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GENETIC ANALYSIS OF THE MULTIDRUG TRANSPORTER

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ABSTRACT

The analysis of how human cancers evade chemotherapy has revealed a rich variety of cell-based genetic changes resulting in drug resistance. One of the best studied of these genetic alterations is increased expression of an ATP-dependent plasma membrane transport system, known as P-glycoprotein, or the multidrug transporter. This transporter actively effluxes a large number of natural product, hydrophobic, cytotoxic drugs, including many important anticancer agents. This review focuses on the genetic and molecular genetic analysis of the human multidrug transporter, including structure-function analysis, pre- and posttranslational regulation of expression, the role of gene amplification in increased expression, and the properties of transgenic and "knock-out" mice. One important feature of the *MDR* gene is its potential for the development of new selectable vectors for human gene therapy.

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INTRODUCTION: MULTIDRUG RESISTANCE IN CANCER

The search for mechanisms of resistance of cancers to chemotherapy has led to the elucidation of many cell-based genetic alterations that reduce accumulation of drugs, alter their metabolism, change cell-cycle responses to drugs, block apoptosis in response to cytotoxic agents, change cytotoxic targets, and enhance repair of drug-induced damage (93). Efforts are in progress to determine whether such alterations are responsible for drug resistance in the cancers of patients who are unresponsive to chemotherapy, either (*a*) because of acquired resistance following treatment with anticancer drugs, or (*b*) as part of the intrinsic drug-resistance mechanisms of the patients' cancer cells. However, overexpression of an energy-dependent transport system known as the multidrug transporter, or P-glycoprotein (P-gp), which blocks uptake and increases efflux of natural product anticancer drugs, has been shown to be a major mechanism of multidrug resistance in cultured cancer cells. The *MDR*1 gene, which encodes P-gp, is expressed at levels thought to be physiologically significant in about 50% of human cancers (91, 96).

Reviews of various aspects of the biochemistry (96), clinical significance and reversal of function (19, 94, 144), evolution (26, 71), and regulation of expression (49) of the P-gps have appeared in the past two years. Thus, this review focuses on genetic analysis of the human *MDR*1 gene, and where necessary reference is made to these and other recent reviews on the biochemistry, pharmacology, physiology, and cell biology of the multidrug transporter encoded by this gene.

Two major mechanisms have been described for the energy-dependent efflux of drugs from mammalian cells. The first to be described was the multidrug transporter, which is the subject of this review. The second results from expression of a gene known as the multidrug resistance associated protein

(MRP), which was found to be amplified and overexpressed in drug-selected cell lines, such as lung cancer cells, in which *MDR*1 gene expression is not usually seen (54, 253). Subsequently, the MRP gene product was identified as the glutathione conjugate transporter, and it became apparent that drugs that are conjugated to glutathione (and perhaps other intracellular compounds) are effluxed from cells by this transport system (121, 133, 167). The widespread expression of the *MRP* gene in many tissues and cancers suggests that it may not be limiting for drug resistance in cancer, but may be essential for drug resistance mediated by alterations in glutathione levels and levels of glutathione transferases and/or other conjugating systems. Both the *MDR*1 and *MRP* genes belong to a superfamily of ATP-dependent transporters (the ATP-Binding Cassette, or ABC family of transporters) (for review, see 112). It seems likely that other types of drug resistance and multidrug resistance are due to other, yet unidentified members of this family.

Identification and Characterization of the Multidrug Transporter

Although simultaneous resistance to many anticancer drugs had been appreciated as a cause of failure of chemotherapy since the introduction of multiagent treatment of cancer, the first cell lines shown to become simultaneously resistant to anticancer drugs were not reported until the late 1960s. The pioneering work of Danø (65) and Juliano & Ling (123) identified both a physiological alteration (reduced accumulation of drug due to efflux) and a biochemical change (increased amounts of a cell surface protein dubbed P-glycoprotein) in multidrug-resistant (MDR) cells selected in colchicine. These early studies also established the pattern of multidrug resistance to many different hydrophobic, amphipathic, natural product drugs, including colchicine, doxorubicin, and vinblastine. This list has grown in time to include dozens of cytotoxic drugs, including the newer chemotherapeutic agents taxol and topotecan; other cytotoxic drugs such as toxic hydrophobic peptides, including gramicidin D and valinomycin; and many other pharmacologic agents that are relatively nontoxic to cells but interact with P-gp, such as verapamil and cyclosporin A (for a more complete list of these agents, see 96).

The identification of extrachromosomal elements (i.e. episomes and double minute chromosomes) in the highly multidrug cell lines suggested that the *MDR*1 genes were amplified in these lines (78). Shortly thereafter, an in-gel renaturation technique was used to identify and isolate amplified segments of DNA in human MDR adenocarcinoma (KB) cells (192) and in DNA-mediated MDR transformants (214), which allowed for the detection of increased levels of a specific *MDR*1 mRNA (217). Cloning and sequencing of the *MDR*1 cDNA and its two homologs in the mouse (*mdr*1a and *mdr*1b, also called *mdr*3 and

*mdr*1) established that the human *MDR*1 cDNA encoded the 1280-amino-acid P-gp that was homologous to other ATP-dependent transporters (the ABC family). The sequence information led to a working model for the structure of the multidrug transporter, including 12 transmembrane segments and two putative ATP-binding/utilization sites (44, 100, 238). This model is shown in Figure 1.

Early speculation that the mechanism of action of P-gp involved transport of carrier proteins for various drugs was rendered unlikely by the demonstration that vinblastine and a vinblastine-based photo-affinity agent (NASV) bound directly to P-gp (57, 59). These studies were followed by the demonstration of ATP-dependent transport of drugs into vesicles containing P-gp, evidence that agents that reverse multidrug resistance also bind to P-gp, and the demonstration that agents that reverse drug resistance block P-gp-mediated drug transport in isolated vesicles (58, 117). These studies established the basic mechanism by which P-gp transports drugs (by direct interaction with the transporter) and of its inhibition (by competition for transport sites). Recent studies in which purified P-gp has been isolated from drug-resistant cells and reconstituted into proteoliposomes have proved conclusively that P-gp is itself an ATP-dependent transporter capable of moving drugs across a lipid bilayer against a concentration gradient (7, 8, 212, 213).

It was not immediately clear why the human genome or the genomes of other mammals would house genes that conferred broad-spectrum resistance to chemically dissimilar anticancer drugs. Through use of specific probes for *MDR*1 mRNA and a specific antihuman P-gp monoclonal antibody (MRK-16) developed in the laboratory of Tsuruo (108), the tissues that normally express P-gp in humans were delineated (55, 77, 232, 233). Based on expression of the human *MDR*1 gene in transporting epithelia of the liver, kidney, intestine, and pancreas, in capillary endothelial cells of the brain and testis, and in adrenocortical cells, it was proposed that the normal functions of the multidrug transporter are to excrete endogenous metabolites and exogenous xenobiotics, to prevent toxic substrates from entering the brain and testis and, possibly, to help transport steroid across the plasma membrane in the adrenal and other steroid-secreting tissues.

Although knowledge of the normal localization and function of the transporter helped clarify the biology and physiology of the multidrug transporter, it still did not provide an explanation for the extraordinary promiscuity of this transport system. There were hints in the literature that P-gp was not a typical efflux pump. Some groups demonstrated that MDR cells show decreased influx as well as increased efflux (227) and that P-gp provided protection against membrane intercalating peptides such as gramicidin D and valinomycin. Unexpected changes in epifluorescence spectra of lipophilic MDR substrates during transport were also found (127). Through the use of energy transfer

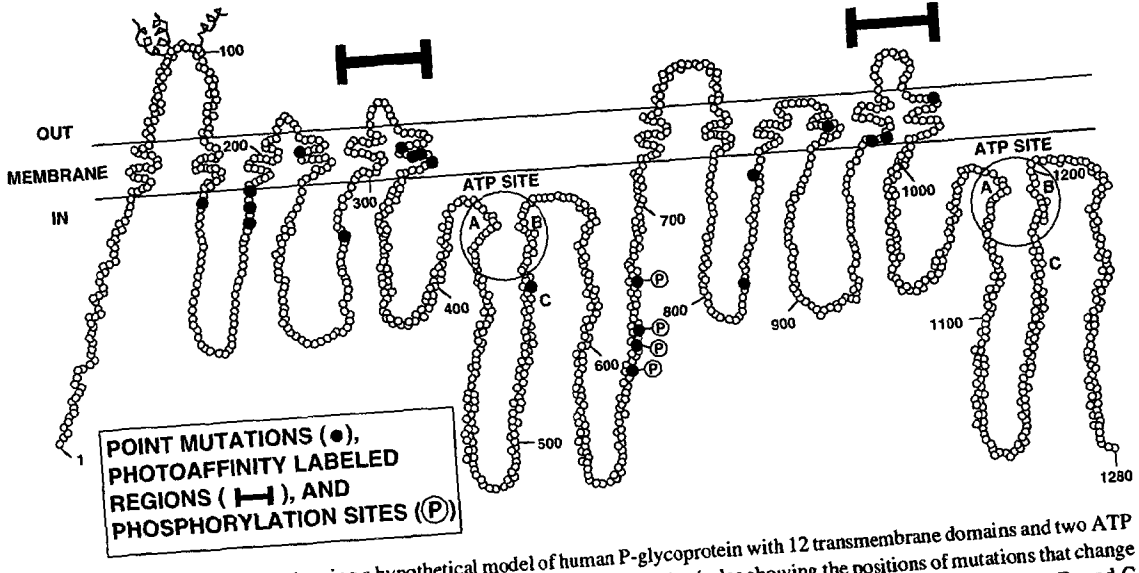

Figure 1 Schematic diagram showing a hypothetical model of human P-glycoprotein with 12 transmembrane domains and two ATP sites. In this diagram, each circle represents an amino acid residue, with filled-in circles showing the positions of mutations that change the substrate specificity of P-glycoprotein; the squiggly lines are glycosylation sites; the ATP sites are circled, with A, B, and C components indicated (see text); the known sites of phosphorylation are marked; and the sites that are photo-affinity labelled are shown in brackets. This figure is modified from (95).

from doxorubicin to a photo-activatable hydrophobic probe, it was demonstrated that an active P-gp pump could reduce the apparent concentration of doxorubicin in the plasma membrane, suggesting the hypothesis that P-gp was a "hydrophobic vacuum cleaner" (182) or a "flippase" (114). This concept has forced a rethinking of the mechanism of action of the multidrug transporter and may have implications for other members of the ABC family of transporters.

MUTATIONAL ANALYSIS OF THE MULTIDRUG TRANSPORTER

Human P-glycoprotein (P-gp) encoded by the *MDR*1 gene is known to encode a 170-kDa integral membrane protein composed of two homologous halves. Each half is thought to span the plasma membrane bilayer six times and to contain an ATP-binding/utilization domain (Figure 1). Although ATP binding and hydrolysis appear to be essential for substrate transport, the mechanism by which the energy of ATP hydrolysis is transduced through the molecule to transport the large variety of hydrophobic agents out of the plasma membrane or the cytoplasm into the extracellular milieu remains unknown. Another compelling yet confounding issue concerning the mechanism of action of P-gp is that it is still not understood how a single molecule can function to transport such a wide variety of agents.

Mutations Affecting Substrate Binding

The major sites of interaction of three P-gp inhibitors, ^{3}H-azidopine, ^{125}I-6-AIPP-forskolin, and ^{125}I-iodoarylazidoprazosin, have been determined by photo-affinity labelling, digestion with proteases or cyanogen bromide, and specific immunoprecipitation with antibodies directed against polypeptide epitopes of P-gp (28–30, 97, 164). Binding of these photo-affinity labels is specifically inhibited by P-gp substrates, including vinblastine, suggesting that they interact with major substrate interaction sites in the multidrug transporter. Equal labelling of the amino-terminal and C-terminal halves of P-gp is observed as well as equal inhibition of labelling by vinblastine (29). Further refinement of the binding site using ^{125}I-6-AIPP-forskolin (164) revealed that a tryptic peptide consisting of transmembrane (TM) regions 5 and 6, the extracytoplasmic loop that lies between them, and a small part of the cytoplasmic region lying between TM 6 and the amino-terminal ATP-binding site is labelled (Figure 1). In mouse P-gp, a comparable region around TM 11 and 12 has been found to be labelled (97). Recently, it has been found that a chimeric *MDR*1 molecule in which the loop between TM 11 and TM 12 was replaced with that of *MDR*2 conferred increased resistance to actinomycin D,

colchicine, and doxorubicin but not vincristine (258). Interestingly, replacements limited to TM 12 itself significantly reduced resistance to actinomycin D, vincristine, and doxorubicin, but not colchicine, and resulted in poor labelling with ^{125}I-iodoarylazidoprazosin. These results suggest that these regions, working either separately or in concert, are either primary binding sites for substrates and inhibitors or define a pathway by which the drugs move through the transporter in the plasma membrane. Importantly, drug-stimulated ATPase activity has been observed in Sf9 insect cells only when both halves of human P-gp are co-expressed, suggesting that the coupling of ATPase activity to drug binding and possibly to transport requires direct interaction between the two halves of the molecule (142).

Although these and other direct biochemical studies have provided many clues as to the mechanism of action of P-gp, one of the most useful and informative experimental approaches to probing substrate specificity and ATP utilization has involved the study of mutant and chimeric molecules that are either naturally occurring or artificially engineered. Figure 1 provides a summary of many, but certainly not all, of the mutations examined (see also 81, 86, 96 for reviews). All of the darkened circles shown in Figure 1 represent amino acids that when mutated alter the substrate specificity of the transporter. Unless otherwise noted, mutations are in the human P-gp molecule and changes in drug specificity are described relative to the wild-type molecule.

The change-of-specificity mutations are found scattered throughout the molecule but are generally localized to the TM regions. Most of the functionally relevant mutations described to date are found in TMs 5 and 6 and 11 and 12. Melera and coworkers identified a mutant form of hamster P-gp in cells selected for resistance to actinomycin D that contains two adjacent mutations, Gly-to-Ala at position 338 and Ala-to-Pro at position 339 in TM 6 (69). These changes render transfected cells most resistant to actinomycin D, whereas the wild-type gene confers highest resistance to colchicine. Loo & Clarke (140) engineered mutations into TM 6 of human *MDR*1 and transfected the cDNA into drug-sensitive NIH3T3 cells. A Val-to-Ala mutation at position 338 resulted in increased resistance to colchicine and doxorubicin and reduced resistance to vinblastine, and had no effect on actinomycin D resistance. Additionally, the mutation of Gly to Val at position 341 resulted in little resistance to colchicine and doxorubicin but retention of resistance to vinblastine and actinomycin D (140). A Phe-to-Ala or -Ser mutation at residue 335 resulted in decreased vinblastine and actinomycin D resistance and increased resistance to colchicine and doxorubicin. A Phe-to-Leu mutation at the same position results in a partial decrease in resistance to vinblastine and actinomycin D but has no effect on resistance to colchicine and doxorubicin. The mutation of Ser to Phe in TM 11 of mouse *mdr*1 (position 941) and *mdr*3 (position 939) also alters the pattern of drug

resistance. In *mdr*1, this mutation caused a drastic reduction of resistance to doxorubicin and colchicine but retention of vinblastine resistance (102). Interestingly, this mutation also appears to reduce the capacity of verapamil and progesterone, two reversing agents, to modulate P-gp activity and the binding of photo-activatable drug substrates (124). In TM 12, a Phe-to-Ala or -Ser mutation at residue 978 of human P-gp resulted in no resistance to colchicine and doxorubicin and in reduced resistance to vinblastine and actinomycin D. A Phe-to-Leu mutation at this residue resulted in an intermediate effect, decreasing resistance to all four drugs but demonstrating a more pronounced phenotype with colchicine and doxorubicin (137).

Loo & Clarke mutated 13 proline residues in human P-gp, five of which are located in putative transmembrane domains (138). Interestingly, only two of these, Pro-to-Ala at positions 223 and 866 in TMs 4 and 10, showed any effect on function. The mutation of either of these residues caused a significant reduction in resistance to colchicine, doxorubicin, and actinomycin D. Vinblastine resistance was retained with the mutation at residue 866 and was increased with the mutation at position 233.

A number of functionally significant mutations have also been found in the putative cytoplasmic loops of P-gp. One of the best-characterized mutations, of Gly to Val at position 185, results in increased resistance to colchicine and etoposide but decreased resistance to actinomycin D, vinblastine, doxorubicin, vincristine, and taxol (52, 63, 129, 197). Interestingly, membrane preparations from Sf9 insect cells expressing this mutant P-gp show an increase in drug-stimulated ATPase activity for verapamil and colchicine but not vinblastine in comparison to wild-type (181). These data suggest that this amino acid is involved in defining the transport site for verapamil and colchicine but not vinblastine. This mutation made in conjunction with an Asn-to-Ser mutation at position 183 results in recovery of resistance to actinomycin D, vinblastine, and doxorubicin and retention of resistance to colchicine (63). Other Gly-to-Val mutations studied include those at positions 141, 187, 288, 812, and 830. All of these mutations demonstrate increased resistance to colchicine and doxorubicin, but only the mutations at 187, 288, and 830 show decreased resistance to actinomycin D (139).

Mutations Affecting ATP Sites

In Figure 1, the N-terminal and C-terminal ATP sites (nucleotide-binding/utilization domains) are circled, and the Walker A and B motifs (249) are designated by the A and B. The "linker dodecapeptide," also called the C motif, is designated by the C. Since these sites are highly conserved among all members of the ABC superfamily, and considering the importance of

ATP binding and hydrolysis for transport, mutational analysis of these regions has been the subject of some interest. Amino acid substitutions from Gly to Ala at positions 431 and 1073 and from Lys to Arg at positions 432 and 1074 in the Walker A motif of mouse *mdr*1 result in no detectable function, suggesting that these residues are essential for function (15). Either of the mutations alone was sufficient for inactivation, suggesting that the two nucleotide-binding domains may act cooperatively or interact physically. These molecules still retained the ability to bind 8-azido-ATP, suggesting that a step subsequent to ATP binding is impaired in these mutants. A Lys-to-Met mutation at either position 433 and/or 1076 in human P-gp severely attenuated drug-stimulated ATPase activity, but similar to the case of the mouse *mdr*1 mutations, the molecules were still able to bind 8-azido-ATP (M Muller, E Bakos, E Welker, A Varadi, UA Germann, MM Gottesman, BS Morse, IB Roninson, B Sarkadi, unpublished data). Additionally, a chimeric molecule in which the C-terminal ATP site of *MDR*1 was replaced with the N-terminal ATP site allowed for partial function of P-gp, but the C-terminal site does not work in the N-terminal position. These results suggest that these two sites are not interchangeable and serve somewhat different, but not entirely unique functions and may, in fact, interact in the native molecule (UA Germann, P Wu, S Currier, I Pastan, MM Gottesman, unpublished results). A structure-function analysis of the nucleotide-binding domains of STE6, the yeast a-factor transporter and member of the ABC superfamily of membrane transporters, revealed that co-expression of each of the half-molecules in yeast led to reconstitution of a functional transporter. Further mutagenic analysis revealed that both ATP-binding domains are critical for function and that the molecule is rendered inactive by many mutations engineered into the Walker A and B regions (20, 21). Several changes made in the C region (see Figure 1) had no significant effect on STE6 activity, however. These changes include a mutation analogous to the most common mutation associated with cystic fibrosis, ΔF508 in the cystic fibrosis transmembrane conductance regulator (CFTR) (188), which is also a member of the ABC transporter family.

An interesting mutation engineered into the C region of human P-gp was recently described by Hoof et al (116). In this study, another common mutation in CFTR was introduced at position 536 in the N-terminal ATP site with the Lys residue being changed to either a Gln or Arg. The Lys-to-Arg mutation increased resistance to colchicine and doxorubicin but had no effect on vinblastine resistance. Mutation of this Lys to Gln resulted in a significant decrease in resistance to colchicine and doxorubicin but only a slight decrease in resistance to vinblastine. These results taken together suggest that the ATP-binding/utilization domains and the C region in particular may be interacting directly with the drug-binding sites of P-gp.

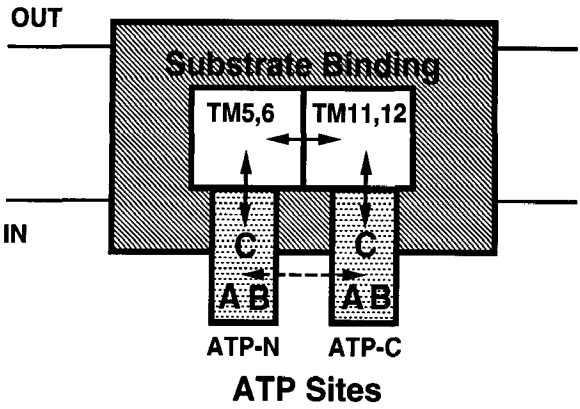

Figure 2 Hypothetical diagram showing how the substrate binding and ATP sites of P-glycoprotein may interact. This diagram is based on the known biochemical and mutational analysis of P-glycoprotein and is subject to verification by additional genetic and biochemical means.

A Model for Interaction of Substrate Binding and ATP Sites

All of the current data from these point mutational analyses, deletion and insertion analyses, and other chimeric molecule studies suggest that transmembrane domains 5, 6 and 11, 12 are the major drug-binding sites and that they in fact most likely form a structure through which the substrates must pass to allow extraction from the plasma membrane or from the cytosol directly by P-gp. Other TMs and cytoplasmic domains are likely necessary for proper folding of the transporter. This general model is illustrated schematically in Figure 2. In this regard, it has also been hypothesized that aromatic residues present in the TMs of P-gp can provide an environment that is sterically compatible with a wide variety of ring-containing compounds (176). It also seems possible that other TMs, such as TMs 4 and 10, interact directly in the native molecule and may be responsible for alternative or overlapping substrate recognition sites for molecules such as vinblastine whose interaction with P-gp differs somewhat from the interaction of doxorubicin, colchicine, and actinomycin D (138). This hypothesis is supported by the change in substrate specificity upon mutation of Pro to Ala at positions 223 and 866, in which only resistance to vinblastine remained unchanged. The results of existing photo-affinity labelling and inhibition studies call into question this idea, however.

The mutational data also suggest a direct interaction between the substrate-binding sites and the C region of the nucleotide-binding domain, since a mutation in this domain alters the substrate specificity profile of P-gp as described above. Additionally, other evidence suggests that there may be some

cooperativity or interaction between the ATP-binding domains themselves, since mutation of highly conserved residues in the Walker A region of one domain attenuates function of the transporter (15). Further mutational studies should help to elucidate the mechanism of action of P-gp and ultimately allow for the development of new inhibitors and reversing agents.

PRETRANSLATIONAL REGULATION OF *MDR*1 GENE EXPRESSION

The tissue-specific expression of P-gp and the potential importance of regulation of the *MDR*1 gene in normal drug metabolism and in cancer cells have stimulated studies of the regulatory biology of the *MDR*1 gene. The major and minor start sites for transcription of the human *MDR*1 gene have been identified (238), as have DNA sequences upstream from the major site of transcription initiation that were shown to function as a promoter in standard reporter assays (239). The mouse, hamster, and rat promoters for *mdr* genes were also identified subsequently (reviewed in 49). Interestingly, there is not a lot of sequence conservation between human and rodent *mdr*1 promoter regions, consistent with the differential expression of rodent *mdr*1a and *mdr*1b genes and with apparent differences between rodent and human cells in sensitivity to induction of *mdr* genes (see below).

In work reported by the laboratories of Cornwell (56, 61), Tsuruo (172), Baas (245), and Cowan (90, 146), the *mdr* promoter region eventually proved to have the following features (reviewed in 92): (*a*) a GC-rich region at approximately −100 to −120 from the transcription start site, which is the site of action of a repressor of *MDR*1 transcription; (*b*) a consensus site of binding for NF-Y transcription factors at −70 to −80 (Y-box), which affects basal transcription; (*c*) a binding site for SP1 and members of the early growth response (EGR) family of transcription factors that overlaps with the consensus site in (*b*); and (*d*) a 13-basepair region around the transcription initiation site (INR) involved in accurate initiation of transcription. Initial reports of a tissue-specific enhancer several kilobases upstream of the *MDR*1 structural gene appear to be in error (87).

Studies on the physiology of expression of the *MDR*1 gene suggested that a variety of stimuli could increase levels of *MDR*1 RNA in different cells and tissues, including partial hepatectomy and xenobiotic treatment in rat liver (74, 234), heat shock and arsenite in certain cell types (50), DNA-damaging and chemotherapeutic agents (42, 48, 149, 236), sodium butyrate (160, 165), extracellular matrix (209), protein kinase C agonists (41), inhibitors of P-gp such as verapamil (166), and growth factors (60). Although in several cases a direct effect on transcription was suggested by nuclear runoff studies, in other cases a direct effect on transcription was either not demonstrated or difficult to detect.

In some cases, indirect evidence suggested that enhanced mRNA stability might be responsible for the increases in mRNA levels seen (147). Failure to detect transcriptional activation by nuclear runoff may not exclude this mechanism of induction of *MDR*1 expression. For example, even though increases in nuclear runoff are not detected in human kidney cells exposed to heat shock (50), the *MDR*1 promoter appears to have a heat-shock-like consensus element that is activated in transient expression experiments (130).

Are there endogenous inducers of *MDR*1 expression? Evidence in rodent systems suggests strongly that certain steroids, particularly progesterone, can induce *mdr* gene expression in the pregnant uterus (14). There is a progesterone-responsive element in the rodent *mdr*1b upstream region (177). In a recent study in which one of two *mdr*1b alleles was inactivated in mouse adrenocorical Y-1 cells, the remaining allele was overexpressed, suggesting feedback control in *mdr*1b-expressing tissues (6). The human adrenal cortex expresses very high levels of the *MDR*1 gene, consistent with induction by steroids in this tissue. The overall impression from these studies (and others not cited) with exogenous xenobiotics and endogenous steroids is that *MDR*1 transcript levels are highly regulated, either at the transcriptional or posttranscriptional amounts, or both, and that the mechanism of this regulation is complex and still very poorly understood.

One striking regulatory feature of *MDR*1 expression is the high frequency of expression in human tumors (91). Three classes of human cancers express P-gp (96): (*a*) those derived from tissues in which the *MDR*1 gene is constitutively activated (e.g. colon, liver, adrenal); (*b*) those that have acquired multidrug resistance after repeated exposure to chemotherapeutic agents (e.g. leukemias and lymphomas, breast and ovarian cancers); and (*c*) those in which the transforming event per se appears to be responsible for activation of the *MDR*1 gene. Transient expression of *MDR*1 promoter fragments fused to a reporter gene such as that encoding chloramphenicol acetyl transferase (CAT) allowed the demonstration that both wild-type Harvey *ras* and mutant forms of p53 can activate the *MDR*1 promoter (51). The p53 responsiveness can also be demonstrated under some conditions with wild-type p53 (89), and appears to require only the core *MDR*1 promoter and initiator region (168, 254). Although the clinical significance of the responsiveness of the *MDR*1 promoter to p53 remains unclear, these studies have defined a role for p53 in regulating gene expression independent of a specific p53-response element.

POSTTRANSLATIONAL MODIFICATIONS OF P-GLYCOPROTEIN

Soon after P-gp was first discovered (123), it was described as a phosphoglycoprotein (32). Initially, experiments were performed to study glycosylation

and phosphorylation of P-gp. Subsequent molecular genetic analyses, however, proved to be indispensable to evaluate the role of these posttranslational modifications for the function of P-gp.

Glycosylation

The apparent molecular weight of mature P-gp may range from 130,000 to 180,000, depending on the species and type of cell in which it is expressed (98, 186). Pulse-chase labelling experiments have revealed that P-gp is synthesized as a nonglycosylated precursor with an apparent molecular weight of 120,000–140,000 (98, 186). Processing to the mature form occurs with a half-life time $t_{1/2}$ = 1–2 h in human cells or $t_{1/2}$ = 20–30 min in mouse cells, and is inhibited by tunicamycin treatment (98, 186). All oligosaccharide side chains in human P-gp appear to be N-linked and can be removed with endoglycosidase F (186). Endoglycosidase F treatment converts mature P-gp to a lower-molecular-weight form that is similar to precursor protein (30, 186). In contrast, no shift in molecular weight results from neuraminidase-, endoglycosidase H-, or O-glycanase-treatment of human P-gp (30, 186). While ^{14}C-labelled N-acetylglucosamine and galactose are efficiently incorporated into human P-gp, labelling with ^{14}C-labelled fucose and mannose is poor (186). For none of the mammalian P-gps, however, has the exact composition of the carbohydrate moiety been elucidated.

Between two and four N-linked glycosylation sites are predicted within the primary structure of mammalian P-gps (44, 68, 72, 100, 218, 242). The number and exact positions of the predicted glycosylation sites differ among human and rodent P-gps, but they are all located in the first putative extracellular loop, a region that is otherwise highly divergent in primary amino acid sequence. Additional glycosylation sites have been postulated based on alternate topological models of P-gp (219, 256, 257), but no evidence for such sites has been gained from molecular genetic or biochemical studies in intact mammalian cells (202; SV Ambudkar, unpublished data). Site-directed mutagenesis and deletion analyses have confirmed that human P-gp contains three N-linked glycosylation sites, which are present in the amino-terminal half in the first extracytoplasmic loop (202).

Both cell biological and molecular genetic studies have indicated that the multidrug transport function of P-gp does not depend on glycosylation. Recombinant human P-gp expressed in *MDR*1-transfected murine cells has a lower apparent molecular weight than native human P-gp owing to altered glycosylation, but its multidrug transporter activity is not affected (SV Ambudkar, UA Germann, I Pastan, MM Gottesman, unpublished data). Recombinant P-gps expressed in baculovirus-infected insect cells, in *Saccharomyces cerevisiae,* or in *Escherichia coli* are underglycosylated or nonglycosylated,

but appear to be functionally similar to their native counterparts in multidrug-resistant mammalian cells (22, 73, 88, 131, 184, 196, 200). It was also reported that multidrug-resistant sublines can be isolated from lectin-resistant mutants, which are partially glycosylation-defective (136). Moreover, blocking N-linked glycosylation by treatment of multidrug-resistant cell lines with tunicamycin does not affect their drug resistance, providing even stronger evidence that glycosylation is not required for the drug efflux activity of P-gp (18, 120).

The most conclusive work on glycosylation involved a series of human P-gp mutants that lack N-linked glycosylation sites in the first extracytoplasmic loop (202). The analysis of selected transfectants revealed that these glycosylation-defective mutants, independent of their state of glycosylation, confer the same pattern of cross-resistance as the wild-type P-gp. The frequency with which drug-resistant transfectants were obtained, however, was drastically reduced for a P-gp mutant that lacks all three N-linked glycosylation sites, suggesting that the carbohydrate moiety may contribute to the correct folding and/or proper routing of P-gp, and/or its stabilization en route to or within the plasma membranes (202). Generally, P-gps are extremely stable proteins with a half-life of 48–72 h for the human and approximately 18 h for the mouse isoforms (53, 141, 186). Although mouse P-gp isoforms differ in their sites and state of glycosylation, the rate of degradation is similar among different mouse isoforms (53), which may be used as an argument against a role of glycosylation in stabilizing the membrane-associated polypeptide chain against proteolytic digestion. A recent analysis of a series of temperature-sensitive mutants of P-gp demonstrated that the core-glycosylated form is associated with the molecular chaperone calnexin in the endoplasmic reticulum and that the duration of this association determines the fate of the polypeptide chain (141). Wild-type P-gp escapes the association with calnexin efficiently and is properly targeted to the plasma membranes via the Golgi apparatus, whereas misfolded mutants are retained by calnexin in the endoplasmic reticulum for prolonged times (141). Similar studies of the biosynthesis of the glycosylation-defective mutants may eventually help to elucidate the exact role of glycosylation of P-gp.

Phosphorylation

Phosphorylation has been established as a characteristic of mature P-gp in intrinsically drug-resistant or drug-selected cell lines of human and rodent origin (e.g. 16, 34, 80, 107, 145, 154, 186, 193, 210, 252). Recombinant *mdr* gene products expressed in mammalian or insect cells are also phosphorylated (88, 210). A change in the state of phosphorylation of P-gp has been associated with differences in relative drug resistance of mammalian cells, and it has been hypothesized that phosphorylation/dephosphorylation mechanisms may be involved in the regulation of the drug efflux activity of P-gp (33, 107).

In an attempt to elucidate the role of phosphorylation of P-gp, many investigators have correlated the levels and/or activities of protein kinases in multidrug-resistant cells with relative drug resistance and/or concentrations of drug(s) accumulated intracellularly. Several studies have revealed that the multidrug-resistance phenotype of cells is frequently associated with elevated levels of protein kinase C (PKC) (e.g. 11, 12, 33, 75, 107, 152, 153, 171, 174, 179, 180). Through use of plasma membranes isolated from multidrug-resistant human KB-V1 cells, partially purified P-gp, or a synthetic human P-gp peptide, it was shown that the human *MDR*1 gene product is a target for in vitro phosphorylation by PKC, supporting the notion that PKC may serve as an important modulator in the development of multidrug resistance in mammalian cells (1, 37, 38). Transfection studies have indicated that overexpression of the PKCα isoform of PKC may confer increased drug resistance to P-gp-expressing cells (2, 252; reviewed in 82). While it is possible that PKCα-mediated phosphorylation of P-gp may control its drug transport activity, indirect effects of PKCα on the composition of the plasma membranes and/or expression of *mdr* mRNA cannot be excluded in these experiments. In this regard, increased levels of PKCα have been detected in nuclei of MCF-7 multidrug-resistant cells (132). Recent data obtained with a baculovirus co-expression system producing high amounts of membrane-associated PKCα and *MDR*1 gene products suggested that PKCα may serve as a positive regulator of the drug-binding and ATPase activities of P-gp (3). It remains unresolved, however, if PKCα also directly affects phosphorylation of P-gp in intact mammalian cells, which express much lower levels of both proteins and quite likely contain higher amounts of other targets of PKCα.

PKC is one among several protein kinases that may phosphorylate P-gp directly (36, 39; reviewed in 82). Other kinases include cAMP-dependent protein kinase (PKA) (152) and at least three different novel kinases that remain to be identified (134, 198, 223; SV Ambudkar, IH Lelong, CO Cardarelli, I Pastan, MM Gottesman, unpublished). Our insights concerning the expression and the role of these different protein kinases in the multidrug-resistance phenotype are rather limited at present, although there is evidence that individual PKC isoforms (e.g. PKCα as described above) may be involved in modulating multidrug resistance (reviewed in 82). The proteins that dephosphorylate P-gp need to be identified as well. Preliminary results pointed to an involvement of the membrane-associated protein phosphatases 1 and 2A (39), but more detailed studies are required to establish the enzymology and the kinetics of the phosphorylation/dephosphorylation cycle of P-gp.

Other studies that addressed the role of P-gp phosphorylation in modulating multidrug resistance have made use of protein kinase agonists and/or antagonists to analyze short-term effects on drug accumulation by multidrug-resistant cells, or long-term effects on drug survival. Several research groups have

demonstrated that upon brief exposure of multidrug-resistant cells to protein kinase activators (such as the phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate, or TPA), phosphorylation of P-gp is enhanced, drug accumulation reduced, and drug resistance increased (e.g. 1, 16, 34, 36, 75, 107). Conversely, protein kinase inhibitors (e.g. staurosporine, calphostin C) were found to decrease phosphorylation of P-gp and increase drug accumulation (e.g. 17, 39, 145). With few exceptions (33, 107), an increase in protein kinase activity and/or phosphorylation of P-gp has been associated with increased drug resistance, and vice versa. Hence, these correlative studies are in agreement with the hypothesis that the state of phosphorylation of P-gp may regulate its drug efflux activity and, as a consequence, modulate multidrug resistance.

Unfortunately, many of the activators and inhibitors of protein kinases used to alter the state of phosphorylation of P-gp are not very specific and often cause multiple cellular effects, which makes interpretation of the data difficult. Three complicating side effects of protein kinase agonists and/or antagonists in multidrug-resistant cells have been defined to date.

1. Various protein kinase activators and inhibitors may affect P-gp expression, suggesting that protein kinase signal transduction pathways may regulate *MDR*1 gene expression. For example, the PKC activators TPA and diacyl glycerol were found to enhance *MDR*1 gene expression, both at the transcriptional and translational level, and this effect could be suppressed with staurosporine (41). Transcriptional activation of the *MDR*1 gene was shown to be attenuated by the PKC inhibitor H7 (236), while the PKA inhibitor H-87 was found to diminish *MDR*1 gene transcription (128). Staurosporine was observed to have dual (opposite) effects on levels of P-gp and/or *MDR*1 mRNA, which appear to be cell-type specific and concentration dependent (41, 199).

2. Many protein kinase modulators are amphiphilic molecules that may bind to P-gp and, therefore, inhibit its drug transport activity. For example, staurosporine and its derivatives have been shown to reverse multidrug resistance independent of their protein kinase inhibitory activities, presumably by interacting with P-gp directly (163, 201, 247). Similarly, calphostin C and certain isoquinolinesulfonamide (H7) derivatives may increase drug accumulation in multidrug-resistant cells by interfering with drug transport (106, 162, 248). Taken together, there is considerable evidence that protein kinase modulators may alter drug accumulation within multidrug-resistant cells independent of, or in addition to, their effects on the phosphorylation state of P-gp by interacting with the multidrug transporter directly, or by affecting levels of expression of the *MDR*1 gene product at the transcriptional and/or posttranscriptional level.

3. Another potential problem in interpreting data obtained from studies involving protein kinase agonists and/or antagonists arises from the time of

exposure of the cells to a particular protein kinase modulator, which may affect its mechanism of action. For example, brief exposure of mammalian cells to TPA may cause activation of PKC, whereas prolonged exposure may reduce levels of PKC by increasing its rate of proteolysis (25, 118, 169, 251).

Recent strategies to assess the role of phosphorylation in multidrug resistance have focused on the biochemical identification of actual sites of phosphorylation within P-gp to provide a basis for molecular genetic approaches involving site-directed mutagenesis. Phosphoamino acid analyses revealed that human P-gp contains phosphoserine exclusively (33, 107). A two-dimensional tryptic phosphopeptide map of human P-gp, obtained after metabolic labelling of multidrug-resistant cells with ^{32}P-orthophosphate, indicated the presence of at least three major phosphopeptides (39). The same two-dimensional map of tryptic phosphopeptides was obtained from human P-gp phosphorylated by PKC in vitro (38). Amino acid sequence analysis of the isolated tryptic phosphopeptides identified serine 661, serine 671, and one or more of serine 667, serine 675, or serine 683 as sites of phosphorylation in human P-gp (38) (see also Figure 1). Using a synthetic peptide encompassing amino acid residues 656–689, serine 667 was demonstrated to be a third PKC phosphorylation site in human P-gp (37). Similarly, serine 667, serine 671, and serine 683 were shown to be phosphorylated by PKA in vitro (37). Experiments with a different, so far unidentified membrane-associated protein kinase isolated from multidrug-resistant KB-V1 cells (V-1 kinase) confirmed serine 661 and serine 667 as major sites of phosphorylation in human P-gp (SV Ambudkar, TC Chambers, I Pastan, MM Gottesman, unpublished data). Moreover, a molecular genetic analysis of glutathione-S-transferase (GST) fusion proteins containing amino acid residues 644–689 of the human *MDR*1 gene product helped to corroborate these biochemical findings (35). A systematic mutational analysis was performed in which the biochemically identified phosphorylatable serine residues were substituted with nonphosphorylatable alanine residues individually or in different combinations. The isolated fusion proteins were subjected to in vitro phosphorylation, followed by tryptic digestion, and subsequent analysis by two-dimensional phosphopeptide mapping. This study confirmed serine 661 and serine 667 as major sites of phosphorylation sites by PKC and the V-1 kinase. Surprisingly, serine 683 was also found to be phosphorylated by PKC, but only in the absence of the major phosphorylation sites. Taken together, both the biochemical and molecular genetic analyses revealed that a cluster of maximally four serine residues is accessible and recognized as targets for phosphorylation by multiple kinases, despite the presence of numerous (>40) consensus sites for PKC and/or PKA phosphorylation distributed throughout the primary structure of human P-gp. These clustered phosphorylation sites are confined to a central cytosolic segment of approximately 60 amino acids that connects the two homologous halves of P-gp and is known

as the linker region (see Figure 1). These phosphorylation data and partial proteolysis data (SV Ambudkar, unpublished data) suggest that this central cytosolic segment of P-gp may be more accessible to soluble enzymes than are other parts of the transporter.

Analyses of the mouse *mdr*1b P-gp corroborated that the linker region represents the preferred target for multisite phosphorylation of *mdr* gene products by several kinases (173). Two serine residues are phosphorylated in the mouse *mdr*1b gene product, namely serine 669 by PKC (analogous to serine 671 in human P-gp), and serine 681 by PKA (analogous to serine 683 in human P-gp) (173). Consensus sites of phosphorylation by PKC and/or PKA are also predicted in the linker region of other mammalian P-gps (e.g. the mouse *mdr*1a and the hamster *pgp*1 and *pgp*2 P-glycoproteins). Although the actual sites of phosphorylation have not been reported for most *mdr* gene products, it is worth noting that the linker region of P-gp resides at an analogous location within the polypeptide chain to the regulatory (R) domain of the cystic fibrosis transmembrane conductance regulator (CFTR). The R-domain of CFTR harbors a large number of potential phosphorylation sites, some of which are involved in the regulation of CFTR's cAMP-dependent chloride channel activity (40, 46, 188). In analogy to the role of the R-domain for CFTR function, the linker region of P-gp has been viewed as a mini-R(regulatory)-domain that may control its drug transport activity (38).

The identification of the major sites of phosphorylation in P-gp provided an opportunity to introduce site-specific mutations within a human *MDR*1 cDNA and to assess their effects on the multidrug transport function. In a recent mutational analysis, five putative phosphorylation sites within the linker region of human P-gp (serines at positions 661, 667, 671, 675, and 683) were substituted either with nonphosphorylatable alanine residues (5A-mutant) or with aspartic acid residues to mimic permanently phosphorylated serine residues (5D-mutant) (83). Transfection studies revealed that both mutant proteins, similar to wild-type P-gp, were expressed at the cell surface and conferred multidrug resistance by diminishing drug accumulation. In contrast to wild-type P-gp, however, the 5A- and 5D-mutants exhibited no detectable levels of phosphorylation in vivo and in vitro. This analysis reconfirmed that the major phosphorylation sites are confined to the linker region of human P-gp. Moreover, these data suggest that phosphorylation/dephosphorylation mechanisms do not play an essential role in the establishment of multidrug resistance mediated by human P-gp. Apparently, phosphorylation of P-gp is not necessarily required for its basal multidrug transporter activity. These experiments do not completely rule out that phosphorylation may modulate P-gp-mediated multidrug resistance. One possibility is that phosphorylation of P-gp may affect its affinity for certain drugs and change the drug resistance profile of multidrug-resistant cells, as studies by Bates and coworkers have suggested (16).

Alternatively, the velocity of drug transport may be affected, as recently suggested by Aftab and coworkers (1). Although the phosphorylation- and dephosphorylation-defective mutants of P-gp make stably expressed proteins, their half-lives may be different from that of wild-type P-gp. Protein kinases may contribute to the stability and/or instability of P-gp directly or indirectly, i.e. via another phosphorylated protein that may prevent proteolysis of P-gp (199), or may contribute to its degradation. Conclusive evidence for a direct role of protein kinases in regulating the drug transport activity of P-gp will eventually be gained from studies involving the functional reconstitution of purified protein (wild-type and mutants) in phospholipid vesicles. It is also possible that phosphorylation of P-gp may be less important for efflux of drugs, but significant for the transport of a putative substrate(s) that still remain(s) to be identified. A recent study suggests that phosphorylation of human P-gp may indirectly regulate an endogenous chloride channel and that P-gp itself may not, as previously hypothesized, possess intrinsic channel activity (111).

MECHANISMS OF GENE AMPLIFICATION IN MULTIDRUG RESISTANCE

The multidrug-resistant phenotype of a cell is defined as the simultaneous acquisition of resistance to multiple drugs that are dissimilar in structure and function and does not result merely from the accumulation of independent genetic mutations, each of which confers specific drug resistances to a single cell. The identification of specific genes whose expression resulted in MDR phenotypes relied almost exclusively on in vitro studies of mammalian MDR cell lines. As outlined in this section, multistep drug selections were most frequently used as the genetic strategy to establish MDR cell lines, because these stepwise drug selections greatly enhanced the expression of specific, but uncharacterized, multidrug-resistance genes. Their high level of expression, which often resulted from gene amplification, was then exploited by a variety of molecular biological approaches to identify and clone specific mammalian multidrug-resistance genes operative in human cancers.

To establish MDR cell lines, cultured cells were selected for resistance to a specific anticancer drug. These drug selections were conducted in either a single step or in multiple steps. Single-step drug selections exposed cells to a relatively high dose of selecting drug for a brief time. Such short-term drug exposure resulted in the survival of a few clones that expressed a stable, low-level MDR phenotype after clonal expansion and propagation in drug-free medium. In contrast to the single-step drug selection, multistep drug selections were conducted over the course of several months to years, during which time the drug was increased in sublethal increments. MDR cell lines that were established with stepwise drug selections resulted in highly drug-resistant cell

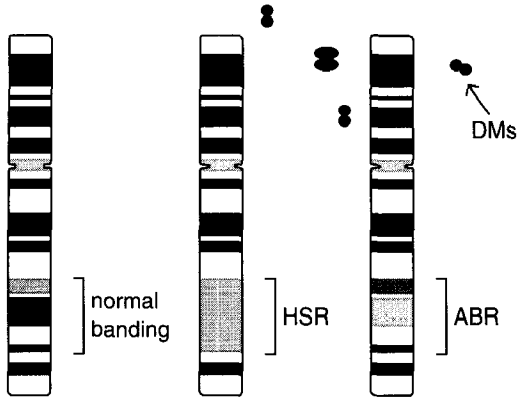

Figure 3 Schematic representation of intrachromosomal and extrachromosomal amplification structures. Trypsin-Giemsa or quinacrine-stained metaphase chromosomes viewed by light microscopy show discernible and characteristic longitudinal differentiation of darkly to lightly stained bands for specific chromosomes. Homogeneous staining regions (HSRs) appear unstained or homogeneous, due to their lack of cross-striational banding patterns, whereas abnormal banding regions (ABRs) show alterations in the specific banding pattern. Extrachromosomal circular DNAs greater than 1000 kb long, referred to as double minute chromosomes (DMs), can also be seen with Giemsa or quinacrine-stained metaphase chromosome spreads.

lines that usually required the presence of drug to maintain their high-level MDR phenotype. The loss of MDR that occurred in these cell lines in the absence of drug selection was due to the loss of unstable DNA amplification structures harboring amplified copies of multidrug-resistance (*mdr)* genes. Subsequent analyses of these cell lines have identified gene amplification events that occurred during the multistep drug selections at the multidrug-resistance (*mdr*) locus and have led to a physical map spanning approximately 1 megabase (Mb) of the native *mdr* genomic region in both rodent and human MDR cell lines.

Molecular Cytogenetics of In Vitro MDR Model Systems

An obvious feature of many of the independently selected multidrug-resistant cell lines was an altered karyotype that showed evidence of gene amplification. With classical cytogenetic procedures, it has been shown that gene amplification is manifested most commonly as either intrachromosomal DNA structures or extrachromosomal circular DNAs (schematically represented in Figure 3). These atypical DNA structures contain one to several genes and are referred to as amplicons. Intrachromosomal DNA structures show either homogeneously staining regions (HSRs) or abnormal banding regions (ABRs), following specific staining procedures of chromosomes, such as trypsin-Giemsa banding

(23). ABRs, which can be more difficult to detect than HSRs, usually represent fewer copies of an amplified chromosomal region. Extrachromosomal DNAs, greater than 1000 kilobases (kb) long, are also visible with Giemsa staining and appear most frequently as circular, paired chromatin structures referred to as double minute chromosomes or DMs (126).

The significance of gene amplification structures in cells was first realized when Biedler & Spengler identified HSRs in methotrexate-resistant Chinese hamster cells (23) and then correlated their presence to increased amounts of the dihydrofolate reductase (DHFR) enzyme in the cells (24). Following their observations, Schimke and colleagues were able to show the selective amplification of *DHFR* genes in methotrexate-resistant variants of cultured murine cells (5) and to specifically localize the *DHFR* amplicon to an HSR on a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line (170). Thus, the presence of gene amplification structures in many of the MDR cell lines provided strong indirect evidence that amplification of unknown *MDR* genes was a prominent mechanism involved in mediating the MDR phenotype in vitro.

Cytogenetic studies that characterized the fate of amplification structures in the MDR cell lines during drug selection and withdrawal showed two interesting observations. First, the length of the HSRs or the number of DMs that were identified in some of the MDR cell lines paralleled the level of MDR, and in some cases the amount of P-gp, expressed by the cell line. Second, gene amplification structures in drug-resistant cell lines were unstable; if the selecting drug was withdrawn, the MDR-resistant phenotype was lost over time concomitantly with a decrease in the length of the HSR or in the number of DMs that had been identified in the MDR cell line prior to drug withdrawal (64, 78, 143, 156, 193, 215).

The presence of gene amplification structures harboring unknown *MDR* genes offered unique opportunities for the genetic and biochemical characterization of those genes. For example, the unstable MDR genotype allowed for the relatively easy isolation of revertant cell lines, which upon prolonged growth in the absence of drug acquired a drug-sensitive phenotype, often comparable to that of the parental cell line from which they were derived, along with the loss of obvious gene amplification structures. These revertant cell lines were used as controls in many of the biochemical and genetic studies that ultimately identified the multidrug-resistance genes and demonstrated that their protein products, the P-gps, were plasma-membrane-localized multidrug efflux pumps. In addition, the knowledge that MDR cell lines harbored amplified gene structures led to the development of novel strategies that exploited the increased copy number of an unknown gene in several of the MDR cell lines to facilitate its cloning and the subsequent identification of specific *mdr* genes in rodents and humans (104, 189, 192, 211).

Isolation of Amplified Gene Sequences in MDR Cells

Two of the cloning strategies employed to isolate novel *mdr* genes were based on the cytogenetic evidence showing the presence of amplified DNAs in the MDR cell lines. In the first approach, Roninson and colleagues cloned amplified genomic DNA sequences directly from agarose gels that contained size-fractionated, enzyme-digested genomic DNA from MDR hamster cells. This technique was based on a series of in-gel denaturation/renaturation steps during which single-stranded DNAs in the gel were destroyed by S1 nuclease digestion. Heteroduplex DNAs formed from the relatively rapid reannealing of amplified DNAs were preferentially retained in the gel (189, 190). Once an amplified DNA sequence was cloned using this novel in-gel renaturation protocol and shown to detect overexpressed mRNAs in an MDR hamster cell line (191), this sequence was used as a DNA probe to isolate amplified human genomic fragments from colchicine-selected MDR KB cells (78, 192), and to identify amplified MDR genomic DNA sequences and cDNAs in MDR murine cell lines (99–101). A second approach involved the direct cloning of amplified *mdr* genomic DNA sequences following differential hybridization of Cot-fractionated DNA isolated from MDR Djungarian hamster cell lines (104). Similarly, a Cot 10–300 probe was used to screen a cDNA library of vincristine-selected Chinese hamster cells, designated DC-3F/VCRd-5L, and a partial cDNA was isolated. Its sequence showed that it was a member of the P-gp gene family (211; reviewed in 151).

The *mdr* clones that were obtained from the various cloning strategies outlined above were used for in situ hybridization studies of chromosomes to confirm the presence of amplified *mdr* genes on the gene amplification structures harbored by many of the MDR cell lines (103, 122, 151, 187, 235). In addition, these *mdr* clones were used as DNA probes in Southern and Northern blot analyses of DNA and RNA, respectively, to show that the evolution of MDR during drug selections occurred concomitantly with increased expression of *mdr* genes. The P-gp RNA transcript is approximately 4.5 kb in size in both human (217) and rodent (66, 101, 231) MDR cell lines.

Human *MDR* genomic DNAs have also been sequenced to locate positions of introns within the two halves of the P-gp molecule (45). The human *MDR*1 gene contains 28 exons, interspersed with introns that together extend over greater than 100 kb of genomic DNA. A comparison of the positions of the introns, which are not conserved between the two halves of the gene, indicates either independent evolution of the two halves of P-gp or a duplication event of a primordial half molecule after which extensive intron rearrangements occurred (183). Such a duplication event is conceivable, since many of the transport proteins identified in the ABC family (i.e. in bacteria and for intracellular eukaryotic transporters) consist only of half molecules with six putative

transmembrane regions and one ATP-binding site or separate TM regions and ATP subunits presumed to function as dimers or multimers (reviewed in 9, 10, 62, 112, 113, 119).

One perplexing feature of MDR cell lines is the altered pattern of drug resistance seen in different selection schemes, even under conditions in which the overexpression of *mdr* genes give a wild-type phenotype. In rodent MDR cell lines, these different MDR profiles have been explained, in part, by the identification of two multidrug-resistance genes, each conferring different, but overlapping, profiles of MDR (68). However, in human MDR cells, where there is only one *MDR*1 gene, MDR profile differences among different MDR cell lines are largely unexplained. Although spontaneously occurring basepair mutations have been shown to alter the MDR profile of human cell lines (see above), the generation of basepair mutations during drug selections does not appear to be common. For example, the preferential adriamycin resistance of the human cell line KB-A1 (216) does not appear to be due to the presence of basepair mutations in the amplified *MDR*1 genes of this cell line, as determined by RNase protection assays recently performed by Fojo and colleagues (161). In fact, Fojo and colleagues screened a number of single-agent-selected MDR cell lines with different MDR profiles and generally did not find basepair mutations in the amplified copies of the *MDR*1 gene. Thus, other mechanisms, either genetic or epigenetic, appear to result in the different MDR profiles of human *MDR*1-expressing cell lines.

Several hypotheses have been offered to explain MDR profile differences among MDR cell lines. First, posttranslational modifications such as glycosylation and protein kinase-mediated alterations of P-gp may be involved (see above). Second, different cross-resistance profiles of *MDR*1-expressing cell lines may result from the independent amplification, altered expression, and/or mutation(s) of a nonlinked gene that confers MDR independent of P-gp. For example, the decreased expression of topoisomerase II (Top II) enzyme and/or the presence of mutations in the *top*II gene in *MDR*1-expressing cell lines has been demonstrated (43, 115, 125, 148). Similarly, in the analysis of in vivo biopsies of malignant ovarian tumors (244), renal cell carcinomas, and non-small cell lung carcinomas (246), both *MDR*1 gene expression and altered Top II activity occur. The co-expression of the *MRP* gene and the *MDR*1 genes also occurs in some MDR cell lines (220), as well as in some refractory lymphomas (255) and breast cancers (70). This complex nature of different MDR mechanisms, which may be operative within single MDR cells, presents a great challenge to the clinician, who must consider concurrent strategies aimed at different MDR mechanisms in human cancers.

A third possibility is that genes that are expressed as a result of their co-amplification with *MDR* genes may contribute to the different cross-resistance profiles of some mammalian MDR cell lines. Physically linked genes

are frequently co-amplified with the *mdr* genes, and their expression contributes to some of the protein changes that have been documented in MDR cells. For example, in human MDR KB cells that were selected independently in three different drugs (adriamycin, vinblastine, and colchicine), two-dimensional gel analysis identified a number of differential protein alterations, including increased amounts of a 21-kDa protein, in only the colchicine-selected cells (185, 216). The 21-kDa protein has since been identified as a calcium-binding protein in human MDR cells that is homologous to the Sorcin protein also overexpressed in MDR rodent cells (155). Recent studies have shown that the overexpression of the 21-kDa protein in the colchicine-derived KB cell lines results from overexpression of its cognate gene, termed CP22, which lies on multigene circular DNA amplicons and is linked to the native *mdr* locus in humans (207) (PV Schoenlein, Y Sugimoto, T Tsuruo, I Pastan, MM Gottesman, unpublished data), as the Sorcin gene has been shown to be linked to the *mdr* locus in rodents (241, 243). To date, no distinct pattern of drug resistance has been correlated to the amplification of the CP22 gene in human MDR cell lines, its sorcin gene homolog in murine cell lines, or any of the other genes that differentially co-amplify with the *mdr* genes during drug selections (described in detail in the next section). Thus, the role, if any, of the genes that are co-amplified with the human *MDR*1 genes will only be conclusively shown by cotransfection experiments in which their cDNAs and the *MDR*1 cDNA are introduced in various combinations into drug-sensitive cells and the resulting profile of MDR in the transfectants is analyzed.

Structural Analysis of MDR1 Amplicons

One interesting feature of *mdr* gene amplification during drug selection is the co-amplification of other genes that are physically linked to the native *mdr* locus (207, 225, 241, 243). Studies that have identified the specific, but differential amplification and expression patterns of these linked genes in many rodent and human MDR cell lines have led to a more complete understanding of the structural and genetic organization of the rodent and human *mdr* locus, the genetics of multidrug resistance, and gene amplification mechanisms in mammalian cells (reviewed in 205). Non-*mdr* cDNAs representing amplified genes that are linked to the native *mdr* locus were initially isolated from a cDNA library based on the overexpression of genes in the MDR Chinese hamster cell line CH^RC5 (66, 211, 241). From these studies, six distinct gene classes, designated class 1–6, were isolated. cDNAs of each of the classes were analyzed with Southern hybridization studies, which included pulsed field gel electrophoresis (PFGE) used to size-fractionate large DNA fragments generated from rare-cutting restriction enzyme digests, Northern hybridizations (241), in situ hybridizations to hamster metaphase chromosomes (122), and/or

DNA sequence analyses (241, 243). Class 2 is composed of cDNAs representing the three multidrug-resistance genes, *pgp*1, *pgp*2, and *pgp*3, in hamster. The other gene classes are largely uncharacterized, with the exception of class 4, which encodes a phosphorylated calcium-binding protein, referred to as Sorcin/VP19 or CP22 (155, 243). The six gene classes were shown to be physically linked at the native *mdr* locus in hamster cells within a 1500 kb region, and their gene order was determined (reviewed in 27). The hamster cDNAs were sufficiently conserved to allow their use in the analysis of gene amplification structures and the corresponding genomic DNA regions in MDR cells from mouse (224) and human (240). Taken together, these physical mapping studies provided strong evidence that gene classes 2–6 are syntenic in both rodents and humans, suggesting a similar overall structure of the human and rodent *mdr* (*PGY*) locus.

The main conclusion from studies analyzing the multigene amplification structures in MDR cell lines expressing P-gp is that only the *mdr* genes are consistently amplified and overexpressed, so the amplification of the non-*mdr* genes is not required to confer an MDR phenotype. This conclusion is supported by DNA transfer studies, in which the full spectrum of the MDR phenotype is conferred on drug-sensitive cells after the introduction of functional *mdr* cDNAs by plasmid DNA-mediated transfer (99, 237) or after retroviral gene transfer (105, 175). Further, no discernible correlation has been reported between the pattern of cross-resistance of an MDR cell line and that of a particular set of co-amplified genes suggesting, but not proving, that the co-amplified non-*mdr* genes that have been identified do not contribute to the variations in cross-resistance patterns (66, 224). However, it may be difficult to discern variations in the MDR profiles of rodent cells resulting from expression of co-amplified non-*mdr* genes, because the two *mdr* genes (designated *mdr*1a and *mdr*1b in mouse and *pgp*1 and *pgp*2 in hamster), which have been shown to encode distinct MDR profiles in mouse cells (68), are also differentially co-amplified upon drug selection (66, 122), although apparently not in direct response to the specific drug used for selection (226). The *mdr*2 gene (*pgp*3 in hamster) is also differentially co-amplified and expressed in MDR cell lines, but it does not appear to confer an MDR phenotype (203).

In studies conducted in the human MDR KB cell lines that were selected in either colchicine, vinblastine, or adriamycin (4, 216), large multigene circular DNA amplicons have been identified (206) (described below and in Figure 4) on which differential patterns of gene amplification of non-*mdr* genes linked to the native MDR locus also occur (207) (PV Schoenlein, Y Sugimoto, T Tsuruo, I Pastan, MM Gottesman, unpublished data). Interestingly, in one MDR KB cell line, a change in the pattern of cross-resistance (as compared to its progenitor cell line established at the previous step in the drug selection) appeared at the same time during drug selection that a specific DNA deletion

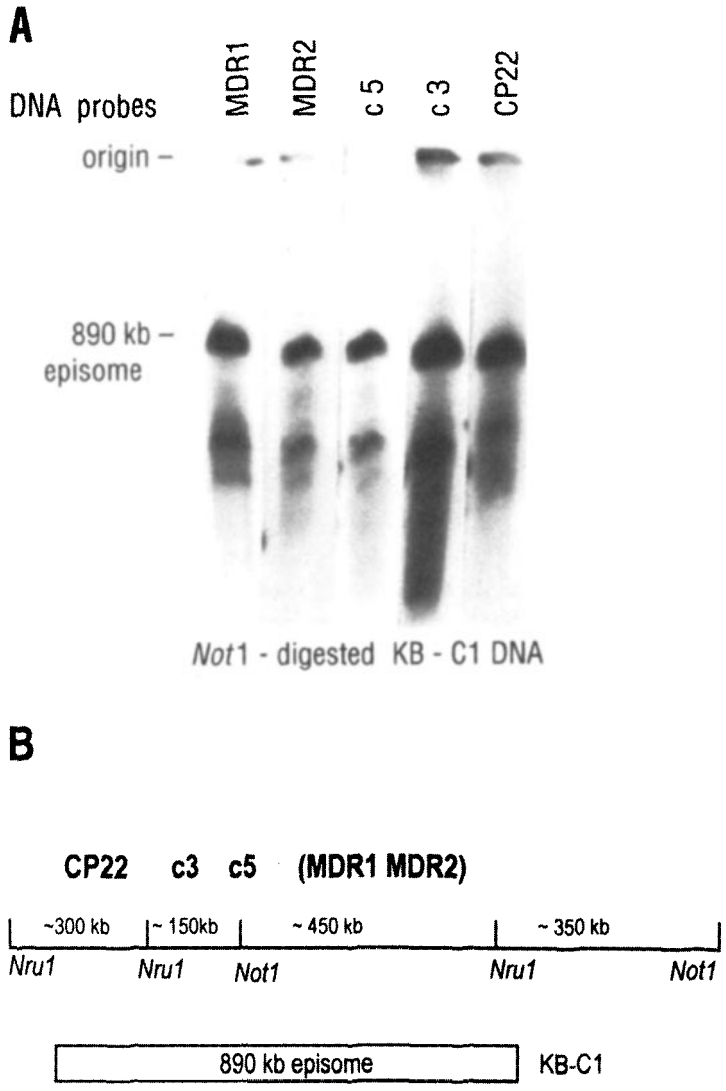

Figure 4 Identification of a five-gene 890 kb amplicon comprising the *MDR*1-containing episomes in KB-C1. Pulsed field gel electrophoresis was performed after *Not*1-enzyme digestion of high-molecular-weight DNA that was embedded in agarose plugs. Following electrophoresis, fractionated DNA was transferred to nitrocellulose and hybridized with the following cDNA probes: pHDR5A, which hybridizes to *MDR*1 and *MDR*2 gene sequences (238), and *MDR*2pvuII, an *MDR*2-specific DNA probe (63). cDNA probes c3, c5, and CP22 were isolated from the multidrug-resistant K562/ADM cell line as a result of their mRNA overexpression (Y Sugimoto, N Asami, S Tsukahara, T Tsuruo, unpublished). Conditions for the PFGE have been described (205). The bottom of the figure shows a restriction map with the relative location of five linked genes at the native MDR locus in HeLa subline KB-3–1. The solid box schematically depicts the five-gene 890 kb amplicon comprising the *MDR*1-containing episomes in the colchicine-selected cell line KB-C1 that were detected with PFGE studies shown in the top of the figure.

occurred in the circular DNA amplicon within a region where two of the non-*mdr* genes map (PV Schoenlein, unpublished). The concurrent timing of the appearance of this DNA deletion and the emergence of the altered MDR profile, which included specific diminished resistance to vinblastine, is intriguing. This observation suggests that further, more direct studies are needed before we can exclude a role for differentially co-amplified non-*mdr* genes in the final MDR profile of human cells.

PFGE techniques (208) were used to identify and determine the approximate size of extrachromosomal circular DNA amplicons in the MDR KB cell lines (Figure 4) that were established in multistep drug selections using three different drugs (4, 216). The size of the circular DNAs are 890 kb in the colchicine-selected KB-C1 cell line (206) and 750 kb in the vinblastine-selected KB-V1 (195, 206) and in the adriamycin-selected KB-A1 cell lines (PV Schoenlein, unpublished). Since these circular DNAs are less than 1000 kb long, they are referred to as episomes. Episomes, unlike DMs, are difficult to detect with Giemsa staining methods; however, fluorescence in situ hybridizations, using a cosmid *MDR*1 DNA probe to metaphase spreads, very easily detect the presence of these circular DNA structures (PV Schoenlein, AM Sanchez, JT Barrett, unpublished data). Recent hybridization studies have determined that the 890-kb episome in the KB-C1 cell line contains the *MDR*2 gene; the human sorcin gene, designated CP22; and at least two other genes of unknown function (PV Schoenlein, Y Sugimoto, T Tsuruo, I Pastan, MM Gottesman, unpublished data). The order of these genes is schematically shown in Figure 4, both in the 890-kb amplicon and at the human native *MDR* locus. These mapping studies are consistent with earlier PFGE studies by Roninson and colleagues, which demonstrated the location of the *MDR*1 and *MDR*2 genes on a 600-kb *Nru*1 DNA fragment that was amplified in colchicine-selected KB cells (47). According to Borst's classification of co-amplified genes (see above), the CP22 cDNA represents class 4; however, the c3 and c5 cDNAs have not been classified. Due to the unequivocal linkage of these genes within a 1-Mb region, as determined by *Not*1 and *Nru*1 restriction digests (Figure 4), it can be inferred that the genes represented by the c3, c5, and CP22 cDNAs map to human chromosome 7, band q21.1, where the *MDR*1 and *MDR*2 genes have been previously localized (31, 76).

TRANSGENIC APPROACHES TO MANIPULATE EXPRESSION OF P-GLYCOPROTEIN IN INTACT ANIMALS

Many of the hypotheses concerning the physiologic function of the P-glycoproteins were based on correlative studies in tissue culture systems. To demonstrate definitively that the expression of the *MDR*1 gene was responsible for

resistance of cells to anticancer drugs in vivo and to determine the physiological function of P-gp in intact animals, it was necessary to introduce *MDR*1 genes into transgenic mice and to ablate endogenous *mdr* genes to create P-gp "knockout" mice. Both of these goals have been achieved, and the properties of these mice confirmed the original hypotheses concerning the function of *mdr*1 genes and yielded new, unexpected information about the function of the closely related *mdr*2 gene in the mouse.

The introduction of a human *MDR*1 cDNA under control of a chicken β-actin promoter into the genome resulted, unexpectedly but serendipitously, in one strain in which the gene was expressed in bone marrow cells (79). This expression resulted in resistance of these animals, and of animals receiving bone marrow transplants from the *MDR*1 transgenic mice, to leukopenia induced by a variety of cytotoxic anticancer drugs (79, 157–159). These results demonstrate that levels of expression of the *MDR*1 gene found in human cancers and in normal human tissues are sufficient to confer multidrug resistance in vivo, and raise the possibility that introduction of a human *MDR*1 gene into the bone marrow of patients might protect their bone marrow against the cytotoxic effects of chemotherapy (see section on GENE THERAPY USING *MDR*1 AS A SELECTABLE MARKER). Unfortunately, expression of the *MDR*1 gene in these mice was lost after several generations, probably owing to extinction of the actin promoter.

The generation of mice with ablated *mdr*1a and *mdr*2 genes has made it possible to determine the physiologic function of these genes in mice (204, 221). The homozygous *mdr*1a knockout has an altered blood–brain barrier, consistent with the expression of this gene in capillary endothelial cells of the brain (204). At least two drugs, the antihelminthic ivermectin and the anticancer drug vinblastine, accumulate to high levels in the brain of *mdr*1a-defective mice. In addition, increased plasma levels of vinblastine due to reduced excretion were found in these animals after intravenous administration of this drug, consistent with a role for the *mdr*1a gene in excretion of vinblastine. Since *mdr*1a knockout mice show increased expression of the *mdr*1b gene in liver, it is possible that other functions of *mdr*1a may be partially compensated for by *mdr*1b expression. Consequently, *mdr*1a *mdr*1b double knockout mice will need to be analyzed to determine whether other hypothesized functions of P-gp in handling of endogenous steroids and in excretion of other xenobiotics are correct.

The *mdr*2 ablated mice develop progressive hepatic sclerosis (221). Analysis of their bile demonstrates reduced levels of phosphatidyl choline, suggesting that the function of *mdr*2 is to transport or "flip" phosphatidyl choline from the plasma membrane of hepatocytes into the bile as a constituent of the micelles, which normally consist of phosphatidyl choline and bile salts. The recent demonstration of a phosphatidyl choline transport activity of the *mdr*2 gene product expressed in yeast vesicles confirms this hypothesis (194).

GENE THERAPY USING *MDR*1 AS A SELECTABLE MARKER

The demonstration that transplantation of *MDR*1 bone marrow to drug-sensitive mice conferred resistance to anticancer drugs suggested that transduction of bone marrow cells with *MDR*1 vectors might result in a selective advantage of such cells in the presence of cytotoxic drugs that are P-gp substrates (158). Through use of a retroviral expression vector in which the human *MDR*1 cDNA was under control of a Harvey sarcoma virus long terminal repeat (LTR) (175), it was possible to show that transduction of mouse bone marrow cells (150) and mouse erythroleukemia cells (67) resulted in resistance in vitro to cytotoxic drugs. When mouse bone marrow transduced with these *MDR*1 retroviruses was transplanted into mice, expression of *MDR*1 mRNA and P-gp was detected on circulating peripheral blood cells, and treatment of mice with taxol enriched these *MDR*1-transduced cells in the peripheral blood (178, 222). The finding of single unique sites of integration of the *MDR*1 retrovirus in multiple hematopoietic lineages (222), and the demonstration that these *MDR*1-transduced bone marrow cells could be serially transplanted and still be resistant to cytotoxic drugs in their new hosts (109), suggested that the *MDR*1 retrovirus was integrating into multipotential stem cells. Isolated mouse stem cells expanded ex vivo can also be efficiently transduced with *MDR*1 retroviruses and transplanted into mice (135). These *MDR*1 retroviruses are also able to transduce human bone marrow CD34 positive cells (a population that includes bone marrow stem cells) with efficiencies of up to 20% (110, 250). These studies provided the basis for the development of *MDR*1-based vectors that could be used to protect the bone marrow of cancer patients from the cytotoxic effects of anticancer drugs. Three such clinical trials involving *MDR*1 retrovirus transduction of autologous bone marrow transplants in patients with breast, ovarian, and brain cancer have been approved by the US Recombinant DNA Advisory Committee and the US Food and Drug Administration and are in progress at the M.D. Anderson Cancer Center, the National Cancer Institute, and Columbia University College of Physicians and Surgeons.

The goal of gene therapy is to modify cells genetically so that they can supply a necessary or useful function to the host. This function is usually the synthesis of a necessary enzyme, growth factor, or other protein that directly or indirectly benefits the host. In the case of *MDR*1-based vectors, the most straightforward application is in the conversion of drug-sensitive bone marrow cells to drug-resistant cells so that higher doses of chemotherapy can be given to patients with cancer. In addition, *MDR*1 vectors can be engineered to carry a passenger gene. If the transfected cells are in the bone marrow, treatment of animals with MDR drugs will have two effects. One is that the drugs kill untransduced cells, creating space in the bone marrow for the transduced cells

to engraft. The other effect is that because the drugs kill normal bone marrow cells, the transduced cells must expand in order to meet the hematopoietic needs of the host. In this application, all bone marrow–derived cells should express both *MDR*1 and the passenger gene. This approach should be useful in producing enzymes necessary to correct all hereditary metabolic disorders that are now curable by bone marrow transplantation. These disorders include both common and uncommon genetic diseases, such as Gaucher disease (deficiency of glucocerebrosidase) and severe combined immunodeficiency due to reduced activity of adenosine deaminase. Passenger genes may also be used to fight viral infections, such as AIDS, by blocking HIV replication or packaging. Dominant negative mutants in regulatory and packaging proteins may serve this purpose. In this case, it is useful to be able to kill cells that have not been transduced by the *MDR*1 vector so that only "cured" or HIV-resistant cells will survive.

As noted, in principle, genes linking other genes to *MDR*1 on gene therapy vectors should allow selection of otherwise unselectable markers in vivo in cells transduced with these vectors. To exploit the potential of *MDR*1-based vectors as in vivo selectable systems for gene therapy, we have developed several different vector systems in which an *MDR*1 cDNA and a nonselectable gene could be co-expressed from a single plasmid. Although systems in which the two genes are under control of separate promoters are useful, expression of the *MDR*1 cDNA does not guarantee expression of the second gene, and often expression of the unselected gene is extinguished. Protein chimeras in which the carboxy-terminus of P-gp were fused to adenosine deaminase proved to be bifunctional (84, 85), but other chimeras with Herpes Simplex Virus (HSV) thymidine kinase (Y Sugimoto, I Pastan, MM Gottesman, unpublished data) were nonfunctional. Interestingly, in one construction in which a potential open reading frame encoding a chimeric P-gp-glucocerebrosidase protein was tested, no chimeric protein was found, but variant mRNAs encoding either P-gp or glucocerebrosidase were produced in transfected cells so that both proteins were found in transfected cells selected for the MDR phenotype (J Aran, MM Gottesman, I Pastan, unpublished data). Whether this approach will prove useful for gene therapy is uncertain, since the efficiency of production of the requisite variant mRNAs appears to be low.

Our most efficient vectors utilize an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus to allow translation of a second open reading frame in a single mRNA transcribed from the Harvey LTR. A series of vectors encoding the *MDR*1 cDNA and HSV thymidine kinase (228, 230), glucocerebrosidase (13), and alpha-galactosidase (229) have been constructed. Several principles emerge from these studies. (*a*) The IRES-based vectors are efficient vectors for co-expression of two genes in virtually all cells that can be selected for the MDR phenotype. (*b*) The cDNA cloned downstream from the

IRES is translated at rates three- to fivefold less than the cDNA translated from the cap-dependent upstream site. Thus, if *MDR*1 is downstream, the number of clones isolated by drug selection is reduced, but each drug-resistant clone expresses more of the upstream protein (and vice versa). (*c*) Selection of populations of MDR cells transduced with these vectors under increasingly stringent conditions results in higher expression of P-gp and coordinately increased expression of the unselected gene. (*d*) Virtually 100% of the cells that express the downstream gene will also express the upstream gene; 95–98% of cells expressing the upstream gene also express the downstream gene. Loss of expression is presumably because of rearrangements occurring during insertion. (*e*) Despite the large size of these bicistronic vectors, retrovirus producing lines with supernatant retroviral titers of up to 1×10^5/ml are easily obtained in the PA317 packaging line. The *MDR*1-HSV-tk bicistronic vector shows some promise as a "suicide" vector, which should make it possible to use the antiviral drug ganciclovir to kill host cells inadvertently transduced to drug resistance, such as cancer cells or other inappropriate cell types.

The future use of selectable markers, such as *MDR*1, in human gene therapy depends on developing solutions to several problems. The safest and most efficient gene delivery systems should be utilized. It is unclear at present whether these will be retroviral vectors, which suffer from the disadvantage of random integration into target cells; vectors that integrate into specific sites (adeno-associated virus is reported to do this, but most transducing AAVs do not integrate efficiently); or vectors that can be maintained as extrachromosomal episomes. In this last case, the presence of a selectable marker, such as *MDR*1, on the episome would be decidedly advantageous. How these vectors will be delivered to cells (either in vivo or ex vivo) is also unclear—either as packaged defective viruses, replication-competent viruses, or as DNA efficiently transfected into cells, utilizing cationic liposomes or some other delivery system. The choice of target cells will vary with the specific selectable markers; for *MDR*1, most tissues are drug-sensitive, but only rapidly dividing cells such as bone marrow cells, skin, and gastrointestinal epithelium will be selectable in vivo. Finally, the choice of selective agent is critical. Drugs that do not produce damage to DNA should be used. Fortunately, the multidrug resistance conferred by *MDR*1 expression is so broad that literally dozens of cytotoxic drugs are candidates as selective agents.

Genetic studies on the multidrug transporter have come full cycle. A genetic mechanism that protects cancer cells from chemotherapy is about to be exploited for treatment of cancer and inborn errors of metabolism.

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