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Functional and Molecular Characterization of the Human Multidrug Transporter

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Multidrug resistance (MDR) remains one of the greatest barriers to the successful treatment of disseminated cancers. The treatment of these diseases is to a large degree dependent upon the effectiveness of cytotoxic anticancer drugs. Chemotherapeutic agents such as the *Vinca* alkaloids, the anthracyclines, the epipodophyllotoxins, taxol and actinomycin D have several different cytotoxic targets including microtubules, DNA and RNA polymerase. Although these natural product compounds share little to none of the same chemistry, they all are amphipathic molecules with planar aromatic rings that preferentially partition to organic solvents. Unfortunately, most cancers either are intrinsically resistant to any initial treatment with these therapeutic compounds or acquire resistance to a broad spectrum of these agents over time[1].

To determine the molecular basis of this resistance, a human cell line, KB (a HeLa subclone), was selected with either colchicine, doxorubicin or vinblastine in a stepwise manner. This selection scheme resulted in a series of well-characterized multidrug-resistant cell lines that were then used to isolate and characterize the *MDR1* gene responsible for the MDR phenotype exhibited by these cells[2,3], and it was determined that the *MDR1* genes were amplified in these cell lines[4]. Cloning and sequencing of the *MDR1* cDNA established that it encoded a 1280-amino-acid polypeptide, termed P-glycoprotein (P-gp), that was homologous to a variety of other ATP-dependent transporters[5–7]. Subsequently, it has been described as a member of the large ATP-binding cassette (ABC) superfamily of membrane transporters[8,9]. Details on the cloning and genetic analysis of the human *MDR1* gene can be found in Chapter 5 and in Gottesman et al[10].

It is well established that the broad-based resistance observed in the KB cell lines as well as in many other cultured cell lines and many human cancers results, in large part but not solely, from the overexpression of *MDR1*. The *MDR1* gene has been found to be both necessary and sufficient for conferring the MDR phenotype to both cells in culture and to cells growing in animals[11,12]. Many different human cancers express *MDR1* at levels sufficient to confer MDR. Based on an analysis of several hundred

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different human cancers, it can be estimated that approximately 50% of human cancers will express *MDR1* at some time during therapy[13].

STRUCTURE AND MECHANISM OF ACTION OF THE MULTIDRUG TRANSPORTER

The sequence information led to a working model for the structure of the multidrug transporter, or P-gp, including 12 transmembrane domains and two putative ATP-binding/utilization sites[1,10] (Figure 2.1). Although there has been some recent controversy about possible topographic variations in this model, in which some transmembrane domains are thought to be a part of large extracellular loops, other studies suggest that the original 12-transmembrane-domain model remains the most likely. Recently, Loo and Clarke[14] have provided additional evidence for this model by assessing the reactivity of reintroduced cysteine residues to membrane-permeant and membrane-impermeant sulfhydryl group reagents in a cysteine-less mutant of human P-gp. Additional characteristics of P-gp include two confirmed substrate-binding regions, several phosphorylation sites in the linker region of the protein and putative glycosylation sites in the first extracellular loop (Figure 2.1).

One of the most intriguing questions that remains unanswered however, pertains to the mechanism of action of P-gp. Phenotypically, P-gp functions as an ATP-dependent pump, reducing the accumulation of drugs within cells and giving rise to drug-resistant populations of cells. It has been shown that vinblastine and a vinblastine-based photoaffinity agent (NASV) bind directly to P-gp[15,16]. Additional labeling studies with [^{3}H]azidopine, [^{125}I]6-AIPP-forskolin and [^{125}I]azidoiodoarylprazosin have further refined our understanding of the substrate-binding sites of human P-gps, localizing them to transmembrane domains 5, 6 and 11, 12[17–20] (see also Figure 2.1). Subsequently demonstration of ATP-dependent transport of drugs into membrane vesicles containing P-gp, evidence that agents that reverse MDR also bind to P-gp, and the demonstration that agents that reverse drug resistance block P-gp-mediated drug transport in isolated vesicles have all provided the basis for our present understanding of the mechanism[21,22]. Additionally, it has been shown that P-gp exhibits both basal and drug-stimulated ATPase activities in both crude and purified/reconstituted systems[23–28], but how the energy of ATP hydrolysis is transduced in the system to result in the efficient transport of this large variety of hydrophobic agents out of the plasma membrane or cytoplasm into the extracellular milieu remains unknown.

One model that has been proposed is that P-gp may reduce intracellular drug concentrations by acting as a 'hydrophobic vacuum cleaner', effectively increasing drug efflux and decreasing drug influx by the recognition and removal of compounds from the membrane before they reach the cytosol to elicit their cytotoxic effects. This model was based on studies demonstrating that an active P-gp pump could reduce the apparent concentration of doxorubicin in the plasma membrane through the use of energy transfer from doxorubicin to a photoactivatable hydrophobic probe[29]. Although several other specific models have been offered to explain this phenomenon, such as the 'flippase' model[30] and the 'water wheel' model[1], none have been sufficiently tested experimentally.

To understand the mechanism of action of P-gp more completely, it is important to understand and appreciate the function and possible interactions of the ABCs and the drug-binding domains in the transport process. For efficient transport, drug binding and transport occur concomitantly with ATP hydrolysis, suggesting an important role for both of these domains. Possibly, the transduced energy of ATP hydrolysis may cause a conformational change in P-gp that allows for the direct interaction of the ABCs with drug-binding sites. Alternatively, an ATPase-associated cooperative conformational change in the protein allowing for drug transport could also be imagined. Additionally, the ABCs may play a role in maintaining the structural integrity of P-gp in the cell. It is of obvious importance, therefore, to fully understand how the energy of ATP hydrolysis is transduced by the P-gp transporter in order to effectively combat the phenomenon of MDR clinically.

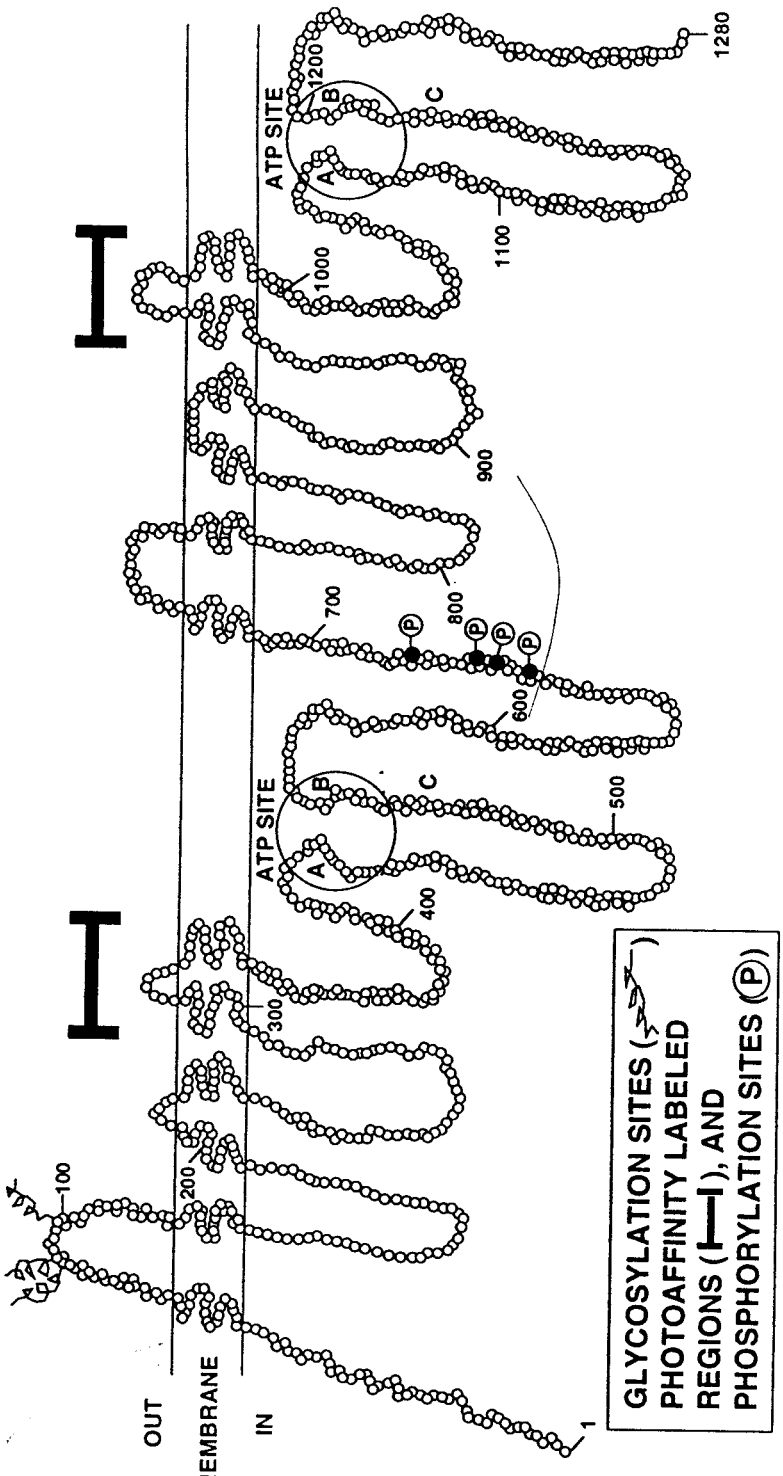

Figure 2.1 Two-dimensional hypothetical model of P-gp structure based on hydropathy plot analysis of primary amino acid sequence. The ATP-binding/utilization domains are circled, with the Walker A, B, and 'linker dodecapeptide' (LSGGQ) motifs designated by the letters 'A', 'B' and 'C'. Putative glycosylation sites are represented by squiggly lines. The regions known to bind photoaffinity drug analogs are designated by the heavy dark bars, and the serine residues at positions 661, 667, 671 and 683 that are known to be phosphorylated are shown as darkened circles with an attached and encircled 'P'. Adapted from Gottesman et al[10] and Gottesman and Pastan[49]

MUTATIONAL ANALYSIS OF HUMAN P-GLYCOPROTEIN

In addition to studying the biochemistry of wild-type human P-gp in either a crude or purified system, one of the most widely used approaches to explore mechanisms is through the use of molecular genetic analysis techniques, namely the study of *mutant and chimeric molecules. The regional* sequence and topologic similarities among various members of the ABC superfamily make these proteins attractive candidates for the exploration of structure–function relationships. By the use of molecular biological and biochemical techniques, it has been possible to dissect these regions on the molecular level in an attempt to fully understand the energy transduction process as it relates to P-gp function.

Many mutations in human P-gp, either naturally occurring or *artificially engineered*, have been studied and were found to have a variety of effects on transporter function, ranging from inactivation to change of substrate specificity (for reviews see Gottesman and Pastan[1], Gottesman et al[10], Germann[31] and Germann et al[32]). These results, taken together with the identification of drug-binding sites by photoaffinity labeling studies, have predicted a model for how the substrate-binding and ATP sites of P-gp may interact[10]. This model suggests that transmembrane domains 5, 6 and 11, 12 are the major drug-binding sites and that they most likely form a structure through which the substrates must pass to be removed from the membrane or cytosol, and also predicts that the ATP sites directly interact in some manner with these drug-binding regions (Figure 2.2). It is presumed that the other transmembrane, cytosolic and extracellular domains are involved either in correct folding of the transporter or may represent alternative overlapping substrate recognition sites[10].

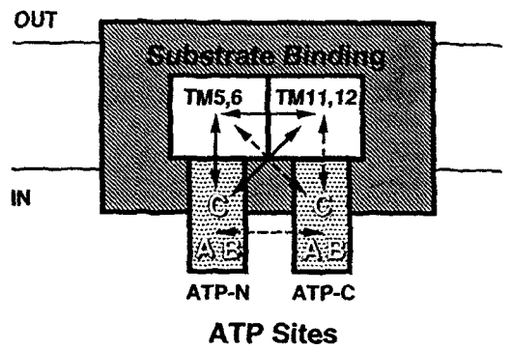

Figure 2.2 Hypothetical model of the interaction between substrate-binding and ATP-binding/utilization sites of P-gp. This model is derived from known biochemical interactions and from mutational studies of P-gp. Adapted from Gottesman et al[10]

STABLE AND TRANSIENT EXPRESSION OF P-GLYCOPROTEIN IN ANIMAL CELLS

Historically, drug selection of mammalian cells transfected or transduced with P-gp constructs in order to drive expression to high levels with MDR substrates has been a necessary step in the analysis of wild-type or mutant P-gp molecules. The imposition of such selection schemes has always been a point of contention because of the unknown effects that drug selection could have on host cell functions. The data must necessarily be interpreted with the understanding that other endogenous drug resistance mechanisms may possibly be activated or selected for in the presence of the cytotoxic agents used for selection. Transient expression systems have not proven useful in the past because of low levels of expression. Recently, however, Loo and Clarke have developed a system in which they transiently expressed human P-gp mutants tagged *with 10 histidine residues at the C-terminus in HEK* 293 cells[33] and subsequently purified the protein by nickel-chelate chromatography. This methodology resulted in significant enrichment of P-gp, allowing for quantitative assessment of the ATPase activity of this protein and correlation of this activity with its drug resistance phenotype. However, the expression levels do not appear high enough to allow for direct assessment of function in whole cells.

EXPRESSION IN INSECT CELLS USING BACULOVIRUS VECTORS

An alternative approach to the study of human P-gp, recently reviewed by Evans et al[34], has been to

express the wild-type and mutant forms of the protein in heterologous expression systems, such as in the yeast *Saccharomyces cerevisiae* and in insect cells infected with recombinant baculovirus vectors. These systems offer the advantages of ease of genetic selection and manipulation and, perhaps more importantly, they allow for a high level of expression of recombinant P-gp. The baculovirus system has proven to be the best for the overexpression of P-gp and for *in vitro* biochemical characterization, since recombinant P-gp represents approximately 3% of the membrane fraction (M. Ramachandra, S.V. Ambudkar, D. Chen, U.A. Germann, M.M. Gottesman, I. Pastan, unpublished observations). However, this system is not ideally suited for studies in intact cells, because infection with baculovirus causes disruption of the membrane, making the cells permeable and leaky. Additionally, baculovirus is not well suited for mutational studies, because of the need to develop a new virus for each construct. However, new technological advances are making it easier to produce mutants much more rapidly[35,36].

EXPRESSION IN YEAST

Recently, several laboratories have attempted to express functional human P-gp in the yeast *Saccharomyces cerevisiae*, an organism that has been used extensively in the past as a powerful, simple and rapid system in which to study higher eukaryotic cell function. Kuchler and Thorner[37] attempted to demonstrate that the human *MDR1* gene product under control of the yeast *STE6* promoter could functionally complement a *STE6* deletion mutant by allowing the export of a-factor mating pheromone. Unfortunately, however, this gene product was unable to complement the *STE6* defect, though it should be noted that the cDNA used was not wild type but instead contained a mutation, Gly185Val, which is known to alter the substrate specificity of P-gp. Recent studies (K. Kuchler and J. Thorner, unpublished observations; B. Ni, I. Pastan and M.M. Gottesman, unpublished observations) have suggested that the wild-type human P-gp has only a very limited ability to complement STE6 function in yeast. In these initial studies, however, human P-gp was found in yeast plasma membranes, increased resistance to valinomycin was observed, and total membranes from yeast could be specifically photoaffinity labeled with 8-azido-ATP, suggesting that P-gp made in yeast was properly folded and functional[37]. Unfortunately, however, it appears that much of the human P-gp expressed in *S. cerevisiae* appears to be localized to the endoplasmic reticulum.

Taking a somewhat similar approach, Saeki et al[38] studied the drug-binding activity of human P-gp expressed in yeast using the acid phosphatase promoter and discovered that the major sterol component of the yeast plasma membrane, ergosterol, inhibited or altered the drug-binding ability of P-gp. Subsequently, utilizing an *erg6* deletion strain deficient in sterol transmethylase activity necessary for ergosterol biosynthesis, the human *MDR1* cDNA has been expressed in both low and high copy number plasmids with no detrimental effects on the cell (B. Ni, I. Pastan and M.M. Gottesman, unpublished observations). Resistance to Adriamycin, daunomycin, actinomycin D and valinomycin was achieved. The pattern of resistance differed between the wild-type and mutant gene products, as predicted by the behavior of the mutant in mammalian cells[39]. Additionally, drug binding was confirmed with the photoaffinity labels azidopine and azidoiodoarylprazosin, and ATP-dependent drug transport was confirmed using plasma membrane vesicles made from cells overexpressing P-gp. However, direct biochemical assessment of drug-dependent ATPase activity has not yet been accomplished, due mainly to high background and endogenous ATPase activity.

Recently, a useful system was developed for the study of yeast plasma membrane ATPase that involved protein expression in a *sec6* mutant of *S. cerevisiae*[40]. This mutant accumulated 95–120-nm post-Golgi secretory vesicles containing an abundance of newly synthesized protein *en route* to the plasma membrane. These vesicles can be purified and offer several advantages over vesicles prepared from plasma membranes. First, these secretory vesicles are tightly sealed, since they are protected from proteolytic attack by the plasma membrane itself during removal of the cell wall. Second, the mem-

brane proteins expressed are uniformly in an 'inside-out' configuration, making them useful for both ATPase studies and drug uptake studies. Third, this vesicle system allows for the expression of large amounts of both wild-type and mutant forms of membrane proteins that can be purified and characterized relatively easily.

Upon shifting to the non-permissive temperature, vesicle transport to the plasma membrane will be blocked due to the *sec6* mutation and secretory vesicles containing the newly expressed P-gp will accumulate in the cytoplasm. These vesicles can subsequently be isolated and analyzed for expression by immunoblotting procedures and for function by assaying for drug-stimulatable ATPase activity, ATP-dependent drug uptake studies and photoaffinity labeling with drug analog[16,22]. Ruetz and Gros have successfully adapted this system for the expression of three isoforms of mouse P-gp[41], including the demonstration of ATP-dependent vinblastine uptake into vesicles that was verapamil sensitive. However, little success was obtained in studies carried out in this laboratory with human P-gp expressed in this system, owing mainly to low expression levels at the plasma membrane[34] (C.A. Hrycyna, I. Pastan and M.M. Gottesman, unpublished observations). Since this methodology exploits the fact that P-gp can be captured in the cytosol in vesicles *en route* to the plasma membrane and if, as is suspected, most of the synthesized protein remains in the endoplasmic reticulum, little to no enrichment of P-gp will be observed in these purified vesicles. Additionally, neither basal nor drug-stimulated ATPase activity of P-gp was detectable in this or the mouse expression system, perhaps due to endogenous ATPase activities or inhibitors. For these reasons, use of this system to directly study the effects of mutations in the ATP-binding/utilization domains on drug-stimulated ATPase activities is not feasible at this time.

Although this system did not prove to be adaptable for our purposes, it seems still possible, though somewhat more cumbersome, to study human P-gp in yeast plasma membranes. Under permissive conditions where P-gp is in the plasma membrane, at least to some degree, it should also be possible to select directly for resistance to actinomycin D, anthracyclines or valinomycin to confirm the function of P-gp mutants and to select for second-site revertants of non-functional variants. Gros and colleagues[42] (Chapter 1) have successfully screened mouse P-gp mutants in yeast using resistance to various antifungal drugs, including FK520. However, the intrinsic resistance yeast has for many MDR substrates coupled with the relatively low level of expression of human P-gp in yeast makes these analyses much more difficult.

Whichever heterologous expression system is used, it will ultimately be essential to express any chimeras and *MDR1* mutants in mammalian cells to confirm the findings. We believe that the new generation of P-gp vectors and vector systems presently being developed both in our laboratory and in others will eliminate concerns about pleiotropic cellular effects due to drug selection, while simultaneously expressing P-gp in its native and functional form in a mammalian cellular environment. Presently, we are developing complementary transient and stable systems that are designed to result in high expression levels of human P-gp.

EXPRESSION BY A RECOMBINANT VACCINIA VIRUS SYSTEM

We have recently developed and successfully characterized a vaccinia virus–bacteriophage T7 RNA polymerase hybrid expression system[43] for the rapid analysis of transiently expressed P-gp in a human osteosarcoma cell line that has no endogenous P-gp expression and low endogenous membrane-associated ATPase activity (Figure 2.3). We monitor expression of human *MDR1* by immunoblot analysis and presence on the cell surface in intact cells by fluorescence-activated cell sorting (FACS) analysis. The functionality of P-gp is evaluated by rhodamine 123 and bodipy-verapamil efflux experiments as well as uptake of [^{3}H]vinblastine and [^{3}H]colchicine in intact cells. *In vitro* assays have also been established to evaluate [^{3}H]azidopine labeling and drug-stimulatable ATPase activity using a variety of drug substrates.

This system has proven to be ideally suited to study almost all aspects of P-gp function. Since all of

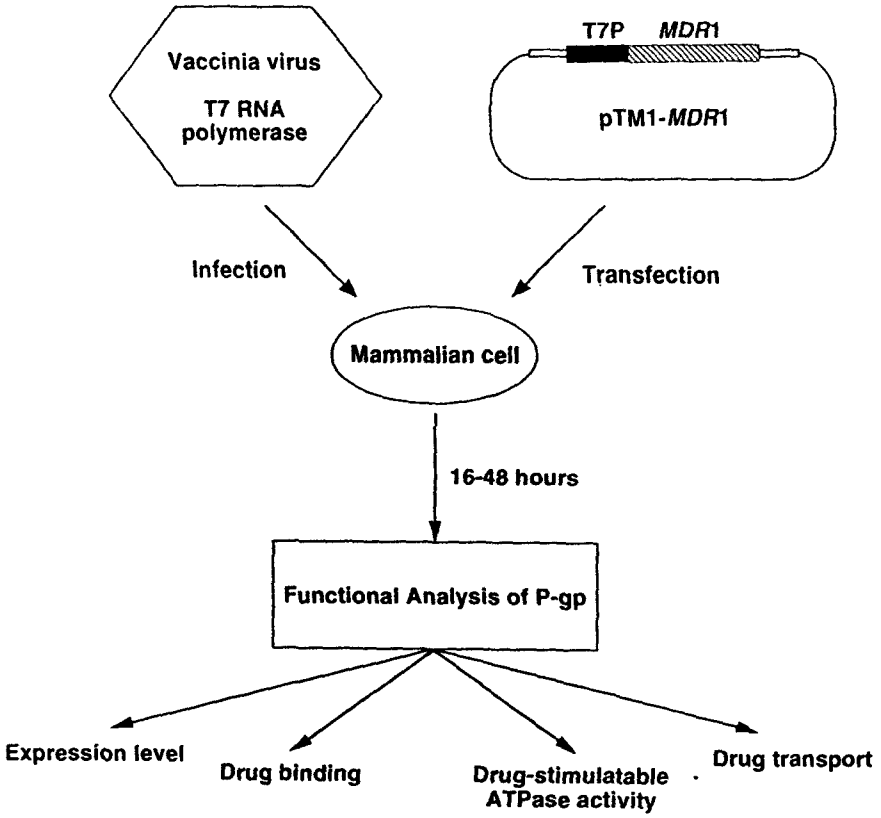

Figure 2.3 Schematic diagram of the vaccinia virus–bacteriophage T7 RNA polymerase hybrid expression system for the functional analysis of transiently expressed P-gp. Mammalian cells are infected with a recombinant vaccinia virus that produces large amounts of T7 RNA polymerase and cotransfected with an *MDR1* gene under control of the T7 promoter. The cells are incubated for 16–48 h and subsequently analyzed for the expression of functional P-gp by a variety of methods, including immunoblot analysis, FACS (fluorescence-activated cell sorting), assessment of cell surface expression, fluorescence-based and radioactive drug transport assays, drug-binding assays with photoaffinity drug analogs and the assessment of drug-stimulatable ATPase activity *in vitro*

the assays can be performed in a single cell line and large amount of protein can be expressed within 16–24 h of transfection without having to impose any drug selection, this system is simple and extremely powerful. It is also well suited for studying large numbers of chimeric/mutant constructs, since the recombinant protein can be expressed by an infection and transfection procedure (Figure 2.3) that eliminates the need to generate recombinant viruses for each mutant construct to be analyzed. One inherent drawback, however, of the vaccinia system is that the infected/transfected cells cannot be examined for changes in substrate specificity by cell proliferation assays.

BICISTRONIC VECTORS FOR ANIMAL CELL EXPRESSION

To complement the vaccinia virus system, we have also developed a stable expression system that does not require MDR drug selection to drive protein expression. A bicistronic retroviral vector has been constructed in which the promoter is the Harvey long terminal repeat (LTR); and *MDR1* cDNA is cloned immediately downstream from the cap-dependent translation site, followed by an internal ribosomal entry site (IRES) from the encephalomyocarditis virus and a mutant cDNA for murine dihydrofolate reductase, a selectable marker that

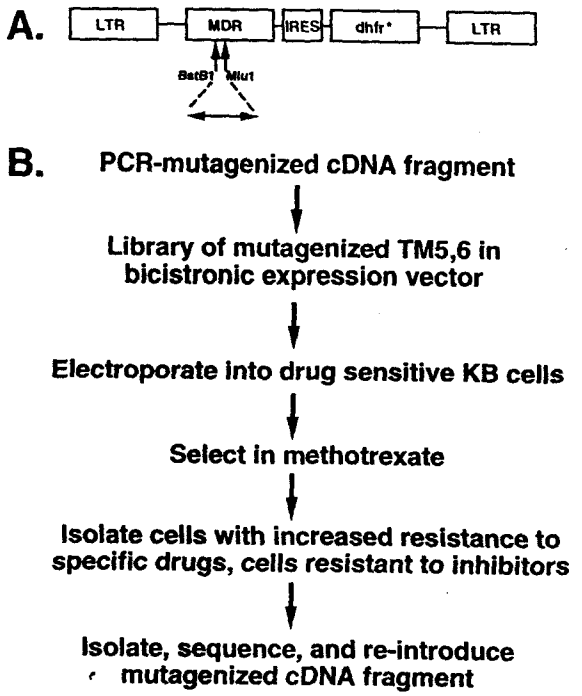

Figure 2.4 (A) Bicistronic retroviral vector (pHa-MDR-IRES-dhfr) containing the Harvey virus LTRs, followed by the *MDR1* gene, the IRES (internal ribosomal entry site) from encephalomyocarditis virus, and a mutant form of the mouse *dhfr* gene that confers methotrexate resistance to otherwise sensitive cells. The advantage of this system is that *MDR1* expression is directly linked to methotrexate resistance, allowing for high levels of *MDR1* expression without needing to impose selection with MDR drugs. (B) Flow chart of the protocol that our laboratory has developed to perform PCR-based mutagenesis studies of the substrate-binding sites in transmembrane domains 5 and 6 of P-gp using the vector depicted in (A)

confers methotrexate resistance to otherwise sensitive cells[44]. The use of this bicistronic vector (pHa-MDR-IRES-dhfr) (Figure 2.4A) guarantees expression of both genes. Because the mutant *dhfr* gene is amplifiable, expression of both gene products is high. *MDR1*-based bicistronic vectors have been developed in the past as potential reagents to be used for gene therapy[45–48], but our present effort represents the first use of the technology in the development of a stable high-level expression system for P-gp that will enable us to isolate and study the function of a variety of human P-gp mutants.

Electroporation of this vector into drug-sensitive cells results in resistance to methotrexate, and virtually all methotrexate-resistant clones express the *MDR1* cDNA at high levels, as shown by vincristine cross-resistance of the clones and high levels of expression of human P-gp by FACS analysis with the human specific monoclonal antibody MRK16. This approach is shown schematically in Figure 2.4B. As shown, a library of mutagenized P-gp molecules can be generated and selected in methotrexate. These mutant transporters can then be screened or selected for properties including altered substrate specificity and resistance to inhibitors.

These versatile expression systems offer the opportunity to study wild-type and mutant P-gp in their native forms without concern about pleiotropic cellular effects due to drug selection. We hope to further our understanding of the mechanism of action of P-gp through the use of these new systems. Ultimately, a complete understanding of the mechanism of action of P-gp could lead to the development of new inhibitory agents that could greatly facilitate the treatment of a large number of human cancers and perhaps help in the therapy of other human diseases which involve the superfamily of ABC transporters.

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