RNA Expression Profiles of the Human ABC Transporter Family in Patient-Derived Leukemia Cell Lines and in HL60 Cells Exposed to Chemotherapeutic Drugs

by

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B.Sc., The University of British Columbia, 1998

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Biochemistry and Molecular Biology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2001

Department of Biochemistry and Molecular Biology

The University of British Columbia
Vancouver, Canada

Date April 18, 2001

ABSTRACT

Poor outcome in Acute Myeloid Leukemia (AML) is associated with the elevated expression of P-glycoprotein (Pgp) and the Multidrug-Associated Resistance Protein (MRP1), members of the ATP-Binding Cassette (ABC) protein superfamily. However, a proportion of AML patient blasts demonstrates drug-efflux that is not correlated with Pgp or MRP1 expression. The AML1, 2 and 3 cell lines, derived from AML patient blasts at relapse, exhibit ATP-dependent daunorubicin accumulation with no Pgp and low MRP1 expression. This evidence strongly suggests the presence of energy-dependent processes distinct from Pgp that contribute to daunorubicin efflux. The large number of human ABC proteins (>50) and the complex nature of drug efflux in Multiple Drug Resistance (MDR) deems it reasonable to expect that currently uncharacterized ABC proteins may contribute to drug efflux in AML.

Our laboratory has developed a Competitive-Reverse Transcription-Polymerase Chain Reaction (C-RT-PCR) assay to examine global mRNA expression of 40 human ABC transcripts to evaluate their contribution to MDR in cancer cell lines. This assay uses RNA competitors for each transcript and the fluorescently labeled PCR products are detected using an automated DNA sequencer.

Surprisingly, C-RT-PCR expression profiles of the AML cell lines revealed that the majority of ABC transcripts examined were detectable (75%) and that considerable patient heterogeneity was evident, providing no clear indication of which ABCs might be relevant to MDR. To address this issue, we examined whether chemotherapeutic drugs could induce expression of ABC genes.

The human HL60 leukemic cell line was exposed for 24 hours to individual chemotherapeutic drugs at low drug concentrations. Using C-RT-PCR, significant expression changes were detected with only 2% of the transcripts examined. Among these, a 6.2 ± 1.1-fold increase in MRP7 ($p<0.01$), a novel member of the Multidrug Resistance-Associated Protein subfamily of ABC transporters, was observed with daunorubicin treatment (1/2 IC_{50}). Efforts then focused on generating a MRP7 cDNA construct to be used in drug resistance assays of transfected cells. The absence of significant expression changes in response to drug exposure might suggest that induction of ABC gene expression may not play a significant role in the development of drug resistance in HL60 cells.

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ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Victor Ling for his guidance, optimism and support. I am indebted to Steven Ralph for his mentorship and I thank him for not only for his critical evaluation of this manuscript, but also for his many contributions to this project, for sharing his enthusiasm for science, his friendship, and for introducing me to sushi. I am grateful to Douglas Hogue for his many helpful suggestions and I also thank Barbara Schmidt for sharing her expertise in cell culture.

I would also like to extend my thanks to my supervisory committee members, Drs. Ross MacGillivray, Michel Roberge and Heather Sutherland, for their insightful comments. I would like to thank Dr. Heather Sutherland for her commitment to provide patient samples.

Finally, I thank the B.C. Foundation for Non-Animal Research for their support in the form of the Evelyn Martin Memorial Fellowship.

Dedication

To my parents, Dan and Cheryl Pahl, for their unending support and encouragement.

ACKNOWLEDGMENT OF COLLABORATIVE WORK

The work represented in Chapter III was conducted in collaboration with Steven Ralph (Ph.D student, Dr. Victor Ling's laboratory). Both authors contributed equally to the design of these experiments and the analysis of the data. Allison Pahl performed 80% of the cell culture, IC_{50} determinations and PCR analyses, with the remainder completed by Steven Ralph. All RNA isolations and operation of the ABI DNA sequencer were performed by Steven.

The work represented in Chapter IV was also conducted in collaboration with Steven Ralph. Both Allison Pahl and Steven Ralph contributed equally to the design of these experiments, labour and analysis of the data. We the authors acknowledge that the above statements are accurate.

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CHAPTER I

Introduction to the ATP-Binding Cassette Gene Family

1.1 General Properties of ATP- Binding Cassette Transporters

Many important cellular processes rely upon the transport of substances across biological membranes. 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 a sensors of external signals. Most membrane channel proteins allow solutes such as water and other non-polar molecules to cross the membrane passively, a process known as facilitated diffusion. However, cells also require membrane proteins that will actively pump polar molecules, such as ions, sugars, amino acids, nucleotides and cell metabolites across the membrane in a directional manner against a concentration gradient. This process requires the coupling of transport with the hydrolysis of ATP.

The characterization of proteins involved in transmembrane transport has been intensively studied over the past 20 years, and it became apparent that the myriad of membrane transport systems could be grouped into a limited number of subfamilies, based upon sequence, functional similarity and mechanism of action (Higgins, 1992). The largest and most diverse of these subfamilies is the ATP Binding Cassette (ABC) superfamily whose members are associated with important physiological processes in many organisms, ranging from bacteria to humans. The first ABC transporters to be characterized were bacterial uptake systems. The hemolysin protein complex, a pathogenicity factor of *Escherichia coli,* was among the first bacterial ABC export proteins to be discovered (Felmlee *et al.*, 1985) and by 1992, 50 ABC proteins had been discovered, the majority from prokaryotic species. Among the first eukaryotic ABC transporters to be characterized were P-glycoprotein (Pgp), an ATP-dependent drug efflux pump found to be overexpressed in tumour cells (Gerlach *et al.*, 1986), the *Saccharomyces cerevisiae* STE-6 protein, necessary for the secretion of the a-mating pheromone (Kuchler *et al.*, 1989), the white and brown gene products of *Drosophila melanogaster,* implicated in the transport of pigment precursors (Dreesen *et al.*, 1988) and the Cystic Fibrosis protein CFTR, which functions as a chloride channel (Riordan *et al.*, 1989). Comparison of these amino acid sequences revealed significant similarity between the prokaryote, yeast and higher eukaryote

open reading frames. This discovery of great homology among these proteins suggested they all 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. Since then, ABC proteins have been found in all kingdoms of life and more than 40 ABC proteins have been identified in humans.

The majority of ABC proteins identified act as transmembrane pumps, however ABC proteins can serve other functions, such as in DNA repair in *E.coli* (UvrA) (Husain *et al.*, 1986), translation 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 within intracellular compartments (across organelle membranes). In contrast, specific bacterial ABC proteins mediate the either the import or export of substrates across the bacterial plasma membrane, including sugars, amino acids, 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 in eukaryotes, where specific ABC proteins transport ions, metals, steroid hormones, sugars, peptides and drugs. Despite the wide range of substrate diversity across the entire superfamily, most individual ABC proteins exhibit limited substrate specificity. Notable exceptions are the human Pgp and the human Multidrug Resistance-Associated Protein-1 (MRP1), each of which transport a variety of structurally unrelated compounds. How these transporters are able to transport a diverse range of substrates, yet retain a high degree of substrate selectivity, remains poorly understood. It is also this diversity which makes it virtually impossible to predict the function or substrate specificity of a particular ABC transporter based on sequence information alone.

1.2 Structural Characteristics of ABC Proteins

ABC proteins are defined by the presence of at least one nucleotide binding domain (NBD) (Klein *et al.*, 1999). Within the NBD region are three consensus sequences: the first two are the ABC Walker A (G-X_2-G-X-G-K-S/T-T/S-X_4-hydrophobic) and ABC Walker B (R-X-hydrophobic$_2$-X_2-P/T/S/A-X-hydrophobic$_4$-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. The third motif is found only in ABC superfamily proteins, called the "linker" (Shyamala *et al.*, 1991) or the "ABC Signature Motif" (hydrophobic-S-X-G-Q-R/K-Q-R-hydrophobic-X-hydrophobic-A). These conserved regions are always found in the order (from the N-terminus to C-terminus): ABC Walker A, followed by a 100-150 amino acid non-conserved stretch, linker, 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 (TMD) and two NBDs. These domains may be present within one polypeptide chain ('full transporters') or homo- or heterodimers 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 helices that are highly divergent among ABC proteins, consistent with the notion that TMDs are important determinants of substrate specificity. Under native conditions full transporters appear to predominantly localize to the plasma membrane, while most half transporters are localised to intracellular organelles, such as the endoplasmic reticulum, peroxisome or mitochondria (Klein et al., 1999).

With respect to gene organization of ABC transporters, the NBDs and TMDs of most prokaryotic ABC transporters are encoded by separate genes, though always in the same operon. In some prokaryotic and all known eukaryotic ABC superfamily members, two or more sequence domains are found within one gene.

1.3 ABC Gene 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 6 distinct subfamilies (Figure 1). An analysis of the recently completed human genome sequencing project suggests there are 52 human ABC genes (Allikmets, 2001) however some of these may be pseudogenes since transcribed open reading frames have not been examined. For the purposes of this research, we have only described ABC genes for which there is definitive transcript data. For a complete up-to-date overview of ABC transporters, please see the webpage of Michael Muller: http://www.med.rug.nl/mdl/humanabc.htm

The existence of several ABC proteins (ABCA5, ABCA6, ABCA8, ABCB5, ABCF2, ABCF3, ABCG3 and ABCG4) have 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. Subfamily A consists of 8 known members, all of which are full-transporters. ABC1 and ABCR are associated with inherited genetic disorders of lipid export processes involving vesicular budding between the Golgi and plasma membrane (Orso, 2000) and transport of protonated N-retinylidene-phosphatidylethanolamine out of retinal disk cells (Weng *et al.*, 1999), respectively (Table I). The function of ABC1 may also be required for engulfment of apoptotic bodies (Luciani & Chimini, 1996). ABCA7 was recently described as a sterol-sensitive protein (Kaminski *et al.*, 2000) and the functions of ABC2 and ABC3 are unknown.

There are 4 MDR1 - like full transporters and 7-half transporters in subfamily B. The MDR1 transporter (P-glycoprotein) can extrude a wide range of structurally unrelated natural product drugs (i.e. vincristine, doxorubicin, mitoxantrone and paclitaxel) from the cell (Germann, 1996). P-glycoprotein is localized to apical membranes of epithelial 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). TAP1 and TAP2 have been localized to the endoplasmic reticulum and actively transport peptides from the cytosol into the lumen of the ER where the peptides associate with Class I molecules for presentation and immunorecognition by cytotoxic lymphocytes (Abele & Tampe, 1999). ABCB9 localizes to lysosomes in transfected SKOV3 cells but its function is unknown (Zhang *et al.*, 2000). The other 4 half-transporters (M-ABC1, ABC7, M-ABC2 and MTABC3) are localized to the mitochondria and although their specific functions are unknown, at least two of these proteins are likely to play a role in iron homeostasis (Allikmets *et al.*, 1999) (Mitsuhashi *et al.*, 2000).

Subfamily C consists of 10 full transporters including CFTR, SUR1, SUR2 and MRPs 1-7. Six proteins of this subfamily (i.e. MRPs 1, 2, 3, and 6, SUR1 and SUR2) form a subcluster within which each member possesses an additional N-terminal transmembrane domain (TMD_0) 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 hyperinsulemic hypoglycemia of

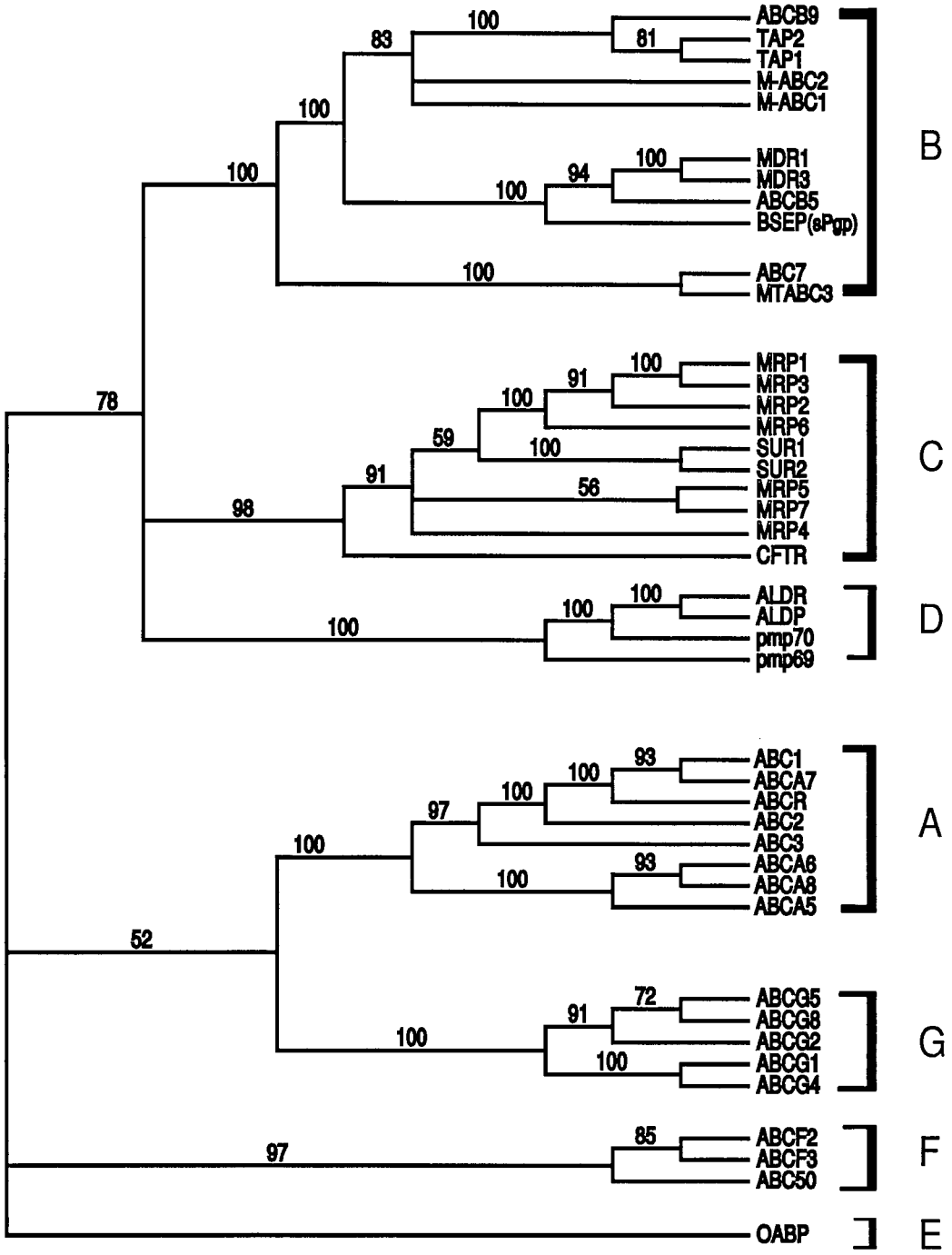


FIGURE 1. Phylogenetic tree of ABC proteins in *H. sapiens*: predicted amino acid sequences were aligned using ClustalX (Thompson et al., 1997.) The cladogram is derived from a neighbour-joining analysis using PAUP*4.0b3a (Swofford, 1997) of the resulting alignment. Bootstrapping (Felsenstein, 1985) 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 results of the first tree (not shown). Bootstrap values are therefore not strictly comparable between the base and the tips of the tree. The figure was prepared using Adobe Illustrator v8.0.1. Letters to the right of the tree indicate membership in the corresponding subfamilies according to the HUGO nomenclature (http://www.gene.ucl.ac.uk/users/hester/abc.html). Tree was contributed by Jonathan A. Sheps.

Table I. Functions and/or Diseases Associated with Human ABC Proteins.

Subfamily	ABC protein	Function or association	Disease or clinical phenotype (N.D. – not determined)
A	ABC1	·Export of cholesterol and phospholipids from cells ·Phagocytosis of apoptotic cells	Tangier disease
	ABC2	·Overexpression confers resistance to estramustine	N.D.
	ABCR	·Transport of N-retinylidene-phosphatidylethanolamine	Stargardt's disease Retinitis pigmentosa
	ABCA7	·Upregulated in the presence of low density lipoprotein	N.D.
B	MDR1	·Broad specificity amphipathic drug transporter ·Overexpression confers resistance to many natural product drugs	MDR in cancer
	MDR3	·Phosphatidylcholine secretion into bile	Progressive familial intrahepatic cholestases
	BSEP	·Bile acid transporter	Progressive familial intrahepatic cholestases
	TAP1/2	·HLA Class I antigen transporter	Herpes simplex virus infection Immune surveillance
	ABC7	·Mitochondrial iron homeostasis	X-linked sideroblastic anemia and ataxia
	MTABC3	·Mitochondrial iron homeostasis	N.D.
C	MRP1	·Organic anion transporter ·Transports cysteinyl leukotriene LTC_4, 17ß-estradiol 17-(ß-D-glucuronide), aflatoxin B_1, hydrophobic anticancer agents, and anionic drug conjugates ·Overexpression confers resistance to many natural product drugs	MDR in cancer
	MRP2	·Organic anion transporter with substrate specificity similar to MRP1 ·Overexpression confers resistance to cisplatin, methotrexate, etoposide, vincristine and doxorubicin	Dubin-Johnson syndrome
	MRP3	·Organic anion transporter with substrate specificity similar to MRP1 with a preference for glucuronide-conjugates and also transports bile salts ·Overexpression confers resistance to etoposide and methotrexate	N.D.
	MRP4	·Overexpression confers resistance to antiviral nucleoside analogs	N.D.
	MRP5	·Organic anion transporter ·Overexpression confers resistance to thiopurines and antiviral nucleoside analogues	N.D.
	MRP6	·Unknown	Pseudoxanthoma elasticum
	CFTR	·Chloride channel	Cystic fibrosis
	SUR1	·Neuronal/pancreatic sulfonylurea receptor subunit (K_{ATP} channel)	Familial persistent hypoglycemia of infancy
	SUR2	·Cardiac/vascular smooth muscle sulfonylurea receptor subunit (K_{ATP} channel)	Familial persistent hypoglycemia of infancy
D	ALDP	·Regulates very long chain fatty acid ß-oxidation (?)	Adrenoleukodystrophy
	PMP70	·Long chain acyl-CoA transporter (?)	Zellweger's syndrome
E	Rnase LI	·Endoribonuclease inhibitor	N.D.
F	ABC50	·Homology to yeast ribosomal proteins involved in translation	N.D.
G	BCRP	·Overexpression confers resistance to mitoxantrone, daunorubicin, doxorubicin, and topotecan	N.D.
	WHITE1	·Regulator of macrophage cholesterol and phospholipid transport	N.D.
	ABCG5	Unknown	Sitosterolemia
	ABCG8	Unknown	Sitosterolemia

infancy (Bryan & 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.*, 1999) (Konig *et al.*, 1999). Whether MRP6 and MRP7 are organic anion transporters has not yet been determined.

Our understanding of multiple drug resistance was broadened upon the discovery that overexpression of the MRP1 protein is frequently a cause of non-MDR1-mediated drug resistance (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 elastica but its substrate(s) is unknown (Bergen *et al.*, 2000). MRP7 has recently been cloned, although its function is unknown (please see Chapter IV). The MRP subfamily and its role in drug resistance will be described in Chapter IV.

The four half-transporters of subfamily D have been localized to the peroxisome and mutant forms of two of these proteins cause peroxisomal disorders (Shani & 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. Zellweger's disease, a disorder of peroxisome biogenesis, is associated with mutations of PMP70. However, the association of PMP70 with disease and the identity of the substrate for this protein are unclear. The functions of the other two peroxisomal ABCs (PMP69 and ALDR) are unknown.

Rnase L inhibitor, the sole member of subfamily E and ABC50/ABCF1 (subfamily F) possess two ATP-binding cassettes without any TMDs suggesting they do not perform transport functions. RNase L inhibitor is a regulatory protein whose expression inhibits the 2-5A-dependent activation of Rnase L, an interferon –inducible pathway that regulates the stability of many mRNA species (Bisbal et al., 1995). ABC50 is predicted to be a ribosomal protein involved in translation, based on homology to the yeast GCN20 (Richard *et al.*, 1998). ABCF2 and ABCF3 are recent additions to subfamily F, however their functions and substrate specificities remain to be determined.

Finally, subfamily G consists of 5 half-transporters. Members of this subfamily are orthologous to the putative pigment transporters in D. *melanogaster* (WHITE, SCARLET and BROWN). Endogenous human WHITE1/ABCG1 is 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/ABCG2) has been shown to be overexpressed in several mitoxantrone-selected cancer cell lines and may contribute to transport of this drug, although its normal physiological role is unknown (Doyle *et al.*, 1998). ABCG5 and ABCG8 are two adjacent, oppositely oriented genes, in which mutations have been found in patients with sitosterolemia, a disease characterized by the accumulation of dietary sterols and atherosclerosis (Berge *et al.*, 2000).

1.4 ABC Proteins and Multidrug Resistant Cancer

Resistance to chemotherapeutic drugs is a common problem in cancer patients and is a major obstacle to the successful treatment of this disease. Cancerous cells are often found to be refractory to a variety of drugs with different structure and function, despite exposure to only a single chemotherapeutic agent, in some cases. This phenomenon has been termed Multidrug Resistance (MDR). MDR is a complex phenotype, often a result of multiple contributing mechanisms including: (1) enhanced drug efflux mediated by members of the ABC protein family (i.e. Pgp, MRPs 1, 2, 4 and 5, and BCRP); (2) increased detoxification of compounds (i.e. glutathione-s-transferase) (O'Brien & Tew, 1996); (3) alteration of drug targets such as DNA topoisomerase II (Nitiss & Beck, 1996) and (4) alteration of drug induced apoptosis, involving genes of the Bcl-2 pathway (Reed, 1995). Other molecular markers have been shown to be associated with MDR, such as the major vault protein LRP (Scheffer *et al.*, 1995).

Decreased drug accumulation due to increased expulsion of drug in 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 cell surface glycoprotein in cells which displayed this classical MDR phenotype, which was later designated P-glycoprotein, encoded by the MDR1 gene. Subsequent gene transfection studies demonstrated that Pgp could confer a classical multidrug resistance phenotype and drug accumulation experiments using Pgp-containing membrane vesicles elucidated the wide array of drugs transported by Pgp. For many years it was widely believed that Pgp was the exclusive cause of drug resistance, 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 cells which overexpressed MRP1 (Cole *et al.*, 1994) (Grant *et al.*, 1994). As observed with Pgp, MRP1-mediated resistance is associated with decreased cellular accumulation of drugs in membrane vesicles that overexpress MRP1. The spectrum of drugs transported by each of these proteins and their role in clinical resistance is discussed below.

1.4.1 Role of P-gp and MRP1 in Drug Resistant Cancer

Pgp and MRP1 transport a wide range of drugs with different cellular targets. Pgp transports hydrophobic natural product drugs in unmodified form, (e.g. vincristine, vinblastine, doxorubicin, daunorubicin, etoposide, teniposide, mitoxantrone, topotecan and paclitaxel (Germann, 1996)). MRP1 transports a similar yet unique spectrum of drugs conjugated to anionic ligands such as glutathione (GSH), glucuronide, or sulfate, or in unmodified form, possibly together with GSH (Loe *et al.*, 1996) (Leier *et al.*, 1994) (Jedlitschky *et al.*, 1996). In contrast to Pgp-mediated MDR, MRP1 overexpressing tumour cells often possess only modest levels of resistance to taxol and colchicine, and are resistant to heavy metal oxyanions which are not within the spectrum of substrates transported by Pgp (Cole et al., 1994). Transfection experiments with eukaryotic expression vectors containing full length cDNAs of the MRP1 or MDR1 genes into sensitive human colon (Ueda *et al.*, 1987), lung (Zaman *et al.*, 1994) and ovarian (Grant et al., 1994) carcinoma cell lines results in resistance to a number of different anticancer drugs, which cells are then able to expel.

In light of the established links between Pgp and 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 Pgp is associated with an increased risk of treatment failure in several types of cancer. Although there is good evidence for a correlation between Pgp expression and clinical outcome in adult hematopoietic malignancies (Leith *et al.*, 1999)(Senent *et al.*, 1998) (Goasguen *et al.*, 1993), the clinical relevance of Pgp in solid tumors remains in question, as a result of the wide range of MDR1 protein and RNA levels observed in these cancers (Ling, 1997) (Fardel *et al.*, 1996). Patients with melanoma or cancers of the lung who express high levels of MRP1 have significantly worse survival following chemotherapy (Berger *et al.*, 1997) (Ota *et al.*, 1995). MRP1 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 have prompted intensive efforts to identify ways in which the function of MRP1 and/or Pgp can be reversed or circumvented. Current pharmacological approaches to this problem include chemosensitizers which enhance the cytotoxicity of known chemotherapeutic drugs and the use of novel drugs and analogues. Some of the most effective modulators of Pgp-mediated MDR include Cyclosporine A (CsA), verapamil and trifluoperazine, however these agents are generally much less effective in at reversing MRP-associated drug resistance (Loe et al., 1996) (Germann, 1996).

1.4.2 Potential Role For Other ABC Transporters in Drug Resistant Cancer

Several studies involving the step-wise selection of a series of drug-resistant cells have demonstrated the overexpression of MRP1 in low level resistant cells, followed by MDR1 overexpression at higher levels of resistance (Jensen *et al.*, 1995) (Hasegawa *et al.*, 1995), suggesting that several drug efflux mechanisms can collectively contribute to MDR. Zaman *et al.*, (1993) reported the existence of 10 multidrug-resistant sublines of a non-small cell lung carcinoma cell line which expressed neither Pgp nor MRP1, suggesting that overexpression of MRP1 might not account for all forms of non-Pgp mediated MDR. Moreover, other ABC proteins, such as MRPs 2, 4, 5 and BCRP are able to transport, and confer resistance to, anticancer drugs (Borst *et al.*, 2000) (Doyle et al., 1998). For a detailed description, please see Chapter IV. Previous studies indicate an MDR phenotype is often expressed in tumours derived from tissues with high MDR1 expression (Germann, 1996), which might also occur with other MDR-associated proteins. It remains to be determined if these and/or other uncharacterized ABC proteins contribute to a multi-component drug-efflux phenotype or if they act alone. Furthermore, the contribution of known ABC proteins to the MDR phenotype is unclear owing to the difficulty in generating transfected cell lines that overexpress these proteins with proper cellular localization (Borst et al., 2000). To define the roles of individual ABC proteins in the MDR phenomenon, a comprehensive understanding of the relationship between expression levels of individual ABC proteins, their collective expression profile and drug resistance is required.

1.5 Rationale for Generating mRNA Expression Profiles of the ABC Transporter Family

To date there has been no systematic attempt to determine which combination of ABC proteins contribute to the transport of individual anti-cancer drugs *in-vitro,* nor has anyone investigated the role of the entire human ABC protein family in MDR patient derived-cell lines nor in MDR clinical samples. To understand these multi-component transport mechanisms it is necessary to examine global changes in expression of human ABC proteins, a difficult task due to the characteristically low expression levels of ABC proteins in human tissues. A sensitive method of detection is necessary since clinically relevant expression levels have not yet been defined and even small changes (<2 fold) may significantly alter the resistance profile of a cancer cell.

To address these issues, our lab has developed a fluorescent competitive-RT-PCR (C-RT-PCR) assay, described in Chapter II, to generate mRNA expression profiles of known members of the human ABC gene family. Chapters II and III describe the application of this assay to the study of MDR in Acute Myeloid Leukemia (AML) and to the study of induction of ABC gene expression in response to exposure to chemotherapeutic drugs in the HL60 leukemia cell line, respectively. Chapter IV describes our attempt to characterize MRP7, a transcript which appeared to be induced in response to the chemotherapeutic drug daunorubicin.

CHAPTER II
mRNA Expression Profiles of the ABC Gene Family in Acute Myeloid Leukemia Cell Lines

2.1 INTRODUCTION

2.1.1 Contribution of ABC Proteins to MDR in Acute Myeloid Leukemia

AML is the most common variant of acute leukemia occurring in adults, comprising 80-85% of cases of acute leukemias diagnosed in individuals over 20 years of age. Combination chemotherapy with cytarabine arabinoside (Ara-C) and an anthracycline antibiotic (daunorubicin, doxorubicin, idarubicin) produces complete remission (CR) in 50% to 80% of adults with AML who have not been treated previously. However, only 15% to 30% of patients who achieve remission can be expected to achieve long-term survival (Schiffer, 1993). The remainder of patients relapse and become unresponsive to chemotherapeutic treatment. Most standard or high-dose chemotherapy used for leukemias that are initially unresponsive to chemotherapy (refractory AML) or for relapsed AML fails to induce prolonged disease-free survival. This "salvage" chemotherapy often includes drugs such as etoposide, cyclophosphamide busulfan, and carboplatin (Dr. Heather Sutherland, personal communication).

It has been demonstrated that resistance to anthracyclines involves multiple mechanisms including enhanced drug efflux, mediated by members of the ABC protein family such as Pgp and MRP1 (Germann *et al.*, 1993) (Loe et al., 1996). Efforts to determine the contributions of Pgp to drug efflux in AML blasts have shown that Pgp is frequently expressed in AML and is associated with lower CR rates and in some studies, with shorter overall survival (Zochbauer *et al.*, 1994) (Guerci *et al.*, 1995) (Leith *et al.*, 1997). In addition, Pgp modulators such as verapamil, cyclosporine A (CsA) and its non-immunosuppressive analogue PSC833 can reverse Pgp-mediated resistance *in vitro* and *in vivo.* Clinical trials have been developed to assess the benefits of incorporation of these Pgp modulators into AML therapy and encouraging results have been presented from a recently completed randomized trial that incorporated CsA in the treatment of patients with high risk AML (List *et al.*, 1999).

In contrast to these reports, conflicting data exist regarding the relationship between expression levels of Pgp and clinical outcome in AML, particularly in childhood acute leukemia (den Boer *et al.*, 1998) (Hart *et al.*, 1993). Several investigators have also reported functional drug/dye efflux in AML patient blasts which do not display the "classic" Pgp positive phenotype, indicating that alternate non-Pgp-mediated efflux mechanisms are present in these patients (Leith *et al.*, 1995) (Lamy *et al.*, 1995) (Xie *et al.*, 1995). Even more striking are the recent findings of a large study of 352 AML patients in which flow cytometry was used to quantify expression of Pgp, MRP1 and LRP combined with functional dye efflux measurements (Leith et al., 1999). As expected, they found Pgp expression and CsA-inhibited dye efflux to be correlated with complete remission, whereas no such correlations were observed for MRP1 or LRP. However, they also observed a distinct phenotype in 18% of these cases where CsA-resistant efflux was not associated with Pgp, MRP1 or LRP expression, implying the existence of other as yet undefined efflux mechanisms in AML.

2.1.2 Patient Derived AML Cell Lines

In a collaborative effort with Dr. David Hedley at the Ontario Cancer Institute, our laboratory compared CsA and potassium cyanide (KCN), an inhibitor of oxidative phosphorylation, for their ability to increase daunorubicin uptake into AML blast cells (Hedley *et al.*, 1997). KCN depletes cellular ATP and as such, inhibits energy-dependent processes, including the activity of ABC transporters. The modulating effects of CsA and KCN on daunorubicin accumulation in a number of cell lines and in AML patient blasts were compared. In some cases, neither agent increased drug accumulation, indicating that energy-dependent processes were not involved, whereas in other cases, the CsA and KCN effects were comparable, implicating the involvement of Pgp or another CsA-inhibitable ABC transporter. A series of AML patient-derived cell lines (OCI/AML 1-5) (without *in vitro* drug selection) and many patient samples showed very little modulation of daunorubicin accumulation by CsA but large increases with KCN. These provided strong evidence for the involvement of energy dependent processes other than Pgp, possibly other ABC transporters mediating the efflux of daunorubicin out of AML blasts.

These early observations by Hedley et al. of a novel (non Pgp, non-MRP1-mediated) energy-dependent drug efflux mechanism in several of the OCI/AML cell lines

encouraged us to examine the expression profiles of other ABC transcripts in three of these cell lines. Using C-RT-PCR we examined mRNA expression levels of 32 ABC transcripts, two MDR-associated markers (GST-π, LRP) and the housekeeping gene β_2-microglobulin in the OCI/AML 1, 2 and 3 cell lines. These cell lines were selected because they show the greatest increase in daunorubicin uptake in the presence of KCN and possess the lowest MRP1 levels among the five OCI/AML cell lines. This work represents the first comprehensive expression profile of ABC transcripts in a hematopoietic cell lineage.

2.1.3 The Competitive-Reverse Transcription-Polymerase Chain Reaction Assay

A number of new technologies have been developed in recent years to perform large-scale expression analysis. Serial Analysis of Gene Expression (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 ribonuclease protection assays are limited by the inability to compare more than a few transcripts due to the relatively large amounts of RNA required.

Conversely, PCR-based approaches are relatively simple and can provide excellent sensitivity with a small amount of material. Semi-quantitative PCR methods are limited by 'tube-to-tube' variation, the need to identify initiation of the plateau phase of PCR and the inability to compare transcripts that have been amplified with different primer pairs (Cross, 1995). These limitations are overcome through the use of competitive-RT-PCR since it employs an internal RNA standard that is co-amplified with the target RNA (Wang *et al.*, 1989).

To determine the mRNA expression levels of ~40 ABC transcripts, our lab has modified a fluorescent C-RT-PCR assay (Pannetier *et al.*, 1993) (Steven Ralph, unpublished data). This assay employs RNA competitor molecules that are identical to

electrophoresis on a 310 automated sequencer, detected using a 5' fluorescent label on the forward primer and quantitated using Genescan 2.1 software (Figure 4a). Initial mRNA copy number is determined from the ratio of the endpoint native (target) and internal standard (competitor) PCR products (Figure 4b). This assay is able to reproducibly detect a 50% change in expression levels across a range of more than five orders of magnitude, permits detection of one target in 1 x 10^8 transcripts and has a coefficient of variation of ~ 15% (Steven Ralph, unpublished data).

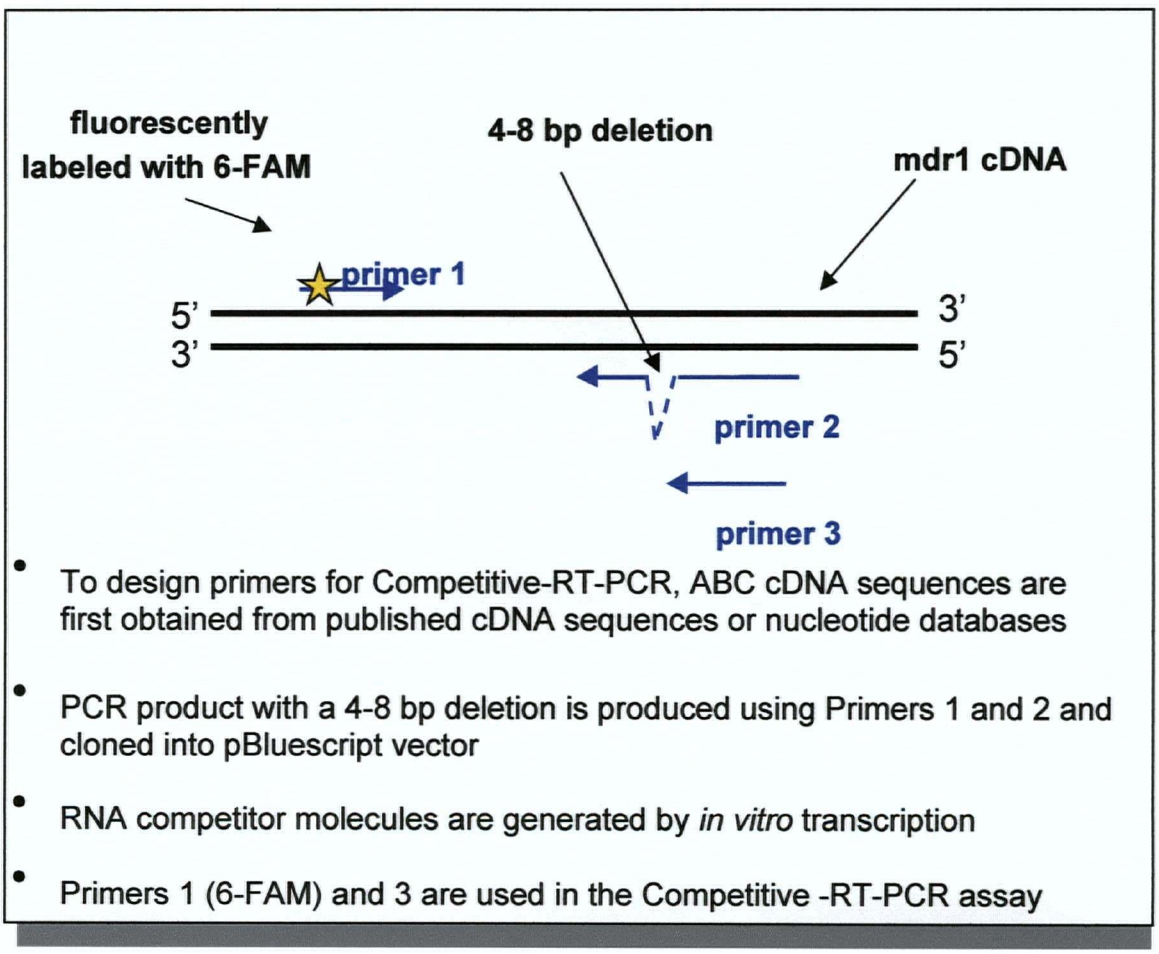


Figure 2. Construction of RNA Competitor Molecules

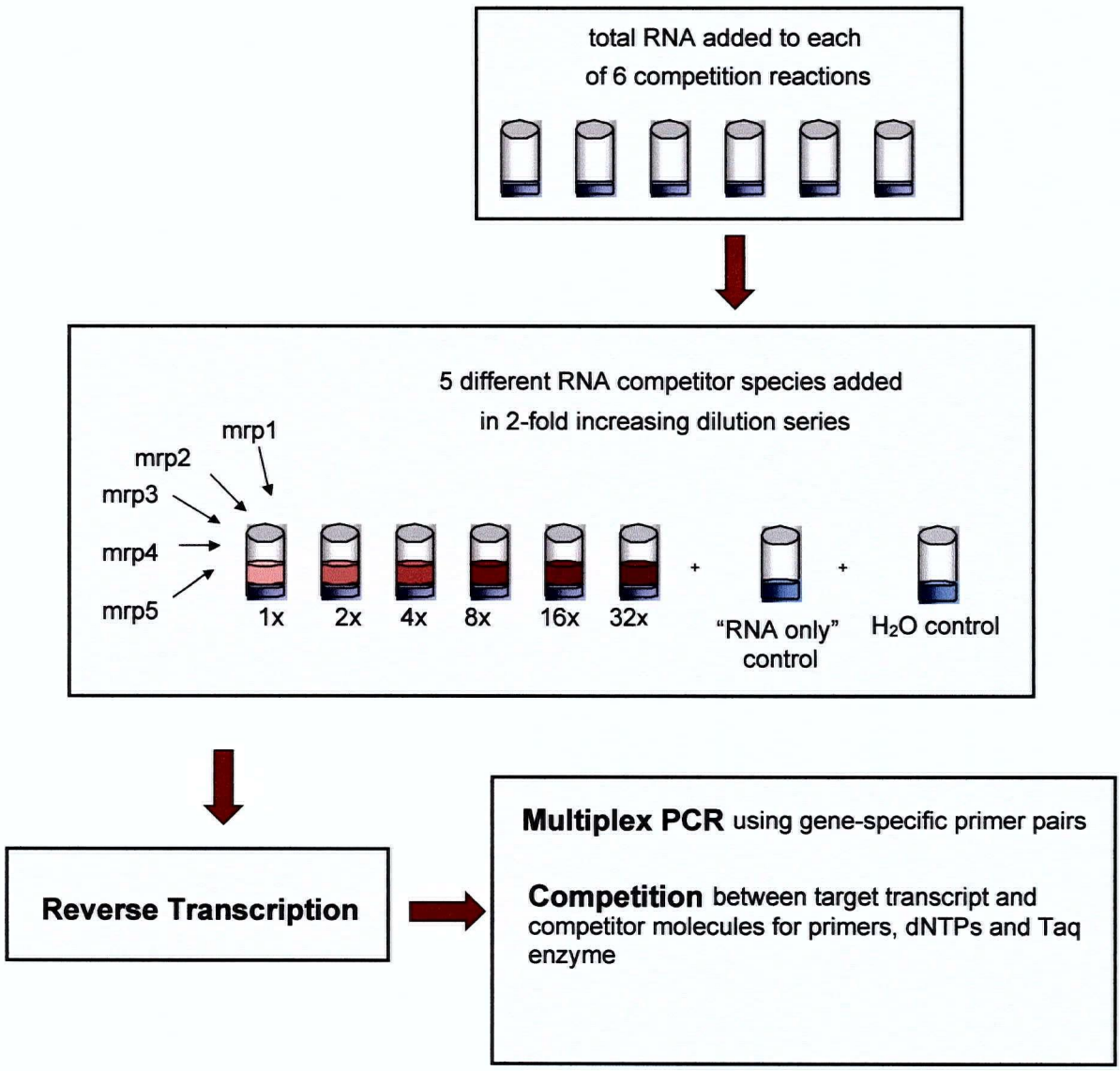
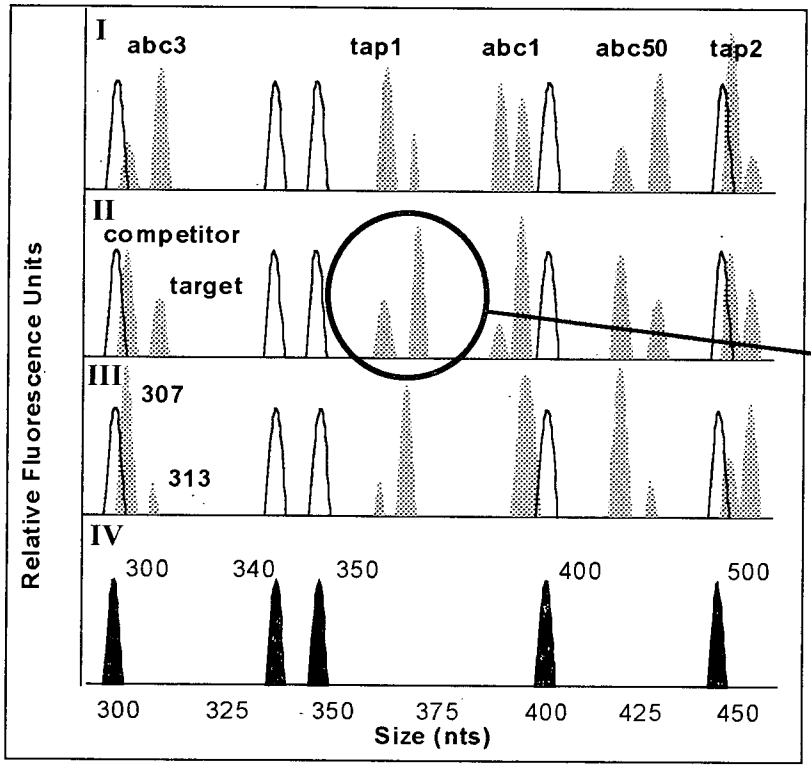


Figure 3. The Competitive RT-PCR Assay: Reverse Transcription and PCR



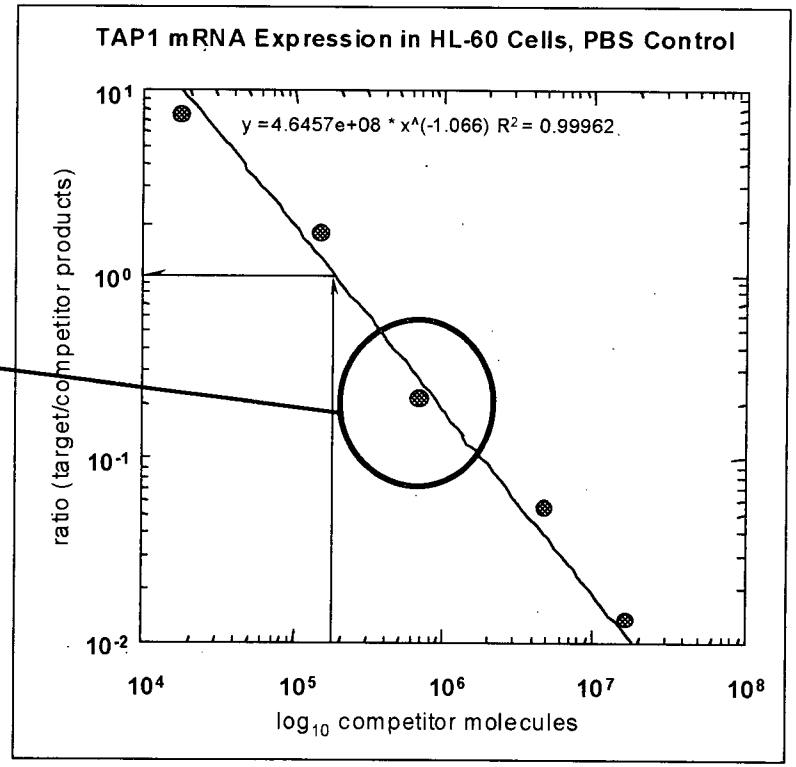


Figure 4a. Electrophoretic Separation and Quantitation
Chromatogram display of RT-PCR products separated via capillary electrophoresis on an ABI 310 DNA sequencer and analyzed with GeneScan2.1 software. Grey peaks represent RT-PCR products fluorescently labeled with 6-FAM and black peaks represent a size standard fluorescently labeled with TAMRA. Rows I-III are a series of competitive RT-PCR reactions and row IV is a H_2O negative control.

Figure 4b. Determination of Transcript Copy Number
Competition plot for TAP2 mRNA based on peak areas obtained using GeneScan2.1 software. The areas under the target and competitor peaks are used to compute the target/competitor ratio, which is plotted against the number of competitor molecules added. The estimated number of target copies is determined from the equation of the line.

2.2 MATERIALS AND METHODS

2.2.1 Cell Lines

The OCI/AML1, 2 and 3 cell lines were obtained from Dr. David Hedley at the Princess Margaret Hospital in Toronto. All cell lines were derived from individual patients at relapse and were morphologically characterized as M2 (myeloid leukemia with maturation) according to the French-American-British (FAB) leukemia classification system (Dr. D.W. Hedley, personal communication). The OCI/AML1 cell line was maintained in RPMI (Gibco BRL) supplemented with 20% Fetal Bovine Serum and 10% conditioned media, and 1% streptomycin/penicillin antibiotic (Gibco BRL). Conditioned media was acquired from flasks containing human 5637 human bladder carcinoma cells in RPMI (Gibco BRL), 10% FBS and 1% streptomycin/penicillin (Gibco BRL). The OCI/AML 2 and OCI/AML3 cell lines were maintained in α-MEM (Gibco BRL) supplemented with 10% FBS and 1% streptomycin/penicillin (Gibco BRL). All cell lines were grown at 37°C with 5% CO_2 in a humidified incubator.

2.2.2 Preparation of Total RNA.

Cells were centrifuged at 500 rpm for 10 minutes at 4°C, and resuspended in 2.5-ml guanidinium HCl containing 0.7% β-mercaptoethanol (Sigma), and frozen at -80°C until use. Total RNA was isolated using a guanidine isothiocyanate/cesium chloride method (Chirgwin *et al.*, 1979), with some minor modifications. The extracted RNA was cleared of genomic DNA by a 15-minute incubation at RT in RNase-free DNase I along with RNase Inhibitor (Gibco BRL) according to manufacturer's instructions followed by phenol-chloroform extraction and precipitation with isopropanol and a wash with 70% ethanol. Pelleted RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O with 10mM Tris-Cl and 1mM EDTA, pH 8.0, quantitated in triplicate by absorbance at 260nm and stored at –80°C.

2.2.3 Assessment of Integrity of Total RNA

Integrity of RNA was assessed by RT-PCR amplification of full-length β_2-microglobulin mRNA (Figure 5). 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 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).

250 ng of total RNA was reverse transcribed under the following conditions: 50°C (50 min), then 70°C (10 min) in a 9600 thermal cycler (Perkin-Elmer). RT solution: 50mM KCl, 20mM Tris-HCl (pH 8.3), 500μM dNTPs, 2.5mM $MgCl_2$, 10 ng/μl random hexamers and 10mM DTT. PCR was performed using the following primers for β_2-microglobulin: (primers: 5'-ATTCGGGCCGAGATGTCTCG-3' and 5'-CCAGATTAACCACAACCATGC-3') with the following cycling parameters: 95°C (2 min), then 24 cycles of 95°C (15 sec), 64°C (15sec), 72°C (90sec) and a final extension period of 72°C (5 min). 5μl of cDNA was added to 20 ul of PCR mix. (PCR buffer: 50mM KCl, 10mM Tris-HCl (pH 8.3), 100μM dNTPs, 2mM $MgCl_2$, 20μg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 2.5U of Taq enzyme and 1μM of forward and reverse primers). PCR products were separated electrophoretically on a 1-% agarose gel, stained with ethidium bromide and visualized on a Eagle Eye II (Stratagene).

2.2.4 Construction of ABC RNA Competitors

Competitor ABC cDNAs were generated by PCR amplification using forward and mutagenizing primers (Table II) to create deletions ranging from 4-8 bp. DNA templates used included commercially available Expressed Sequence Tagged 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 and ABCG3 cDNAs generously provided by Dr. Rando Allikmets, Columbia University, New York). The PCR protocol varied depending on the template used. PCR products were digested with the EcoRV restriction endonuclease (Gibco BRL) and ligated overnight at 14°C into pBluescript II using T4PNK and DNA Ligase (Gibco BRL). Plasmids were transformed into *E.coli* and grown overnight. Several colonies were dissolved in water and colony PCR was performed to confirm the presence of the insert. For PCR, colonies were picked and diluted in 10μl H_20, 2μl of this mixture was used as template. PCR was performed

using primers T7 universal (5' CCCTATAGTGAGTCGTATT3') and M13 reverse (5' CAGGAAACAGCTATGA 3'). PCR buffer: 50mM KCl, 10mM Tris-HCl (pH 8.3), 100μM dNTPs, 2mM $MgCl_2$, 20μg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 4U of Taq polymerase, 0.5μM of T7 and M13 primers and 2.0μl of the colony mixture in a 15 μl volume. PCR cycling parameters were 95° (5 min) and 30 cycles at 95° (30sec), 56° (15s), 72° (45s). Products were separated on a 1-% agarose gel and visualized after ethidium bromide staining. Colonies containing vectors with the appropriate insert sizes, as determined by PCR, were grown overnight and plasmids were extracted using the Quantum Prep Plasmid Miniprep Kit (Biorad). Plasmids were sequenced using 4 ul of Big Dye Terminator Mix (Applied Biosystems) and 2 ul of plasmid template and 4ul of 0.8 μM of one of the following primers: T7 universal (described previously), M13 Reverse (described previously) or T3 (5' ATTAACCCTCACTAAAG 3'). Typical sequencing PCR conditions were: 95° (2 min) and 28 cycles of 95° (10s), 47° (5 sec), 60° (3 min). Annealing temperature varied depending on the primer used. PCR products were analyzed on an ABI 310 DNA sequencer. Sequencing PCR conditions were: 95° (2 min) and 28 cycles of 95° (10s), 47° (5 sec), 60° (3 min). Competitor constructs were then linearized with the ScaI restriction endonuclease. In-vitro transcription was performed at 50°C (50 min), then 70°C (10 min) in a 9600 thermal cycler (Perkin-Elmer) using a Megascript T7 in-vitro transcription kit (Ambion).

2.2.5 Reverse Transcription

Each reverse transcription consisted of 6 competition reactions and a H_2O-only negative control. Competition reverse transcription reactions contained 200ng 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 concentrations to span a 3125-fold range or 32-fold range, respectively. RT solution: 50mM KCl, 20mM Tris-HCl (pH 8.3), 500μM dNTPs, 2.5mM $MgCl_2$, 10 ng/μl random hexamers and 10mM DTT. RT reactions were heated at 70°C (10 min), chilled to 4°C (5 min) and incubated at RT (10min). 180U of Superscript II RT and 2U of RNase Inhibitor (Gibco BRL) were then added to a final volume of 40μl. Reverse transcription was performed at 50°C (50 min), then 70°C (10 min) in a 9600 thermal cycler (Perkin-Elmer).

2.2.6 Amplification and Analysis of PCR Products

PCR amplification for each ABC transcript consisted of six competition reactions, a H_2O-only negative control and an untranscribed total RNA control. Uniplex PCR conditions were: 50mM KCl, 10mM Tris-HCl (pH 8.3), 100μM dNTPs, 2mM $MgCl_2$, 20μg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 2U of Amplitaq Gold (Perkin-Elmer), 2μM of forward (5'-6FAM labeled) and reverse primers (Table II) and 4μl of the appropriate cDNA mixture or 40 ng of untranscribed total RNA in a 20μl volume. Multiplex PCR employed 3.5U of Amplitaq Gold, scaling the amount of primer to product size (100-150bp/0.8μM, 151-200/1.15μM...451-500bp – 3.25μM) and adding 8 μl of cDNA in a final volume of 40μl. Uniplex cycling parameters were: 95°C (7 min), 45 cycles at 95°C (15s), 64°C (15s) and 72°C (90s) and a final extension at 72°C (90 min). Multiplex cycling parameters were: 95°C (7 min), 45 cycles at 95°C (15s), 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 and competitor products) were routinely pooled and diluted 2- to 50-fold in H_20. Mixture (2μl) was added to 11μl of template suppression reagent and 0.5μl of TAMRA-500 DNA size standard (PE 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 6-FAM labeled DNA peaks were quantitated using GeneScan2.1 software.

Table II. Oligonucleotides and PCR Product Sizes

Nomenclature + (common name)	Genbank Accession Number	Native/ Competitor size (nts)	Forward primer (5'to3')	Reverse primer (5' to 3')	Mutagenizing primer (5' to 3')
ABCA1 (ABC1)	NM 005502	246/241	GGCAATCATGGTCAATGGAAGG	GGAGAGGATGCTGAATATCCTG	GGAGAGGATGCTGAATATCCTGAGAAGATAATG
ABCA2 (ABC2)	AB028985	140/136	GTGGTGCTGACATCACACAGC	ACCGTGATCATGTAGCCATCTC	ACCGTGATCATGTAGCCATCTCCCCGGTTCTTC
ABCA3 (ABC3)	NM 001089	313/307	CATCCAGTACCAGGAGAACTTC	CTGCTCGTACACCTTGGAGAG	CTGCTCGTACACCTTGGAGAGGATAATCAG
ABCA4 (ABCR)	NM 000350	166/162	CATCGGCATCAACAGCAGTGC	ATCTGTCACAGCCTGGCTCAG	ATCTGTCACAGCCTGGCTCAGAGGTCAATG
ABCA5	U66672	295/291	GTGTTGTGAGGAGAAACCATCC	AGGACAGTAACCCATACACTTC	AGGACAGTAACCCATACACTTCAATCATCATC
ABCA6	U66680	353/349	CTCAAGAGAACGTGCTGTGGC	TTCCGCCTCAGCCAGGTTATG	TTCCGCCTCAGCCAGGTTATGGTCAGGAGG
ABCA8	NM 007168	297/289	TGAAGAACGAATGGATGTACAGC	TGCATACTCCTTGCGTAGACAG	TGCATACTCCTTGCGTAGACAGGATGACTG
ABCA10	AB020629	193/187	GAGGTTGAGATGTATCGGTTCC	TTGCACATCTTCCACTGGCAAC	TTGCACATCTTCCACTGGCAACACCATCAG
ABCB1 (MDR1)	NM 000927	128/123	TCCTATGCTGGATGTTTCCGG	AATGAACTGACTTGCCCCACG	AATGAACTGACTTGCCCCACGGGCACCAAAG
ABCB2 (TAP1)	NM 000593	373/369	TGCCTACAGTTCGAAGCTTTGC	TGTAAGGGAGTCAACAGACCAC	TGTAAGGGAGTCAACAGACCACTGGGCAGCG
ABCB3 (TAP2)	NM 000544	456/452	GATGCTGATGCTGAGCTGTGG	CAGTGTTCATACTGTGAGATGG	CAGTGTTCATACTGTGAGATGGTTCATCCAGC
ABCB4 (MDR3)	NM 000443	285/277	GGTGCATATCTCATTGTGAATGG	CTGAAGCACTGGCACGTTTGC	CTGAAGCACTGGCACGTTTGCGATAGTTGAACACG
ABCB5	AC002486	Not done			
ABCB6 (PRP)	AF070598	428/424	TCACAGCTGGGAATGATGAGG	GGTTTCTTCCTGTCCCTGCTG	GGTTTCTTCCTGTCCCTGCTGTGCCACATGTC
ABCB7 (ABC7)	NM 004299	402/397	GAAGTGATGCTTGTCAGTGGTG	TAGATCTCCAACAGTAAGGGTAC	TAGATCTCCAACAGTAAGGGTACACAATTCCC
ABCB8 (M-ABC1)	NM 007188	349/341	CCAAGGGCTTTCCAACATCGC	GGTGAAGTCTTTCAGCACCTC	GGTGAAGTCTTTCAGCACCTCGGCGGCAGG
ABCB9	U66676	195/190	TCTACAGTGGCCTGATGCAG	ACAGGCTGAAGGAGACATTCTG	ACAGGCTGAAGGAGACATTCTGCCTGGGTGTG
ABCB10 (M-ABC2)	AA318189	270/264	CTCTCCGGAAACCTGATCGTG	CTCCAAAGCACCCTGGAAGC	CTCCAAAGCACCCTGGAAGCCATTTAAGATG
ABCB11 (BSEP)	NM 003742	268/262	TCAGTAACATCCGCACTGTTGC	TCTTCCAAGAGCTGTTGCACTC	TCTTCCAAGAGCTGTTGCACTCACTGCAGAG
ABCC1 (MRP1)	NM 004996	214/208	CTTCGTCCAGAGGTTCTACG	CCACCTGTTGGCCACGATG	CCACCTGTTGGCCACGATGTAATAGGCC
ABCC2 (MRP2)	NM 000392	186/181	GGCTGGTGAGGATGACATCAG	CCAGATCCAGCTCAGGTCGG	CCAGATCCAGCTCAGGTCGGCACTTGGTAG
ABCC3 (MRP3)	NM 003786	345/337	GAGACCTGAGTCTGCATGTGC	CCTCTGAGCACTGGAAGTCC	CCTCTGAGCACTGGAAGTCCCGGCTGGGAG
ABCC4 (MRP4)	NM 005845	238/234	GAAACCATTGAAGATCTTCCTGG	TCAATCTGTGTGCAATGGTTAGC	TCAATCTGTGTGCAATGGTTAGCTGCAGTGGGC
ABCC5 (MRP5)	NM 005688	150/142	GGTCTCGCCATCTCTTATGC	CTTAATTCTGGCAGGTGCTTCC	CTTAATTCTGGCAGGTGCTTCCAGTCTTAATG
ABCC6 (MRP6)	NM 001171	326/318	GGCAGATCGAGTTCCGGGAC	AGATAGCCTCGTCCGAGTGC	AGATAGCCTCGTCCGAGTGCCAGGTCGAGG
ABCC7 (CFTR)	NM 000492	485/481	TCCAGATCGATGGTGTGTCTTG	CTGGATGGAATCGTACTGCCG	CTGGATGGAATCGTACTGCCGTTGTTCTCTTC
ABCC8 (SUR1)	NM 000352	Not done			
ABCC9 (SUR2)	NM 005691	Not done			
ABCC10 (MRP7)	AL133613	257/253	CCTGAGTGAGGTGATTACATCC	TCAGGATCGTGTTGAGCCTATG	TCAGGATCGTGTTGAGCCTATGATGGTCAGC
ABCD1 (ALDP)	NM 000033	357/353	ATCAACCTCATCCTTCTGGAAC	CTTGAAGTGACAGCGCTGAAC	CTTGAAGTGACAGCGCTGAACTCAAATACC
ABCD2 (ALDR)	AF119822	365/361	TTTGCAGATGGTGAGGATGGC	TCTCCTGCTGGTGTAATTATGG	TCTCCTGCTGGTGTAATTATGGATTTTCAC
ABCD3 (PMP70)	NM 002858	287/283	GGTGCTGGAGAAATCATTATTGC	ATCACTTGATCTCGAAGTGTTCC	ATCACTTGATCTCGAAGTGTTCCGTCATGTAAG
ABCE1 (RNase LI)	NM 002940	Not done			
ABCD4 (PMP69)	NM 005050	278/274	AGATTCGGGTGAATGCGGAGC	GAGGTAGATGCACACAAAGGC	GAGGTAGATGCACACAAAGGCTTGCTGACC
ABCF1 (ABC50)	AF027302	420/415	TGAAGGAGCTGAAGGCAGGC	CATACTGCTGGTTGAAGAAGCC	CATACTGCTGGTTGAAGAAGCCTCAGCCGG
ABCF2	NM 005692	410/406	CACATGAAGAACTACATTGCGAG	AGCTGCTCTTGTAAATGCTGATG	AGCTGCTCTTGTAAATGCTGATGCGCCCTATC
ABCF3	AK002060	177/173	TCTGTGTCTGCTGATCTCGAG	TAGGTCCAGCTGCTCCACATG	TAGGTCCAGCTGCTCCACATGTGGCTGAAATAG
ABCG1 (WHITE1)	NM 004915	33/329	TCCTATTCGGTTCCTGAAGGAC	TCTGCCTTCATCCTTCTCCTG	TCTGCCTTCATCCTTCTCCTGTTCAGATGTGC
ABCG2 (BCRP)	NM 004827	454/447	CACAGAGATCATAGAGCCTTCC	CACTCTGTAGTATCCGCTGATG	CACTCTGTAGTATCCGCTGATGGTATGAAGAG
ABCG3	Not submitted	261/255	CTGTGCGCTATGGCTGAGAAG	TGCTGGCATCGTCGCCAATATG	TGCTGGCATCGTCGCCAATATGGAGGAGGC
ABCG4	Not submitted	216/209	TACGCGTCTCCTTCAGAATCTG	GCCAGCATCATCTGCCACTTC	GCCAGCATCATCTGCCACTTCGGCCGTC
$\beta_2$microglobulin		227/220	CCGACATTGAAGTTGACTTACTG	CATCTTCAAACCTCCATGATGC	CATCTTCAAACCTCCATGATGCATGTCTCGATC
LRP	X79882	159/155	CACAACTACTGCGTGATTCTCG	CTCCGACAGCACATACACATC	CTCCGACAGCACATACACATCGCCTTGTTC
TOPOIIα	J04088	494/489	TCTGGTCCTGAAGATGATGCTG	GTATCGTGGACTAGCAGAATCC	GTATCGTGGACTAGCAGAATCCACCATGTAG
GST-π	X15480	275/270	CTGTACCAGTCCAATACCATCC	GTAGTCAGCGAAGGAGATCTG	GTAGTCAGCGAAGGAGATCTGCCACAATG

2.2.7 IC_{50} Measurements in the AML 1, 2 and 3 Cell Lines

IC_{50} values were 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. To first determine the optimal number of cells per well (in a 150µl volume), we generated standard curves relating the number of cells plated to the absorbance value of solubilized MTT for each cell line examined (Figure 7). Cells were plated in duplicate wells in a 1/3 dilution series over a 130-fold range of cell concentrations in 96-well flat bottomed plates and were grown for 3 days in their respective media (section 2.2.1). 20 µl of 2-mg/ml sterile-filtered MTT solution was added to each well and plates were incubated for 18 hours at 37°C. The resulting precipitate was solubilized using 75 µl of 1:24 HCl/isopropanol solution, pipetted 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_{50} values of the cells to various drugs, cells were plated at 4.5 x 10^5 cells/ml in 100 ul of appropriate media in 96-well flat-bottomed plates, including media only, media+drug only, cell+medium, cell+drug+medium and cell+medium+solvent (either PBS or DMSO) controls. 50 ul 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_{50} values were determined using the MTT assay as described above and values were obtained in triplicate for each drug examined (Figures 8a, b, c).

2.2.8 Drugs

Etoposide (Sigma) was dissolved in DMSO at a stock concentration of 15801 µM and diluted in PBS to the appropriate concentration. Cytosine Arabinoside (Ara C) (ICN Biomedical), and daunorubicin (Sigma) were dissolved in PBS at concentrations of 35753µM and 3723µM respectively, and diluted with PBS to the appropriate concentrations.

2.3 RESULTS

2.3.1 Assessment of RNA Integrity

As expected, the control lanes containing non-reverse-transcribed RNA are blank, as is the H_20 control (Figure 5) and the PCR product representing full-length β_2-microglobulin is present at approximately equal levels in all three cell lines.

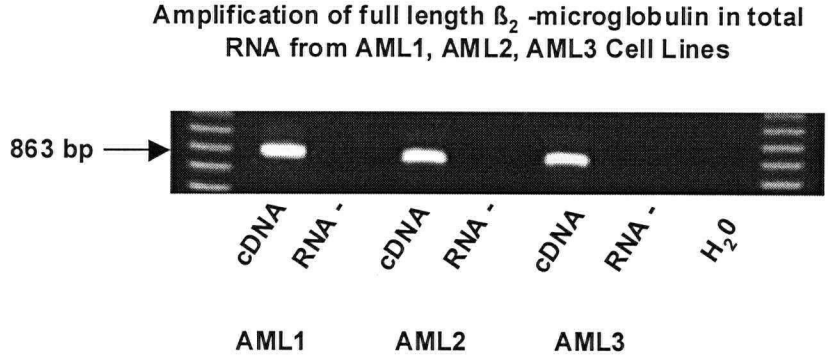


Figure 5. **RT-PCR of the Full-Length β_2-microglobulin Housekeeping Gene in Total RNA from the AML 1, 2 and 3 Cell Lines.**

2.3.2 C-RT-PCR Expression Profiles of the AML1, AML2 and AML3 Cell Lines

We have applied competitive-RT-PCR to examine expression profiles of 32 ABC transcripts, LRP, GST-π and β_2-microglobulin in three patient-derived AML cell lines (Figure 6). The most striking feature observed was that the majority of transcripts examined, approximately 75%, were expressed at detectable levels. More than half of these were below the detection limits of Northern blot or cDNA microarray analyses (1000 copies/ng).

The expression levels of TAP2 and β_2-microglobulin were done in triplicate as controls for reproducibility. The average coefficient of variation (C.O.V.) for TAP2 and β_2-microglobulin in the three cell lines was 30%.

The expression levels of some of the drug-resistance associated ABC transporters are noteworthy. The transcript representing the major vault protein, LRP, was expressed at relatively low levels in each of these cell lines. GST-π was expressed at moderate to high levels in each of the cell lines. BCRP, the putative mitoxantrone transporter, was

not detectable in any of the AML cell lines. Among the MRPs, neither MRP3, nor MRP6, were detectable, whereas MRP2 was expressed at low levels and MRPs 1, 4 and 5 were expressed at moderate to high levels. MDR1 was expressed at very low levels or was undetectable.

In addition, many striking differences amongst the profiles of the three cell lines were evident. For example BSEP, the human bile salt export pump, was not detectable in the AML1 and 2 cell lines, however it was exclusively expressed at a moderate level in the AML 3 cell line and this change was verified by an additional C-RT-PCR experiment. MRP7 was detectable only in the AML2 cell line and MDR1 was detectable only in AML1. Finally, MDR3 was expressed, albeit at low levels, only in AML 2 and 3. The potential significance of these differences will be discussed.

2.3.3 Standard Curves for AML1, AML2, AML3 Cell Lines Using the MTT Assay

From the standard curves generated in triplicate for each of the cell lines using the MTT assay, we observed on average that the linear range of absorbance values ended at approximately $1x10^4$ cells/well for each of the cell lines (Figure 7). 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 $x10^4$ cells/well as the optimal number of cells to be used in the MTT assays.

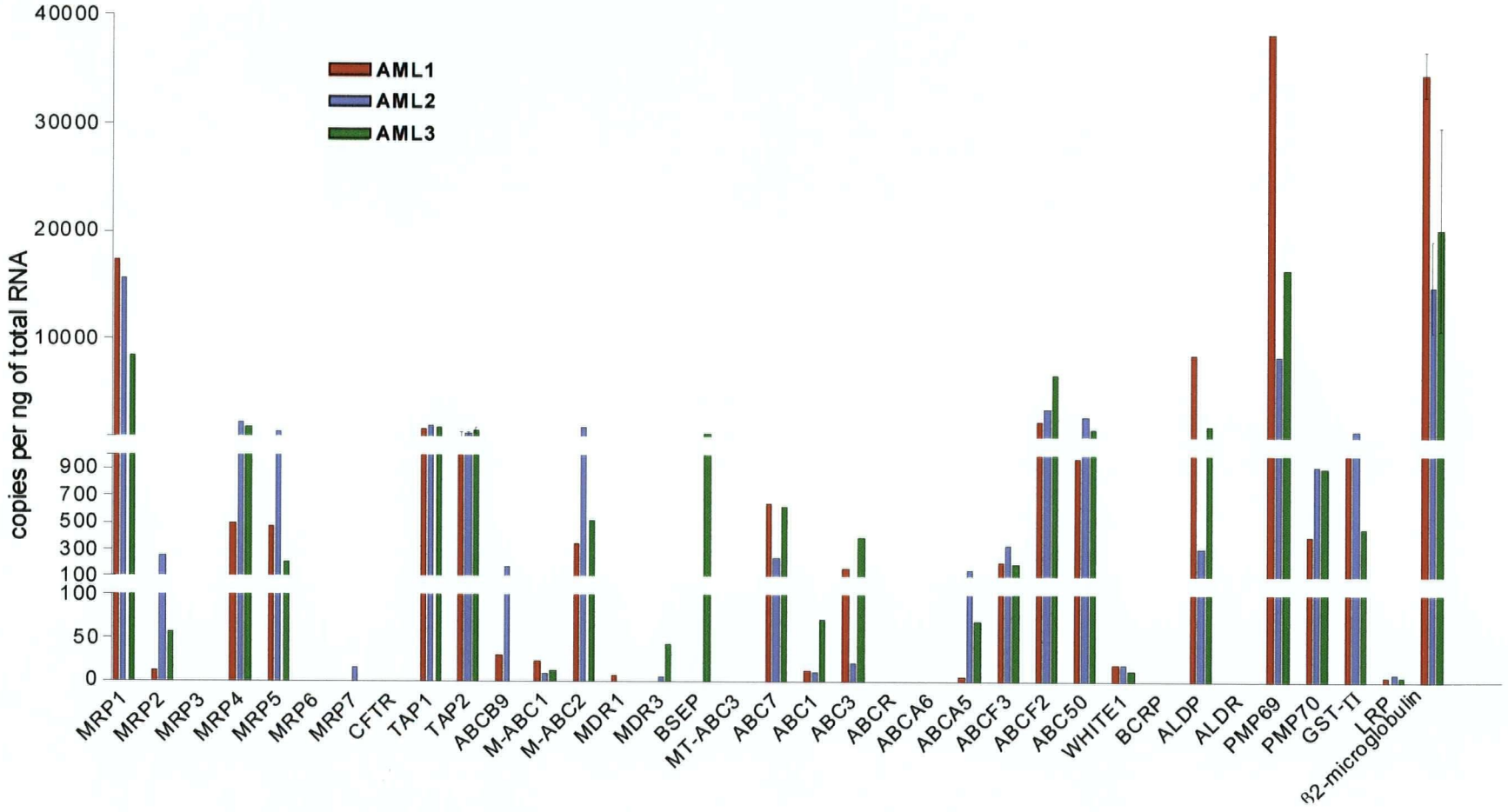
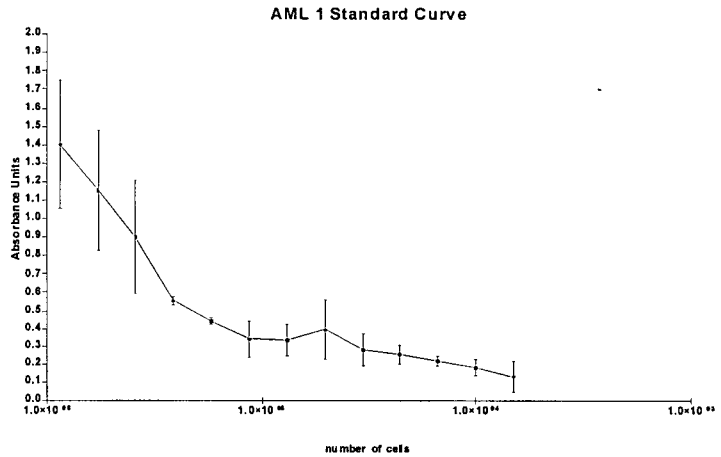
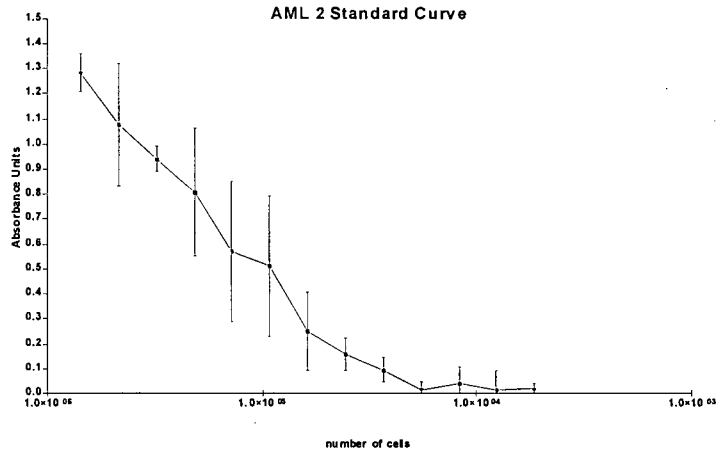


Figure 6. C-RT-PCR Expression Profile of AML Cell Lines

Profile of 32 ABC transcripts, LRP, GST-π and the housekeeping gene β_2-microglobulin. All data are normalized relative to β_2-microglobulin levels. Error bars for β_2-microglobulin and TAP2 represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable.





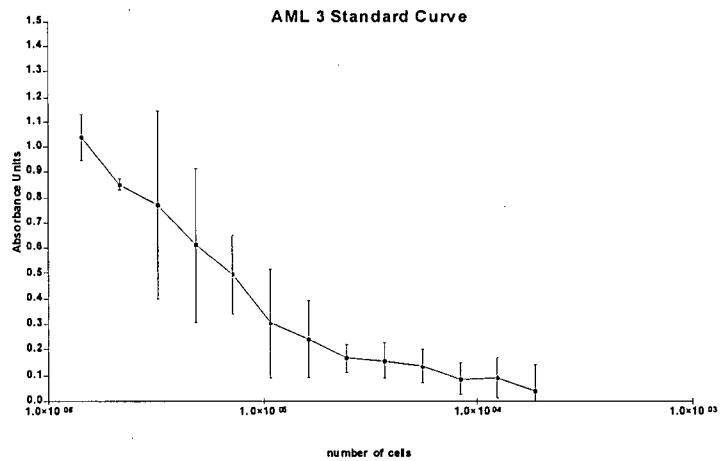


Figure 7. Standard Curves for the AML 1, 2, 3 Cell Lines Using the MTT Assay. Absorbance values were obtained using a range of cell concentrations in the absence of any chemotherapeutic drug. Error bars represent the standard deviation of three independent experiments.

2.3.4 IC_{50} Values for Growth Inhibition of the AML1, 2 and 3 Cell Lines by Daunorubicin, Etoposide and Ara C.

To discern if the unique expression profiles of the three cell lines might be related to differences in drug resistance properties, IC_{50} values of three drugs used in the treatment of AML, Ara C, daunorubicin and etoposide, were determined in triplicate using the MTT Assay (Figure 8). The AML3 cell line was more resistant to all three drugs than either AML2 or AML1, with AML1 the most sensitive (Table III). Fold-wise differences in drug resistance among the three cell lines include a 46000-fold difference with Ara C, an 11.9-fold difference with daunorubicin and an 18-fold difference with etoposide.

Table III. Drug Resistance Profiles of the AML1, AML2 and AML3 Cell Lines Generated Using the MTT Assay.

	Drug		
Cell Line	Ara C $IC_{50}(\mu M)$ +/- S.D.	Daunorubicin $IC_{50}(\mu M)$ +/- S.D.	Etoposide $IC_{50}(\mu M)$ +/- S.D.
AML 1	**0.010** +/- 0.08	**0.026** +/- 0.005	**0.198** +/- 0.05
AML 2	**2.0** +/- 0.21	**0.066** +/- 0.02	**0.48** +/- 0.02
AML 3	**460** +/- 98	**0.31** +/- 0.14	**3.6** +/- 1.5

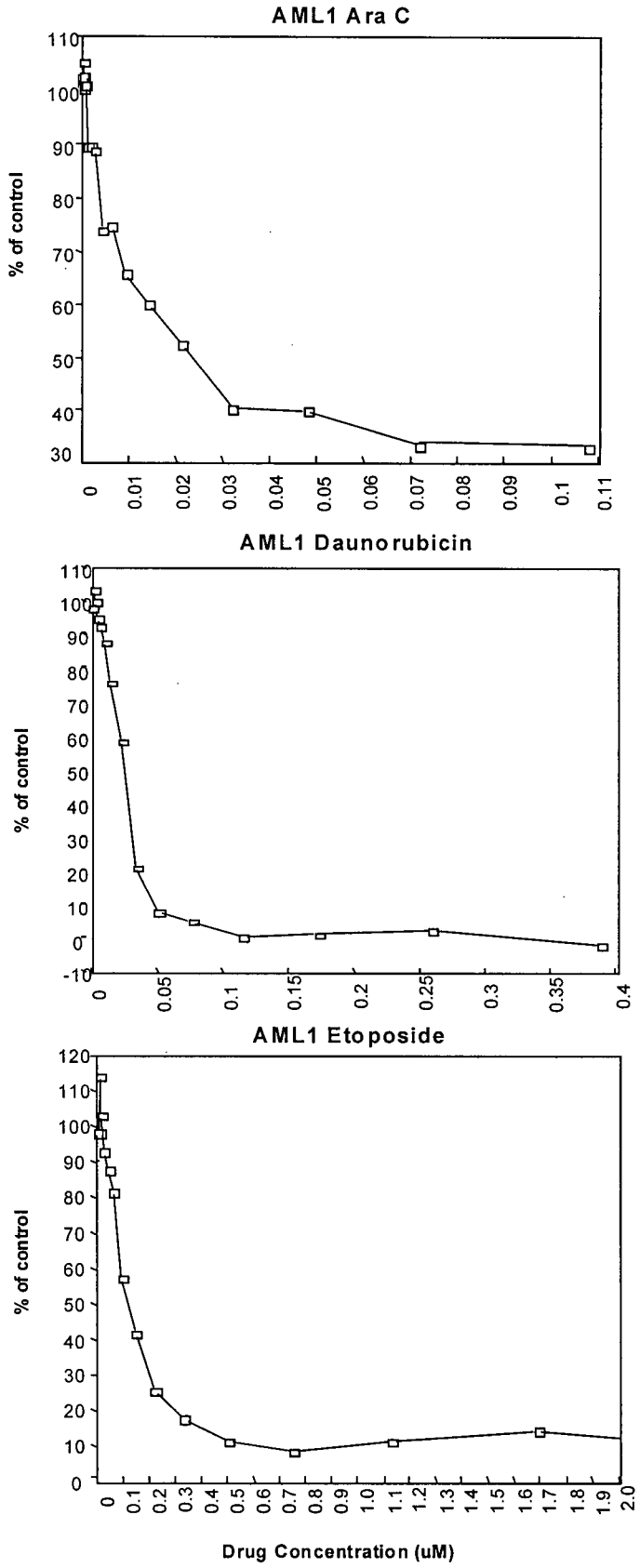


Figure 8a. **Representative Dose-response Curves used to Determine IC_{50} Values of the AML1 Cell Line to Ara C, Daunorubicin, and Etoposide using the MTT Assay.** Each graph represents single trial measurements for each drug.

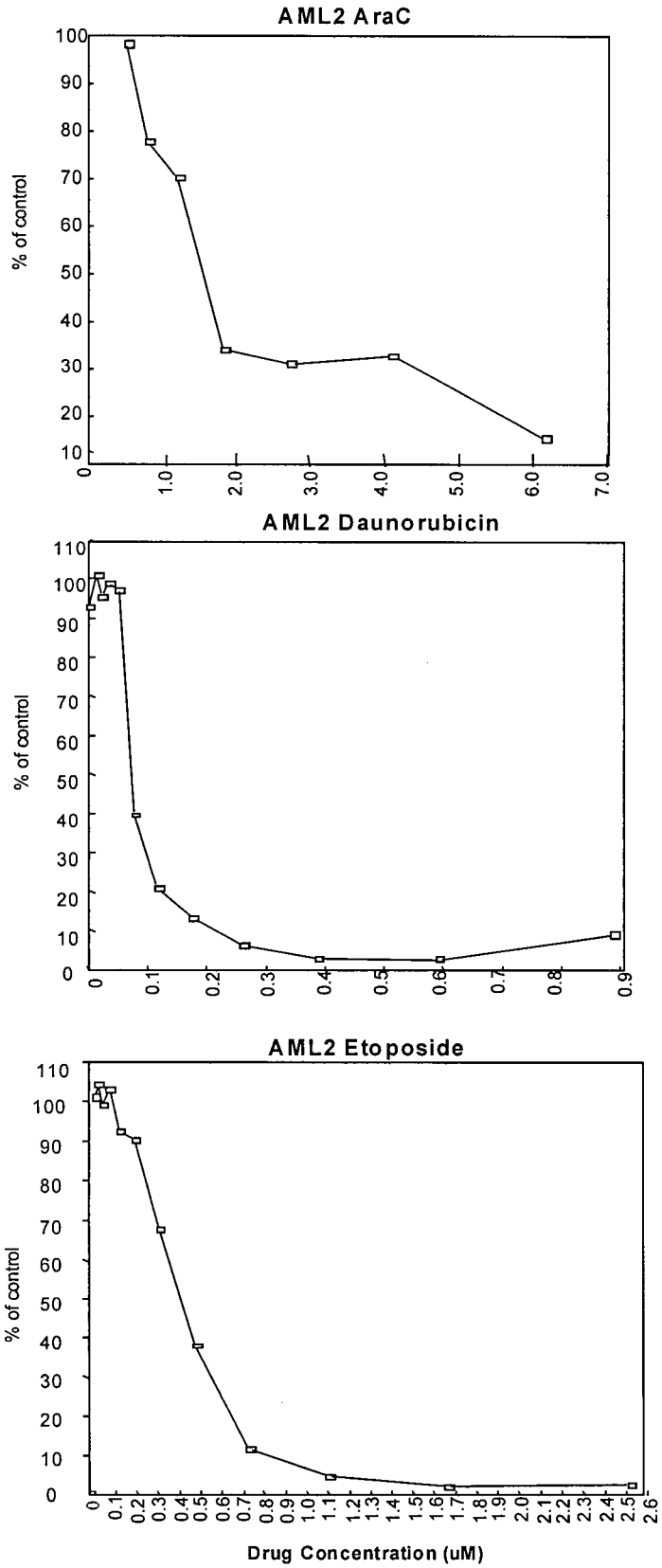


Figure 8b. **Representative Dose-response Curves used to Determine IC_{50} Values of the AML2 Cell Line to Ara C, Daunorubicin, and Etoposide using the MTT Assay.** Each graph represents single trial measurements for each drug.

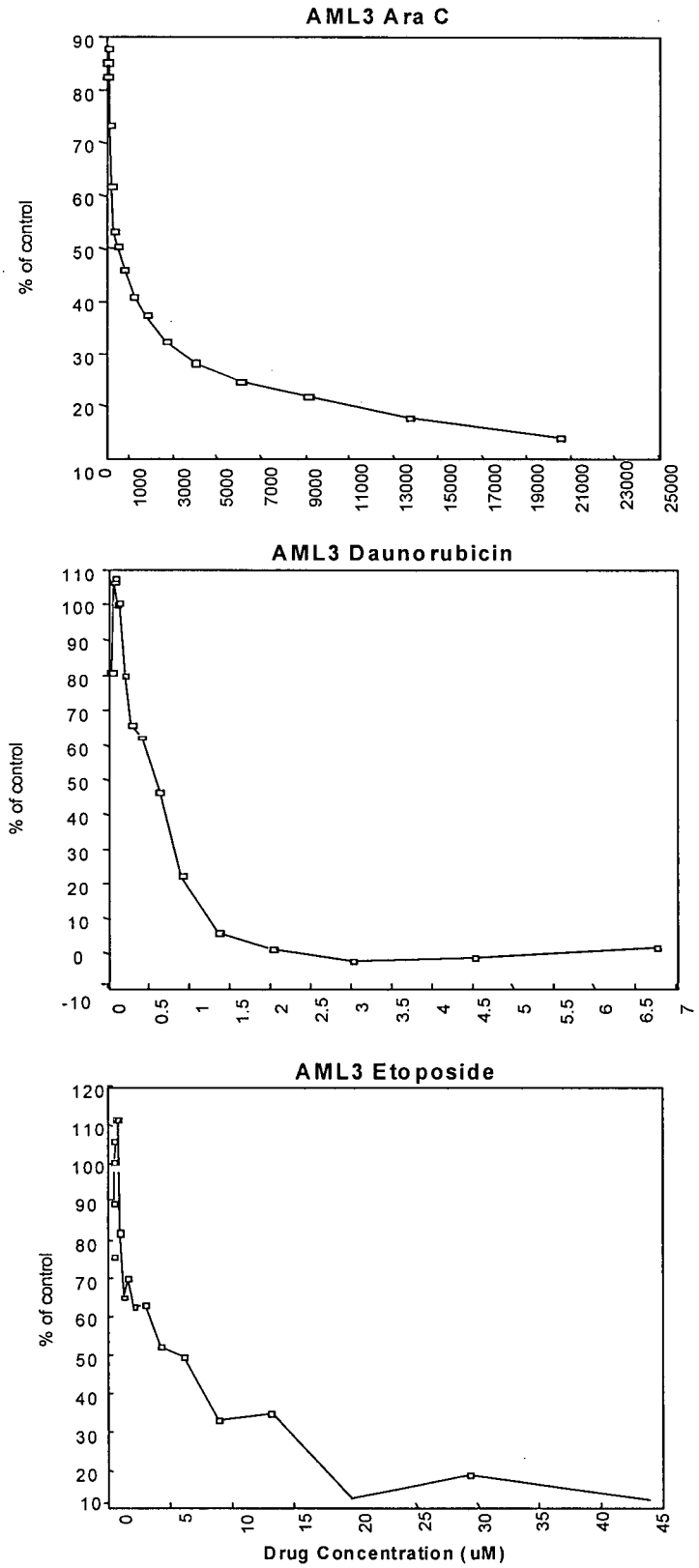


Figure 8c. **Representative Dose-response Curves used to Determine IC_{50} Values of the AML3 Cell Line to Ara C, Daunorubicin, and Etoposide using the MTT Assay.** Each graph represents single trial measurements for each drug.

2.4 DISCUSSION

By the mid- to late 1990s, only a handful of ABC genes had been characterized to any significant extent. Though the expression patterns of these ABCs in various tissues had been reported, only a few groups had analysed expression in hematopoietic cells. The limited information available revealed that MRP1 was expressed at a high level, MRPs 4 and 5 were expressed at low levels, and MRP2, MRP3 and MDR1 were not expressed in hematopoietic cells (Kool *et al.*, 1997) (Abbaszadegan *et al.*, 1994). Consequently, we expected to find a similar pattern of expression of these ABC transcripts when we began mRNA expression profiling of the AML cell lines. The expression pattern of the majority of the remaining 20-30 cloned ABCs in hematopoietic cells, however, had not yet been studied. Based on the available information, it was anticipated that a limited number of ABC transcripts would be expressed in these cell lines and that these might contribute to daunorubicin efflux. Unexpectedly, we observed that more than 75% of the transcripts were expressed, many of them at high to moderate levels (Figure 6). This rendered the interpretation as to which ABC's might be relevant to daunorubicin efflux very difficult, if not impossible.

As expected, the retina-specific ABCR, the brain-specific ALDR and liver specific MRP3 were not detectable in the AML cell lines. The absence of MRP6 expression agreed with a recent study that failed to detect expression of MRP6 in normal human bone marrow cells (van Der Kolk *et al.*, 2000). The canalicular organic anion transporter of the liver, MRP2, was expressed at low levels in all of the AML cell lines and the liver specific bile salt-export pump, BSEP, was expressed exclusively in the AML3 cell line at relatively high levels. MRPs 4 and 5 were expressed at moderate levels.

M-ABC2, known to be expressed at high levels in erythroid hematopoietic cells (Shirihai *et al.*, 2000) and in bone marrow (Zhang et al., 2000), was detected in each of the AML cell lines. It is speculated that M-ABC2 may mediate critical mitochondrial transport functions related to heme biosynthesis (Shirihai et al., 2000).

PMP69, a peroxisomal half-transporter, known to be expressed in many tissues, including leukocytes (Holzinger *et al.*, 1997) was one of the most highly expressed transcripts in the AML cell lines. The role of this protein in hematopoietic cell biology is unknown.

Overall, 37% of the transcripts examined showed variations greater than 5-fold in magnitude in expression among the three cell lines. Although most of these expression differences among the cell lines are due to genetic heterogeneity among patients, a few

expression changes stand out, in particular, the high level of BSEP in the AML3 cell line, which is absent in the more drug sensitive AML1 and AML2 cell lines. Transfectants of the rat homologue of BSEP, sister of P-Glycoprotein (sPgp), display a low level of resistance to taxol, but not to any other anti-cancer drugs that comprise part of the multidrug resistance phenotype (Childs *et al.*, 1998). However, BSEP shares a very similar amino acid structure (51% amino acid identity) with the multidrug transporter Pgp (Figure 1). Unlike Pgp, BSEP has not been observed to be a common mechanism of defense against chemotherapeutic drugs, although it could play a role in specialized circumstances, such as in the case of AML3. We chose not to pursue this observation further as there is no information to support a role for BSEP in drug transport in human cells.

Other notable differences in expression among the cell lines include ABCB9, a lysosomal half-ABC, expressed only in AML1 and 2, and MRP7, a novel member of the MRP subfamily, found exclusively in AML2. MDR3 was detected only in the AML2 and 3 cell lines. Both cell lines were more resistant to drug than AML1, suggesting a possible role for this ABC transporter in drug resistance. Earlier studies have reported overexpression of MDR3 (and not of MDR1) in certain drug resistant leukemias and an increase in daunorubicin accumulation after treatment of the cells with CsA (Herweijer *et al.*, 1990) (Nooter *et al.*, 1990). A more recent study using MDR3-transfected cells reported the transport of several Pgp substrates, such as digoxin, paclitaxel, vinblastine, daunorubicin and ivermectin, yet transfection of MDR3 did not confer the MDR phenotype (Smith *et al.*, 2000). Other numerous attempts to obtain drug resistance by transfection of MDR3 into sensitive cells have also met with failure and it is generally accepted that MDR3 overexpression occurs only in specialized circumstances, such as observed in the AML2 and AML3 cell lines.

The putative mitoxantrone transporter, BCRP, was not detected in the AML cell lines. Another study which examined the mRNA expression levels of BCRP in AML patient samples found half of the samples to possess barely detectable levels of BCRP, the other half with moderate to high expression (Ross *et al.*, 2000). Furthermore, they found that the levels of BCRP varied more than 1000-fold among the 20 patients examined. Our findings of a 2.1-fold range in MRP1 expression corresponds to a similar range of variability found for this transcript among several AML patient samples (0.07-1.74 fold) (Ross, 1996) (Beck *et al.*, 1996). We observed MDR1 expression in only the AML1 cell line and other studies have reported significant heterogeneity in both Pgp and MDR1 expression in AML patient blasts. A large study of AML

blasts from groups of elderly and younger AML patients failed to detect Pgp protein expression in 29% (Leith et al., 1997) and 65% (Leith et al., 1999) of patients, respectively.

The expression levels of non-ABC transcripts, such as GST-π and LRP are noteworthy because of their association with alternative mechanisms of drug resistance. Moderate levels of GST-π mRNA were detected in the AML cell lines and this transcript has also been detected in AML patient samples, often concomitantly overexpressed with MDR1 mRNA (Russo *et al.*, 1994). Very low levels of LRP mRNA were detected in the AML cell lines and other studies have shown frequent expression of LRP mRNA and protein in patient blasts, however conflicting data exists regarding the role of LRP as a prognostic indicator in AML (Xu *et al.*, 1999) (Leith et al., 1999).

In accordance with results of Hedley et al., which demonstrated a lack of MRK-16 staining with flow cytometric measurement of Pgp, we did not detect MDR1 mRNA in the AML2 and AML3 cell lines, however a very low level of MDR1 mRNA was detected in the AML1 cell line. MRP1 mRNA, however, was the most highly expressed of the ABC transcripts examined, albeit at levels similar to those found using C-RT-PCR in normal human tissues and significantly lower than MRP1 mRNA levels in drug–selected cell lines (Steven Ralph, unpublished data). The relative expression levels of MRP1 found using C-RT-PCR among the three cell lines corresponded with the relative MRP1 protein levels found by Hedley et al. using Western blotting.

Our daunorubicin resistance data, however, did not correspond to the daunorubicin accumulation data of Hedley et al. The largest percent increase in daunorubicin accumulation in the presence of KCN was observed in AML2, with decreasing uptake in AML3, followed by AML1. Surprisingly, AML2 is 5-fold less resistant to daunorubicin than the AML3 cell line. This suggests that drug efflux may not be a significant mechanism of resistance in these AML cell lines.

The complex nature of the data, owing to patient heterogeneity and the large percentage of transcripts expressed, complicates the identification of ABC transporters that contribute to MDR in these cell lines. The ultimate goal of this work was to apply knowledge gained from expression profiles of the AML cell lines to identify a limited number of ABC transcripts to be studied using C-RT-PCR in paired blood samples of AML patients, at presentation and relapse. A collaboration with Dr. Heather Sutherland at the B.C. Cancer Research Center was

established and >40 paired AML patient samples were made available for our use. Unfortunately, the complexity of the cell line data did not allow us to clearly identify target transcripts relevant to AML and the prospect of generating full expression profiles in a number of patient samples was simply not practical. Future experiments could include a cDNA microarray approach, containing a comprehensive list of ABC genes, as well as several hundred cDNAs implicated in apoptosis or DNA repair. This array is currently under construction (Steven Ralph, personal communication) and might prove more helpful in identifying mechanisms of drug resistance in AML patient samples. To further examine the role of ABC transporters in MDR AML, a new approach was devised, described in Chapter III, which involved the brief exposure of the human promyelocytic cell line, HL60, to low doses of single chemotherapeutic drugs, followed by expression analysis of ABC transcripts using C-RT-PCR.

CHAPTER III

Induction of ABC Gene Expression in Response to Chemotherapeutic Drug Exposure

3.1 INTRODUCTION

3.1.1 Intrinsic vs. 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). Cancers of the kidney and pancreas are typically intrinsically unresponsive to chemotherapy (Hartmann & Bokemeyer, 1999) (Borst, 1999), and as such are among the most difficult cancers to successfully treat. Expression levels of MDR1 in normal kidney are among the highest of any tissue (Steven Ralph, unpublished data), and are high in kidney and pancreatic tumours prior to treatment (Kakehi *et al.*, 1988). Conversely, other types of cancer, such as testicular cancer and leukemias, are generally responsive to drug in the initial stages of treatment, with resistance arising only after prolonged therapy or relapse. Myeloid and testicular cells possess very little or no MDR1 in the normal physiological state (Steven Ralph, unpublished data), however expression levels can increase dramatically after treatment (Nooter & Sonneveld, 1994), at which point the cancer can become refractory to further therapy.

There appears to be a direct correlation between the expression of MDR1, prior to treatment, and survival in Acute Myeloid Leukemia (Del Poeta *et al.*, 1996), suggesting intrinsic drug resistance contributes to treatment failure in this disease. However, evidence also exists to demonstrate the role of acquired resistance in AML, with the observation that the expression level of the MDR1 gene is commonly increased after chemotherapy (Grogan *et al.*, 1993) (Nooter & Sonneveld, 1994). In other types of cancer, MDR1 expression is usually low or undetectable prior to treatment, but it is frequently increased during disease progression and, most noticeably, after chemotherapy (Chan *et al.*, 1990) (Holzmayer *et al.*, 1992).

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 selection can be a result of the natural heterogeneity present in a population of cells, whereby a small number of the cells inherently possess genetic differences that confer a survival advantage in the event that an insult such as drug is presented. Selection can also arise due to the activity of the drug itself. Depending on the mechanism of action of the drug, exposure may cause point mutations or genomic rearrangements such as gene amplification or deletions, or may disrupt post-translational mechanisms, such as the targeting of proteins within the cell and cell signaling events. For example, mitoxantrone causes DNA strand breaks by binding directly to GC-rich regions of DNA (Kirkwood, 1994) whereas the Her-2/Neu antibody targets a transmembrane tyrosine kinase (Kumar *et al.*, 2000). These genetic alterations typically are stable over very long periods of time, and are sometimes permanent, such as the case with genomic rearrangements. Repeated exposures to these drugs often produce a subpopulation of genetically altered cells resistant to the selecting agent. Eventually this "selected" cell population predominates and the result is a drug resistant tumour. This process of drug selection typically requires repeated drug exposures and a prolonged period of time to develop.

A second mechanism for development of drug resistance occurs with exposure to chemotherapeutic drugs which can induce changes in gene expression. It has been well established that regulation at the level of gene transcription is the primary means by which cells rapidly respond to changes in their environment, and it has been shown that many genes can be induced in response to drug exposure. For example, altered expression of the transcription factor RNA Polymerase II, the transcriptional enhancers AP-3 and AP-4, the apoptosis genes cytochrome C and Bcl-2, and genes of the ubiquitin-protease pathway occurred in response to brief (1-15 hours) exposure to the anthracyline doxorubicin (Kudoh *et al.*, 2000).

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. AML patients, for example, typically receive several rounds of chemotherapy with intervening recovery periods. It is reasonable to hypothesize that initial rounds of chemotherapy might induce expression changes in several genes, conferring some short-

term survival advantage on a small population of cells which could persist through to subsequent rounds of chemotherapy. These expression changes are usually only transient and may return to normal levels a few hours later (Manzano *et al.*, 1996) or several weeks later (Chaudhary & Roninson, 1993). If these genes are induced repeatedly, this population of cells becomes "selected" for survival in the presence of drug and will eventually predominate during the later stages of treatment, resulting in drug resistant cancer. This process is poorly understood because of difficulties in acquiring serial tumour samples from patients for expression analysis.

3.1.3 MDR1 Gene Expression is Inducible by Anticancer Drugs

It is generally accepted that a major cause of Pgp-mediated drug resistance during chemotherapy is due to the selection of drug resistant cells, whereby a small number of tumour 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) (Chen *et al.*, 1994) (Gekeler *et al.*, 1994) (Manzano et al., 1996). However, this increased expression is frequently a result of gene amplification in cultured cells which have been selected with drug concentrations hundreds of times higher than would be used in the clinic. Furthermore, the degree of genomic amplification of MDR1 does not always adequately explain the increased Pgp expression and drug resistance (Kohno *et al.*, 1994) and has not been documented in clinical samples.

An interesting observation made by a number of investigators is that the level of MDR1 gene 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 UV irradiation (Uchiumi *et al.*, 1993). An early reporter gene study by (Kohno *et al.*, 1989) demonstrated that the human MDR1 promoter could be activated by anticancer agents in human cell lines. In addition, Chin *et al.*, (1990) and Chaudhary & Roninson, (1993) demonstrated that brief exposure to clinically-relevant concentrations of a number of chemotherapeutic drugs, including Pgp and non-Pgp substrates, resulted in up-regulation in MDR1 mRNA expression in several cell lines.

More recent studies using a human multidrug resistant leukemia cell line showed rapid induction of MDR1 after as few as 4-24h exposure to anthracyclines (Hu *et al.*, 1995) and anthracycline analogues (Hu *et al.*, 1999b). Differential up-regulation of

MDR1 expression has been observed with *ex vivo* exposure (16h) of anthracyclines and the nucleoside analogue Ara C to Pgp-negative and Pgp-positive AML blasts (Hu *et al.*, 1999a). Induction of MDR1 has also been shown to occur *in vivo* following short term exposure (50 minutes) to doxorubicin in human metastatic sarcomas using an isolated chemotherapy perfusion circuit, followed MDR1 mRNA analysis of tumour tissues obtained by biopsy (Abolhoda *et al.*, 1999). From these studies it is apparent that induction of ABC genes can occur with clinically relevant doses of numerous Pgp and non-Pgp drug substrates.

3.1.4 Induction of Other ABC Genes

Although our understanding of the contributions of ABC transporters to MDR has expanded over recent years, very little information is known about the potential induction of these genes by chemotherapeutic drugs or other agents. Several ABC proteins are capable of drug transport (i.e. MRPs 1-5, MDR3, BSEP and BCRP) and it is important to determine the conditions under which these genes may be up- or down -regulated.

There are a few examples of induction of ABC genes 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 can be induced by heavy metals and cisplatin in the human HL60 leukemic cell line (Ishikawa *et al.*, 1996) (Akimaru *et al.*, 1996). Some ABC genes are also induced by exposure to biological compounds. For example, the human White1 and ABCA1 genes are transcriptionally activated when macrophages accumulate excess lipids (Schmitz *et al.*, 1999) (Venkateswaran *et al.*, 2000). Finally, the promoter activity of the adrenoleukodystrophy-related gene ALDR is upregulated by 9-cis-retinoic acid and forskolin (Pujol *et al.*, 2000).

Given this evidence and the knowledge of the involvement of other ABC genes in multidrug resistance, it is possible that exposure of cancer cells to chemotherapeutic drugs might result in the simultaneous induction of several ABC genes. To investigate this hypothesis, we have generated expression profiles of 25 ABC genes, using C-RT-PCR, in HL60 leukemia cells following 24-hour exposure to individual chemotherapeutic drugs. This brief exposure time was chosen to minimize cell division and expansion of any subpopulation that might be inherently resistant. This would enable us to focus on

expression changes that are a result of direct exposure to the drug rather than changes due to drug selection.

The chemotherapeutic drugs chosen for these experiments represent a variety of different mechanisms of action. Mitoxantrone is a substituted anthracenedione, which exerts a cell-cycle-specific cytotoxic effect on cells, by binding to GC-base-pair-rich regions and causing DNA strand breaks. It also induces cell membrane and mitochondrial lipid peroxidation and inhibition of glutathione synthesis. 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. Ara C is an antimetabolite that interferes with DNA synthesis. Cellular kinases convert Ara C to active nucleotide metabolites (e.g. ara-CTP), 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. Vincristine belongs to the class of vinca alkaloid drugs, which are unique among chemotherapy drugs in that they do not target DNA. Its major mechanism of action appears to be the arrest of cells as they enter metaphase by binding to tubulin. Finally, All-Trans-Retinoic Acid (ATRA/vitamin A) 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 (Kirkwood, 1994).

This work represents, to our knowledge, the first attempt to study, in a comprehensive manner, the effects of short-term chemotherapeutic drug exposure on ABC gene expression.

3.2 MATERIALS AND METHODS

3.2.1 Cell Lines

The promyelocytic HL60 leukemia cell line was a kind gift from Dr. Connie Eaves' laboratory at the B.C. Cancer Research Centre, and was maintained in RPMI (Gibco BRL) without phenol red, 10% charcoal/dextran-stripped FBS (Hyclone) and 1/100

streptomycin/penicillin (Gibco BRL). 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 compounds. Cells were grown at 37°C with 5% CO_2 in a humidified incubator.

3.2.2 Preparation of Total RNA.

RNA was prepared as described in Chapter II.

3.2.3 Assessment of Integrity of Total RNA

The integrity of the RNA was assessed as described in Chapter II (Figure 11).

3.2.4 IC_{50} Measurements of the HL60 Cell Lines:

The standard curve for the HL60 cell line using the MTT assay (Figure 9) was generated as described in Chapter II. IC_{50} values (Table IV) were determined as described in Chapter II. The concentration of All-Trans Retinoic Acid (ATRA) used was based upon the minimal level required to induce promoter activity in HL60 cells, as detected by luciferase assays, for a family of lysosome-associated transport proteins (Douglas Hogue, personal communication).

3.2.5 Drugs

Etoposide (Sigma) and mitoxantrone (Sigma) were dissolved in DMSO (Sigma) at stock concentrations of 15801 μM and 2252μM, 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 5417, 1397μM, 35753μM , 79951μM and 3723μM respectively, and diluted with PBS to the appropriate concentrations. ATRA (Sigma) was dissolved in ethanol at a concentration of 1265μM and diluted with PBS to the appropriate concentration.

3.2.6 24 Hour Exposure of HL60 Cells to Chemotherapeutic Drugs

HL60 cells were plated at a density of $2x10^5$ cells/ml in 50ml (T125) cell culture flasks, incubated at 37°C for 24 hours and then treated with varying concentrations of single chemotherapeutic drugs or ATRA for 24 hours. Controls included cells without drug, and cells treated with the carriers PBS, ethanol or DMSO.

3.2.7 Cell Viability Measurements

Following the 24 hour incubation period with drug, cell viability measurements were measured by diluting cells 1/20 in Trypan Blue Exclusion Dye (Sigma), incubating for 5 minutes at room temperature, plating 10μl of cells on a hemacytometer and performing two counts of 100 cells (Table V). Only cells with normal morphology were counted and those that completely extruded the dye were considered viable.

3.2.8 Reverse Transcription.

Reverse transcription was performed as described in Chapter II.

3.2.9 C-RT-PCR

C-RT-PCR and analysis of PCR products were performed as described in Chapter II.

3.2.10 Statistical Analysis

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

3.3 RESULTS

3.3.1 Standard Curve for the HL60 Cell Line Using the MTT Assay

The optimal number of HL60 cells to use in the MTT assay (Figure 9) was determined as described in section 2.3.3.

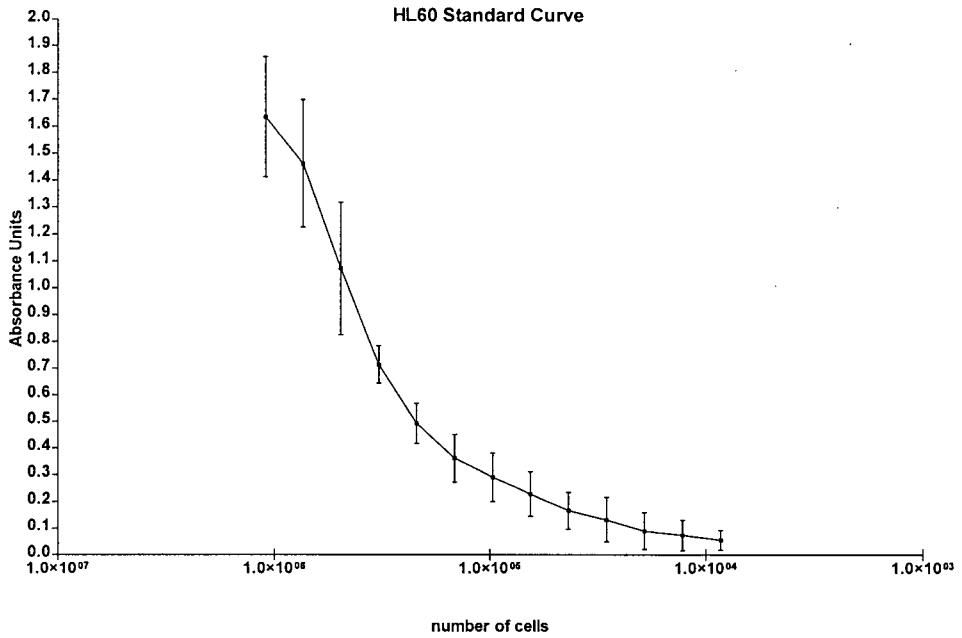


Figure 9. Standard Curve for the HL60 Cell Line Using the MTT Assay
Absorbance values were obtained using a range of cell concentrations in the absence of any chemotherapeutic drug. Error bars represent the standard deviation of three independent experiments.

3.3.2 IC_{50} Determinations

The results of the MTT assays for determining IC_{50} values of the HL60 cell line to various chemotherapeutic drugs are shown in Figure 10 and Table IV.

Table IV. Drug Resistance Profile of the HL60 Cell Line Using the MTT Assay

	Drug Concentration (μM)		
Drug	IC_{10} Mean ± SD	IC_{50} Mean ± SD	IC_{90} Mean ± SD
Ara C	0.025 +/- 0.0097	0.2 +/- 0.079	0.95 +/- 0.21
Etoposide	0.25	0.72 +/- 0.1	2.4 +/- 0.89
Daunorubicin	0.068	0.18 +/- 0.05	0.45 +/- 0.17
Vincristine	0.002 +/- 0.0016	0.0082 +/- 0.0024	0.04 +/- 0.02
Mitoxantrone	0.053	0.14 +/- 0.01	0.33
Cisplatin	0.93 +/- 0.14	1.8 +/-0.25	7.9 +/- 3.8
5-FU	5.0	37 +/- 18	800

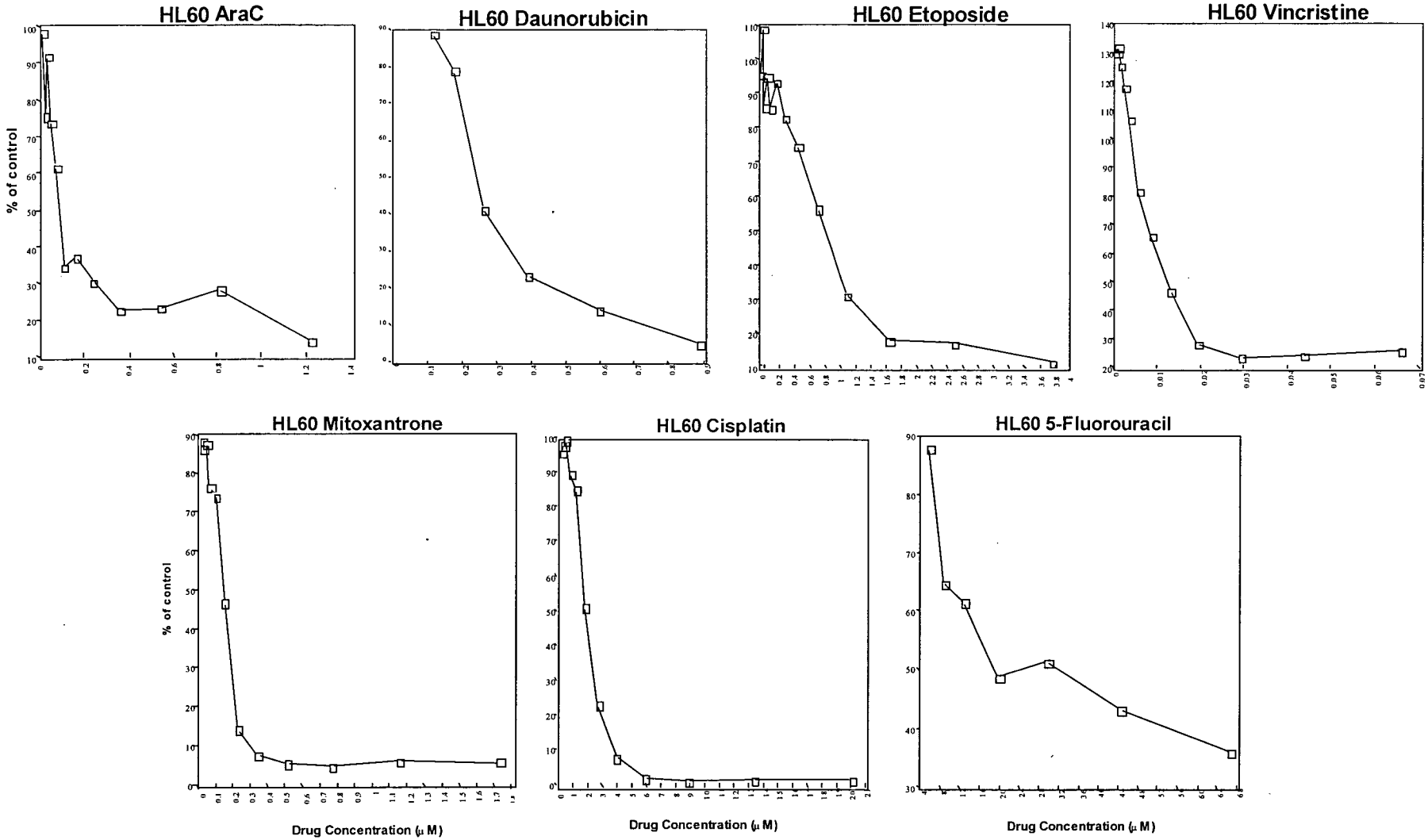


Figure 10. Representative Dose-response Curves to Determine IC_{50} Values of the HL60 Cell Line to Several Drugs, Generated Using the MTT Assay. Each graph represents single trial measurements for each drug.

3.3.3 Cell Viability Measurements

The drug concentrations used in the 24-hour drug exposure experiments were determined by IC_{50} values as well as trial and error based on cell viability measurements. We initially decided that a minimum viability level 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 (Table V).

Table V. Viability and Total RNA Yields of HL60 Cells Treated 24h with Varying Concentrations of Drug.

Drug Treatment	Viability (%)	Total RNA Yield (as % of PBS Control)
PBS (no drug)	95	100
DMSO	97.5	86.8
Vincristine (IC_{50})	97	67.0
Vincristine ($3xIC_{50}$)	73	50.6
ATRA	98	100.3
5-FU (IC_{50})	96.5	33.9
Ara C (IC_{50})	93	92.6
Ara C ($3xIC_{50}$)	83.5	56.3
Etoposide (IC_{50})	96	87.6
Mitoxantrone (IC_{50})	95.5	56.8
Cisplatin (IC_{50})	95	79.1
Daunorubicin ($1/2\ IC_{50}$)	82	22.1

In addition to cell viability, we also examined RNA yield and integrity as indicators of the cytotoxic effects of the drug. Daunorubicin treatment at a concentration of $\frac{1}{2}IC_{50}$ yielded 80% viability, however we found viability to be a misleading indicator in that RNA yield and quality from these cells were poor (Table V and Figure 11). HL60 cells treated with higher doses of daunorubicin were viable (>80%) but had RNA yields of <20% of the control. Though they appeared viable, cells treated with daunorubicin were morphologically distinct compared to the other drug treated cells and a great deal of cell debris was usually present. Possible explanations may be that the RNA was partially degraded or that daunorubicin may cause cross-linking between RNA strands, 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.

3.3.4 Assessment of RNA Integrity of Drug-Treated HL60 Cells

As expected, the control lanes containing non-reverse-transcribed RNA are blank, as is the H_20 control (Figure 11). PCR product is present for all samples examined, with weaker signals for daunorubicin and mitoxantrone-treated cells.

3.3.5 C-RT-PCR Expression Profiles of Drug Treated HL60 Cells

We have applied C-RT-PCR to examine expression profiles of 25 ABC transcripts, LRP, GST-π, Topoisomerase II-α and β_2-microglobulin in HL60 cells exposed to IC_{50} (Figure 12) and higher concentrations (Figure 13). In addition to chemotherapeutic drugs, we have also examined expression of 35 ABC transcripts after treatment of HL60 cells with 100nM ATRA (Figure 14). Several drugs with different mechanisms of action, including drugs used in the treatment of leukemias (e.g. daunorubicin, mitoxantrone, Ara C and All-Trans Retinoic Acid) were chosen for these experiments.

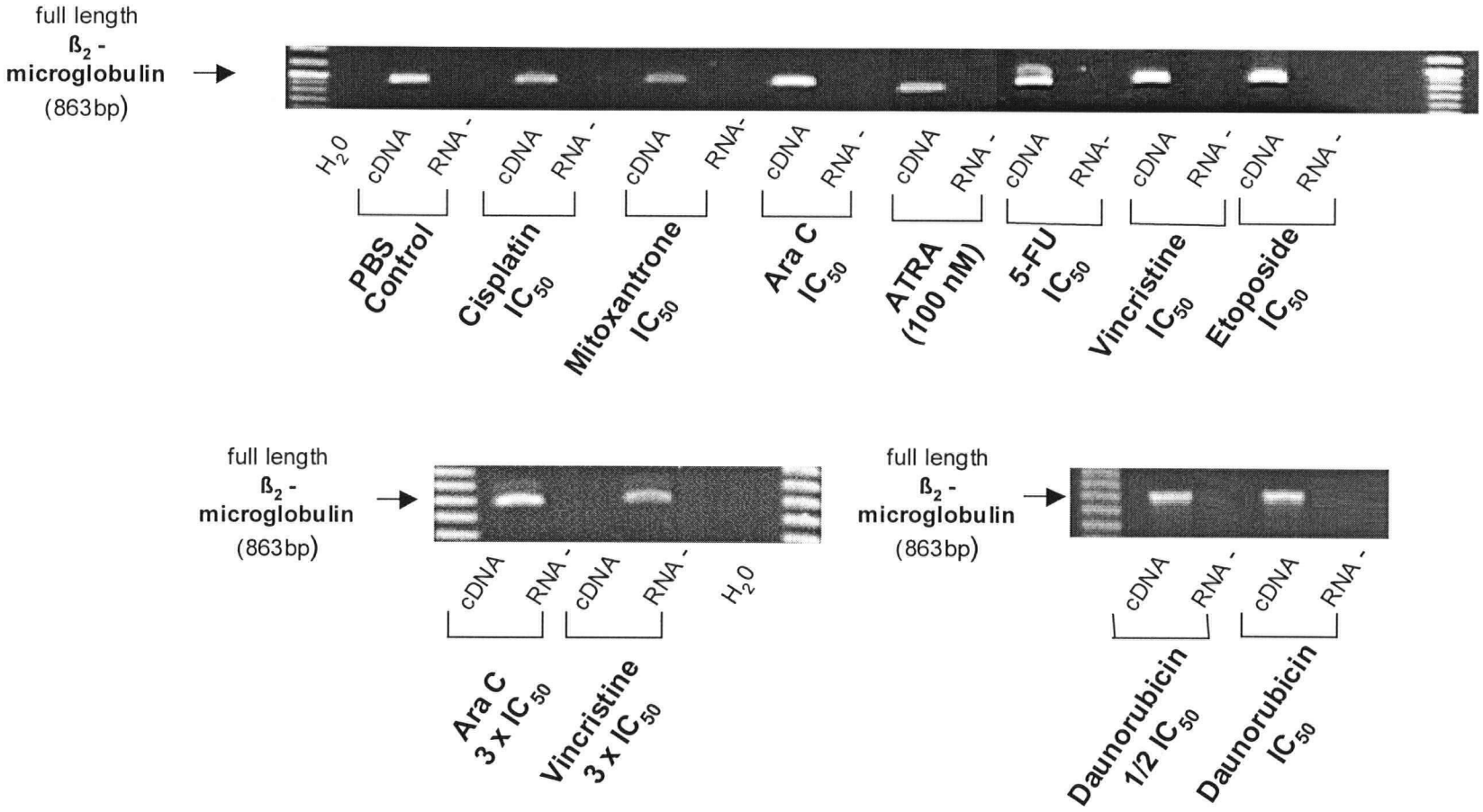


Figure 11. **RT-PCR of Full-Length β_2-microglobulin in Total RNA from HL60 Cells Exposed 24 Hours to Various Concentrations of Drugs.** Lower two panels are from the same experiment.

The native expression levels of the drug-resistance associated ABCs, MRP1, MRP4, MRP5 and GST-π were moderate, whereas MDR1, BCRP, and LRP were not detectable. The retina-specific ABCR transcript and the liver specific BSEP, MRP3, MRP6 and MDR3 transcripts were not detectable. M-ABC2, known to be expressed in hematopoietic cells, was expressed at moderate levels.

Among the 28 transcripts examined in four drug treatment conditions at the IC_{50} concentration (Figure 12), only 7 statistically significant changes in expression were observed. Furthermore, only two of these changes were greater than two-fold in magnitude: a 2.3±0.1-fold increase ($p<0.01$) (mean ± SEM) in the expression of the mitochondrial half-transporter M-ABC2 with vincristine treatment and a 3.2±0.4-fold increase ($p<0.01$) in GST-π expression with mitoxantrone treatment. Only two statistically significant changes were observed with ATRA exposure: a 3.2±0.9-fold increase ($p<0.05$) in MRP7 expression and a 1.8±0.1 fold increase ($p<0.05$) in ABC1 expression (Figure 14).

The experiment was repeated using higher drug concentrations (Figure 13), based on the hypothesis that this might be required to induce larger changes in gene expression. Drug concentrations were increased to 2x IC_{50} for mitoxantrone and 3x IC_{50} for vincristine and Ara C. Daunorubicin at ½ IC_{50} is also included in this analysis, as we were initially unable to isolate sufficient quantities of RNA after treatment with higher concentrations of daunorubicin.

Even at high doses of drugs, there were only a few expression changes observed. Overall, only 2% of the transcripts examined, in both the low and high drug concentration conditions (Figures 13 and 14), showed expression changes greater than 2-fold in magnitude. The ability to discern such a small number of changes verifies the reproducibility of the assay. The most notable expression changes were: a 3.6 ± 0.7 fold reduction ($p<0.05$) in ABC1 mRNA with Ara C treatment (3x IC_{50}), a 4.8 ± 1.1 fold increase ($p<0.01$) in MRP2 and a 6.2 ± 1.1 fold increase ($p<0.01$) in MRP7 mRNA with daunorubicin (½ IC_{50}) treatment. No alterations in expression occurred with exposure to higher concentrations of vincristine.

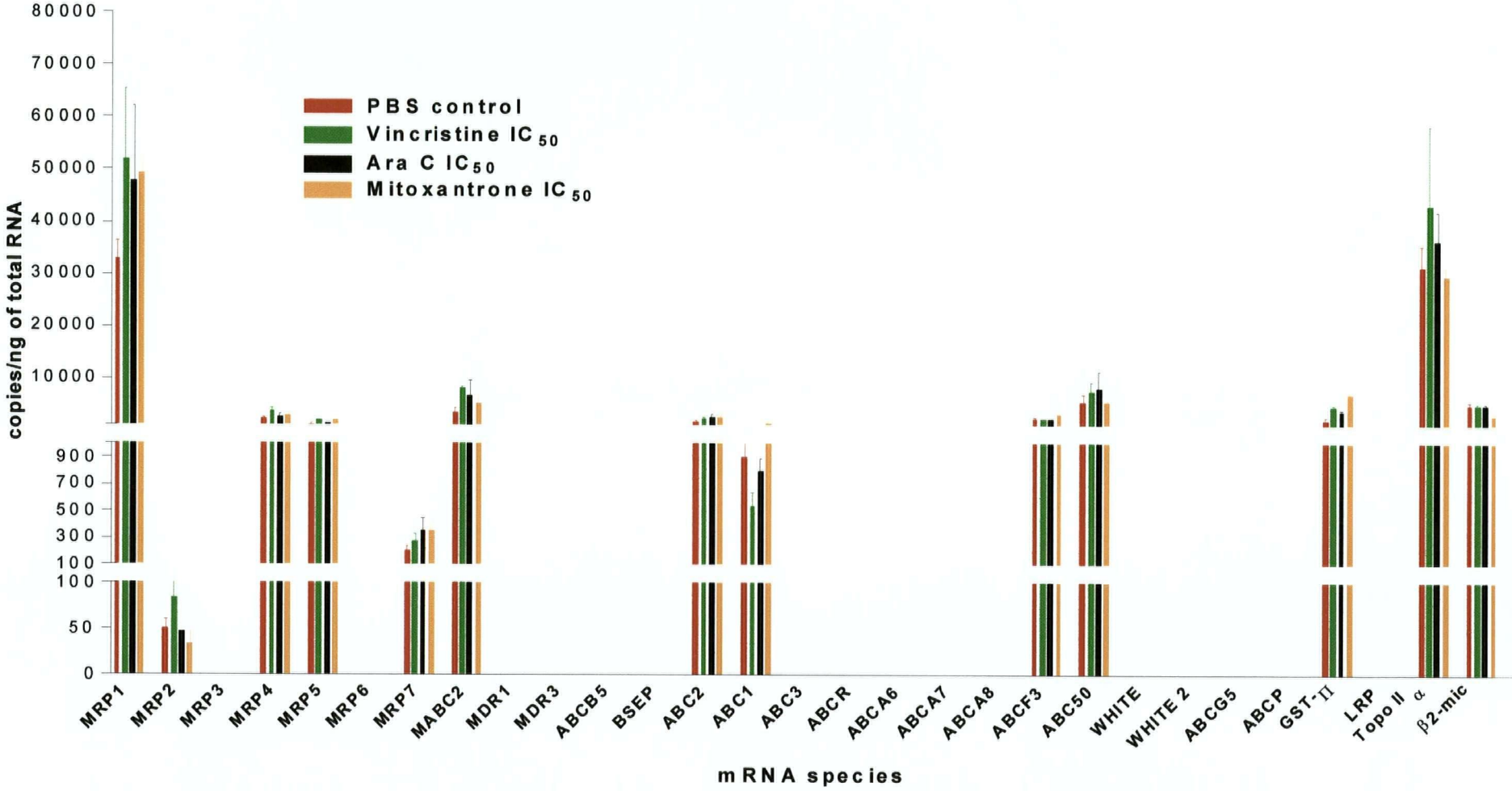


Figure 12. **C-RT-PCR Expression Profile of HL60 Cells after 24hr Treatment with Chemotherapeutic Drugs (IC_{50})** Profile of 25 ABC transcripts, LRP, GST-π and Topoisomerase II-α. All data are normalized relative to β_2-microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable.

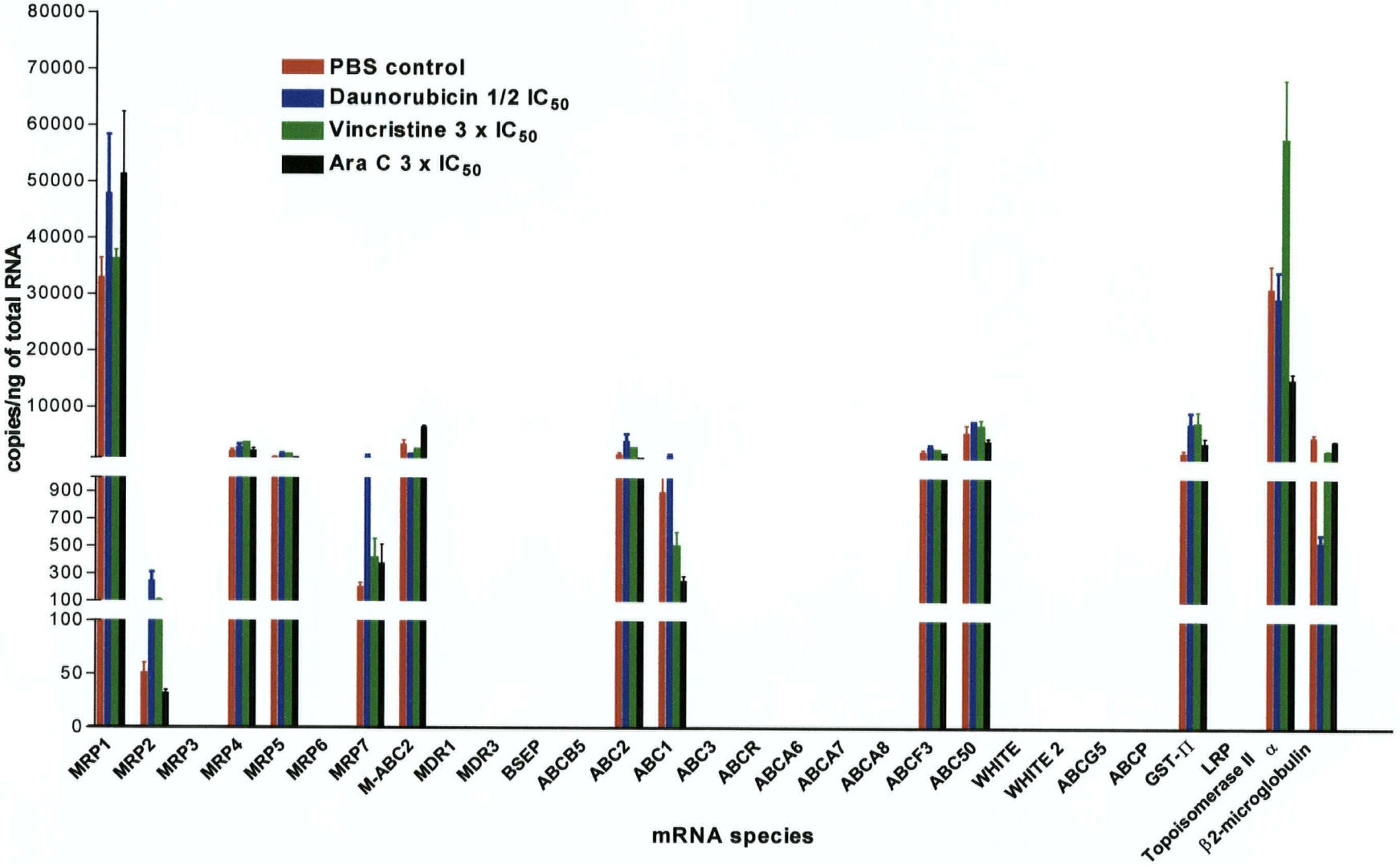


Figure 13. **C-RT-PCR Expression Profile of HL60 Cells after 24h Treatment with Increased Concentrations of Chemotherapeutic Drugs and Daunorubicin @ 1/2 IC_{50}.** Profile of 25 ABC transcripts, LRP, GST-π and Topoisomerase II-α and β_2-microglobulin. All data are normalized relative to β_2-microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable.

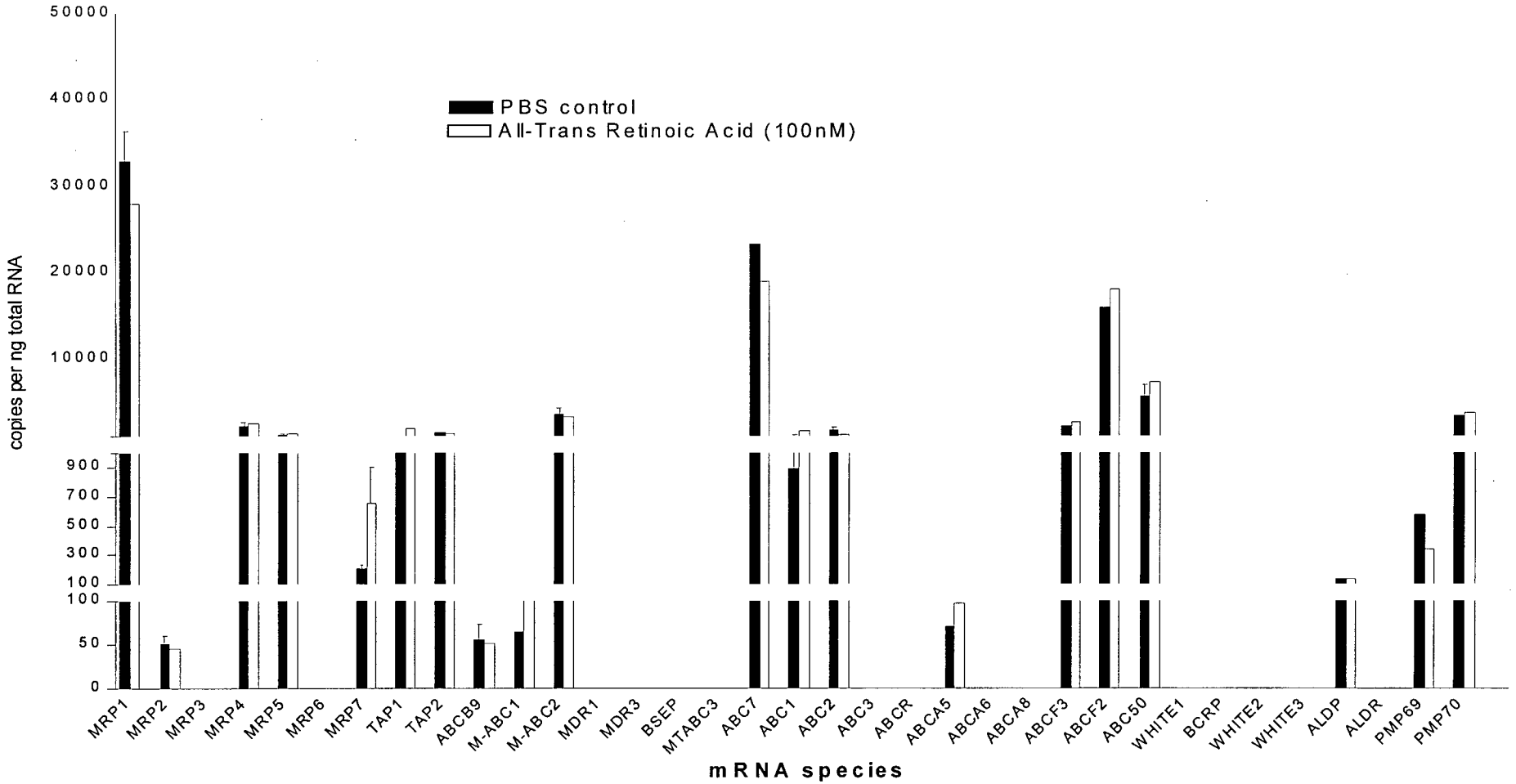


Figure 14. C-RT-PCR Expression Profile of HL60 Cells after 24hr Treatment with 100nm ATRA

Profile of 35 ABC transcripts. All data are normalized relative to β_2-microglobulin levels. Error bars represent SEM of three independent experiments. The absence of a bar denotes the transcript was not detectable.

3.4 DISCUSSION

For several of the drugs, our IC_{50} values (Table IV) are similar to those reported by other groups (Wada *et al.*, 1999) (Ogretmen & Safa, 2000). Differences between clones and experimental procedure may account for the disagreement of IC_{50} values for some drugs.

The expression profile of untreated HL60 cells is comparable to expression reported in the literature. Using a similar C-RT-PCR technique, (Wada et al., 1999) et al. report extremely low expression of MDR1, whereas another study was unable to detect MDR1 (Ogretmen & Safa, 2000). Our results indicate that MDR1 is not detectable in control or drug-treated HL60 cells. MRP1 and Topoisomerase II-α were expressed at high levels in our experiments and these transcripts were also detected by Wada et al.

A comparison of the expression profiles of the HL60 and AML 1, 2 and 3 cell lines reveals a high degree of similarity among these myeloid-derived cell lines. In contrast, the C-RT-PCR expression profile of the human ovarian carcinoma cell line SKOV3 differs significantly from the profiles of these cell lines (Steven Ralph, unpublished data). Differences among the two types of myeloid cells include the presence of moderate levels of ABC3 in the HL60 cell line, which is undetectable in AML 1,2 and 3, and the presence of low levels of LRP in the AML cell lines, which is undetectable in HL60. The reason for the striking difference in ABC3 expression is unknown since its function has not been determined and it is only highly expressed in lung (Connors *et al.*, 1997).

There is no previous evidence for drug resistance to ATRA via a drug transport mechanism, nor is there evidence for induction of ABCs by treatment with this compound. Hence it was not surprising to find so few significant changes in expression with ATRA treatment.

The decision to pursue further study of any of the expression changes greater than 2-fold was based upon current knowledge of the specific ABC gene and whether it might have a potential role in drug resistance. The increase in MABC2 expression with vincristine treatment was not a candidate for further analysis since it is unlikely that a mitochondrial half-ABC involved in iron transport contributes to drug efflux at the plasma membrane. Furthermore, there is no evidence for its role in drug resistance, however it is possible that MABC2 might contribute to non-efflux mechanisms of drug resistance. The role of GST-π with respect to drug detoxification is well established, hence the increase in GST-π expression with mitoxantrone

treatment was not considered for further analysis. ABC1 mediates export of cholesterol and phospholipids from the cell (Orso, 2000) and no evidence exists to support a role for ABC1 in drug transport. MRP2, an anion transporter in 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., 1999), and its role in drug resistance has already been well characterized. The most intriguing alteration in expression was that of MRP7 with daunorubicin treatment. This 6.2-fold increase was the largest we had observed and at the time of this observation, no full-length cDNA had been reported in the literature. Chapter IV describes the rationale behind the decision to further characterize the expression change of MRP7 and the results of this work.

There was only one ABC transcript for which we observed a significant expression change at a low dose of drug, and there was no corresponding change in expression at high doses. The remainder of the expression changes occurred only at the higher drug concentrations. For example, we observed a 2.3-fold increase in MABC2 expression with the IC_{50} dose of vincristine, however expression actually decreased to levels below that of the PBS control at a higher dose of vincristine, suggesting the alteration with vincristine is likely biologically insignificant. Alternatively, this alteration could be a random induction of expression in response to drug exposure rather than as part of a common defense mechanism used by cells. Experiments to examine whether the expression changes of MRP2 and MRP7 show dose dependency with higher doses of daunorubicin are underway.

In retrospect, the surprisingly low number of 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. There is evidence, however, of altered expression of MDR1 and MRP1 in drug selected HL60 cell lines. MRP1 expression was increased by 8-fold in doxorubicin selected HL60 cells that were 13-fold more resistant, relative to the parental cell line, to doxorubicin, and cross-resistant to a number of other structurally unrelated drugs (Wada et al., 1999). (Ogretmen & Safa, 2000) found increased expression of MDR1 in a HL60 cell line displaying high (2300-fold) resistance to vincristine. This data suggests that ABC transporters are a potential mechanism of defense against chemotherapeutic drugs in the HL60 cell line. Other researchers using the K562 erythroleukemia cell line have observed induction of MDR1 with a variety of drugs (Chaudhary & Roninson, 1993) and induction of MDR1 by anthracyclines has been documented in the T Cell CEM cell line (Hu et al., 1995), in contrast to our results with HL60

cells. These cell lines, unlike HL60, express a basal level of MDR1 expression, which appears to be a necessary prerequisite for up-regulation of MDR1 expression (Kantharidis, 2000). Whether expression changes in ABC genes are more likely to be induced by chemotherapeutic drugs in only specific cell lines remains to be determined. This data might also suggest that induction of ABC gene expression by drugs, at least in the HL60 cell line, may not play as great of a role in the development of drug resistance as we had anticipated.

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 1h up to 24h since it is believed that longer exposure times might allow sufficient time for cell division and expansion of an inherently resistant subpopulation of cells. As a result, it would be difficult to determine whether the observed expression changes are a result of drug selection or induction under these conditions.

Current work involves generating HL60 cell lines selected with low concentrations of the same drugs to enable comparison of the effects of short-term drug exposure vs. drug selection on ABC gene expression. This protocol consists of 4 to 5 sequential 3-day exposures at low drug concentrations with an intervening recovery period of approximately 1-2 weeks in duration. Thus far, we have generated an Ara-C resistant (2600-fold) cell line using $3xIC_{50}$ concentrations. RNA has been isolated and analysis of ABC expression profiles is underway. A drug resistant cell line has also been generated using ¼ of the IC_{50} concentration of daunorubicin. It is our hope that these experiments will elucidate selection mechanisms that emerge without long-term stable selection.

CHAPTER IV

Characterization of MRP7 mRNA and cDNA

4.1 INTRODUCTION

4.1.1 The MRP Subfamily and Drug Transport.

In 1996, an EST with significant homology to members of the 'C' subfamily of ABC transporters was generated as part of a larger project whose aim was to discover novel ABC transporters. This EST was localized to chromosome 6p21 and a Northern Blot predicted a mRNA transcript size of 5.5 Kb, suggesting that this was a full ABC transporter (Allikmets et al., 1996). Based on the high degree of sequence similarity specifically with the six MRP 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 until very recently.

The C subfamily of ABC transporters includes MRPs 1-7, CFTR, SUR1 and SUR2, however the MRP members bear a greater degree of amino acid sequence similarity among themselves than with the remaining members of the family. Several MRP family members are capable of transporting anticancer drugs out of cells and are implicated in drug resistance. MRP1, the first member of the family to be characterized, transports a vast spectrum of chemotherapeutic drugs conjugated to glutathione, glucuronate, or sulfate and its role in MDR has been described in Chapter I. MRP2 is found predominantly in the canalicular membrane of hepatocytes, hence it was originally known as the canalicular multispecific organic anion transporter. In transfected cells, overexpression of MRP2 resulted in resistance to cisplatin, etoposide, doxorubicin (Cui *et al.*, 1999) and to several antifolates including methotrexate (Hooijberg *et al.*, 1999).

Another member of the human MRP family, MRP3, when transfected into ovarian carcinoma cells, confers resistance to etoposide, teniposide and methotrexate (Kool *et al.*, 1999b). MRP3 displays a substrate specificity distinct from that of MRP1 and MRP2 in that it preferentially uses glucuronate as a substrate conjugate over glutathione (Hirohashi *et al.*, 1999). MRP4 has been observed to function as an organic anion efflux pump of the anti-human immunodeficiency virus (HIV) drugs 9-(2-phosphonylmethoxyethyl) adenine (PMEA), azidothymidine monophosphate and other nucleoside analogues (Schuetz *et al.*, 1999).

The recent characterization of MRP5 elucidated its role as a transporter of glutathione - conjugated nucleotide analogues. Overexpression of MRP5 in transfected polarized cells resulted in resistance to two thiopurine anticancer drugs, 6-mercaptopurine and thioguanine, in addition to the anti-HIV drug PMEA (Wijnholds *et al.*, 2000). The potential involvement of MRP6 in drug resistance remains unclear. Mutations in this gene cause the connective tissue disorder pseudoxanthoma elasticum (Bergen et al., 2000). Overexpression and/or amplification of MRP6 is found only in drug resistant cell lines with high overexpression and amplification of MRP1 (Borst et al., 2000). The general consensus is that it is unlikely that MRP6 plays a role in drug resistance and that it is co-amplified with MRP1 due to its close proximity to MRP1 on chromosome 16 (Kool *et al.*, 1999a). The three remaining subfamily members, SUR 1, SUR 2 and CFTR have not been associated with drug transport.

With this knowledge and our observation of increased MRP7 expression with daunorubicin exposure, we attempted to further characterize this gene with the hypothesis that it may contribute to drug resistance. Our approach consisted of the construction of full-length epitope-tagged cDNA constructs for use in transfection studies and to determine whether the change in MRP7 expression was dose or time-dependent.

4.2 MATERIALS AND METHODS

4.2.1 Search of the Expressed Sequence Tag Database (dbEST) and the human total genomic sequence (htgs) Database for a Full-Length cDNA or Genomic Sequence Representing MRP7

Using EST U66684 as bait, we used the BLAST 2.1.2 program (National Center for Biotechnology Information Website) to search the dbEST and htgs databases for novel cDNA clones and genomic sequence representing MRP7. Default parameters were used for all BLAST 2.1.2 queries.

4.2.2 MRP7 cDNA Clone

The 5023 bp cDNA clone (AK000002) matching the EST representing MRP7 (U66684) was a generous gift from the Kazusa DNA Research Institute, Chiba, Japan. The cDNA was inserted into the *SalI*/*Not*I site of the pBluescript II SK^+ vector.

4.2.3 5' RACE

To confirm that clone AK000002 represented the complete open reading frame and 5' untranslated region of MRP7, 5' Random Amplification of cDNA Ends (RACE) was performed using Clontech's Marathon Ready cDNA Kit (Figure 15). Nested PCR amplification consisted of primers AP1 (5' CCATCCTAATACGACTCACTATAGGGC 3') and a MRP7 gene-specific primer, 370 RACE1 MRP7 (5' GTAGGATGTAATCTGGACTCCTCGG3') which were used to amplify cDNA clones from the Clontech Marathon Ready placental cDNA library in the first round of PCR. This PCR consisted of one 50μl reaction and a H_2O negative control. PCR conditions were: 50mM KCl, 10mM Tris-HCl (pH 8.3), 100μM dNTPs, 2mM $MgCl_2$, 20μg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 3.5U of Amplitaq Gold (Perkin-Elmer), 2μM of AP1 and 370 RACE1 MRP7 primers and 2.5μl of the cDNA mixture in a 50μl volume. Touchdown PCR cycling parameters were 95° (12 min), 5 cycles at 95° (5 sec), 72° (2.5 min), 5 cycles at 95° (5 sec), 70° (2.5 min), and 25 cycles at 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 μl of 1/50 diluted PCR product from round one and primers AP2 (5' ACTCACTATAGGGCTCGAGCGGC3') and 341 RACE2 MRP7 (5' CCAAGTAACAGGCACTGAGCACGG3'). PCR conditions were performed as described above.

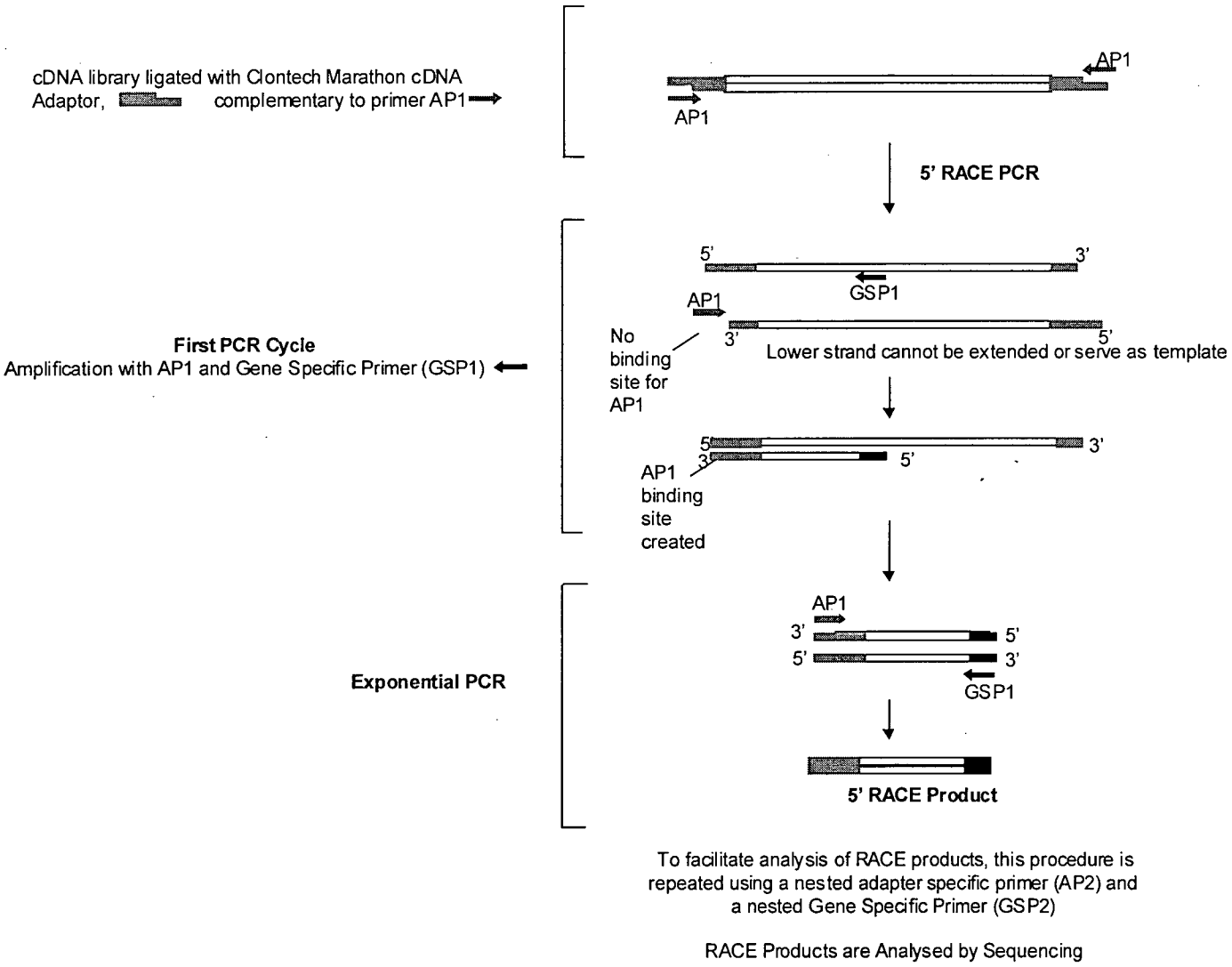


Figure 15. 5' RACE Procedure

(adapted from Marathon RACE Manual, Clontech laboratories, Palo Alto, California, U.S.A.)

4.2.4 Cloning and Sequencing of 5' RACE Products

50μl of 5' RACE PCR products from the second amplification were run on a 1.0% agarose gel, excised and gel purified using the Qiaex II gel extraction kit (Qiagen), then cloned into the pCR-XL-TOPO vector (Invitrogen) and transformed into competent cells using the TOPO-XL PCR Cloning Kit (Invitrogen). Cells were grown overnight on agar plates containing kanamycin and colony PCR was performed to confirm insert size. For PCR, colonies were picked and diluted in 10μl H_20, 2μl of this mixture was used as template. PCR was performed using primers T7 universal (5' CCCTATAGTGAGTCGTATT3') and M13 reverse (5' CAGGAAACAGCTATGA 3'). PCR buffer: 50mM KCl, 10mM Tris-HCl (pH 8.3), 100μM dNTPs, 2mM $MgCl_2$, 20μg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 4U of Taq polymerase, 0.5μM of T7 and M13 primers and 2.0μl of the colony mixture in a 15 μl volume. PCR cycling parameters were 95° (5 min) and 30 cycles at 95° (30sec), 56° (15s), 72° (45s). Products were separated on a 1% agarose gel and visualized after ethidium bromide staining. Colonies containing vectors with the appropriate insert sizes, as determined by PCR, were grown overnight and plasmids were extracted using the Quantum Prep Plasmid Miniprep Kit (Biorad). Plasmids were sequenced using 4 ul of Big Dye Terminator Mix (Applied Biosystems) and 2 ul of plasmid template and 4ul of 0.8 μM of one of the following primers: T7 universal (described previously), M13 Reverse (described previously) or T3 (5' ATTAACCCTCACTAAAG 3'). Typical sequencing PCR conditions were: 95° (2 min) and 28 cycles of 95° (10s), 47° (5 sec), 60° (3 min). Annealing temperature varied depending on the primer used. PCR products were analyzed on an ABI 310 DNA sequencer.

4.2.5 C-RT-PCR Expression Profile of MRP7 in Total RNA from Normal Human Tissues

Total RNA from Clontech's Human Total RNA Panels I and III were used to evaluate MRP7 expression in 11 normal human tissues. Clontech RNA was extracted from tissues collected from single individuals (brain, heart, liver and lung) or from tissues pooled from several individuals (trachea, mammary gland, prostate, skeletal muscle, testis and uterus). C- RT-PCR was performed as described in Chapter II.

4.2.6 Semi-quantitative PCR Analysis of MRP7 and Beta-2 Microglobulin in cDNA from a Panel of Normal Human Tissues

cDNA from Clontech's Multiple Tissue cDNA Panel was used to amplify a 250 bp MRP7 cDNA using a 35 cycle PCR. This cDNA panel has been normalized by the manufacturer according to the expression levels of 4 housekeeping genes. A high cycle number was chosen based on the low level of MRP7 expression observed by C-RT-PCR. PCR cycling conditions consisted of 95°C (2 min), 5 cycles: 95°C (30s) 52°C (15s) 72°C (45s) and 30 cycles: 95°C (30s) 64°C (15s) 72°C (45s). Full-length β_2-microglobulin was amplified as described in Chapter II. PCR products were resolved on a 1% agarose gel, stained with ethidium bromide and visualized using the Eagle Eye II (Stratagene).

4.2.7 Amino Acid Similarity of MRP7 with Other MRP Subfamily Members

The percent amino acid identity of MRP7 with other MRP subfamily members was performed using the BLASTP 2.1.2 program (NCBI website) using default settings. For NCBI accession numbers, please see Table I.

4.2.8 Predicted Protein Structure of MRP7

To compare the predicted protein structure of MRP7 with that of the other MRP subfamily members, hydrophobicity plots were generated with the TopPred II 1.3 program using the sequence of clone AK000002 and published full-length sequences of MRPs 1-6, using default settings. For NCBI accession numbers, please see Table I.

4.2.9 Expression Pattern of MRP7 Using Clontech's MTE Array

To assess mRNA expression of MRP7 in a broader variety of adult tissue sources, embryonic tissues and cell lines, we used Clontech's Human Multiple Tissue Expression (MTE) Array, which contains normalized loadings of poly A^+ RNA from 76 different human tissues and 8 different control RNAs and DNAs. A 791bp cDNA probe, generated using PCR (see below) was randomly labeled with ^{32}P (Amersham) and hybridized to the MTE Array according to the manufacturer's protocol.

4.2.10 PCR Amplification of a MRP7 mRNA/cDNA Probe

A 791 base-pair cDNA probe for use in the MTE Array hybridization and Northern Blot protocols was generated by PCR amplification using the AK000002 clone in pBluescript as template. PCR amplification consisted of one 50µl reaction containing 1µl of 1/10 diluted template and an H_2O negative control. PCR conditions were: 50mM KCl, 10mM Tris-HCl (pH 8.3), 100µM dNTPs, 2mM $MgCl_2$, 20µg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 2.5U of Taq Polymerase, 1.5µM of 967 MRP7 (5' TAGGTTGGCTGCAGTCTGTG3') and EcoRI-M-MRP7 (5'GAGCTAGAATTCGCGTCCATGGAACGACTTC3') primers. PCR cycling parameters were 95° (2 min), 5 cycles at 95° (15 sec), 52° (15s), 68° (2 min), 25 cycles at 95° (15s), 68° (2 min), and a final extension period of 68° (10 min). The PCR product containing the probe was run on a 1.0% agarose gel and purified using the Qiaex II gel extraction kit (Qiagen). This cDNA was solubilized in 10ul of water, giving an estimated yield of 35 ng.

4.2.11 Northern Blot of MRP7 Using RNA From a Panel of Human Cancer Cell Lines

Total RNA was extracted using the method described in Chapter II from a panel of 9 human cancer cell lines (please see section 4.2.13). 5µg of total RNA from each cell line and one H_20-negative control were denatured in 2µl 1x MOPS, 10µl formamide, and 3.5 µl 37% formaldehyde, in a total volume of 20.5µl per reaction. Samples were heated to 65°(15 min), then snap cooled on ice (2 min). 2µl of loading dye (90% RNA loading dye, 5% ethidium bromide) was added to each sample. Samples were loaded on a 1% agarose gel composed of 1X MOPS and 2.2M formaldehyde and run at 70 Volts for 2.5h. RNA was transferred to a nylon membrane (Hybond) overnight, baked at 80° (2hrs) and stored at -80°C.

4.2.12 Epitope-Tagged MRP7 cDNA Constructs for Transfection Experiments

We attempted to generate 3' and 5'epitope-tagged MRP7 full-length cDNAs for cellular localization studies of MRP7 and for use in drug resistance assays. To engineer a MRP7 construct with a 5' or 3' FLAG epitope in the pcDNA 3.1/ Zeo expression vector, we

employed a cloning strategy which would incorporate a Sac II site at the 5' or 3' end of MRP7, into which FLAG oligomers would be ligated. This restriction site was chosen because it was not found in either the MRP7 sequence or in the expression vector pcDNA3.1/Zeo.

This strategy consisted of several steps (Figures 16, 17, 18 a,b). With respect the 5'-epitope tagged MRP7 cDNA, the first step consisted of removing the 5' UTR and the addition of an *Eco*RI site immediately 5' to the start codon/Kozak sequence using PCR (Figure 16 step 1).

PCR primers *Eco*RI-M (5'GAGCTAGAATTCGCGTCCATGGAACGACTTC3') and 1678 MRP7 (5'AGTCGCCCCAGCTCTCGAGCCCG 3') were used to amplify a 1430 bp fragment from the 5' end of the 5023 bp MRP7 cDNA clone (AK000002) in pBluescript II. PCR reactions included two 50µl reactions containing 1 µl of undiluted, 1µl of 1/10 diluted AK000002 MRP7 template and one 50µl water negative control. PCR Buffer: PCR PfX Buffer (Gibco BRL), 10 mM dNTPs, 50 mM $MgSO_4$ and 2U Pfx Taq (High-fidelity enzyme, Gibco BRL). PCR cycling parameters were 95° (2 min), 5 cycles at 95° (15 sec), 52° (15s), 68°(2 min) and 25 cycles at 95° (15 sec), 68° (2 min), and a final extension period of 68° (10 min) on a Perkin- Elmer 9600 thermocycler. To ensure the addition of a 3' A-overhang for cloning purposes, Taq polymerase was added at the end of the PCR cycling and incubated 10 min at room temperature.

The PCR product (MRP7-EcoR1) was run on a 1% agarose gel and stained with ethidium bromide. Bands were excised and gel-purified using the Qiaex II gel purification kit (Qiagen). Fragments were subsequently cloned into the pCR-XL-TOPO vector (Invitrogen) and transformed into competent cells using the TOPO-XL-PCR Cloning Kit (Invitrogen). Colony PCR (described previously) was performed to confirm insert size. Several clones were chosen for sequencing analysis to confirm identity and orientation. Sequencing was performed as described in section 4.2.4. The second step (Figure 16) was the incorporation of a *Sac*II restriction site into which FLAG oligomers would be inserted (Figure 16, step 2). The pCR-XL-TOPO vector containing the MRP7-

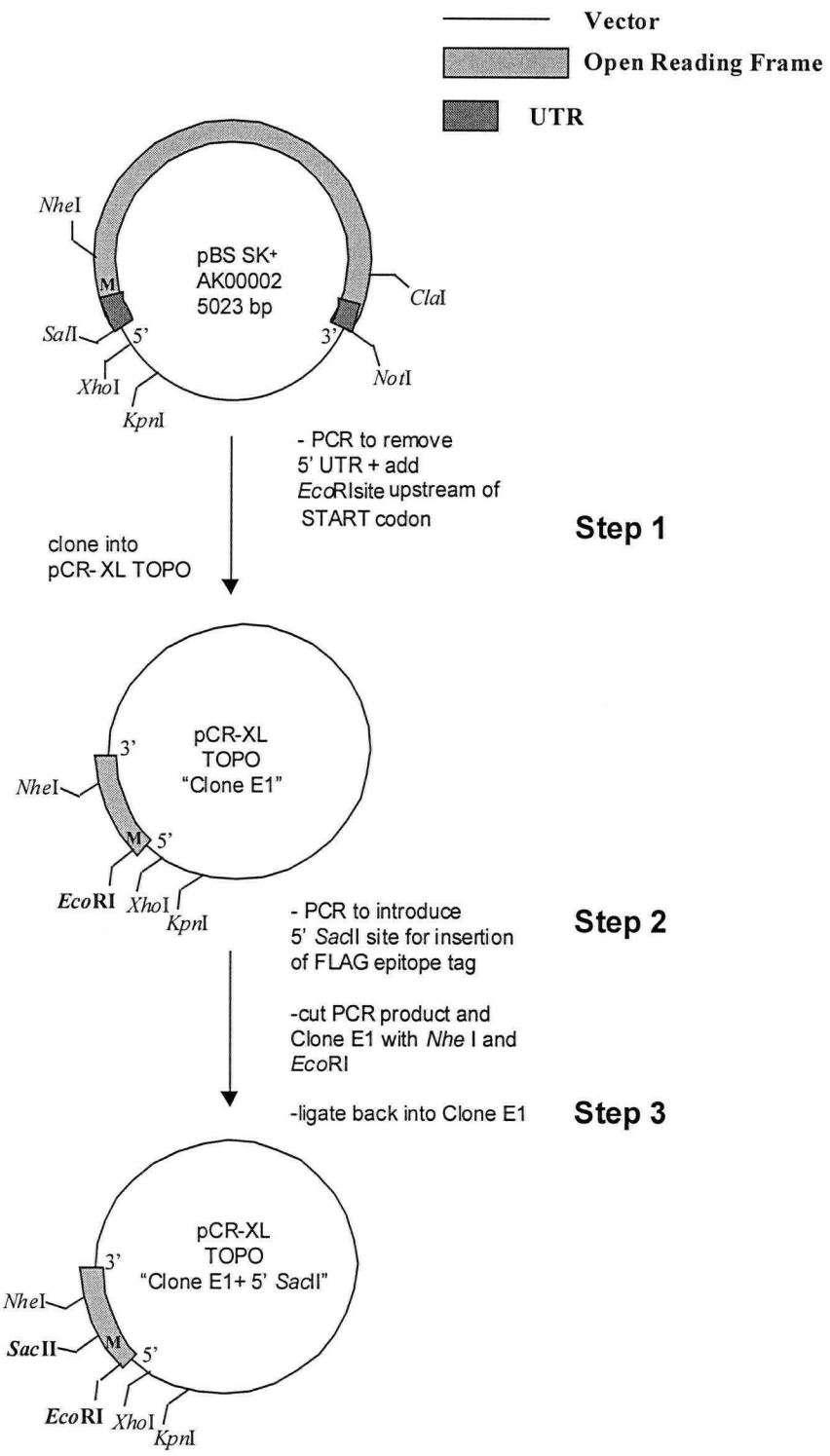


Figure 16. Steps 1-3 of Cloning Strategy for Generation of 5' Epitope-tagged MRP7 Construct

*Eco*RI fragment (Clone E1) was amplified with primers 1678 MRP7 (5'AGTCGCCCCAGCTCTCGAGCCCG3') and *Eco*RI/*Sac*II MRP7 (5'GCTAGAATTCGCGTCCATGCCGCGGGAACGACTTCTG3') to insert a *Sac*II site immediately 3' of the Kozak consensus sequence. This fragment containing the 5' *Sac*II site was cloned into PCR XL-TOPO and was designated clone E1 (Figure 16, step 3). Colony PCR and sequencing were performed as described previously.

To reassemble the full-length cDNA with the incorporated *Sac*II site, (Figure 18a), Clone E1 was digested with *Eco*RI/*Nhe*I (Gibco BRL) and ligated to an *Eco*RI/*Xba*I-cut pcDNA3.1/Zeo vector and a *Nhe*I/*Xba*I-cut Clone 9 (full-length MRP7 cDNA with a *Xba*1 site incorporated at the 3' end, generated during construction of 3'SacII MRP7 clone, described below). All digests were performed in the appropriate buffer (Gibco BRL) incubated for 4h at 37°C, separated on a 1% agarose gel by electrophoresis and gel-purified using the Qiaex II gel purification kit (Qiagen). Colony PCR and sequencing were performed as described previously.

Finally, FLAG oligomers encoding the cDNA sequence for antigenic binding site (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) of the FLAG antibody were annealed and ligated into the *Sac*II site of the appropriate MRP7 pcDNA3.1/Zeo cDNA construct. 10 µl of FLAG1 oligomer (5'CGGACTACAAGGACGACGACGACAAG3') (with a *Sac*II overhang engineered at the 5' end) and 10ul of FLAG2 oligomer (5'CGCTTGTCGTCGTCGTCCTTGTAGTC3') (with a *Sac*II overhang at the 3' end) were phosphorylated with 5 Units of T4 Polynucleotide Kinase (New England Biolabs), 3 ul 10X PNK Buffer (New England Biolabs) and 4 µl of 10 mM ATP in a total volume of 30µl and incubated at 37°C for 1h.The oligomers were then heat denatured in boiling water for 5 minutes. The denatured oligomers were allowed to cool to room temperature to facilitate annealing of FLAG1 and FLAG2. The oligomers were then ligated into the appropriate vector (Figure 18a and b) which had been cut with the SacII restriction endonuclease (Gibco). The SacII digest was performed as described previously. Ligations were performed as follows: 4.5µl of undiluted, 1/10-diluted or 1/100-diluted FLAG1/2 oligomer and a water-negative control were incubated with 1000 units of DNA ligase (Gibco BRL), DNA ligase Buffer (Gibco BRL), 3 µl of appropriate Sac-II-digested vector and incubated at 14°C overnight. Ligated plasmids were transformed into

competent cells and grown overnight on agar plates containing ampicillin. Colony PCR, described previously, was performed to verify the presence of the FLAG oligomer. Colonies were chosen and sequenced as described previously.

The 3' epitope-tagged MRP7 cDNA construct was designed in similar fashion. The first step consisted of the removal the 3' UTR and the incorporation of a *Xba*I site immediately 3' of the stop codon. This restriction site was chosen because it was present in the multiple cloning site of the expression vector pcDNA3.1/Zeo and was not present in the MRP7 sequence.

PCR Primers MRP7 *Xba*I-Stop (5'GAGCTATCTAGATCAGGGACCTCCGAGTGAG3') and 4409 MRP7 (5'ATGCCAAGATCCTGTGTATCGATGAG3') were used to amplify a 290 bp fragment from the 3' end of the 5023 bp MRP7 cDNA in pBluescript II (Figure 17, step 1). PCR reactions included two 50µl reactions containing 1 µl of undiluted, 1µl of 1/10 diluted AK000002 MRP7 plasmid template and one 50µl H_20-negative control. PCR Buffer: PCR PfX Buffer (Gibco BRL), 10 mM dNTPs, 50 mM $MgS0_4$ and 2U Pfx Taq (high-fidelity enzyme, Gibco BRL). PCR cycling parameters were 95° (2 min), 5 cycles at 95° (15 sec), 52° (15s), 68°(2 min) and 25 cycles at 95° (15 sec), 68° (2 min), and a final extension period of 68° (10 min) on a Perkin- Elmer 9600 thermocycler. To ensure the addition of a 3' A-overhang for cloning purposes, Taq polymerase was added at the end of the PCR cycling and incubated 10 min at room temperature.

The PCR product (MRP7-XbaI) was run on a 1% agarose gel and stained with ethidium bromide. Bands were excised and gel-purified using the Qiaex II gel purification kit (Qiagen). Fragments were subsequently cloned into the PCR-XL-TOPO vector (Invitrogen) and transformed into competent cells using the TOPO-XL PCR Cloning Kit (Invitrogen). Colony PCR (described previously) was performed to confirm insert size. Several clones were chosen for sequencing analysis to confirm identity and orientation. Sequencing was performed as described in section 2.2.4. To reassemble the full-length cDNA, a double digest with the *Cla*I and *Not*I restriction endonucleases was performed (Figure 17, step 2) on the pCR-XL-TOPO vector containing the MRP7-XbaI fragment (Clone X3) and the full length MRP7 cDNA in pBS Bluescript (AK000002). The digested fragments were run on a 1.0% agarose gel and excised, purified and ligated (Figure 17, step 3) as described above. Colony PCR and sequencing were performed as

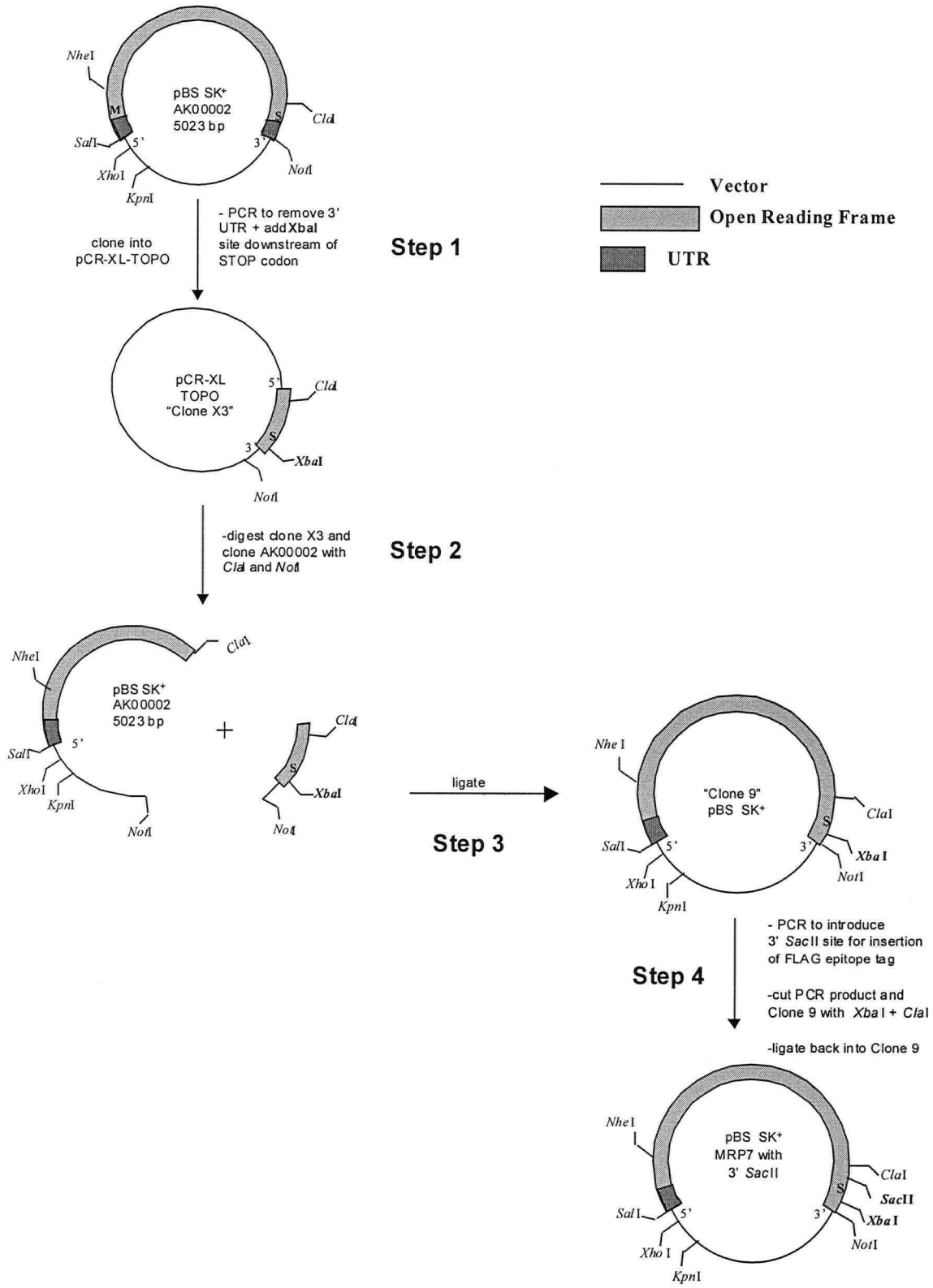


Figure 17. Steps 1-4 of Cloning Strategy for Generation of 3' Epitope-tagged MRP7 Construct

described previously. This pBS SK Bluescript plasmid now contained the full length MRP7 with a 3' end containing a *Bx* I site (designated as Clone 9).

Clone 9 was re-amplified with primers 4409 MRP7 and XbaI/SacII MRP7 (5'GCTATCTAGATCACCGCGGACCTCCGAGTGAG3') to incorporate a SacII site immediately 5' to the stop codon into which a FLAG oligomer would be inserted (Figure 17, step 4). PCR conditions: PCR buffer (50mM KCl, 10mM Tris-HCl (pH 8.3), 100μM dNTPs, 2mM $MgCl_2$, 20μg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 5U of Taq polymerase, 0.5 μM of forward and reverse primers and 1 μl of the appropriate cDNA mixture in a 50μl volume). PCR cycling conditions were as described above. PCR products were run on a 1% agarose gel and stained with ethidium bromide. Bands were excised and gel-purified using the Qiaex II gel purification kit (Qiagen).

The resulting fragment containing the *Sac*II site was cut with the *Cla*I and *Not*1 restriction endonucleases (Gibco) and re-ligated back into the *Cla*I/*Not*1-digested Clone 9 vector, yielding "pBS SK^+ MRP7 with 3'*Sac*II" (Figure 18b).

To reassemble the full-length cDNA with the incorporated SacII site (Figure 18b), "pBS SK^+ MRP7 with 3'*Sac*II" was digested with *Nhe*I/*Xba*I (Gibco BRL) and ligated to an *Eco*RI/*Xba*I-cut pcDNA3.1/Zeo vector and a *Nhe*I/*Eco*RI -cut Clone E1 (full-length MRP7 cDNA with an *Eco*RI site incorporated at the 5' end, generated during construction of the 5'SacII MRP7 full-length clone, described previously). All digests were performed in the appropriate buffer (Gibco BRL) incubated for 4h at 37°C, separated on a 1% agarose gel by electrophoresis and gel-purified using the Qiaex II gel purification kit (Qiagen). Colony PCR and sequencing were performed as described previously. FLAG oligomers were incorporated into the 3' *Sac*II site of the pcDNA3.1Zeo MRP7 expression vector as described previously.

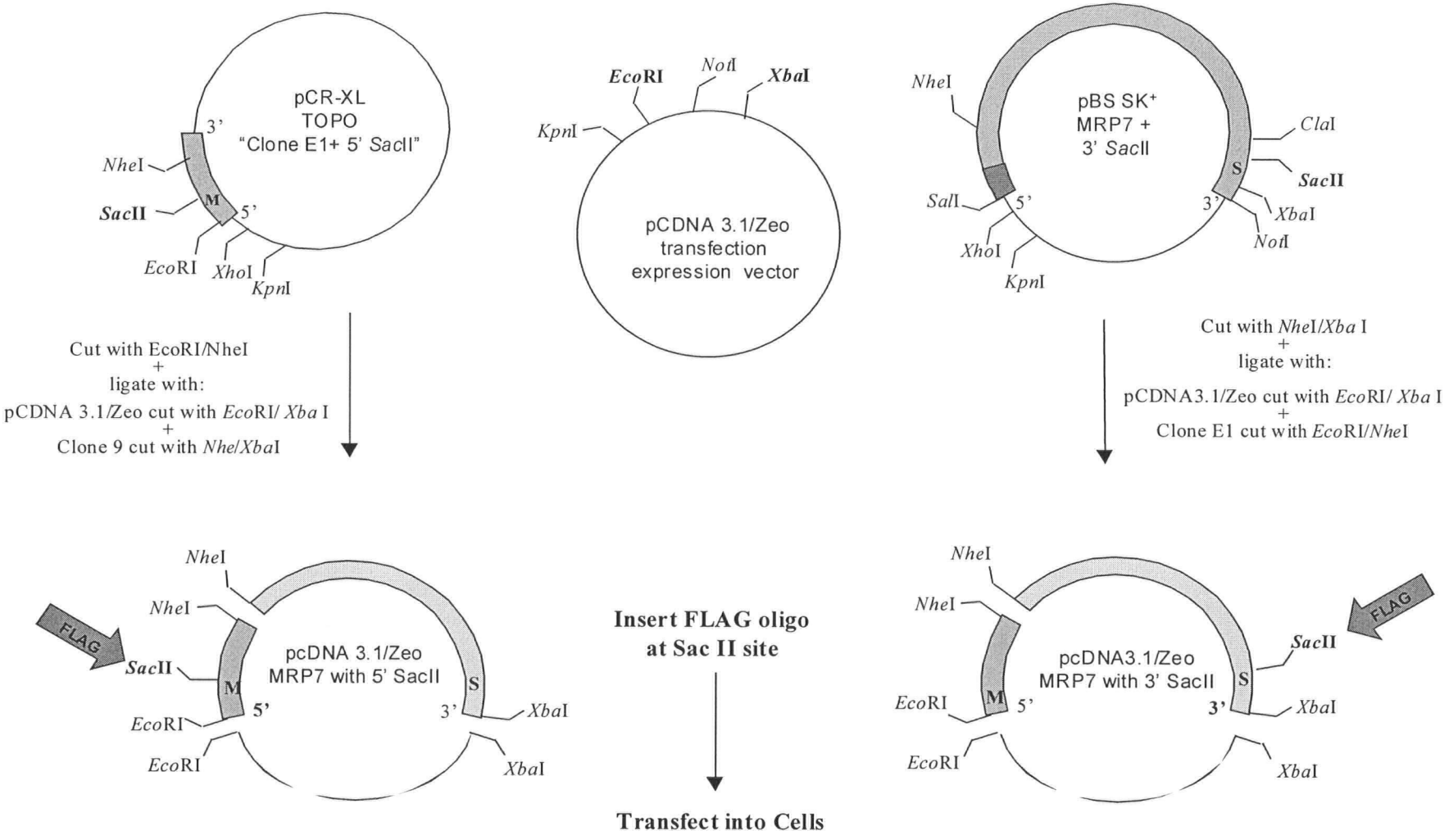


Figure 18a. Final Step of Cloning Strategy for 5' FLAG-tagged MRP7 Construct

Figure 18b. Final Step of Cloning Strategy for 3' FLAG-tagged MRP7 Construct

4.2.13 Cell Lines

Cell lines used for Northern Blot: A498 (renal carcinoma cell line), a generous gift from Dr. Wan Lam, B.C. Cancer Research Centre, was maintained in DMEM (Gibco BRL) and 10% FBS, HL60 (promyelocytic leukemia cell line, described in section 3.2.1), SKOV3 (ovarian carcinoma cell line) was maintained in α-MEM (Gibco BRL) and 15% FBS, SW620 (colon carcinoma) and CEM (leukemia cell line) were maintained in α-MEM (Gibco BRL) and 10% FBS, HS683 (glioma) was maintained in DMEM (Gibco BRL) with 4.5 g/L glucose and 10% FBS, SKUT1 (uterine mesodermal tumour) was maintained in RPMI (Gibco BRL) and 10% FBS, DU145 (prostate carcinoma) and H1299 (lung carcinoma).were maintained in RPMI (Gibco BRL) and 10% FBS. All cell lines were grown without antibiotic.

To determine if the change in expression of MRP7 with daunorubicin treatment was dose-dependent, we exposed HL60 cells to a range of daunorubicin concentrations for 24 hours. HL60 cells were maintained as described in section 3.2.1.

4.2.14 24 Hour Drug Exposures

HL60 cells were exposed to drug as described in section 3.2.6. Cells were exposed 24 hours to daunorubicin concentrations of ¼ IC_{50}, IC_{50} or $2xIC_{50}$. Since we had difficulty isolating sufficient quantities of quality total RNA with our first experiments using daunorubicin (section 3.3.3), we used a greater number of flasks per treatment with the same number of cells per flask as done previously.

4.2.15 Preparation of Total RNA.

RNA from all cell lines was prepared as described in section 2.2.2.

4.3 RESULTS

4.3.1 The Discovery of a Novel EST Representing a Full-Length MRP7 cDNA

In 1998, during a routine search of the dbEST for novel members of the ABC gene family, Steven Ralph found a 1914-base-pair EST (U66684) which had been identified as the putative MRP7 and localized to chromosome 6p21 using FISH analysis (Allikmets et

al., 1996). Upon searching of the non-redundant and htgs databases for full-length clones and genomic sequences matching EST U66684, we identified four cDNA clones. The clones had all been submitted to the databases within the past six months and included: AL1222095 (1003 bp), AK024446 (4538 bp), AL133613 (2472 bp) and AK000002 (5023 bp) (Figure 19). Each of these clones matched perfectly with the 3' end of the original EST. Among these, the 5023 bp clone (AK000002) was of the greatest interest due to its large size. A comparison of the sequence of the AK000002 cDNA against htgs using BLAST 2.1.2 revealed 100% identity with 23 sequence stretches of 2 human unordered Bacterial Artificial Chromosomes (BACs): clones AC021391 and AL359813, located at the same position on the chromosome, 6p21 (Steven Ralph, personal communication). These stretches of identity ranged in size from 75 to 454 nucleotides and were the same in both BACs. These analyses revealed the potential exon/intron structure, although we did not investigate further.

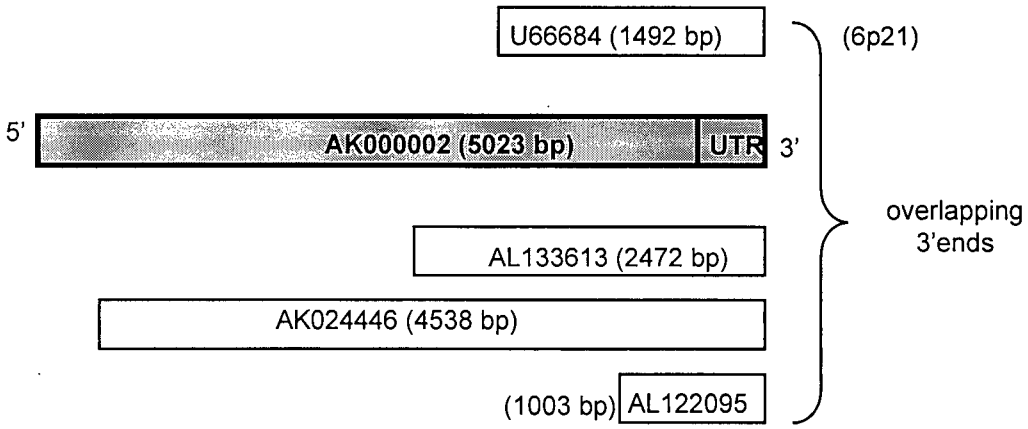


Figure 19. cDNA Clones Representing MRP7
Four recently identified clones from the genomic databases which match EST U66684, representing MRP7.

4.3.2 Choosing the Putative Start Site of the AK000002 cDNA

A 3-phase amino acid translation of the cDNA was performed using the DNA Strider 1.2 program to identify putative translation initiation codons (AUG). A methionine (AUG) was identified at nucleotide position 214 (amino acid position 72) 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 4479 bp, suggesting clone AK000002 contains a complete Open Reading Frame of 1493 amino acids. This length is comparable with the length of other MRP proteins with the same structure.

Using this reading frame we identified the Walker A, linker and Walker B consensus motifs (please refer to section 1.2). The first putative Walker A sequence, located at amino acid (a.a.) position 704, matches perfectly with the Walker A consensus motif and 13 of 14 residues at a.a. position 1351 also match this consensus motif, forming the second putative Walker A region. The first putative linker motif, (a.a. position 796) matches 9 of 12 residues of the consensus, and 11 of 12 residues of the second putative linker (a.a. position 1453) match the linker consensus sequence. Finally, 19 of 20 residues at both a.a. positions 810 and 1465 match the consensus and comprise the first and second Walker B motifs.

The nearest alternative downstream initiation codon occurred at a.a. position 368, with 4 of 6 residues immediately upstream matching the Kozak consensus sequence. However, this putative initiation site would encode a truncated, likely non-functional, protein lacking a large portion of the first transmembrane domain (336 residues), which would be unlike any other MRP protein structure. As such it was unlikely that it was 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'. The two alternative reading frames contained stop codons within 300 a.a. residues of the predicted start site, confirming that we had identified the correct open reading frame.

4.3.3 5' RACE Results

We attempted 5' RACE to confirm that clone AK000002 represented the complete open reading frame and 5' untranslated region (Figure 20). The first attempt produced 4 distinct bands (200bp, 275 bp, 350 bp and 400 bp). Since the gene specific primer from which they were amplified (341 RACE2 MRP7) was located 127 bp downstream of the initiation codon, these bands correspond to products which extend beyond the putative

initiation codon by only 73, 148, 223 and 273 bp, respectively. Repeating the assay yielded 5 bands (150 bp, 250 bp, 350 bp, 475 bp, 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 bands from the first experiment confirmed that they contained MRP7 sequence. These results, and the absence of any larger bands, would suggest that the putative initiation codon identified on 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 open reading frame of MRP7 and a portion of the non-coding 5'UTR. We do not have sequencing analysis data from the second experiment as a result of our decision to terminate the MRP7 project (please see discussion).

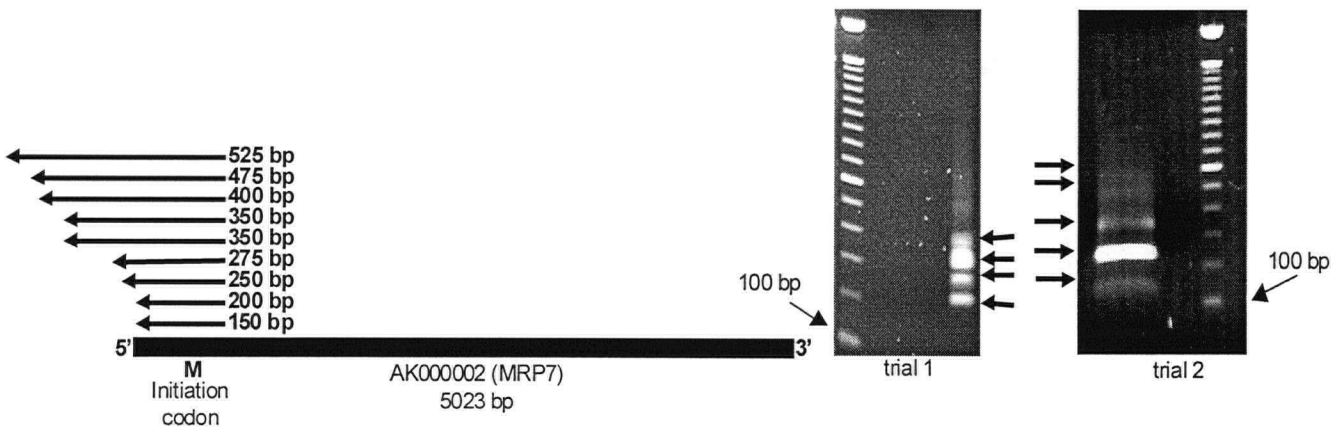


Figure 20. 5' RACE of MRP7.
5'RACE products from two separate attempts are shown in relation to the putative initiation codon (M) of the MRP7 cDNA AK000002.

4.3.4 Amino Acid Similarity of MRP7 with other MRP subfamily members

Using the sequence of this cDNA and the published full-length sequences of the MRP subfamily members, we first determined the extent of amino acid similarity of MRP7 with the other MRP subfamily members **(Table VI)**. MRP7 appeared to be more closely related to other MRPs than to SUR1, SUR2 and CFTR.

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

Table VI. Percent amino acid identity between fully sequenced human MRP subfamily members and MRP7. Percent identity was obtained using BLASTP2.1.2.

4.3.5 Predicted Protein Structure of MRP7

Analysis of hydrophobicity plots of MRP7 and MRPs 1-6 (Figure 21) revealed the presence of an N-terminal domain composed of 5 transmembrane helices at the N-terminus in addition to the 12 membrane spanning regions typical of full ABC proteins. This additional domain is also present in MRPs 1, 2,3 and 6.

4.3.6 Expression Pattern of MRP7 in Normal Human Tissues Using C-RT-PCR

C-RT- PCR analysis of ABC transcripts in total RNA from a panel of 10 normal human tissues showed that MRP7 is expressed at low levels in all tissues examined (Figure 22a). This was confirmed by semi-quantitative PCR analysis of cDNA from a panel of 16 normal tissues (Figure 22b).

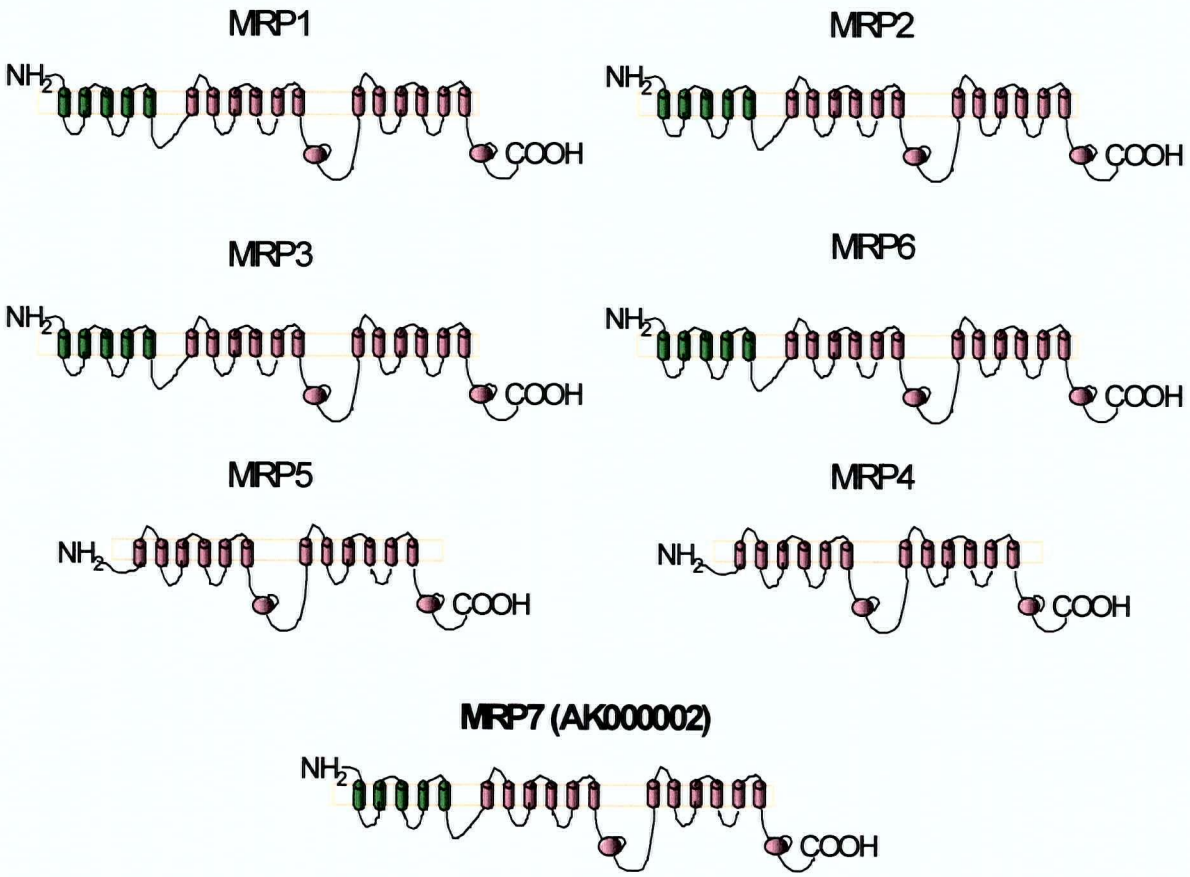


Figure 21. Predicted Protein Structure of MRP7
Figure is based on hydropathy plots of clone AK000002, representing the putative MRP7 and published full-length DNA sequences of MRPs 1-6.

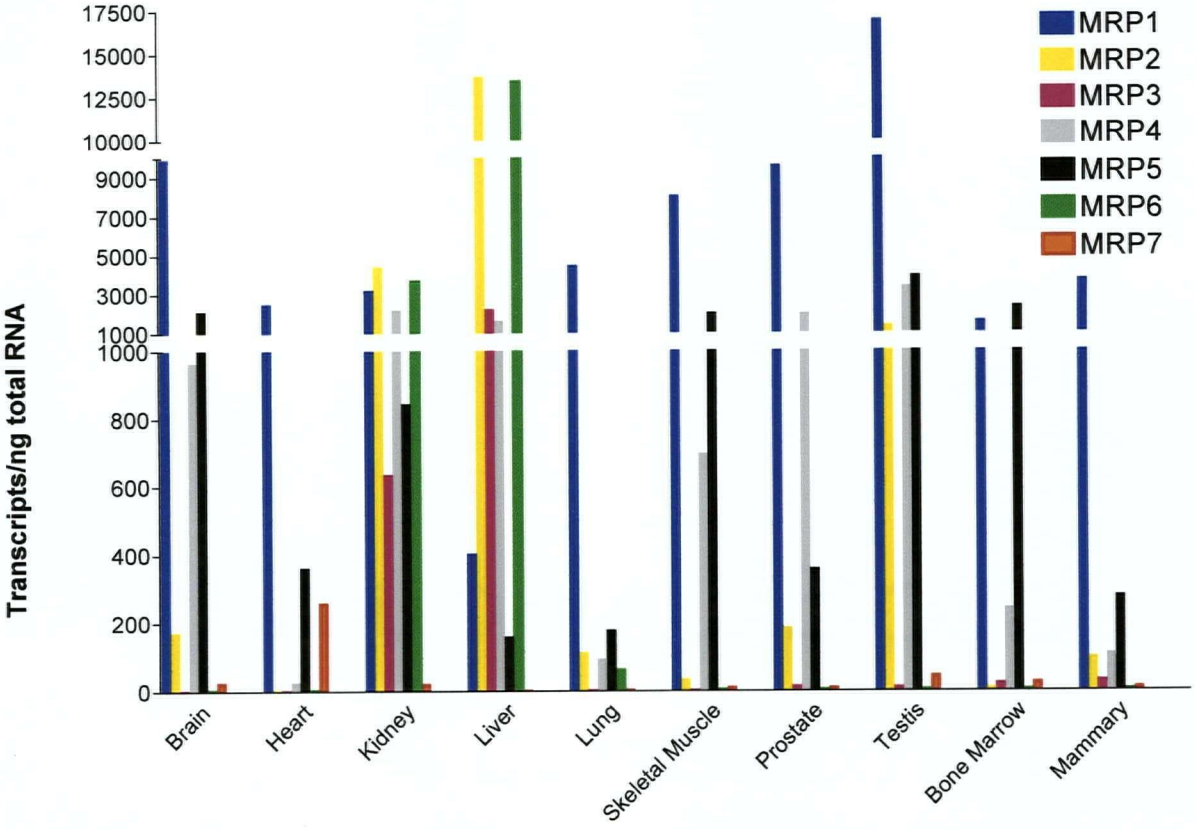


Figure 22a. C-RT-PCR Expression Profile of MRP7 in Normal Human Tissues

C-RT-PCR expression profile of MRP's 1-7 in a total RNA panel of normal human tissues(Clontech). All data are normalized relative to β_2-microglobulin levels. The absence of a bar denotes the transcript was not detectable.

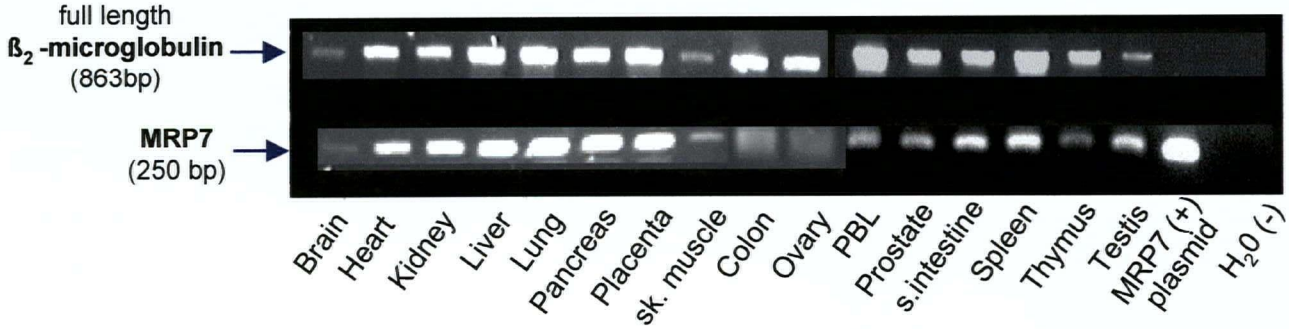


Figure 22b. Semi-quantitative PCR Expression Profile of MRP7 and Full-length β_2-microglobulin in Normal Human Tissues

cDNA is from a panel of normal human tissues (Clontech)
PBL= periperhal blood leukocytes

4.3.7 Expression Pattern of MRP7 Using Clontech's MTE Array

Hybridization of the radiolabeled MRP7 probe to the MTE Array revealed that MRP7 is expressed in all tissues examined (Figure 23). Hybridization of the MRP7 probe also occurred with the *E.Coli* DNA and human C_0t-1 DNA. As expected, hybridization did not occur with yeast total RNA, yeast tRNA or *E.Coli* rRNA. Because of the high background present on the blot, we 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).

4.3.8 Northern Blot of MRP7 Expression in a Panel of Human Cancer Cell Lines

A Northern Blot containing total RNA from a panel of 9 human cancer cell lines was prepared, however hybridization of the Northern blot was not completed because of our decision to terminate the MRP7 project (please see discussion).

4.3.9 Epitope-Tagged MRP7 cDNAs for Transfection

Construction of the FLAG-epitope cDNA constructs was approximately 80% finished, however these experiments were not completed as a result of our decision to terminate the MRP7 project (please see discussion).

4.3.10 Expression of MRP7 in total RNA from HL60 Cells Exposed to a Range of Daunorubicin Concentrations

RNA was isolated from HL60 cells treated with varying concentrations of daunorubicin to determine if the MRP7 expression change was dose dependent. We did not proceed with C-RT-PCR expression analysis of this total RNA for the reasons described previously.

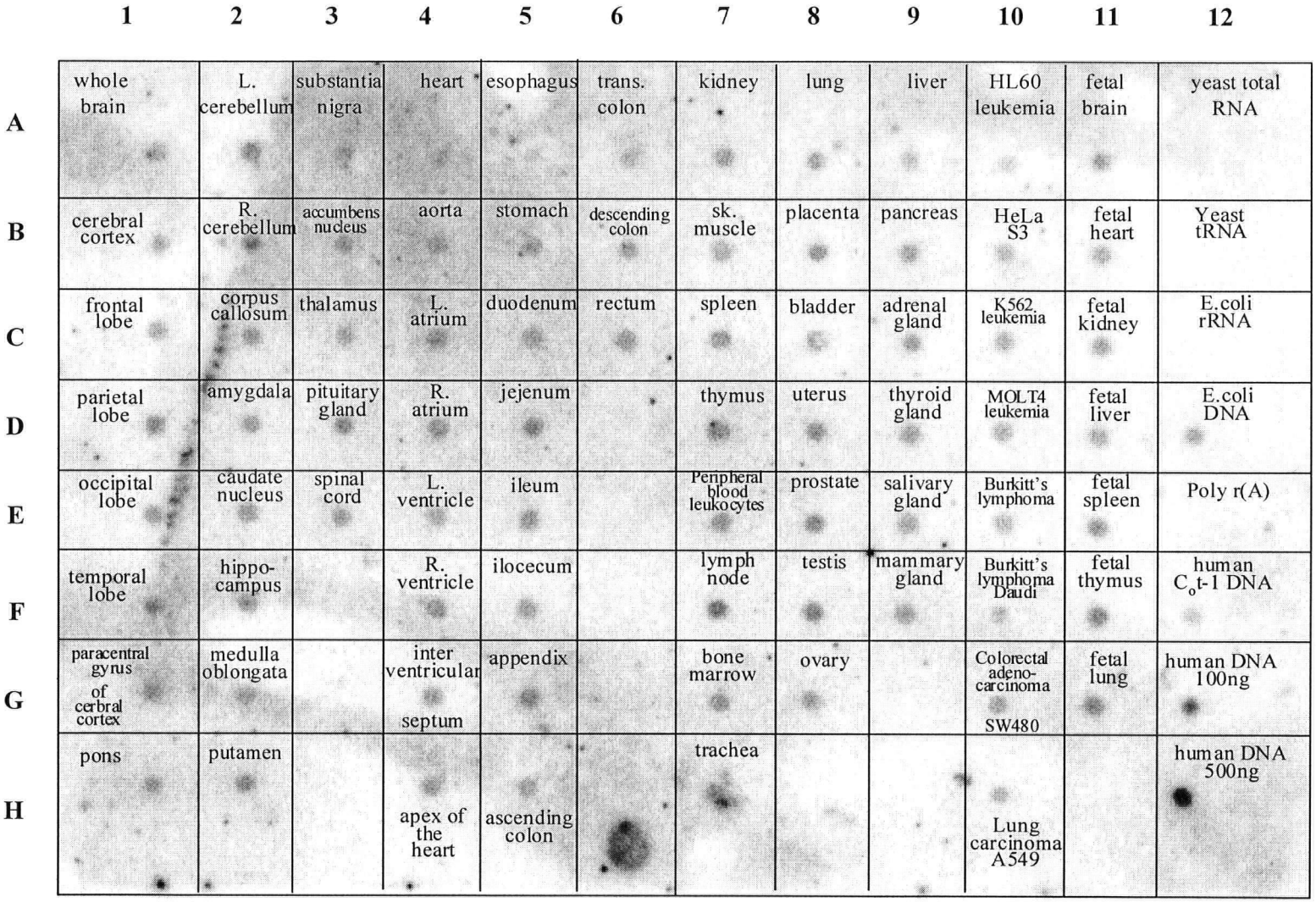


Figure 23. MRP7 mRNA Expression in a Panel of Human Tissues Using the MTE Array (Clontech). Boxes without labels contain no tissue RNA.

4.4 DISCUSSION

In January of 2001, two months after we had begun the cloning and characterization of MRP7, a paper by (Hopper *et al.*, 2001) was published which described the cloning, structural analysis and expression pattern of MRP7, using the same full length clone we had obtained, AK000002. This group analyzed the open reading frame of this cDNA and reported identification of the positions of the putative initiation codon, the length of reading frame and 3' untranslated regions, all of which agreed with our analysis. However, they did not perform 5'RACE to confirm the completeness of the open reading frame and 5'untranslated region. Their assignment of the putative initiation start site and the integrity of the predicted open reading frame were confirmed using an in vitro transcription/translation assay, which yielded a protein product with the predicted size.

This group also performed an overall amino sequence analysis comparison of MRP7 with the other members of subfamily C, and reported that MRP7 was more closely related to other MRP proteins than to CFTR, SUR1 or SUR2, which agreed with our data. The analysis by Hopper et al. of the transmembrane structure of MRP7 predicted an additional N-terminal transmembrane domain, which agrees with our hydrophobicity plot data. In addition, our predictions of the amino acid locations of the motifs composing the nucleotide binding domains of this protein are similar to those reported by Hopper et al.

Our expression analysis of MRP7 using C-RT-PCR and the MTE Array revealed that this transcript is expressed at low levels in all tissues examined. This agreed with the findings of Hopper et al. of MRP7 expression in a limited number of tissues using RT-PCR. Several other ABC genes are known to be expressed in a wide range of tissues. Both MRP1 and MRP5, for example, are expressed ubiquitously, with little variation between tissues (Kool et al., 1997) and at higher levels than MRP7. Several members of Subfamily A, such as ABCA1 and members of the G-Subfamily, such as White 1 are also expressed in many tissues (Steven Ralph, unpublished data). Both ABCA1 and White1 contribute to cholesterol metabolism and it is possible that they may perform other housekeeping duties. Since these genes are usually expressed at low levels, it is apparent that very little protein is required to perform these functions. Our finding that MRP7 is expressed ubiquitously, albeit at low levels, in tissues of both adult and embryonic origin suggests that this protein might also perform general housekeeping functions crucial to all cell types. The normal physiological role of MRP7 is still unknown, but given our knowledge of other MRP subfamily members, we can speculate that MRP7 plays a role in the export of

glutathione conjugates or other organic anions. We can also speculate that specific circumstances, such as exposure to drugs or other insults, may result in the overexpression of this protein as part of a cellular defense mechanism.

Personal communication with Gary Kruh at a recent conference revealed that MRP7-epitope-tagged stably-transfected cell lines had been established and that drug resistance studies were underway. This laboratory also has extensive experience in drug transport studies using MRP-subfamily transfectants (Paul *et al.*, 1997) (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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