75-1 and T47 D (breast carcinoma) A-431 and KB-3-1 (epidermoid carcinoma) HeLa (cervical carcinoma) HT 1080 (fibrosarcoma) Raji (Burkitt lymphoma) SW 613 S and C13 (colon carcinoma) KHOS-24 (osteosarcoma) KB-3-1 (adriamycin-resistant)	not detected not detected not detected not detected not detected 21,000 per ng each 1,500 per ng 1.4x10 <sup>6</sup> per ng	Debuire et al.(1995)
KB-3-1 (Pgp-negative) KB-8-5 (Pgp-positive)	4 per ng 1960 per ng	Lyttelton et al. (1994)
8226/S (multiple myeloma – sensitive) 8226/dox6 (doxorubicin-resistant) 8226/dox40 (doxorubicin-resistant)	not detected 3,200 per ng 89,000 per ng	Futscher et al. (1993)
8226/S 8226/dox40	not detected 20,000 per ng	Norgaard et al. (1998)
CEM-CCRF (T-lymphocyte) CEM-A7 (doxorubicin-resistant) CEM-A7R (revertant)	not detected 10,350 per cell 272 per cell	El-Osta et al. (1999)
MOLT-3 (acute lymphocytic leukemia) MOLT-3/TMQ <sub>70</sub> (trimetrexate-resistant/70-fold) MOLT-3/TMQ <sub>800</sub> (trimetrexate-resistant/800-fold) MOLT-3/VCR <sub>1000</sub> (vincristine-resistant/1000-fold)	marginally detectable 19,000 per ng 60,000 per ng 190,000 per ng	Kobayashi et al. (1997)

<sup>a</sup>Per ng refers to per ng of total RNA.

For many years there has been great interest in evaluating the role of Pgp and MRP1 in drug resistance in hematological neoplasms. The use of competitive-RT-PCR has been a valuable tool for determining MDR1 and MRP1 mRNA levels in these clinical samples due to its sensitivity and relatively simple and inexpensive application (Table IV.IV). Altogether, the MDR1 mRNA levels in these studies are generally low, although they span a broad range. Since we have not examined ABC transcript levels in clinical samples we have no direct comparison. However, the levels of MDR1 mRNA reported in most of these studies are similar to the range of MDR1 expression we observed in other tissues.

**Table IV.IV MDR1 and MRP1 mRNA Expression in Hematologic Malignancies**

**Determined Using Competitive RT-PCR.**

Study	MDR1			MRP1			Reference
	Average # of copies <sup>a</sup>	Range	N	Average # of copies <sup>a</sup>	Range	N	
AML blasts (disease presentation)	55 per ng	0-1,810 (all categories)	30	N.D. <sup>b</sup>			Lyttelton et al. (1994)
AML blasts (relapse)	130 per ng		14	N.D.			Lyttelton et al. (1994)
AML blasts (refractory)	140 per ng		7	N.D.			Lyttelton et al. (1994)
AML blasts (disease presentation)	360 per ng	0-2,000	31	N.D.			Norgaard et al. (1998)
Various leukemic malignancies	8,500 per ng	0-60,200	69	N.D.			Kobayashi et al. (2000)
AML blasts (mixed stages)	1.5 per cell	0.02-5.4	10	1.8 per cell	0-6.0	10	Xu et al. (1996)
AML blasts (mixed stages)	1.3 per cell	0-15.8	128	7.9 per cell	0.1-36.2	128	Xu et al. (1999)
B-cell tumours	1,280 per ng	0-3,400	10	N.D.			Futscher et al. (1993)
B-cell tumours	N.A. <sup>c</sup>	113-1,240 per cell	4	N.D.			El-Osta et al. (1999)

<sup>a</sup>Per ng refers to per ng of total RNA.

<sup>b</sup>N.D. = not determined.

<sup>c</sup>N.A. = not available.

⊙

Several studies have examined ABC mRNA expression using competitive-RT-PCR in normal and cancerous solid tissues (Table IV.V). In comparison to the data of Zollner et al. (2001), we observed mRNA levels of 13,604 (MRP2), 560 (BSEP), 353 (MDR3), 454 (MDR1), and 2154 (MRP3) in normal liver RNA from a single individual. The observation by Debuire et al. (1995) of an average MDR1 mRNA level of 815 copies in normal liver biopsies is also comparable to the 454 copies per ng total RNA that we observed. However, Debuire et al. observed higher average MDR1 levels in breast tumours (4,300 copies) than we observed in pooled normal breast (91 copies per ng of total RNA). Although we have no data that is directly

comparable to that of Hashida et al. (2001), the range of MDR1 mRNA levels is comparable to what we have observed in other tissues.

**Table IV.V ABC Transcripts Expression in Normal and Cancerous Tissues Determined Using Competitive RT-PCR.**

Study	ABC transcript	Average # of copies per ng of total RNA	Range	N	Reference
Normal liver biopsy	MRP2	1,600	N.A. <sup>a</sup>	13	Zollner et al. (2001)
	BSEP	560	N.A.	13	
	MDR3	400	N.A.	13	
	MDR1	360	N.A.	13	
	MRP3	110	N.A.	13	
Normal liver biopsy	MDR1	815	180-1,800	8	Debuire et al. (1995)
Breast tumours	MDR1	4,300	0-14,700	8	
Intestinal mucosa of liver transplant patients	MDR1	247	12-3,000	48	Hashida et al. (2001)

<sup>a</sup>N.A. = not available.

In comparison to competitive-RT-PCR, only a few studies have utilized real-time-PCR to quantify ABC mRNA species (Khokhar et al., 2001; Faneyte et al., 2001; Kielar et al., 2001; Taipalensuu et al., 2001; Yoshida et al., 2001; Takamura et al., 2000 and Hinoshita et al., 2000). Unfortunately, most of these studies have presented mRNA levels as a ratio to a housekeeping gene, preventing a direct comparison with our own results. One exception is the work of Taipalensuu et al., (2001) who conducted the most comprehensive study of ABC mRNA expression to date. They examined expression of 10 ABC mRNAs in jejunal biopsies from 13 healthy human subjects, as well as in human intestinal epithelial Caco-2 cells. The average mRNA expression in copies per ng of total RNA were as follows: BCRP (2,700), MRP2 (2,400), MDR1 (790), MRP3 (460), MRP6 (250), MRP5 (210), MRP1 (180), MRP4 (65), MDR3 (5.2)

and ABCB5 (undetectable). In general, the expression levels of these mRNA transcripts in jejunum correlated with that of Caco-2 cells, although BCRP mRNA exhibited a 100-fold lower transcript level in the latter. We cannot directly compare these data to our results since we did not examine intestinal tissues; however, the range of expression levels reported by Taipalensuu et al. is comparable to what we have observed in other tissues. A second report by Kielar et al. (2001) examined ABC1 mRNA expression in normal human tissues. They reported ABC1 mRNA levels (copies per ng of total RNA) of approximately 30,000 in liver, 6,000 in lung, 2,500 in trachea and kidney, 1,500 in brain, and 1,000 in heart. However, they also reported that Northern blot analysis with these same tissues required a 1-week exposure to visualize these bands, suggesting that the real-time-PCR values may be over-estimated. As discussed previously, we observed ABC1 mRNA to be ubiquitously expressed at low levels.

Another approach to evaluate our competitive-RT-PCR data is to compare these results to available SAGE data. One of the most notable strengths of the SAGE method is that the data represents absolute expression levels, based on the digital enumeration of transcript tags in a total population of transcripts. This allows the expression level of any gene to be compared with that of any other gene, from among many libraries of different sources and sizes. The mRNAs of a typical human somatic cell are distributed in three frequency classes (Davidson and Britten, 1979 and Bishop et al., 1974). In a typical cell, 5-10 species of abundant mRNA comprise at least 20% of the total mass of mRNA (~5,000 copies per cell), 500-2,000 species of intermediately expressed mRNAs comprise 40%-60% of the total mRNA mass (15-5,000 copies per cell), and 10,000 to 20,000 rare messages may account for < 20%-40% of the total mRNA mass (< 15 copies per cell). A summary of SAGE data in 84 libraries revealed detectable expression levels ranging from 0.3 to 9,417 transcript copies per cell (Velculesu et al., 1999). Our expression analysis of 34 ABC transcripts in 12 normal human tissues revealed an average of 24 copies per cell (range: 0 – 561), placing this group of mRNAs near the upper range of the

third, rare class of transcripts. This is not surprising given that cytoplasmic proteins are typically expressed at much higher levels than nuclear or membrane proteins. For example, Drawid et al. (2000) summarized SAGE data from a number of published yeast studies and reported the average expression of cytoplasmic mRNAs was 14.4 copies per cell whereas for membrane mRNAs it was 2.4 copies per cell. Due to the low-level expression of ABC transcripts in most RNA sources, it is difficult for researchers using genome-scale expression analysis methods (e.g. SAGE and cDNA microarray) to detect these species.

In an attempt to compare our competitive-RT-PCR data with SAGE data for each ABC gene, we searched the "Tag to Gene" site at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/SAGE/>). Searching against a database of 100 SAGE libraries and > 4 million total tags, we observed that there were no tags present in any library for a number of ABC mRNAs (data not shown). Furthermore, for those ABC mRNAs for which tags were present, the number of tags per library was in most cases below 5, and these transcripts were present in only a handful of libraries, negating the potential for statistical comparison of expression levels. In contrast, the housekeeping gene  $\beta_2$ microglobulin averaged ~100 tags per library and was detectable in every library.

In conclusion, we have applied competitive-RT-PCR to compare, in a quantitative manner, RNA expression levels among 34 ABC transcripts in cell lines (chapter III) and normal tissues. The expression profiles of each tissue were unique. Overall, ABC transcripts were weakly expressed in comparison to the housekeeping gene  $\beta_2$ microglobulin, and could be categorized among the third class of rare transcripts in cells, as would be expected for membrane proteins. Detailed knowledge of ABC transcript levels will have important implications in determining the relative contribution of ABC proteins to MDR, defense against xenobiotic toxins, phospholipid and cholesterol homeostasis and dimeric interactions. The expression level of a transcript in bulk tissues is, however, only one of several factors that contribute to the

functional activity of an ABC transporter. Other factors include: the expression pattern among sub-compartments within tissues and in specialized cell types; the subcellular localization of the protein; the affinity of the transporter for its substrate(s); protein half-life; and the efficiency of substrate turnover of the transporter. Therefore, the rate of efflux of a specific substrate out of a cell is dependent on the steady-state mRNA levels of relevant transporters, as well as the relative affinity and efficiency of each transporter. Future experiments should attempt to determine not only RNA expression levels, but also the functional activity of ABC transporters in a manner that permits quantitative comparisons among groups of transporters present in the same tissues.

## CHAPTER V

### BAX MEDIATED DOWN-REGULATION OF MDR1 mRNA and Pgp

#### INTRODUCTION

##### 5.1 Apoptosis Mediates the Cytotoxicity of Chemotherapeutic Agents

The growth of any tissue, whether normal or malignant, is determined by the quantitative relationship between the rate of cell proliferation and the rate of cell death. Since a cell must acquire several mutations to progress to the malignant phenotype, and because these mutations are often induced by compounds that damage the cell over an extended period of time, any events that alter this kinetic balance in favor of an enhanced survival of damaged cells promote the carcinogenic process. A successful cancer therapy requires shifting the quantitative relationship between cell proliferation and cell death such that the latter exceeds the former. Detailed knowledge of the process, including identification of critical molecules, which regulates the balance between cell proliferation and cell death in diseased tissues is critical to maintain the balance in favour of cell death (for review see Denmeade and Isaacs, 1996).

The terms apoptosis or programmed cell death are used to distinguish the active, orderly and cell-type specific death observed in organisms from necrotic cell death (Kerr et al., 1972). Necrotic death is a response to pathologic changes initiated in the extracellular environment such as mechanical damage or exposure to toxic chemicals. When cells are damaged by acute injury, the ability of the plasma membrane to control the passage of ions and water is disrupted resulting in increased plasma membrane permeability. This increased permeability results in cellular edema and in the eventual osmotic lysis of the cell and inflammation of surrounding tissues. In necrotic cell death, the cell has a passive role in initiating the process of death, whereas, in

apoptosis, a cell undergoes an energy-dependent cellular death program initiated by specific signals in an otherwise normal extracellular environment.

Apoptosis plays an integral part in a variety of biological events involving selective elimination of mammalian cells including morphogenesis, cell turnover and removal of harmful cells. Apoptosis is initiated in specific cell types by both endogenous tissue-specific agents (hormones or locally diffusing chemicals) and exogenous, cell-damaging treatments (e.g. radiation, chemotherapeutic drugs and viruses). Endogenous activation of apoptosis can occur due to the positive presence of a tissue-specific inducer. In other cases, cells appear to have a default death pathway that must be actively blocked by a survival factor to prevent apoptosis. Once initiated, apoptosis leads to a cascade of biochemical and morphological events that result in the irreversible degradation of the genomic DNA and destruction of the cell.

The temporal sequence of events of apoptosis comprises chromatin aggregation, nuclear and cytoplasmic condensation and eventual fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies). Once formed, these apoptotic bodies are rapidly recognized, phagocytized and digested by macrophages or adjacent epithelial cells. Apoptosis, like the cell cycle of proliferation, can be divided into distinct temporal phases. During the first phase, D<sub>1</sub>, a series of genes is either repressed or activated. These epigenetic changes result in the activation of double-stranded DNA fragmentation during the next step, the F phase. It is during the F phase that the nuclear morphology changes, although cell membranes remain intact and mitochondria are still functional. During the final phase (D<sub>2</sub>) a series of proteases is activated to catalyze degradation of lamins, nuclear fragmentation and plasma membrane blebbing, leading to the eventual formation of apoptotic bodies.

In general, there are three types of gene products involved in the process of apoptosis. The first are those involved in generating the signal transduction for activation of apoptosis. This list includes growth factors (androgens/estrogens, interleukins and TGF- $\beta$ 1), growth factor

receptors and oncogenes (c-myc). The second type of apoptosis gene encodes proteins involved in determining the sensitivity to activation of apoptosis after the process is initiated by pathologic damage to the cell. Examples of this second class of genes include p53 and BCL-2 family members (see below). Functional expression of p53 increases the sensitivity of cells to activation of apoptosis induced by a wide variety of damaging agents, whereas functional expression of BCL-2 decreases the sensitivity of cells to activation of apoptosis by these same agents. A third group of genes encode proteins that carry out the programmed steps of cell death. These include endonucleases and proteases (ICE family and calpain).

For many years, the identity of the molecules essential for apoptosis remained unknown until genetic analysis of *C. elegans* revealed two loci, *ced-3* and *ced-4*, essential for the initiation of apoptosis during worm development, and a third loci, *ced-9*, that could negatively regulate their action (Ellis and Horvitz, 1986 and Hengartner et al., 1992). The first mammalian regulator emerged when BCL-2 transfected B cells were shown to be resistant to apoptosis normally induced in B cells by IL-3 withdrawal (Vaux et al., 1988). For the first time it was shown that cell survival and proliferation were under separate genetic control and that disturbances in both were likely to contribute to cancer.

The mechanism of apoptosis is highly conserved. Expression of the human BCL-2 gene in *C. elegans* demonstrated that *Ced-9* and BCL-2 are functional and structural homologs (Vaux et al., 1992). The survival function of these genes is opposed either by close relatives such as BAX (Oltvai et al., 1993) or by more distantly related proteins. The actual machinery needed to carry out the program of apoptosis was identified when *ced-3* proved to be orthologous to mammalian interleukin-1 $\beta$ -converting enzyme (Yuan et al., 1993). Members of this new family of proteases, now called caspases, are sequentially activated and function in both cell disassembly and in initiating the disassembly in response to pro-apoptotic signals (Thornberry and Lazebnik, 1998). Synthesis of caspases as minimally active precursors precludes their

premature activation. *Ced-4* and its mammalian homolog APAF-1 interact with cytochrome c and facilitate the autocatalysis of procaspase-9 that initiates the proteolytic cascade (Zou et al., 1997).

Members of the BCL-2 family are somehow able to register diverse forms of intracellular damage, gauge whether other cells have provided a positive or negative stimulus, and integrate these competing signals to determine if a cell is to undergo apoptosis. All members of the BCL-2 family possess at least one of four conserved motifs known as BCL-2 homology domains (BH1-BH4). Anti-apoptotic members such as BCL-2, BCL-x<sub>L</sub>, BCL-w, MCL-1, A1 and BOO share sequence homology within regions BH1 and BH2, and to a lesser extent within BH3 and BH4. Pro-apoptotic members include BAX, BAK, BAD, BOK and DIVA, and these proteins share homology in BH1, BH2 and BH3, but not BH4. "BH3-only proteins", which are also pro-apoptotic, include BIK, BID, BIM, HRK, BLK, BNIP3 and BNIP3L, and share sequence homology only in BH3 (see Adams and Cory, 1998 and Tsujimoto and Shimizu, 2000 for reviews).

Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate one another's function, suggesting that their relative concentration may act as a rheostat for the suicide program (Oltvai et al., 1993). A crystal structure of BCL-x<sub>L</sub> demonstrated that this heterodimerization is likely mediated by the insertion of a BH3 region of a pro-apoptotic protein into a hydrophobic cleft composed of BH1, BH2 and BH3 from an anti-apoptotic protein (Muchmore et al., 1996). Over-expression of the pro-apoptotic BAX protein has been shown to accelerate apoptotic death induced by cytokine deprivation in an interleukin3-dependent cell line, an effect that is associated with the formation of BAX/BAX homodimers (Oltvai et al., 1993). Conversely, over-expression of anti-apoptotic proteins such as BCL-2 and BCL-x<sub>L</sub> represses the death function of BAX, which is associated with the formation of BCL-2/BAX or BCL-x<sub>L</sub>/BAX heterodimers. Thus, the ratio of BAX to either BCL-2 or BCL-x<sub>L</sub> appears to be a critical

determinant of a cell's threshold for undergoing apoptosis (Oltvai and Korsmeyer, 1994). The specificity of these interactions is variable and the tendency of individual proteins to interact may range from promiscuous to specific.

Although different classes of chemotherapeutic drugs and ionizing radiation have distinct mechanisms of actions, their cytotoxic activity is frequently mediated through a final common pathway that involves the activation of apoptosis (Lowe et al., 1993). For instance, exposure of cells to a variety of cytotoxic agents including platinum analogs, taxanes, anthracyclines and *vinca* alkaloids leads to internucleosomal DNA fragmentation and activation of the cell-death machinery (da Silva et al., 1996; Dole et al., 1995 and Bhalla et al., 1993). These observations have led to the hypothesis that an intact apoptotic pathway is necessary for chemotherapy-induced death and conversely, that abnormalities in the ability of a cell to undergo apoptosis may lead to the development of resistance to chemotherapy. When intact, this pathway rids the body of abnormal, pre-cancerous cells by triggering a cellular self-destruction mechanism. When this pathway is disrupted (by the loss of p53 function, for example), pre-cancerous cells survive and proliferate resulting in cancer. It has been shown that cells that are functionally deficient in p53 or that express elevated levels of either BCL-2 or BCL-x<sub>L</sub> are relatively resistant to cytotoxic agents such as platinum analogs (Lowe et al., 1993; Dole et al., 1995; and Lowe et al., 1994).

Inactivation of the p53 tumour suppressor gene occurs in over half of all human tumours, implying the loss of this gene represents a fundamentally important step in the pathogenesis of cancer (Hollstein et al., 1991 and Levine et al., 1991). Presumably, the significance of p53 loss in cancer treatment can be explained by the fact that many chemotherapeutic drugs induce tumour cell death through apoptosis; and that the relative sensitivity of tumour cells to induction of apoptosis by drugs and radiation is modulated by p53 in many cases. The p53 protein functions at least in part as a transcriptional regulator and can transactivate cellular genes through interactions with conserved regions within their promoters. For instance, it has been

shown that wild-type p53 is capable of both down-regulating BCL-2 and up-regulating BAX, thereby predisposing cells to programmed cell death (Miyashita et al., 1994 and Miyashita and Reed, 1995).

The pro-apoptotic BAX protein directly promotes apoptosis by inserting into mitochondrial membranes and forming channels for the release of cytochrome c, which initiates the cascade of caspase activity (see Green and Reed, 1998 for a review). A number of findings implicate the loss of function of the pro-apoptotic BAX in oncogenesis. First, the BAX promoter contains several consensus p53-binding sites and can be a direct transcriptional target of p53 (Miyashita and Reed, 1995). Animal studies also support BAX as an effector of p53-dependent apoptosis. BAX deficiency decreases apoptosis and accelerates oncogenesis in truncated SV40 Tag<sup>3</sup> transgenic mice susceptible to brain tumors (Yin et al., 1997). Accelerated tumourigenesis is also observed in *bax*<sup>+/-</sup> Tag transgenic mice susceptible to mammary tumors (Shibata et al., 1999). *In vitro* studies of fibroblasts expressing the E1A oncogene, a setting where apoptosis is dependent on endogenous p53, also show that BAX deficiency decreases apoptosis and promotes transformation (McCurrach et al., 1997). Together these findings provide evidence that BAX partly mediates the p53-dependent apoptosis induced by these two potent viral oncogenes. Adding further support to the importance of BAX in the carcinogenesis process, Zhang et al. (2000c) generated human colorectal cancer cells lacking functional BAX and demonstrated these cells to be partially resistant to the apoptotic effects of 5-fluorouracil. Furthermore, BAX-deficient cells were completely lacking in apoptotic response to the chemopreventive agent sulindac and other non-steroidal anti-inflammatory drugs. It has also been shown that BAX is a direct transcriptional target of c-MYC through binding of the latter to an E-box element within the BAX promoter and in doing so, BAX contributes to c-MYC-induced apoptosis (Mitchell et al., 2000). Finally, Knudson et al. (2001) compared tumour development in *bax*-deficient and BAX-transgenic mice in the presence or absence of p53. *bax*-deficiency did not accelerate

oncogenesis with or without wild-type p53. BAX-transgenic mice showed increased apoptosis consistent with the pro-apoptotic function of BAX, however these mice did have an increased incidence of T-cell lymphomas and an increased percentage of cells in cycle. Knudson et al. concluded that BAX can either accelerate or inhibit tumourigenesis depending on the genetic context.

Several clinical studies have examined the expression level of BAX, as well as the presence of mutations within the coding region of BAX, in normal and cancerous tissues. These data have implicated BAX as an important tumour suppressor. For example, frameshift mutations in BAX are found frequently in tumours (colorectal cancer and gastric carcinoma) with the microsatellite mutator phenotype (Yamamoto et al., 1999 and Rampino et al., 1997). Mutations in BAX have been described in a number of human hematopoietic tumor cell lines (Meijerink et al. 1998) as well as directly from gastrointestinal tumours (Gil et al., 1999). Retrospective studies examining the relationship between BAX expression and clinical outcome have demonstrated that reduced expression of BAX is associated with poor clinical outcome in ovarian cancer (Tai et al., 1998), metastatic breast adenocarcinoma (Krajewski et al., 1995) and squamous cell carcinoma (Xie et al., 1999). In contrast, increased BAX expression correlated with a high rate of relapse in childhood acute lymphoblastic leukemia (Hogarth and Hall, 1999).

To investigate the role of BAX in chemotherapy-induced apoptosis, Strobel et al. (1996) transfected the SW626 ovarian carcinoma cell line, which lacks functional p53, with a hemagglutinin (HA) tagged cDNA encoding for murine BAX (HA-BAX). SW626 BAX-transfectants were significantly more sensitive to paclitaxel, vincristine and doxorubicin compared to SW626 cells transfected with the pSV2-neo plasmid alone (neo-transfectants). Conversely, the cytotoxicity profile of carboplatin, etoposide and hydroxyurea was unchanged; suggesting that the ability of BAX to sensitize cells to apoptotic cell death is stimulus-dependent. The authors also demonstrated by morphologic and flow cytometric criteria that paclitaxel-

induced cytotoxicity of BAX-transfectants was associated with enhanced apoptosis. In a subsequent study, Strobel et al. (1997) demonstrated that stable over-expression of BAX in SW626 cells was also not capable of enhancing apoptotic cell death in response to ionizing radiation. These results suggest over-expression of BAX promoted apoptosis, upon exposure to specific chemotherapeutic drugs, via a p53-independent pathway.

The mechanism by which BAX over-expression sensitized cells to specific chemotherapeutic drugs was unclear. All six clones (3 HA-BAX transfected and 3 control) expressed similar levels of BCL-2 and BCL-x<sub>L</sub> by western blot, which did not change in the presence of paclitaxel, making it unlikely that diminished expression of these molecules is responsible for the observed effect. The fact that none of the cell lines expressed significant levels of Pgp or MRP1, as assessed by flow cytometry and immunoblot analysis, excludes a role for these drug transporters in the observed effects of BAX on paclitaxel cytotoxicity. The growth and viability of HA-BAX transfectants was indistinguishable from that of neo-transfected cells, suggesting that the presence of high levels of BAX by itself is not sufficient to trigger apoptosis without an additional stimulus produced by a cytotoxic agent.

Most studies have focused on the ability of BAX to influence the most distal steps in the apoptotic pathway, especially those involving mitochondrial function (e.g. permeability transition and cytochrome c release). However, the fact that BAX can sensitize ovarian cancer cells to paclitaxel and not to classical DNA-damaging agents led Strobel et al. (1998) to propose that this protein may function indirectly through a mechanism that is proximal to the mitochondria, specifically by affecting the early events that are associated with paclitaxel-mediated cytotoxicity. In SW626 BAX-transfectants, multiple, seemingly disparate proximal actions of paclitaxel are enhanced in the presence of elevated levels of BAX protein. These include: enhanced G<sub>2</sub>-M phase arrest in BAX-transfectants which leads to the majority of cells exhibiting a large sub-G<sub>0</sub> content; enhanced paclitaxel-induced tubulin polymerization in BAX-

transfectants; and more rapid and extensive BCL-2 phosphorylation in BAX-transfectants (Strobel et al., 1998). A unifying explanation for these observations is that diminished drug efflux by these cells results in enhanced intracellular paclitaxel levels. This effect was documented by both [<sup>3</sup>H]-paclitaxel uptake studies and independently validated through the use of confocal microscopy. This drug efflux mechanism, when inhibited by verapamil, resulted in elevated intracellular paclitaxel levels and the restoration of drug sensitivity in neo-transfectants (Strobel et al., 1998). The precise nature of this pump was uncertain, and as mentioned, known candidate molecules were excluded based on insignificant expression levels.

To determine if differential expression of an ABC transporter other than Pgp or MRP1 could be responsible for the observed sensitivity of BAX-transfectants to paclitaxel, we applied competitive-RT-PCR to examine the ABC RNA expression profile of BAX- and neo-transfected SW626 cells.

## MATERIALS AND METHODS

### 5.2.1. Cell Lines

Stable SW626 human ovarian epithelial carcinoma clones transfected with either pSV2-neo plasmid (A10, B10 and F8) or murine BAX cDNA present in a HA<sup>3</sup>/pSFFV expression vector (A9, D7 and D8) were used for transfection and expression studies and have been described previously (Strobel et al., 1996). Cells were cultured in Dulbecco's modified eagle's medium (DMEM, Stem Cell Technologies) supplemented with 10% FBS (Gibco BRL) and 1/100 streptomycin/penicillin (Gibco BRL) at 37°C with 5% CO<sub>2</sub> in a humidified incubator. Both neo-transfectants and HA-BAX-transfectants were selected for neomycin resistance in the presence of 500 µg/ml G418 every third cell passage. The three SW626 HA-BAX-transfected cell lines used in these studies have been characterized previously and express a mean 10-fold higher level of BAX protein than neo-transfected cells (Strobel et al., 1996).

### 5.2.2 Plasmids

The pGL2-basic, pGL2-basic(-434/+105), as well as all other pGL2-basic plasmids containing 5' deletions of the 539 bp MDR promoter were kindly provided by Dr. Marilyn Cornwell, Fred Hutchinson Cancer Research Center, Seattle. The pGL2-basic vector contains the luciferase gene without the SV40 promoter and serves as a promoter-less control. The MDR1 promoter 5'-deletion constructs have been cloned upstream of the luciferase gene in the pGL2-basic vector.

The pRL-TK vector and pcDNA3 vector containing a full-length cDNA for  $\beta$ -galactosidase were generous gifts from Dr. Douglas Hogue, Dalhousie University, Halifax. The pRL-TK vector contains the herpes simplex virus thymidine kinase promoter region upstream of the *renilla* luciferase gene. This promoter provides low-level, constitutive expression.

All plasmids used for transfection studies were prepared using the Qiafilter midi kit according to manufacturer's instructions (Qiagen).

### **5.2.3 Preparation of Total RNA**

Total RNA was prepared as described in section 2.2.2 of this thesis.

### **5.2.4 Reverse Transcription**

Reverse transcription was performed as described in section 2.2.4 of this thesis.

### **5.2.5 Amplification and Analysis of PCR Products**

Amplification and analysis of PCR products was performed as described in section 2.2.5 of this thesis.

### **5.2.6 Preparation of Total Cell Lysate and Total Membrane Protein**

SW626-transfectant cell lines were grown to ~80% confluence in 150 mm plastic dishes. The media was removed via aspiration and cells were rinsed with PBS containing 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>. To obtain total cell lysate, 6 mL of lysis solution [50 mM Tris-HCl (pH 8.0), 2% (v/v) SDS and ½ tablet of complete protease inhibitor cocktail (Roche)] was added to the cell monolayer (one 150 mm dish) and cells were lifted using a plastic scraper. To shear DNA and reduce the viscosity of the solution, lysates were passed through a 22-gauge needle 5 times at 4°C. Cell debris was pelleted at 5,000 revolutions per minute (rpm) for 2 min at 4°C and the remaining supernatant containing cellular protein was stored at -80°C until needed. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich) according to the manufacturer's instructions. A portion of the total cell lysate was boiled for 5

min to solubilize proteins prior to resolution using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

To isolate the total membrane protein fraction, the cell monolayer (one 150 mm dish) was removed using a plastic scraper. All subsequent steps were conducted at 4°C. Cells were pelleted at 400 rpm for 2 min. After discarding the supernatant, cells were resuspended in ice-cold RSB solution [10 mM Tris-HCl (pH 8.0), 10 mM NaCl and 1.5 mM MgCl<sub>2</sub>] and incubated for 10 min to permit cell swelling. Cells were homogenized using a tight-fitting dounce (50 strokes). After cell disruption, 1.4 mL of 2.5x MSB solution was added [525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl (pH 8.0), 2.5 mM EDTA and ½ tablet of complete protease inhibitor cocktail per 10 mL of 2.5x MSB solution] and inverted to mix, followed by the addition of 1x MSB solution to a final volume of 14 mL. Lysates were centrifuged at 2,400 rpm for 5 min to pellet nuclei and unbroken cells. The recovered supernatant was transferred to a new tube, with the volume adjusted with 1x MSB, and then centrifuged a second time at 12,900 rpm for 15 minutes to pellet mitochondria. The supernatant containing cytosol and other lighter membranes was transferred to new tubes, with the volume adjusted with 10 mM Tris-HCl (pH 8.0), 2 mM EDTA and 50 mM NaCl, and centrifuged a third time at 35,000 rpm for 60 min to pellet the remaining cellular membranes. The pellet was resuspended in 10 mM Tris-HCl (pH 8.0) and 50 mM sucrose. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich) according to manufacturer's instructions. To prevent the formation of aggregates of high molecular weight membrane proteins, this protein fraction was not boiled prior to loading on a SDS-PAGE gel.

### **5.2.7 Antibodies**

Primary antibodies used for the characterization of SW626-transfectants in immunoblot analysis were as follows: Murine C219 anti-human Pgp monoclonal antibody [1:2,000 dilution;

generated in the Ling laboratory (Kartner et al., 1985)]; mouse HA.11 anti-HA monoclonal antibody (1:1,000 dilution; BAbCO); rat anti-human MRP1 monoclonal antibody (1:1,000 dilution; Research Diagnostics). Secondary antibodies were either sheep anti-mouse Ig conjugated to horseradish peroxidase (1:10,000 dilution; Amersham) or rabbit anti-rat Ig conjugated to horseradish peroxidase (1:12,000; Amersham), as appropriate.

#### **5.2.8 Immunoblotting**

Total cell lysates (50 µg per lane) and total membrane preparations (300 µg per lane) were resolved by one-dimensional 13.5% (cell lysate) or 6% (membrane protein) SDS-PAGE under reducing conditions (Laemmli, 1970), followed by transfer onto a 0.45 µm polyvinylidene difluoride membrane (Millipore) in transfer buffer at 0.2 A for 2 hours. After transfer, residual binding sites were blocked by incubating the membrane in blocking buffer [Tris-buffered saline (TBS)/0.2% Tween-20] with 5% milk powder for 2 hours at rt. Membranes were washed with TBS and were then incubated with 1% milk powder/blocking buffer with polyclonal antibodies for 1 hour at rt. The blots were then washed three times for 10 min in TBS, followed by incubation with secondary antibody conjugated to anti-mouse (Pgp and HA) or anti-rat (MRP1) Ig for 30 hours at rt. After three washes for 10 min in TBS, the blots were developed using the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's protocol and exposed to X-ray film (Eastman Kodak). The protein levels were quantified by densitometry of the autoradiograms.

#### **5.2.9 Luciferase Reporter Assay**

Initial transfection experiments using SW626-transfectants and pGL2-basic(-434/+105), pGL2-basic and pcDNA3 containing the β-galactosidase cDNA under a constitutive promoter indicated poor efficiency (3-5%) using the transfection reagent Lipofectamine (Invitrogen). β-

galactosidase staining was evaluated 24 hours after the plasmid DNA was added to the cells. Briefly, cells were rinsed with 1 mL PBS, 1 mL of fix solution (PBS with 2% formaldehyde and 0.2% glutaraldehyde) was added and cells were placed on ice for 5 min. After cells were washed 3 times with 2 mL PBS, 1 mL of stain solution (PBS with 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, 2 mM  $MgCl_2$  and 1 mg/mL X-galactosidase) was added and cells were incubated at 37°C for 4-5 hrs until blue staining developed. The percentage of  $\beta$ -galactosidase staining cells was determined from two counts of 100 cells each.

In these experiments, MDR1 promoter activity measured as a function of luciferase activity was normalized to protein content as determined using the BCA assay. Using this approach, we found the data to be inconsistent and did not observe any difference in reporter activity among the BAX- and neo-transfected cell lines (data not shown). To improve our transfection efficiency we repeated these experiments using another lipid-based commercial reagent, FuGENE 6 (Roche). We observed a transfection efficiency similar to that with Lipofectamine; however, due to the easier application of FuGENE compared to Lipofectamine (FuGENE is less toxic to cells), we opted to use this reagent for all subsequent experiments. We later determined the optimal ratio of FuGENE:plasmid DNA to be 2:1 (evaluated a range 3:2, 2:1, 3:1, 6:1 and 9:1) based on  $\beta$ -galactosidase activity.

In our optimized protocol, exponentially growing SW626 cells (neo- or BAX-transfectants) were trypsinized, counted and seeded into 6-well plates at  $2 \times 10^5$  cells/well, with 2 mL of 10% FBS and DMEM without antibiotics. Cells were grown for 24 hours until ~70% confluence was achieved. Transfection was conducted according to the manufacturer's instructions (Roche). Briefly, a total of 1,500 ng of plasmid DNA was added per well with a 10:1 ratio of pGL2-basic or pGL2basic(5' deletion construct):pRL-TK. In addition, each well received 3  $\mu$ L of FuGENE and DMEM up to a final volume of 100  $\mu$ L. The reagents were gently mixed and incubated at rt for 30 min before adding to cells. After addition of transfection

mixture, cells were allowed to grow for an additional 24 hours. Unlike most transfection reagents, fresh medium does not need to be added prior to the 24-hour incubation since the toxicity of FuGENE is relatively low (Roche).

Cells were harvested by first aspirating the media, then adding 1 mL of PBS per well, washing and aspirating. Next, 1 mL of PBS with 1 mM EDTA was added to the cells and incubated for 2-3 min at rt until cells became detached and could be transferred to an eppendorf tube. Cells were pelleted by spinning at 3,500 rpm for 1.5 min at 4°C. After removal of the supernatant, cells were frozen at -80°C prior to processing. Cells were lysed by adding 100 µL of Promega Passive Lysis Buffer and luciferase analysis was conducted using the Stop and Glo system according to the manufacturer's instructions (Promega). A Microplate Luminometer L8 96V (EG&G Berthold) was used for all analysis. For each independent experiment, all transfections were performed in triplicate wells.

## RESULTS

### 5.3.1 Drug Resistance Profile of SW626 BAX- and Neo-transfectants

Table V.I reports the levels of resistance, to a panel of cytotoxic drugs, of SW626 BAX- and neo-transfectants. The BAX-transfectants are significantly more sensitive to the Pgp substrates paclitaxel, vincristine and doxorubicin compared to neo-transfectants, whereas there is little difference for the Pgp substrate etoposide and non-Pgp substrates carboplatin and hydroxyurea.

**Table V.I Levels of Drug Resistance in SW626 BAX- and Neo-transfectants<sup>a</sup>.**

<u>Drugs</u>	<u>LD<sub>50</sub><sup>b</sup>(<math>\mu</math>M)</u>			
	<u>Neo-transfectants</u>	<u>BAX-transfectants</u>	<u>P value<sup>c</sup></u>	<u>LD<sub>50(neo)</sub>/LD<sub>50(BAX)</sub></u>
Paclitaxel	0.20 $\pm$ 0.03	0.02 $\pm$ 0.01	0.001	10.0
Vincristine	0.142 $\pm$ 0.11	0.006 $\pm$ 0.001	0.0001	23.7
Doxorubicin	0.21 $\pm$ 0.07	0.035 $\pm$ 0.02	0.001	6.0
Carboplatin	9.5 $\pm$ 2.0	12.0 $\pm$ 3.0	0.2	0.79
Etoposide	2.8 $\pm$ 0.8	2.6 $\pm$ 0.9	0.2	1.08
Hydroxyurea	0.025 $\pm$ 0.005	0.026 $\pm$ 0.009	0.2	0.96

<sup>a</sup>Data taken from Strobel et al., 1996.

<sup>b</sup>Cells were grown at sub-confluent density in plastic wells over a 3-day period in the presence of various drugs as indicated, followed by trypsinization and assessment of viability by trypan blue exclusion. LD<sub>50</sub> values reflect the amount of drug necessary to achieve 50% cell kill compared to cells grown in medium alone. Data are expressed as mean  $\pm$  SEM LD<sub>50</sub> for either neo-transfected cell lines (B10, A10 and F8) or BAX-transfected cell lines (D8, A9 and D7).

<sup>c</sup>P values determined by two-sided Student's *t* test.

### 5.3.2 Assessment of RNA Integrity

The quality of total RNA isolated from SW626 BAX- and neo-transfectants was assessed by RT-PCR amplification of full-length  $\beta_2$ microglobulin (Figure 5.1). There was no PCR product in the control lanes containing non-reverse-transcribed RNA or H<sub>2</sub>O as template and the PCR product representing full-length  $\beta_2$ microglobulin was present at approximately equal levels in all 6 cell lines. Cell viability was determined to be greater than 95% for each of the four cell lines.



**Figure 5.1. RT-PCR of Full-Length  $\beta_2$ microglobulin in Total RNA From SW626 Neo- or BAX-transfected Cell Lines.**

See Materials and Methods section 2.2.2 for details.

### 5.3.3 Competitive-RT-PCR Expression Profile of SW626 Neo- and BAX-transfected Cell Lines

We have applied competitive-RT-PCR to examine the expression profile of 30 ABC transcripts and the housekeeping gene  $\beta_2$ microglobulin in total RNA from neo-transfected (A10)

and BAX-transfected (D8) cell lines (Figure 5.2). The percentage of ABC transcripts expressed at detectable levels was 83% in both cell lines. This is similar to the average observed in normal tissue RNA (90%, Chapter IV) and SKOV3 and HL60 cells (75% and 60%, respectively, Chapter III). The percentage of ABC transcripts expressed at less than 1000 copies per ng of total RNA, the detection limit of Northern blot or cDNA microarray analyses, was 86% in A10 cells and 90% in D8 cells. Comparable values from normal tissues, SKOV3 and HL60 cell lines were 81%, 77% and 69%, respectively. This value is relatively high in SW626 cells, in part, because we chose not to examine the expression of ABC50, ABCF2 and ABCF3 mRNAs, which are ubiquitously expressed at high levels. These genes likely do not encode membrane proteins and therefore would be incapable paclitaxel transport. Transcripts associated with MDR (MDR1, MRPs 1-5, BCRP, LRP and GST- $\pi$ ), along with  $\beta_2$ microglobulin, were measured in triplicate with an average COV of 21% and 26% in A10 and D8 cell lines, respectively. This is similar to the COV for the assay (20%) that was determined using full-length ABC7 transcript spiked into an *E. coli* background (Chapter II), as well as the average COV for 39 transcripts in SKOV3 cells (31%) and HL60 cells (27%) (Chapter III).

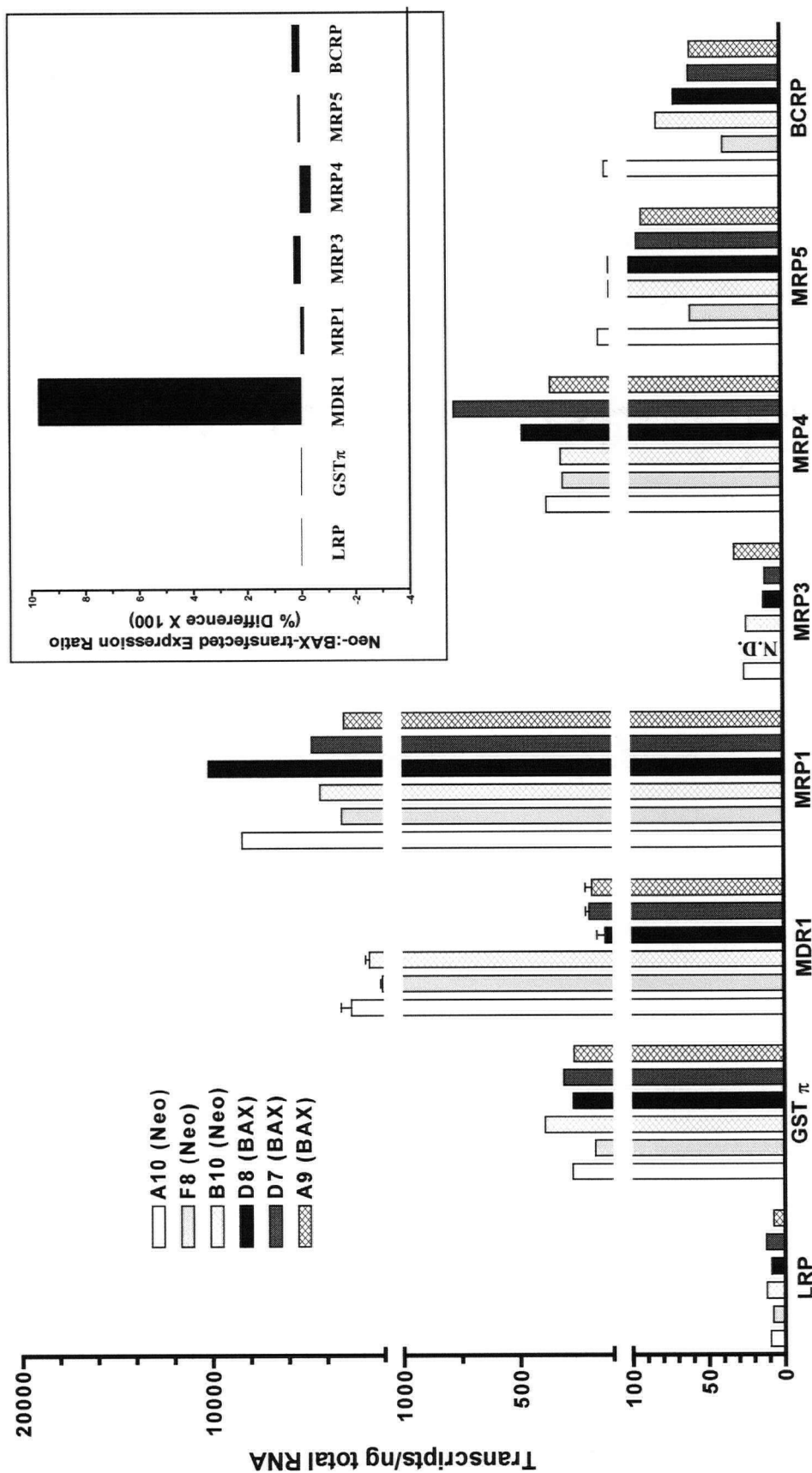
The expression levels of several transcripts in SW626-transfectants are noteworthy. A number of transcripts, including ALDR, ABCR, MDR3, BSEP, MRP6 and CFTR, have expression patterns known to be limited to one or only a few tissues and are, not surprisingly, absent or at very low levels in SW626-transfectants. Among ABC transcripts associated with MDR, MRP3 was very weakly expressed, whereas MRP1 and MDR1 were both very highly expressed. Other ABC transcripts associated with MDR such as MRPs 2, 4 and 5 and BCRP were expressed at low levels. Among transcripts associated with drug resistance, GST $\pi$  was weakly expressed and LRP was marginally detectable.

To identify any expression changes that occur upon stable transfection with a plasmid containing the BAX cDNA, we compared the competitive-RT-PCR expression profiles of A10

(neo-transfected) and D8 (BAX-transfected) cells (Figure 5.2). Overall, there were relatively few expression differences between these cell lines. Using a threshold of a 2-fold difference in mRNA levels, 25 of 30 transcripts examined were expressed at similar levels in both cell lines. The levels of two transcripts are slightly reduced in D8 cells compared to A10 cells. ABCB9 mRNA was 2.2-fold lower and MRP7 was 2.1-fold lower. Larger reductions in mRNA levels in D8 cells were observed for ABC3 (4.3-fold) and ABCR (6.6-fold). However, all of these transcripts were expressed at marginally detectable levels in both cell lines (all measurements performed at least in duplicate). In contrast, MDR1 mRNA was expressed at  $2,718 \pm 534$  copies/ng of total RNA (mean  $\pm$  SEM, triplicate measurements) in A10 cells, but was present at only  $130 \pm 35$  copies in D8 cells, a 20-fold reduction.

To determine if the reduction in MDR1 mRNA in the BAX-transfected D8 cell line was unique to the clone, we examined the mRNA expression levels of 8 MDR-associated transcripts in two other BAX-transfected (A9 and D7) and neo-transfected (F8 and B10) cell lines (Figure 5.3). The mRNA levels of each of the 8 transcripts were very similar among the 6 cell lines. Other than for MDR1 mRNA, there was no significant difference in individual transcript levels between BAX-transfected and neo-transfected clones. MDR1 mRNA expression was  $1,759 \pm 201$  and  $1,082 \pm 82$  copies/ng of total RNA, respectively, for B10 and F8 cells, which when combined with the level in A10 cells, gives an average MDR1 mRNA level of 1,853 copies in neo-transfectants. MDR1 expression in BAX-transfectants was  $200 \pm 14$  and  $187 \pm 29$  copies, respectively, in D7 and A9 cells, which gives an average level of 172 copies. Therefore, the MDR1 mRNA was reduced by an average of 10.8-fold among the three pairs of clones. The extent of decreased MDR1 mRNA in BAX-transfectants can be seen even more clearly by plotting the same data as a ratio of the average RNA expression level for each transcript in neo-transfectants:BAX-transfectants expressed as percent difference (Figure 5.3, inset graph).



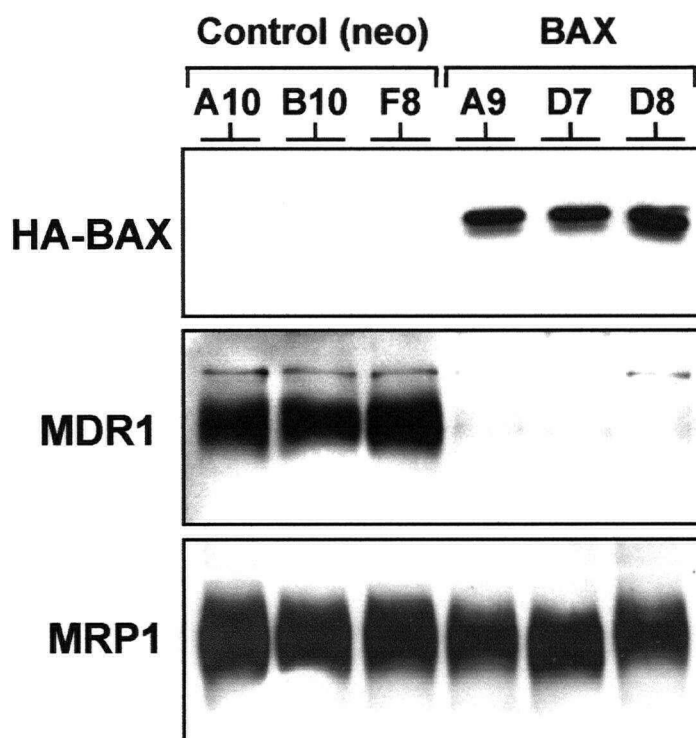


**Figure 5.3 Competitive-RT-PCR Expression Profile of MDR-associated RNA Transcripts in BAX- and Neo-transfected SW626 Cell Lines.**

Expression profile of 6 ABC transcripts, LRP and GST $\pi$ . All data are normalized to  $\beta_2$ microglobulin levels. MDR1 levels were determined in triplicate, whereas all other transcripts were determined in duplicate. Error bars represent SEM of three independent experiments. ND = not determined (**Inset graph**) Ratio of average transcript expression in 3 neo-transfected cell lines:3 BAX-transfected cell lines. See Materials and Methods sections 2.2.4 and 2.2.5 for details.

#### **5.3.4 Immunoblot Analysis of HA-BAX, MRP1 and Pgp Expression in SW626-Transfectants**

We next performed a Western blot analysis to determine if the decrease in MDR1 mRNA corresponded to a change in Pgp levels (Figure 5.4). Both total cell lysate and total membrane protein fractions were isolated from all 6 cell lines. To verify the identity of each clonal cell line we probed with an antibody for the HA tag on the BAX protein and observed a single band migrating at approximately 22 kDa in the A9, D7 and D8 cell lines, as expected (top panel). The position of the HA-BAX protein product is slightly less than the 24 kDa reported previously (Strobel et al., 1997). It has been demonstrated previously that the total amount of BAX protein (HA-BAX plus endogenous BAX) in HA-BAX transfected clones (A9, D7, D8) was approximately 10-fold greater than BAX levels observed in neo-transfected cell lines A10 and B10 (Strobel et al., 1997). To control for variations in sample loading, we also probed for MRP1 (bottom panel). We observed a strong band of similar intensity in all cell lines migrating at approximately 190 kDa, as expected for MRP1 (Hipfner et al., 1994). In contrast to our results, Strobel et al. (1998) previously reported insignificant expression of MRP1 protein as assessed using the anti-MRP1 antibody MRPM6 (Flens et al., 1994) with both flow cytometric and immunoblot analysis. Finally, we probed for Pgp expression and observed a band migrating at approximately 170 kDa, as expected for Pgp (Kartner et al., 1985), with markedly greater expression in the neo-transfected controls (A10, B10 and F8) in comparison to the BAX-transfectants (middle panel). Densitometric analysis revealed a 20.8-fold greater expression of Pgp in neo-transfected cell lines. This is similar to the 10.8-fold greater MDR1 mRNA expression in neo-transfectants compared to BAX-transfectants. In contrast to our results, Strobel et al. (1998) previously reported insignificant expression of Pgp protein as assessed using the anti-Pgp antibody 4E3 (Arceci et al., 1993) in flow cytometric analysis.



**Figure 5.4. Characterization of HA-BAX, Pgp and MRP1 Protein Levels in SW626-transfectants.**

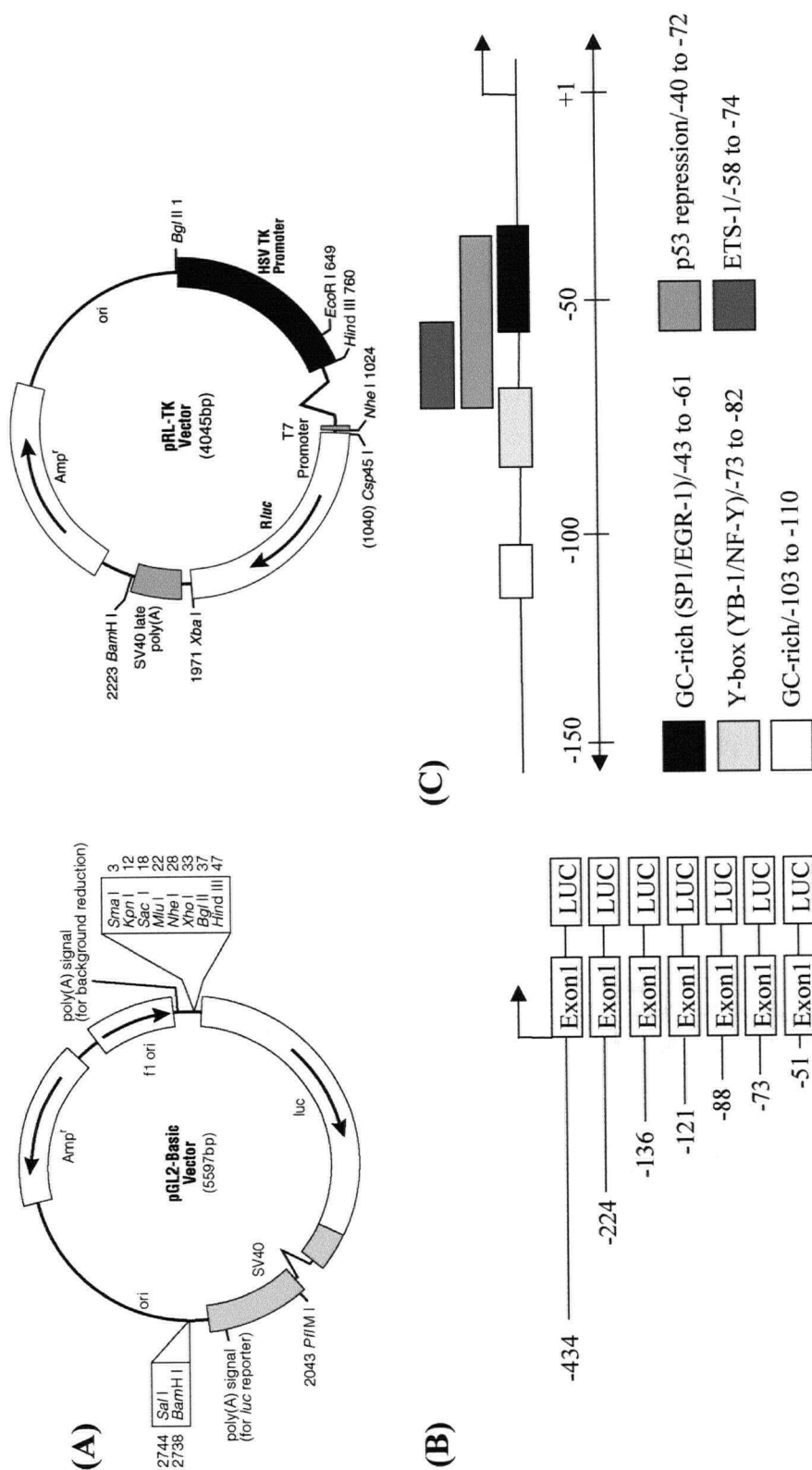
Immunoblot analysis was performed to compare HA-BAX, Pgp and MRP1 levels in neo-transfected clones to those in HA-BAX-transfected clones as indicated. Total cell lysate (HA-BAX) and total membrane preparations (Pgp and MRP1) were resolved on 13.5% and 6% SDS-PAGE gels, respectively. The molecular masses of HA-BAX, Pgp and MRP1 were approximately 22, 170 and 190 kDa, respectively. See Materials and Methods sections 5.2.6, 5.2.7 and 5.2.8 for details.

### 5.3.5 Analysis of MDR1 Promoter Activity in SW626-transfectants

To determine whether the transcriptional control of MDR1 is mediated by decreased promoter activity in BAX-transfected cells, a pGL2-basic vector containing the 539 bp MDR1 promoter DNA fragment spanning the -434 to +105 region upstream of the Firefly luciferase gene was tested (Figure 5.5, panels A and B). The construct was transiently co-transfected into each of the 6 SW626 clones, along with the pRL-TK vector containing a cDNA encoding *Renilla* luciferase under the transcriptional control of the herpes simplex virus thymidine kinase promoter, which provides low-level constitutive expression (Figure 5.5, panel A). MDR1 promoter activity was measured as a function of luciferase activity, normalized to *Renilla* luciferase to control for transfection efficiency. In three independent experiments, the ratio of pGL2-basic(-454/+105):pGL2-basic, normalized to *Renilla* luciferase levels, was consistently higher in neo-transfectants compared to BAX-transfectants. A representative experiment is shown in Figure 5.6. Luciferase activity (relative luciferase units/20,000 *Renilla* units) was greater in neo-transfectants by 1.32-fold in trial 1 ( $6.80 \pm 0.92$  versus  $5.14 \pm 2.21$ , mean  $\pm$  SEM), 2.27-fold in trial 2 ( $44.78 \pm 25.46$  versus  $19.68 \pm 4.78$ ) and 2.79-fold in trial 3 ( $165.75 \pm 102.65$  versus  $59.35 \pm 2.49$ ) for an average of 2.12-fold. Although the relative luciferase activity varied with each transfection experiment, we consistently observed the same rank-order for relative luciferase levels among cell lines. Highest to lowest this was: F8, A10 and B10 for neo-transfectants and A9, D7 and D8 for BAX-transfectants. These results suggest that MDR1 promoter activity is upregulated by positive regulatory transcription factor(s) in neo-transfectants, but not in BAX-transfectants.

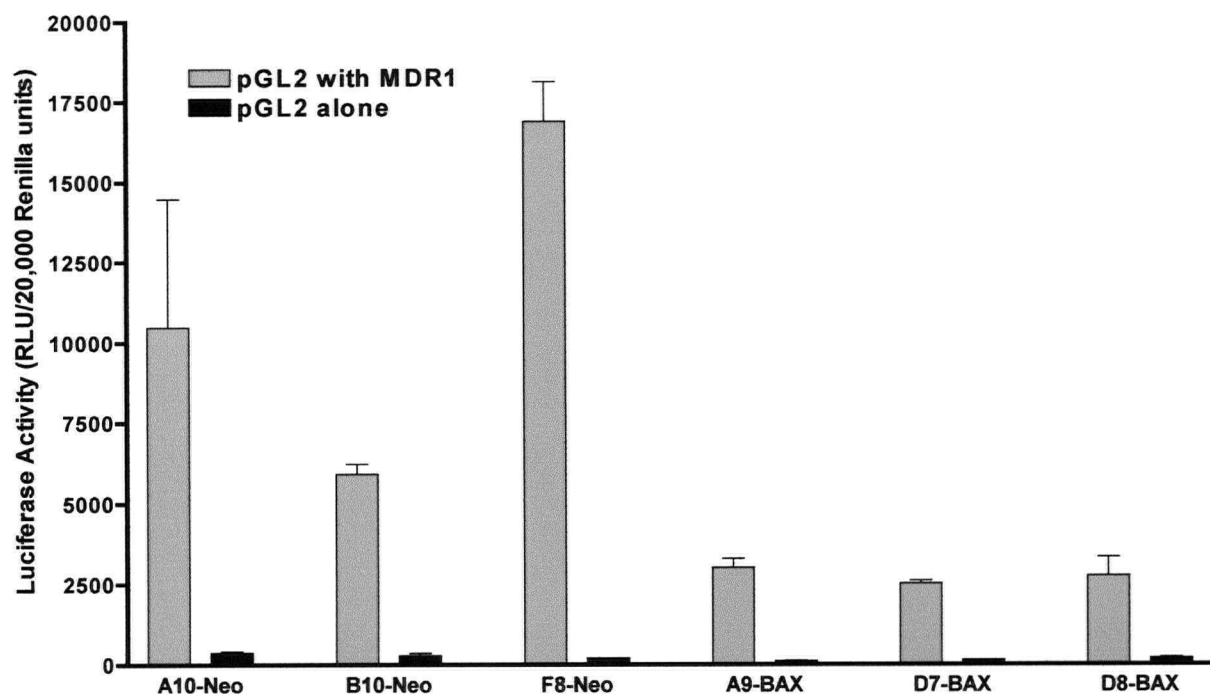
In an attempt to identify the region(s) of the 539 bp MDR1 promoter involved in up-regulating this activity, we repeated these experiments using a series of pGL2-basic promoter constructs containing 5' deletions of the 539 bp MDR1 promoter (Figure 5.5, panel B). In agreement with our results using the pGL2-basic(-454/+105) construct, in three independent

experiments, the ratio of pGL2-basic(MDR1 5' deletion constructs):pGL2-basic, normalized to *Renilla* luciferase levels, was consistently higher in neo-transfectants compared to BAX-transfectants. A representative experiment is shown in Figure 5.7. The ratio of luciferase activity (relative luciferase units/20,000 *Renilla* units) in neo- versus BAX-transfectants for the successive 5' MDR1 promoter deletions averaged over 3 trials were as follows:  $3.56 \pm 1.6$  (-454) (mean  $\pm$  SEM);  $4.36 \pm 1.9$  (-226);  $2.83 \pm 1.7$  (-136);  $3.21 \pm 2.0$  (-121);  $2.37 \pm 0.3$  (-88);  $2.69 \pm 0.4$  (-73) and  $1.54 \pm 1.1$  (-51). The ratio of luciferase activity with the 539 bp MDR1 promoter was higher in these trials (3.56) than our earlier experiments (2.12). A deletion of about 75% of the 5'-flanking sequence (from -454 to -121) had little effect on promoter activity. However, further deletion to -73 reduced promoter activity to about 40% of the -454 (wild type) control level.



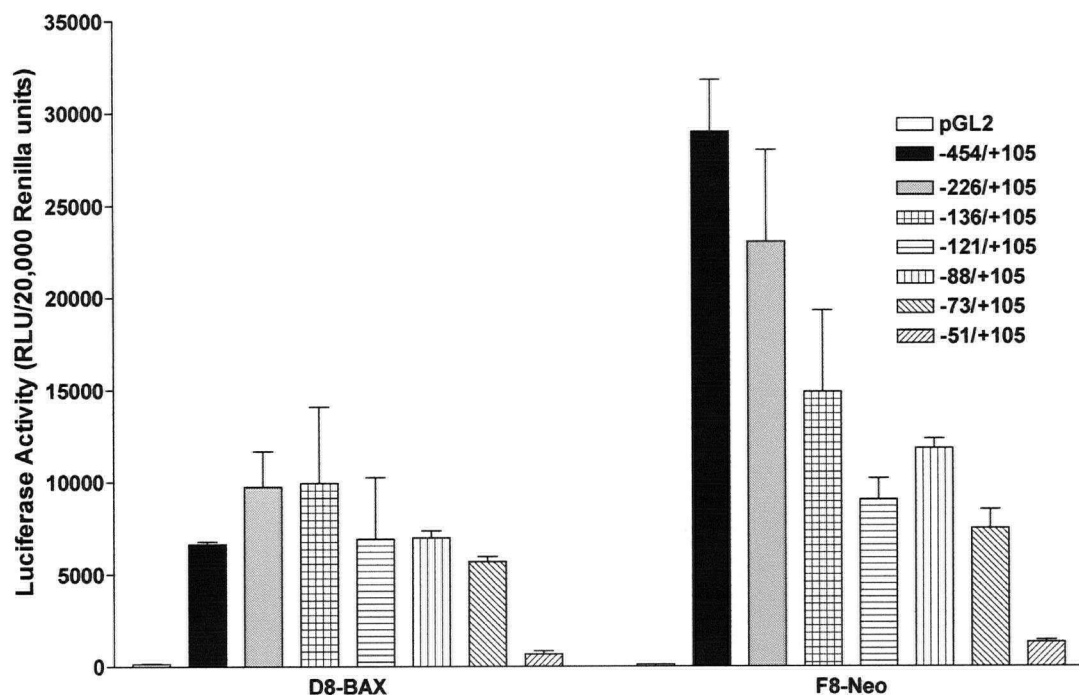
**Figure 5.5 Design of Constructs Used to Analyze Activity of the MDR1 Promoter.**

(A) Plasmid constructs used to analyze MDR1 promoter activity. The pGL2-basic vector contains the luciferase gene without the SV40 promoter and serves as a promoter-less control. The pRL-TK vector contains the herpes simplex virus thymidine kinase promoter region upstream of the *renilla* luciferase gene. This promoter provides low-level, constitutive expression. (B) Series of MDR1 promoter 5'-deletion constructs that have been cloned upstream of the luciferase gene in the pGL2-basic vector and the resulting constructs have been named accordingly [e.g. pGL2-basic(-434/+105)]. (C) Diagram of the location of important regulatory elements within the proximal MDR1 promoter. See Materials and Methods sections 5.2.2 and 5.2.9 for details.



**Figure 5.6. Analysis of MDR1 Promoter Activity in Neo- and BAX-transfected SW626 Cells.**

Each cell line was co-transfected with the 539 bp MDR1 promoter fragment [pGL2-basic(-454/+105)] or the pGL2-basic control vector, as well as the pRL-TK vector containing the *Renilla* luciferase cDNA. After co-transfection, firefly luciferase activity was measured and normalized to *Renilla* luciferase levels. All transfections were performed in triplicate wells. Error bars represent SEM. See Materials and Methods sections 5.2.2 and 5.2.9 for details.



**Figure 5.7. Analysis of MDR1 Promoter 5' deletions.**

Each cell line was co-transfected with one MDR1 promoter construct [e.g. pGL2-basic(-121/+105)] or the pGL2-basic control vector, as well as the pRL-TK vector containing the *Renilla* luciferase cDNA. After co-transfection, firefly luciferase activity was measured and normalized to *Renilla* luciferase levels. All transfections were performed in triplicate wells. Error bars represent SEM. See Materials and Methods sections 5.2.2 and 5.2.9 for details.

## DISCUSSION

Using competitive-RT-PCR we have demonstrated that the levels of MDR1 mRNA are dramatically reduced in BAX-transfected versus neo-transfected SW626 cells. This difference is striking considering that MDR1 mRNA is the second most highly expressed ABC transcript in neo-transfected cells. There were 4 other transcripts differentially expressed more than 2-fold; however, these differences were smaller than observed for MDR1 and these transcripts were weakly expressed in both cell lines. Immunoblot analysis revealed a significant reduction in Pgp levels in BAX-transfectants, in agreement with our competitive-RT-PCR results. In contrast to Strobel et al. (1998), we observed strong expression of Pgp and MRP1 in all 3 neo-transfected SW626 cell lines, as well as the 3 BAX-transfected cell lines in the case of MRP1. Unfortunately, no data (i.e. western blot image) or detailed protocols were provided in their publication. Although it is possible that our usage of different antibodies may account for this discrepancy, both of the antibodies used in their studies, 4E3 for Pgp (Arceci et al., 1993) and MRPM6 for MRP1 (Flens et al., 1994), are commonly employed by various investigators. It is also possible that appropriate controls such as cell lines over-expressing Pgp or MRP1 were not used in their studies to validate their immunoblot and flow cytometry results.

A comparison of the competitive-RT-PCR expression profiles of the ovarian carcinoma cell lines SKOV3 (Chapter III) and SW626 revealed an overall high degree of similarity. Among ABC transcripts associated with MDR, MRPs 1-7 were expressed at similar levels relative to one another, although all of these transcripts were expressed at lower levels in SW626 cells. One of the most striking differences was the high expression of MDR1 in SW626 cells in comparison to its marginally detectable expression in SKOV3 cells. Among other transcripts associated with MDR, LRP was marginally detectable in both cell lines, but GST $\pi$  was considerably higher in SKOV3 cells. Another transcript of interest is ABC3, which was

surprisingly highly expressed in SKOV3 cells, but at low levels in SW626 cells. The significance of these few expression differences among ovarian carcinoma cell lines is unknown.

In an attempt to identify a mechanism responsible for the observed MDR1 mRNA and Pgp changes, we evaluated MDR1 promoter activity using a luciferase reporter assay. We consistently observed greater luciferase activity in neo-transfectants compared to BAX-transfectants; however, this difference was small. In addition to transcriptional regulation, other mechanisms, such as differences in mRNA or protein stability, may also contribute to the observed differences in accumulation of MDR1 mRNA and Pgp.

Considering the importance of Pgp in drug-resistant cancers, there has been a concerted effort to characterize mechanisms of MDR1 transcriptional regulation. In human cells, two promoters produce MDR1 transcripts; however, in most normal tissues, transcripts are driven from the MDR1 proximal promoter (-434 to +1, relative to the transcription initiation start site) (Cornwell, 1990; Ueda et al., 1987a and Ueda et al., 1987b). As was discussed in chapter III, there is ample evidence that MDR1 transcription is regulated by cellular signals in response to cellular stress, development and differentiation. The human genomic MDR1 clone containing sequences 5' to the MDR1 transcription start site has been shown by Ueda et al. (1987b) to contain the proximal promoter activity. Sequence analysis of the proximal promoter has shown that it lacks many of the sequence elements associated with more active promoters, such as a recognizable TATA box (Ueda, 1987b) or high CpG content (Bird, 1986) upstream of the initiation site (Ueda et al., 1987b). These data suggest that the MDR1 promoter is relatively weak, consistent with the relatively low level of MDR1 RNA expression in most cell types (chapter IV).

Our luciferase reporter assay analysis using 5' deletion constructs of the MDR1 promoter revealed that deletion of the proximal promoter from -454 to -121 had a very limited effect on luciferase activity. In agreement with our results, Cornwell and Smith (1993) demonstrated that

deletion of promoter sequences to -121 also exerted very little effect on promoter activity in transiently transfected cells. Likewise, Goldsmith et al., (1993) reported that deletion of promoter sequences to -89 had little effect on expression. We also observed that deletion of the promoter from -121 to -51 caused a significant reduction in luciferase activity, suggesting that important regulatory elements may be present in this region. The data of Cornwell and Smith (1993) suggest that regions between -121 and -88 and between -73 and -51 contain sequences that modulate promoter activity. Within these two regions, mutation of two GC-rich stretches, from -110 to -103 and from -61 to -43, alters promoter activity (Figure 5.5, panel C). Furthermore, the authors demonstrated that the transcriptional activators SP1 and EGR-1 bind to the -50 GC-box. A subsequent study showed that 12-O-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C agonist, increases MDR1 transcription and activates the MDR1 promoter, and that promoter activation by TPA requires the binding of the EGR-1 transcription factor to the overlapping SP1/EGR-1 site (McCoy et al., 1995). This response is inhibited by the Wilms' tumor suppressor, WT1, likely through competitive binding at the SP1/EGR-1 site (McCoy et al., 1999).

Goldsmith et al. (1993) used promoter deletion/mutation constructs and DNaseI footprint analysis to demonstrate that a region from -85 to -70 was essential for basal transcriptional regulation of the MDR1 promoter (Figure 5.5, panel C). The sequence from -82 to -73 is perfectly homologous with the 10-base pair Y-box consensus sequence, which contains the sequence CCAAT in reverse order, found in the promoters of all major histocompatibility complex class-II genes (Dorn et al., 1987), as well as many other genes. Bargou et al. (1997) have reported that the nuclear, rather than cytoplasmic, localization of the Y-box transcription factor YB-1 is closely associated with MDR1 gene expression and MDR in a human breast cancer cell line. In addition, in a subset of untreated primary breast cancers, nuclear localization of YB-1 protein was associated with intrinsic MDR. Other studies have shown that the Y-box,

and possibly YB-1, may be a critical *cis*-regulatory element regulating UV or chemotherapeutic drug-induced MDR1 gene expression (Ohga, et al., 1998 and Ohga et al., 1996). Another transcription factor, NF-Y, has also been shown to interact with the Y-box and has been implicated in constitutive regulation of the MDR1 promoter (Sundseth et al., 1997 and Jin and Scotto, 1998). Furthermore, Hu et al. (2000) have shown that activation of the MDR1 promoter by UV irradiation is dependent on the Y-box, as well as the proximal GC-rich element (-56 to -43). They demonstrated using gel shift assays that NF-Y interacted with the Y-box element while SP1 was bound to the GC-rich element.

Currently, there is no evidence in the literature to suggest that BAX, like p53, can function as a transcriptional regulator by binding directly to elements within promoter regions. There is also no evidence that BAX is able to directly regulate any of the transcription factors known to bind the MDR1 promoter. However, it is reasonable to propose that BAX may directly or indirectly regulate a transcription factor important for the control of MDR1 transcription in SW626 cells. Future experiments using gel shift assays to detect differences in proteins bound to the MDR1 promoter in neo- and BAX-transfectants should be undertaken as a first step towards identifying potential regulatory transcription factors in SW626 cells.

In an attempt to determine if BAX-mediated down-regulation of Pgp is unique to SW626 cells or is a broader phenomenon, we transiently co-transfected the pGL2-basic(-434/+105) and pRL-TK plasmids, as well as a pSFFV expression vector containing the HA-BAX cDNA into several p53-deficient cell lines (SW620 colon carcinoma, DU145 prostate carcinoma and SKOV3 ovarian carcinoma). In doing so, we expected to increase BAX expression transiently and concurrently evaluate MDR1 promoter activity using the luciferase reporter assay. Unfortunately, co-transfection with BAX consistently reduced transcription from the pRL-TK promoter, regardless of the cell line used or ratio of plasmids in the transfection mixture (data not shown). Similar results were observed using the chloramphenicol acetyl transferase (CAT) assay

system (data not shown). We are currently generating stable BAX-transfected cell lines to circumvent these difficulties.

There is considerable evidence that MDR1 transcription is regulated by another mediator of apoptosis, p53. As mentioned previously, deletion or mutation of the p53 gene is the most frequent alteration in human malignancies. p53 is a nuclear phosphoprotein and is a tumour suppressor whose inactivation is strongly correlated with cancer. Loss of p53 represses transcription of some genes resulting in decreased susceptibility to apoptotic stimuli. For example, mutations in p53 correlate with chemotherapeutic resistance (Aas et al., 1996 and Perego et al., 1996) and relapse in p53-expressing tumours (Lowe et al., 1994). Fibroblasts from p53-deficient mice are also relatively more resistant to several chemotherapeutic agents (Lowe et al., 1993). Although this would appear to establish that p53 status predicts sensitivity to chemotherapeutic agents, several reports have linked inactivation of p53 with enhanced chemosensitivity (Fan et al., 1995 and Wahl et al., 1996). It has been proposed by Thottassery et al. (1997) that the unpredictable relationship between p53 and drug sensitivity might be explained by tissue-specific effects of p53 on MDR1 gene expression. It has been demonstrated that mutant p53 stimulates MDR1 promoter activity, whereas wild-type p53 either had no effect or repressed MDR1 gene transcription (Chin et al., 1992 and Zastawny et al., 1993). The MDR1 promoter does not contain the consensus binding site for p53 (El-Deiry et al., 1992). The data of Zastawny et al. (1993) indicate a p53-responsive region is located within the sequences spanning from -5 to +25, suggesting that the mechanism by which p53 modulates MDR1 transcription does not entail direct interactions with *cis*-acting regulatory elements; rather p53 would repress MDR1 transcription indirectly by inhibiting the basal machinery. In contrast to these results, Goldsmith et al. (1995) observed that wild-type p53 stimulated MDR1 promoter activity and mutant p53 repressed this activity. They mapped this stimulatory effect to nucleotide sequences between -39 and +53, which overlaps with the region reported by Zastawny et al. (1993).

Thottassery et al. (1997) demonstrated that cell lines engineered to over-express trans-dominant negative p53 and having impaired p53-mediated transactivation and transrepression, had markedly elevated levels of MDR1 mRNA and Pgp, and this increased Pgp-conferred resistance, specifically to drugs that are Pgp substrates. These data suggest that, at least in some tissues, p53 inactivation likely leads to selective resistance to chemotherapeutic agents because of up-regulation of MDR1 expression. This is similar, although opposite in effect, to the data reported by Strobel et al. (1996) using BAX-transfected cell lines. In an analogous fashion, Wang and Beck (1998) and Sullivan et al. (2000) have shown that wild-type p53 represses MRP1 transcription. Many clinical studies have shown that mutant p53 is associated with increased MDR1/Pgp and/or MRP1 expression (Fukushima et al., 1999; Linn et al., 1996 and Ralhan et al., 1999), consistent with the notion that loss of p53 repression may lead to up-regulation of MDR1 and/or MRP1. Furthermore, Sampath et al. (2001) recently demonstrated that mutant p53 strongly up-regulates the MDR1 promoter, as shown by other investigators. In addition, the authors determined that this activation required an ETS binding site (nucleotide sequence -58 to -74 of the proximal MDR1 promoter) and that mutant p53 and the transcription factor ETS-1 synergistically activated MDR1 transcription (Figure 5.5, panel C). It has also been shown that induction of wild-type p53 activity in human cancer cells using ribozymes that repair mutant p53 proteins results in reduced activity of the MDR1 promoter, as would be expected (Watanabe and Sullenger, 2000). Finally, Johnson et al. (2001) have shown that p53 can repress MDR1 transcription directly by binding to a novel head-to-tail site within the MDR1 promoter (nucleotide sequence from -40 to -72) (Figure 5.5, panel C). Collectively, these data strongly suggest that the apoptotic and drug transport machinery act coordinately to determine resistance to chemotherapeutic drugs.

In conclusion, our data demonstrate that MDR1 mRNA and Pgp levels are regulated by the level of BAX expression in SW626 cells. In addition to the reduction in Pgp levels in BAX-

transfectants, these cells display reduced [ $^3\text{H}$ ]-paclitaxel uptake that is blocked by verapamil, a commonly used inhibitor of Pgp (Strobel et al., 1998). Also, the three drugs to which BAX-transfectants are considerably more sensitive than neo-transfectants are all known Pgp substrates. Taken together, these results strongly implicate Pgp as the BAX-regulated paclitaxel transporter in SW626 cells. These results also establish a previously unreported connection between an important pro-apoptotic molecule and chemotherapeutic resistance mediated by a drug transport protein.

## CHAPTER VI

### SUMMARY

At the outset of this research project it was our objective to develop an assay for quantitative RNA profiling of all members of the human ABC superfamily. Ideally this assay would be relatively inexpensive, it would require only equipment already existing in our laboratory and would be considerably more sensitive, accurate and reproducible than currently used hybridization methods (e.g. Northern blots). We intended to apply this assay to the study of MDR in cancer cell lines and tumour biopsies, as well as to gain insight into the normal biological functions of ABC transporters.

In chapter II we presented our data describing the development and characterization of a fluorescent competitive-RT-PCR assay. We examined the effect of a broad range of parameters on RT and PCR efficiency, as well as the performance of capillary electrophoresis. It was also shown that this assay was highly sensitive, typical of PCR methods. The use of homologous competitive standards allowed us to overcome the inconsistency of amplification inherent to PCR and achieve accurate and reproducible quantification of transcripts. The use of a DNA sequencer for capillary electrophoresis provided single nucleotide resolution of PCR products with semi-automated operation.

In our first application of the assay, we demonstrated that multiple ABC mRNAs are up- or down-regulated upon selection of ovarian carcinoma cells in the presence of the chemotherapeutic drug vincristine. This data suggests that a comprehensive examination of ABC transcript expression is warranted when searching for potential mediators of MDR in cancer cell lines or tumour specimens. To our surprise, short-term exposure of a leukemia cell line to several chemotherapeutic drugs resulted in only a few expression changes, suggesting that altered expression of ABC mRNA species may not be common response to chemotherapeutic drug exposure.

In chapter IV, expression profiling of ABC transcripts in normal human tissues demonstrated that, in most cases, ABC transcripts could be classified as being of low abundance relative to the population of transcripts expressed in a cell. ABC transporters themselves could be grouped into three classes of expression: relatively tissue-specific, ubiquitously expressed at variable levels or housekeeping. The expression profile of ABC transcripts in each tissue has provided insight into the contribution of individual transporters to MDR in cancer, protection against xenobiotics, distribution of cholesterol and lipids and dimeric interactions among half-transporters.

Finally, in chapter V we showed that enforced over-expression of the pro-apoptotic BAX cDNA leads to down-regulation of MDR1 mRNA and Pgp, resulting in increased sensitivity to a range of chemotherapeutic agents. Luciferase reporter assays using the MDR1 promoter demonstrated that this down-regulation was, at least in part, due to decreased MDR1 transcriptional activity. These data provide another connection between MDR and apoptosis and the interaction of these pathways in mediating the cytotoxic effects of chemotherapeutic agents.

Our application of competitive-RT-PCR has provided an important step towards the comprehensive evaluation of ABC transporter activity; however, additional research is needed. This includes more extensive RNA expression data from other bulk tissues, sub-compartments within tissues and specialized cells, as well as a complete set of competitors for all 48 human ABC genes. In addition to RNA expression, complementary information needs to be obtained using other approaches such as protein expression, generation of knockout mice, defining half-transporter associations and characterizing transport kinetics and substrate affinity. It is only through such an integrated approach that we can expect to eventually define the detailed ABC transporter network within human cells.

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## APPENDIX I

### CHARACTERIZATION OF THE HUMAN MRP7 mRNA and cDNA

#### INTRODUCTION

##### A.1.1 Early EST Analysis of the MRP7 Transcript

In 1996, an EST (R12184) of 221 bp in length and with significant homology to members of the 'C' subfamily of ABC transporters was submitted to GenBank. This EST was generated from a human infant brain library as part of a larger project with the aim to characterize the human transcriptosome (Hillier et al., 1996). Subsequently, additional sequencing of this clone was performed by Allikmets et al. (1996) and the expanded sequence data (1914 bp) was submitted to GenBank as EST U66684. This EST was also localized to chromosome 6p21 using fluorescent *in situ* hybridization (FISH) and Northern blot analysis demonstrated a single mRNA transcript of 5.5 kb in size, suggesting this mRNA encoded a full ABC transporter (Allikmets et al., 1996). Based on the high degree of sequence similarity with the six MRP-like members of this subfamily, this EST was designated by the research community as MRP7 (ABCC10); however, no further work on this ABC transporter was published during the ensuing 5 years.

##### A.1.2 The MRP-like Subfamily and Drug Resistance

Subfamily C consists of 12 full-transporters including CFTR, SUR1, SUR2, multidrug resistance-associated proteins (MRPs) 1-7 and ABCC11-12. CFTR is a cAMP activated chloride channel that when mutated causes the inherited disease cystic fibrosis (Riordan et al., 1989). SUR1 and its isoform, SUR2, are regulatory components of the potassium channels responsible for glucose-related insulin release that when mutated can cause the rare genetic disease,

persistent hyperinsulinemic hypoglycemia of infancy (Bryan and Aguilar-Bryan, 1999). There is currently no evidence that any of these 3 proteins contribute to multidrug resistance (MDR). MRPs 1, 2, 3, 4 and 5 are organic anion transporters that differ in their tissue distribution and substrate preference (Borst et al., 1999; Konig et al., 1999). Each of these proteins has been shown to be capable of mediating drug transport and/or drug resistance. Detailed descriptions of the normal functions and transport/resistance profiles of these proteins have been provided in chapter I of this thesis. It remains to be determined whether MRP6 is also an organic anion transporter. Mutations in MRP6 have recently been associated with the inheritable connective tissue disorder pseudoxanthoma elasticum but its substrate(s) is unknown (Bergen et al., 2000). The cDNA sequences of ABCC11 and ABCC12 have recently been published; however the function of these genes is unknown (Tammur et al., 2001).

The fact that 5 of 6 members of the MRP-like subfamily are associated with drug resistance provides strong justification for the characterization of additional ESTs representing a new member of the MRP-like subfamily. In addition, we observed a significant increase in MRP7 mRNA expression after exposure of human leukemia HL60 cells to  $\frac{1}{2}$  IC<sub>50</sub> concentration of the chemotherapeutic drug daunorubicin for 24 hours. Based on these facts, we decided to pursue the identification and characterization of a full-length cDNA complementary to EST U66684.

## **MATERIALS AND METHODS**

### **A.2.1 Identification of Human ESTs and Genomic DNA Clones Matching MRP7 EST**

#### **U66684**

EST clones and bacterial artificial chromosome (BAC) clones with high sequence similarity to EST U66684 were identified as described in section 2.2.1 (chapter II) of this thesis using the sequence of EST U66684 as bait. The chromosome location of BAC clones AC021391 and AL359813 was determined using the fingerprinted contigs (FPC) software available at the Washington University Genome Sequencing Center website ([www. genome.wustl.edu/gsc/](http://www.genome.wustl.edu/gsc/)). FPC is a program for building contigs from fingerprinted clones, where the fingerprint for a clone is a set of restriction fragments (Soderlund et al., 2000).

### **A.2.2 cDNA Clone AK000002 (MRP7)**

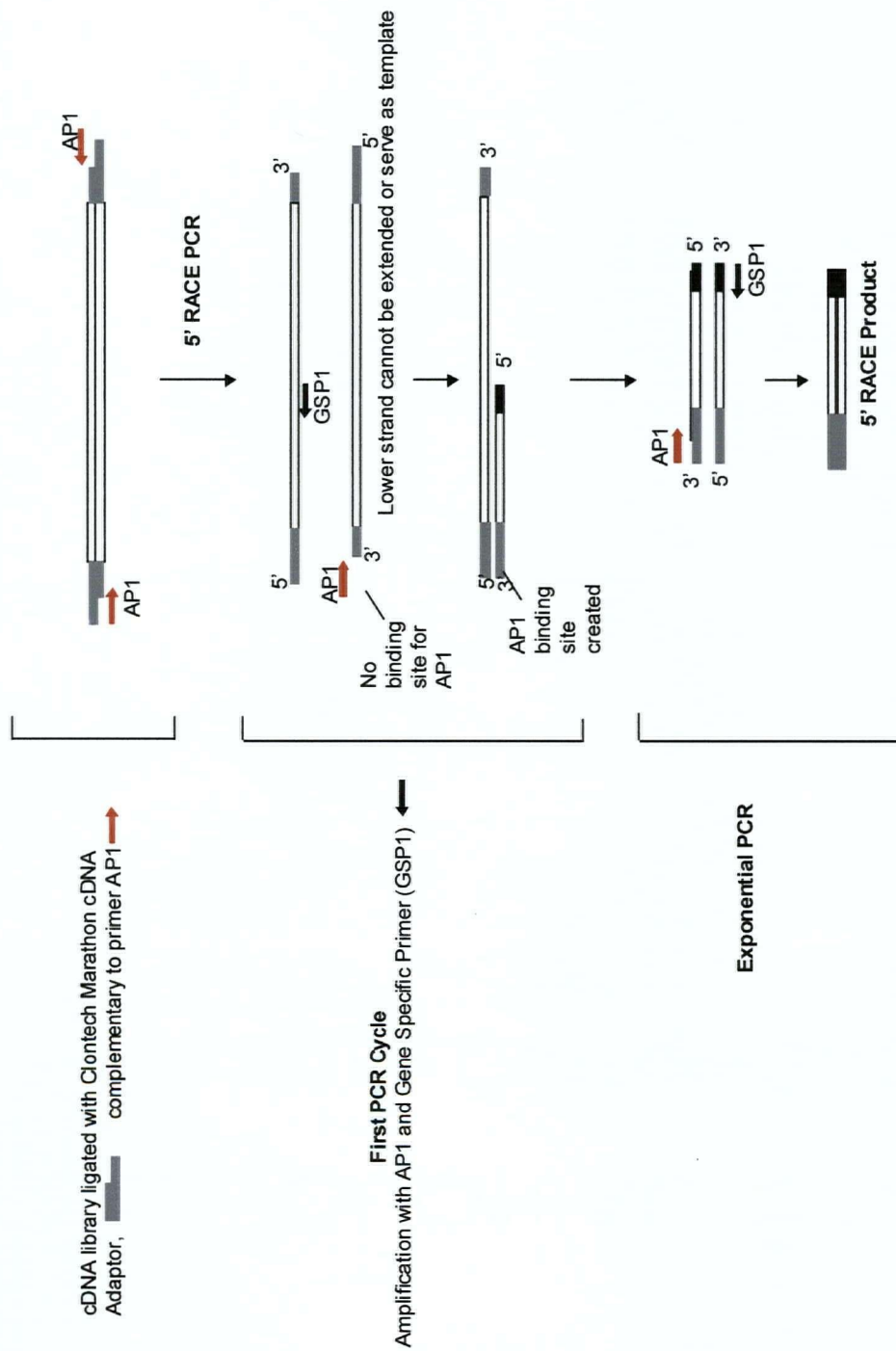
The AK000002 cDNA clone was a generous gift from the Kazusa DNA Research Institute, Chiba, Japan. The cDNA was inserted into the *SalI/NotI* site of the pBluescript II SK<sup>+</sup> vector.

### **A.2.3 5' RACE**

To determine if clone AK000002 represents the complete open reading frame (ORF) and 5' untranslated region (UTR) of MRP7, 5' random amplification of cDNA ends (RACE) was performed using Clontech's Marathon Ready cDNA Kit (Figure A.1). The first round of PCR amplification utilized primers AP1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') and an MRP7 gene-specific primer, 370 RACE1 MRP7 (5'-GTAGGATGTAATCTGGACTCCTCGG-3') to amplify cDNA clones from the Clontech Marathon Ready placental cDNA library. The PCR assay

consisted of one 50  $\mu$ L reaction with cDNA template and an H<sub>2</sub>O negative control. PCR conditions were as follows: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100  $\mu$ M deoxynucleotide-triphosphates (dNTPs), 2 mM MgCl<sub>2</sub>, 20  $\mu$ g/mL bovine serum albumin (BSA), 0.1% (v/v) Triton X-100, 3.5 U of Amplitaq Gold (Perkin-Elmer), 2  $\mu$ M of AP1 and 370 RACE1 MRP7 primers and 2.5  $\mu$ L of the cDNA mixture in a 50  $\mu$ L volume in a Perkin- Elmer 9600 thermocycler. Touchdown PCR cycling parameters were as follows: 95° [12 minutes (min)], then 5 cycles of 95° [5 seconds (sec)], 72° (2.5 min), 5 cycles of 95° (5 sec), 70° (2.5 min), and finally 25 cycles of 95°(5 sec), 68° (2.5 min) and a final extension period of 72° (45 min).

A second round of PCR was performed using 2.5  $\mu$ L PCR product (diluted 1  $\mu$ L template in 49  $\mu$ L H<sub>2</sub>O) from round one and primers AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') and 341 RACE2 MRP7 (5'-CCAAGTAACAGGCACTGAGCACGG-3'). PCR was performed as described for round one.



To facilitate analysis of RACE products, this procedure is repeated using a nested adapter specific primer (AP2) and a nested Gene Specific Primer (GSP2)

RACE Products are Analysed by Sequencing

**Figure A.1 5' RACE Procedure.**

Summary of the RACE procedure (adapted from the Marathon RACE Manual, Clontech laboratories, Palo Alto, California, U.S.A.). See Materials and Methods section A.2.3 and A.2.4 for details.

#### A.2.4 Cloning and Sequencing of 5' RACE Products

5' RACE PCR products from the second amplification were resolved on a 1.0% agarose gel, excised and gel purified using the Qiaex II gel extraction kit (Qiagen). The purified PCR products were then cloned into the pCR-XL-TOPO vector and transformed into competent *Escherichia coli* (*E. coli*) cells using the TOPO-XL PCR Cloning Kit according to the manufacturer's instructions (Invitrogen). Cells were grown overnight at 37°C on agar plates containing kanamycin and X-galactosidase (blue/white selection) and colony PCR was performed to confirm insert size. Colonies were picked and diluted in 10 µL H<sub>2</sub>O. PCR was performed using primers T7 universal (5'-CCCTATAGTGAGTCGTATT-3') and M13 reverse (5'-CAGGAAACAGCTATGA-3'). PCR buffer consisted of: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100 µM dNTPs, 2 mM MgCl<sub>2</sub>, 20 µg/ml BSA, 0.1% (v/v) Triton X-100, 4 U of *Taq* polymerase, 0.5 µM of T7 and M13 primers and 2.0 µL of the colony mixture in a 15 µL volume. PCR cycling parameters were as follows: 95° (5 min) and then 30 cycles at 95° (30 sec), 56° (15 sec), 72° (45 sec). Products were separated on a 1% agarose gel and visualized after ethidium bromide staining. Colonies containing plasmids with the appropriate insert size, as determined by PCR, were re-plated on kanamycin-resistant plates containing X-galactosidase and grown overnight at 37°C. The next day, a single white colony was selected and used to inoculate an overnight culture of LB media containing kanamycin. Plasmids were then extracted using the Quantum Prep Plasmid Miniprep Kit according to the manufacturer's instructions (Biorad).

Insert identity was determined by DNA sequencing on an ABI 310 automated DNA sequencer. Plasmids were sequenced using 4 µL of Big Dye Terminator Mix (Applied Biosystems), 2 µL of plasmid template and 4 µL of one of the following primers (0.8 µM each): T7 universal (described previously), M13 Reverse (described previously) or T3 (5'-ATTAACCCTCACTAAAG-3'). Typical sequencing PCR conditions were as follows: 95° (2 min)

and then 28 cycles of 95° (10 sec), 47° (5 sec) and 60° (3 min). Annealing temperature varied depending on the primer used. PCR products were precipitated with isopropanol, washed with 70% ethanol, resuspended in 13 µL Template Suppression Reagent (Applied Biosystems) and processed via a 310 DNA sequencer.

#### **A.2.5 Comparison of Amino Acid Similarity Between MRP7 and Other MRP Subfamily Members**

The percent amino acid (AA) identity of clone AK000002 (MRP7) compared with other MRP subfamily members was determined using the BLASTP 2.1.2 program available at the NCBI website (<http://www.ncbi.nlm.nih.gov/>) using default settings. For GenBank accession numbers, please see Table II.I (chapter II).

#### **A.2.6 Predicted Protein Structure of MRP7**

To compare the domain organization of MRP7 with that of other MRP subfamily members, hydrophobicity plots were generated with the TopPred II 1.3 program using default settings. The sequence of cDNA clone AK000002 was used, as well as the published full-length cDNA sequences of MRPs 1-6. For GenBank accession numbers, please see Table II.I. (chapter II).

#### **A.2.7 Semi-quantitative PCR Analysis of MRP7 and $\beta_2$ microglobulin**

cDNA from Clontech's Multiple Tissue cDNA Panel was used as template to amplify a 250 bp MRP7 product using 35 cycles of PCR. This cDNA panel has been normalized by the manufacturer according to the expression level of 4 house-keeping genes. A high cycle number was chosen based on the low level of MRP7 expression observed using competitive-RT-PCR

(chapter IV). PCR cycling conditions consisted of 95°C (2 min), 5 cycles of 95°C (30 sec), 52°C (15 sec), 72°C (45 sec) and then 30 cycles of 95°C (30 sec), 64°C (15 sec), 72°C (45 sec). The composition of the PCR reaction (buffer, enzymes etc.) and amplification of full-length  $\beta_2$ microglobulin were as described in section 2.2.2 (chapter II). Primers 435 MRP7 (5'-CCGTCTTCCCGCTGCTAGACC-3') and r672MRP7 (5'-AGTGTGCCTCGCTGGCAATGC-3') were used for the amplification of MRP7. 1  $\mu$ L of cDNA was used as template and MRP7 and  $\beta_2$ microglobulin were amplified in separate tubes. PCR products were resolved on a 1% agarose gel, stained with ethidium bromide and visualized using the Eagle Eye II (Stratagene).

#### A.2.8 Expression Pattern of MRP7 Determined Using Dot Blot Analysis

To assess MRP7 mRNA expression in a broader variety of adult and embryonic tissues and cell lines, we used Clontech's Human Multiple Tissue Expression (MTE) array. This dot blot contains normalized loadings of poly A<sup>+</sup> RNA from 76 different human tissues and 8 different control RNAs and DNAs. A 791 bp cDNA probe was generated by PCR amplification using the AK000002 cDNA clone as template. PCR amplification consisted of one 50  $\mu$ L reaction containing 1  $\mu$ L of 1/10 diluted template (~10 ng), as well an H<sub>2</sub>O negative control. PCR conditions were as follows: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100  $\mu$ M dNTPs, 2 mM MgCl<sub>2</sub>, 20  $\mu$ g/ml BSA, 0.1% (v/v) Triton X-100, 2.5 U of *Taq* Polymerase, 1.5  $\mu$ M of 967 MRP7 (5'-TAGGTTGGCTGCAGTCTGTG-3') and EcoRI-M-MRP7 (5'-GAGCTAGAATTCGCGTCCATGGAACGACTTC-3') primers. PCR cycling parameters were 95° (2 min), 5 cycles of 95° (15 sec), 52° (15 sec), 68° (2 min), 25 cycles of 95° (15 sec), 68° (2 min), and a final extension period of 68° (10 min). The PCR product was resolved on a 1.0% agarose gel and purified using the Qiaex II gel extraction kit (Qiagen). The purified PCR product was

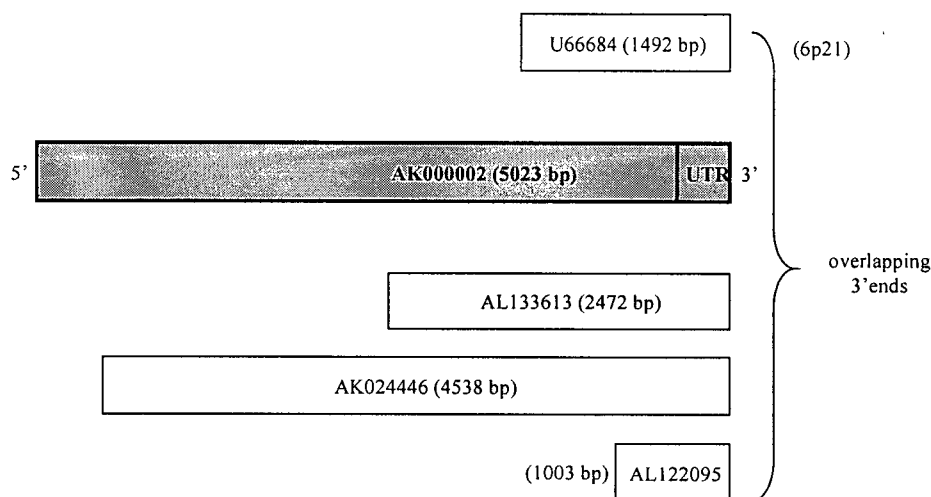
randomly labeled with  $^{32}\text{P}$  according to the manufacturer's instructions (Amersham). The labeled probe was then hybridized to the MTE array according to the manufacturer's protocol (Clontech).

## RESULTS

The experiments described in this appendix were conducted in collaboration with Allison Pahl (MSc. student, Dr. Victor Ling's Laboratory).

### A.3.1 The Discovery of a Novel EST Representing a Full-Length MRP7 cDNA

In 1998, during a routine search of dbEST for novel members of the ABC gene family, we identified a 1914 bp EST (U66684) that had been putatively assigned as MRP7 (Allikmets et al., 1996). In the summer of 2000, a search of the non-redundant (nr) database revealed 4 other cDNA clones with high sequence similarity to EST U66684. These clones had been submitted to the nr database within the past six months and included: AL1222095 (1003 bp), AK024446 (4538 bp), AL133613 (2472 bp) and AK000002 (5023 bp) (Figure A.2). Each of these cDNA clones matched perfectly with the 3' end of EST U66684. Among these, the cDNA clone AK000002 was of the greatest interest due to its large size (5023 bp). A search of the human total genome sequence (HTGS) database using the complete AK000002 cDNA sequence as bait revealed two BAC clones (AC021391 and AL359813) with 100% identity over 23 sequence stretches. Using the FPC software, we determined that these 2 BAC clones were indeed overlapping and located on chromosome 6p21, in agreement with the FISH data reported previously by Allikmets et al. (1996). The stretches of identity (putative exons) between the 2 BAC clones and EST U66684 ranged in size from 75 to 454 nucleotides and were identical in both BACs. The AK000002 cDNA clone was acquired from the Kazusa DNA Research Institute (Japan).



**Figure A.2. cDNA Clones Representing MRP7.**  
See Materials and Methods section A.2.1 for details.

### A.3.2 Choosing the Putative Start Site of the AK000002 cDNA

A 3-phase amino acid translation of the AK000002 cDNA was performed using the DNA Strider 1.2 program to identify putative translation initiation codons. A methionine (AUG) was identified at AA position 22 (Figure A.3) for which four out of the six bases preceding this presumptive initiation codon (GCG TCC) were identical to the Kozak consensus sequence (GCC ACC) (Kozak, 1987). The corresponding stop codon was followed by 331 bp of untranslated sequence, leaving a coding sequence with a length of 4473 bp, suggesting clone AK000002 contains a complete ORF of 1491 amino acids. This length is comparable to that of other MRP proteins with a similar structure.

Using this reading frame we identified the Walker A, Linker and Walker B consensus motifs (Figure A.3). The putative N-terminal Walker A sequence, starting at AA position 654, matches perfectly with the Walker A consensus motif (see section 1.1.3, chapter I) and 13 of 14 residues starting at AA position 1300 also match this consensus motif, forming the putative C-terminal Walker A region. The putative N-terminal Linker motif, (AA position 747) matches 9 of 12 residues of the consensus, and 11 of 12 residues of the putative C-terminal Linker (AA position 1402) match the Linker consensus sequence. Finally, 19 of 20 residues at both AA positions 810 and 1465 match the consensus and comprise the putative N-terminal and C-terminal Walker B motifs, respectively.

The closest alternative downstream initiation codon occurs at AA position 318 (Figure A.3), with 4 of 6 residues immediately upstream matching the Kozak consensus sequence. However, this putative initiation site would encode a truncated protein lacking a large portion of the first transmembrane domain (296 residues), which would be unlike any other MRP protein structure. As such, it was unlikely that it is the preferred translation initiation site.

Stop codons were found 5' to the predicted start site in all 3 frames, suggesting the start site was not more 5' (data not shown). The two alternative reading frames contained stop codons within 300 AA residues of the predicted start site, confirming that we had identified the correct ORF (data not shown).

### **A.3.3 5' RACE Results**

We applied 5' RACE to determine if clone AK000002 represented the complete ORF and 5' UTR (Figure A.4). After the first round of PCR amplification there were no visible products (trial 1, lane 1). After a second round of PCR using nested primers we observed 4 distinct PCR

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1      GSGCLGAEKR EGKNRWQGEA SMERLLAQLC GSSAAWPLPL WEGDTTGHCF TQLVLSALPH
61     ALLAVLSACY LGTPRSPDYI LPCSPGWRLR LAASFLLSVF PLLDLLPVAL PPGAGPGPIG
121    LEVLAGCVAA VAWISHSLAL WVLAHSPHGH SRGPLALALV ALLPAPALVL TVLWHCQRGT
181    LLPPLLPGPM ARLCLLILQL AALLAYALGW AAPGGPREPW AQEPLLPEDQ EPEVAEDGES
241    WLSRFSYAWL APLLARGACG ELRQPQDICR LPHRLQPTYL ARVFQAHWQE GARLWRALYG
301    AFGRCYLALG LLKLVGTM LG FSGPLLLSLL VGFLEEGQEP LSHGLLYALG LAGGAVLGAV
361    LQNQYGYEVY KVTLQARGAV LNILYCKALQ LGPSRPPTGE ALNLLGTDSE RLLNFAGSFH
421    EAWGLPLQLA ITLYLLYQQV GVAFVGGLIL ALLLVPNKV IATRIMASNQ EMLQHKDARV
481    KLVTELLSGI RVIKFCGWEQ ALGARVEACR ARELGRLRVI KYLDAACVYL WAALPVVISI
541    VIFITYVLMG HQLTATKVFT ALALVRMLIL PLNNFPWVIN GLLEAKVSLD RIQLFLDLPN
601    HNPQAYYSPD PPAEPSTVLE LHGALFSWDP VGTSLET FIS HLEVKKGMLV GIVGKVGCGK
661    SSLLAAIAGE LHRLRGHVAV RGLSKGFGLA TQEPW IQFAT IRDNILFGKT FDAQLYKEVL
721    EACALNDDLS ILPAGDQTEV GEKGVTLSSG QRARIALARA VYQEKELYLL DDPLAAVDAD
781    VANHLLHRCI LGMLS YTT RL LCTHRTEYLE RADAVLLMEA GRLIRAGPPS EILPLVQAVP
841    KAWAENGQES DSATAQSVQN PEKTKEGLEE EQSTSGRLLO EESKKEGAVA LHVYQAYWKA
901    VGQGLALAIL FSLLLMQATR NAADWWLSHW ISQLKAENSS QEAQPSTSPA SMGLFSPQLL
961    LFSPGNLYIP VFPLPKAAPN GSSDIRFYLT VYATIAGVNS LCTLLRAVL F AAGTLQAAAT
1021   LHRRLHRLV MAPVTFFNAT PTGRILNRFS SDVACADDSL PFILNILLAN AAGLLGLLAV
1081   LGSGLPWLLL LLPPLSIMYY HVQRHYRASS RELRRLGSLT LSPLYSHLAD TLAGLSVLRA
1141   TGATYRFEED NLRLLELNQR CQFATSATMQ WLDIRLQLMG AAVVSAIAGI ALVQHQQGLA
1201   NPGLVGLSLS YALSLTG LLS GLVSSFTQTE AMLVSVERLE EYTCDL PQEP QGQPLQLGTG
1261   WLQGGVEFQD VVLAYRPGLP NALDGVTF CV QPGEKLGIVG RTGSGKSSLL LVLFRLL EPS
1321   SGRVLLDGVD TSQLELAQLR SQLAIIPQEP FLFSGT VREN LDPQGLHKDR ALWQALKQCH
1381   LSEVITSMGG LDGELGEGGR SLSLGQRQLL CLARALLTDA KILCIDEATA SVDQKTDQLL
1441   QQTICKRFAN KTVLTIAHRL NTILNSDRVL VLQAGRVVEL DSPATLRNQP HSLFQQLLQS
1501   SQQGV PASLG GP

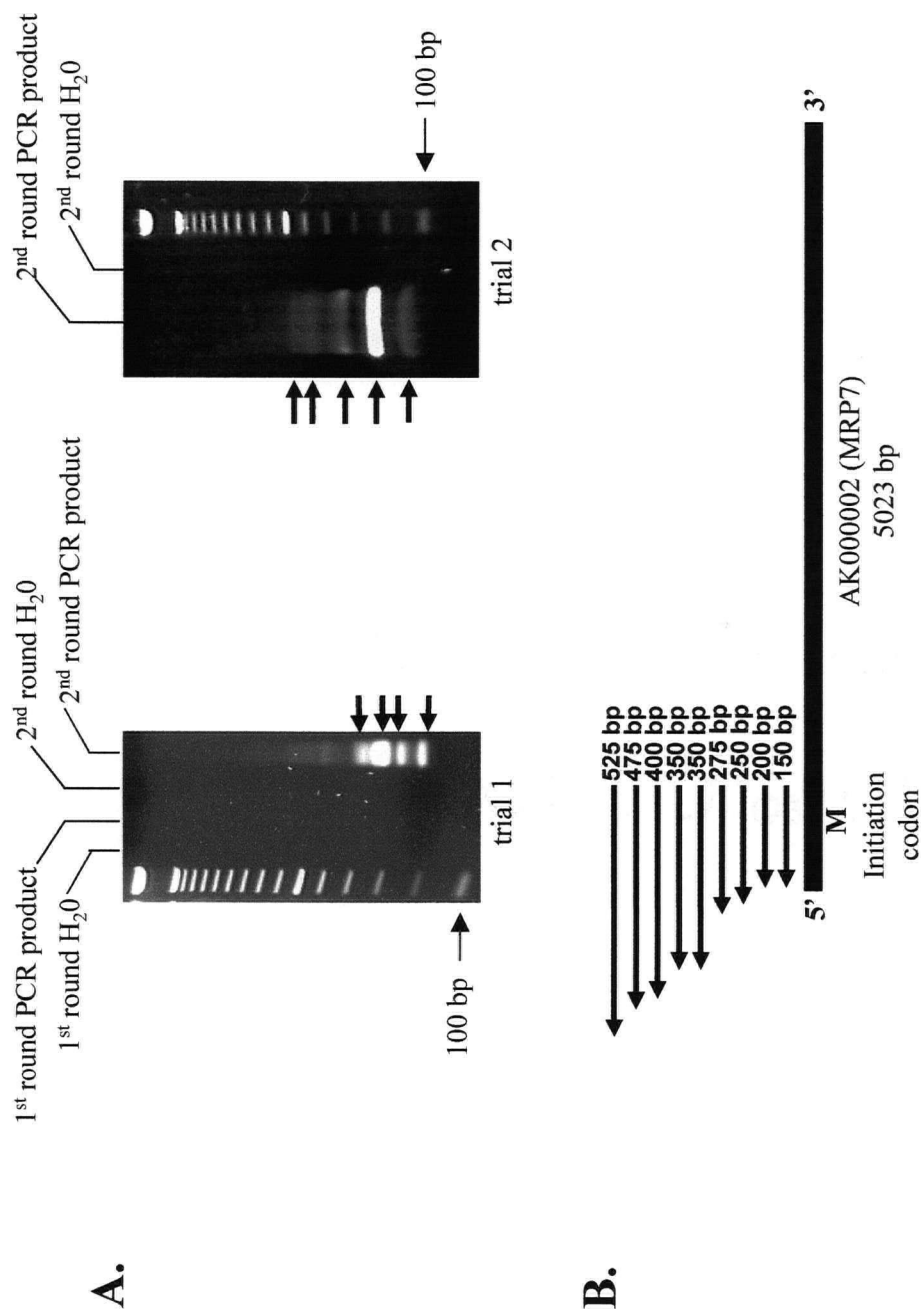
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**Figure A.3. Predicted Amino Acid Sequence of cDNA Clone AK000002 (MRP7).**

Longest open reading frame based on amino acid translation of the AK000002 cDNA nucleotide sequence. Potential methionine start sites are highlighted in red. Underlined text indicate the Walker A, Walker B and Linker motifs. See Materials and Methods section A.2.2 for details.

products (200, 275, 350 and 400 bp), whereas no products were visible in the negative control lane. Since the gene specific primer from which they were amplified (341 RACE2 MRP7) is located 127 bp downstream of the putative initiation codon, these bands correspond to products which extend beyond the putative initiation codon by 73, 148, 223 and 273 bp, respectively. Repeating the assay yielded 5 distinct PCR products (150, 250, 350, 475 and 525 bp), corresponding to products that extend beyond the initiation codon by 23, 148, 223, 348 and 398 bp, respectively.

Sequence analysis of the gel-excised PCR products confirmed that they were identical to the 5' end of the AK000002 cDNA sequence. These results, and the absence of any larger PCR products, would suggest that the putative initiation codon identified in clone AK000002, which is flanked by 211 bp of upstream (5') sequence, is likely the true initiation codon. We concluded that clone AK000002 contains the full ORF of MRP7 and a portion of the non-coding 5'UTR.



**Figure A.4. 5' RACE Analysis of cDNA Clone AK000002 (MRP7).**

(A) 5' RACE PCR products were separated on a 1% agarose gel. (B) Approximate position of sequenced PCR products from panel A relative to the nucleotide sequence of AK000002. See Materials and Methods sections A.2.3 and A.2.4.

### A.3.4 Comparison of Amino Acid Similarity Between MRP7 and Other MRP Subfamily Members

Using the sequence of the AK000002 cDNA and the published full-length sequences of the MRP subfamily members (see Table II.I, chapter II), we determined the extent of AA similarity of MRP7 with the other MRP subfamily members (Table A.I). MRP7 appears to be more closely related to MRPs 1-6 than to SUR1, SUR2 and CFTR; although the distinction is minor.

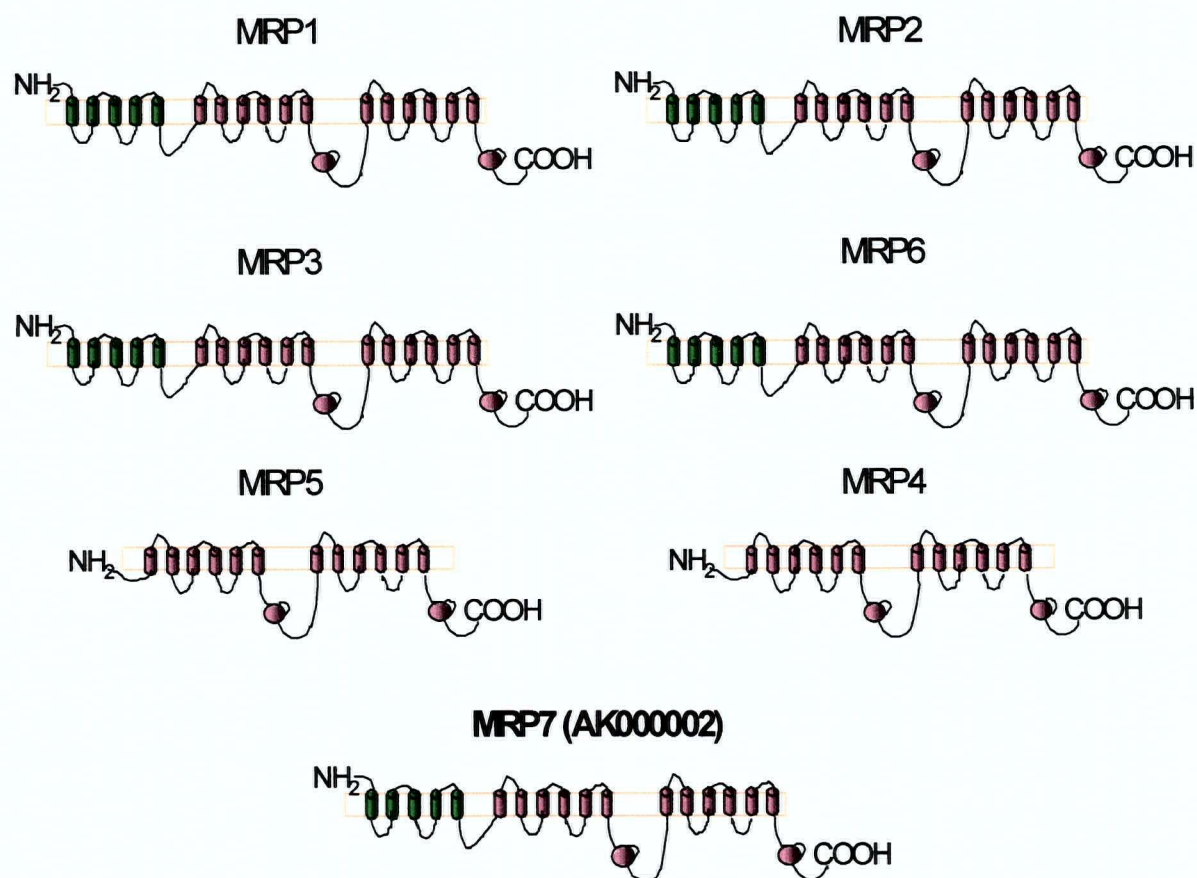
	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	SUR1	SUR2	CFTR
MRP7	34	32	36	32	33	33	---	24	31	30

**Table A.I Percent Amino Acid Identity Among Human MRP Subfamily Members and MRP7.**

Percent identity among MRP subfamily members was determined using BLASTP2.1.2. See Materials and Methods section A.2.5 for details.

### A.3.5 Predicted Protein Structure of MRP7

It has previously been demonstrated by various research groups that MRP-related transporters can be classified into two structural groups, with the first consisting of MRP1, 2, 3 and 6 and the second group consisting of MRP4 and MRP5. This division is based on the presence (MRP1, 2, 3 and 6) or absence (MRP4 and MRP5) of an N-terminal domain composed of 5 transmembrane helices in addition to the 12 membrane spanning domains typical of ABC full-transporters (see section 1.1.3, chapter I). Hydrophobicity plot analysis of MRP7 (Figure A.5) revealed the presence of an N-terminal domain composed of 5 transmembrane helices, as well as two membrane spanning domains and two nucleotide domains typical of a full-transporter.



**Figure A.5 Predicted Domain Organization of MRP7 and Other MRP Subfamily Members.**

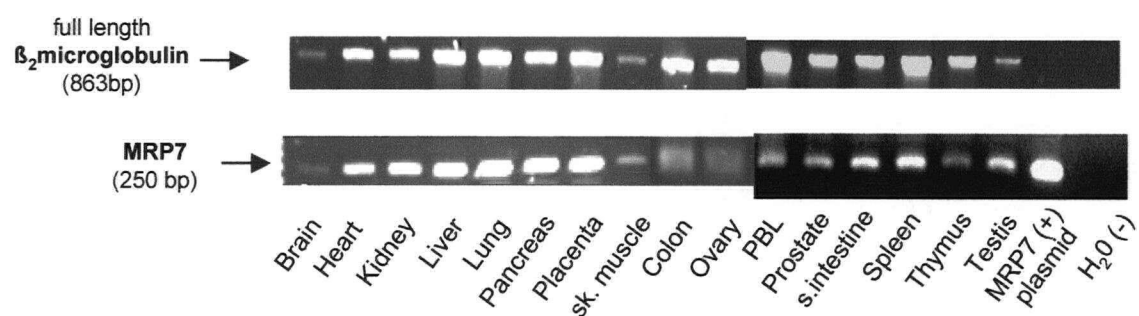
The schematic of domain structures is based on hydropathy plot analysis of clone AK000002, representing the putative MRP7, and published full-length cDNA sequences for MRPs 1-6. See Materials and Methods section A.2.6 for details.

### **A.3.6 Expression Pattern of MRP7 in Normal Human Tissues Using RT-PCR**

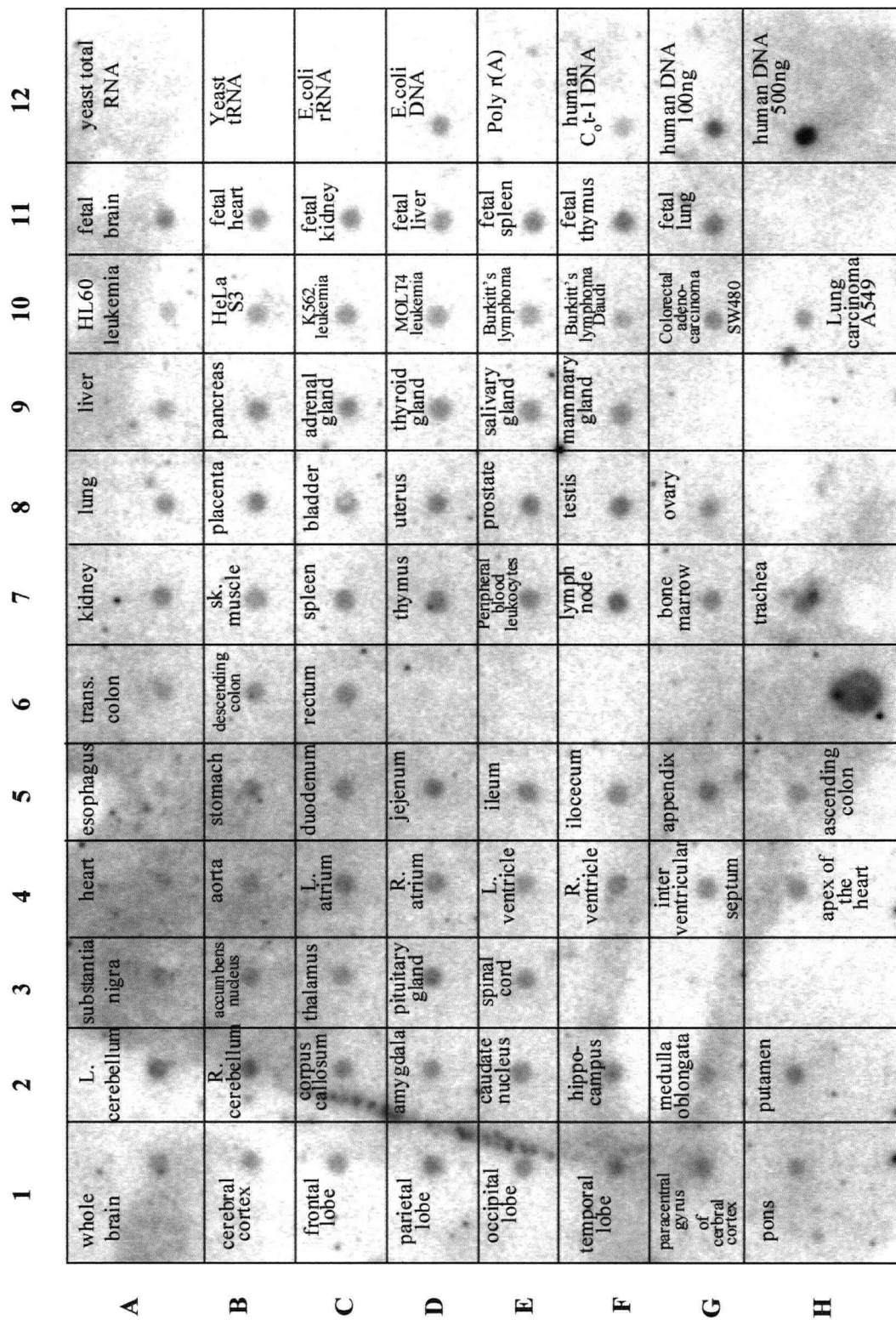
Competitive-RT-PCR analysis of MRP7 expression (chapter IV) revealed the transcript was ubiquitously expressed at low levels in all 12 tissues examined. To confirm these results, semi-quantitative-PCR was performed using a commercial cDNA panel from normal human tissues (Figure A.6). In agreement with our competitive-RT-PCR results, we observed ubiquitous expression of the MRP7 transcript.

### **A.3.7 Expression Pattern of MRP7 Determined Using Dot Blot Analysis**

To further assess MRP7 mRNA expression in an expanded set of tissues we used a commercial dot blot array. Hybridization of a radiolabeled MRP7 probe to the MTE array (Clontech) revealed that MRP7 was expressed in all tissues examined at approximately equal levels (Figure A.7). The MRP7 probe also hybridized with *E. Coli* DNA and human C<sub>0</sub>t-1 DNA; however, hybridization did not occur with yeast total RNA, yeast tRNA or *E. Coli* rRNA. Because of the high background present on the blot, we had planned to repeat this experiment using other washing conditions; however, this was not completed due to our decision to terminate the MRP7 project (please see discussion).



**Figure A.6 Semi-quantitative PCR Expression Analysis of MRP7 in Normal Human Tissues.**  
 A cDNA panel of normal human tissues (Clontech) was used as template for amplification of MRP7 and  $\beta_2$ microglobulin. PBL = peripherhal blood leukocytes. See Materials and Methods section A.2.7 for details.



**Figure A.7 MRP7 mRNA Expression in a Panel of Human Tissues Using Dot Blot Analysis.**

A radiolabeled MRP7 probe was hybridized to an MTE dot blot (Clontech). Boxes without labels are regions of the blot without RNA. See Materials and Methods section A.2.8 for details.

## DISCUSSION

In January of 2001, two months after initiating the cloning and characterization of MRP7, a paper by Hopper et al. (2001) was published describing the cloning, structural analysis and expression pattern of MRP7, using the same cDNA clone as ourselves (AK000002). Hopper et al. analyzed the ORF of this cDNA and reported the position of a putative initiation codon, as well as the 5' and 3' UTRs, all of which agreed with our analysis. However, 5'RACE analysis was not performed. Their assignment of the putative initiation start site and the integrity of the predicted ORF were confirmed using an *in vitro* transcription/translation assay, which yielded a protein product migrating at 158 kD, in agreement with the calculated molecular weight of 162 kD.

Hopper et al. also performed AA sequence comparisons of MRP7 and the other members of subfamily C, and reported that MRP7 was more closely related to the MRP-like proteins than to CFTR, SUR1 or SUR2, in agreement with our data. The analysis by Hopper et al. of the transmembrane structure of MRP7 predicted an additional N-terminal transmembrane domain, as we observed. In addition, our predicted location of the Walker A, Walker B and Linker motifs are similar to those reported by Hopper et al.

Our expression analysis of MRP7 mRNA using competitive-RT-PCR, semi-quantitative-PCR and dot blot analysis revealed the transcript was expressed at low levels in all tissues examined. In agreement with our data, Hopper et al. detected the MRP7 transcript in all tissues examined using RT-PCR, but observed no detectable expression using Northern blot analysis. Compared to other MRP-like transcripts, as well as most other ABC mRNAs, the expression of MRP7 is very low (chapter IV). Since MRP7 is detectable in a very broad range of both adult and embryonic tissues, this protein might belong to the ABC "house-keeping" class of genes

(chapter IV). The low mRNA expression of MRP7 may be compensated for by high affinity for its substrate(s), an efficient rate of substrate turnover, strong transcriptional response to stimuli and/or long protein half-life. The normal physiological role of MRP7 is still unknown; however, based on high sequence similarity to other MRP-like transporters, we can speculate that MRP7 contributes to the export of glutathione conjugates or other organic anions.

In addition to the data described here, we initiated several other MRP7-related projects including Northern blot analysis, generation of FLAG epitope-tagged full-length cDNA for transfection studies, identification of intron and exon boundaries in mouse and human genomic DNA and analysis of MRP7 mRNA expression in drug-exposed cell lines (see Allison Pahl's Master's thesis for details). Personal communication with Gary Kruh at an ABC conference in February of 2001 revealed that stable-transfected cell lines expressing epitope-tagged MRP7 cDNA had already been established in their laboratory and that drug resistance studies had been underway for several months. The Kruh laboratory also has extensive experience in drug transport studies using MRP-subfamily transfectants (Zeng et al., 1999) (Lee et al., 2000). In light of this information and due to time constraints, we regretfully chose not to pursue this project further.

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