O.-G. (1989) *Biochem. Biophys. Res. Commun. 163*, 635–641.
Sober, H. A., Ed. (1970) *CRC Handbook of Biochemistry*, 2nd ed., Cleveland, OH.
Taylor, S., Buechler, J. A., & Knighton, D. R. (1990) in *Peptides & Protein Phosphorylation* (Kemp, B. E., Ed.) CRC Press, Inc., Boca Raton, FL.
Tuzimura, K., Konno, T., Meguro, H., Hatano, M., Murakami, T., Kashiwabara, K., Saito, K., Kondo, Y., & Suzuki, T. M. (1977) *Anal. Biochem. 81*, 167–174.
Wong, P., Barbeau, A., & Roses, A. D. (1985) *Anal. Biochem. 146*, 191–198.

Functional Analysis of Chimeric Proteins Constructed by Exchanging Homologous Domains of Two P-Glycoproteins Conferring Distinct Drug Resistance Profiles†

Rajan Dhir and Philippe Gros*
Department of Biochemistry, McGill University, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6
Received January 15, 1992

ABSTRACT: P-Glycoproteins (P-gps) encoded by the mouse *mdr1* and *mdr3* (Phe^{939}, *mdr3F*) genes confer distinct drug resistance profiles. While the *mdr1* and and *mdr3F* clones confer comparable levels of vinblastine (VBL) resistance, *mdr3F* confers actinomycin D (ACT) resistance levels 2-fold greater than *mdr1*, while *mdr1* confers resistance to colchicine at levels 7-fold greater than *mdr3F*. We wished to identify in chimeric proteins discrete protein domains responsible for the distinct drug resistance profiles of *mdr1* and *mdr3F*. Homologous protein domains were exchanged in hybrid cDNA clones, and the specific drug resistance profiles conferred by chimeric proteins were determined in stably transfected cell clones expressing comparable amounts of wild-type or chimeric P-gps. Immunoblotting experiments showed that all chimeras were found expressed in membrane-enriched fractions of transfected cell clones and all conveyed cellular drug resistance at levels above the background of nontransfected drug-sensitive LR73 cells. For VBL, all chimeric constructs were found to convey similar levels of resistance. For COL and ACT, the levels of resistance conferred by the various chimeras were heterogeneous, being similar to either the parental *mdr1* or the parental *mdr3F* clones, or in many cases being intermediate between the two. The preferential COL and ACT resistance phenotypes of *mdr1* and *mdr3F*, respectively, did not segregate in chimeric proteins with any specific protein segment. Taken together, our results suggest that the preferential drug resistance phenotypes encoded by the *mdr1* and *mdr3F* clones implicate complex interactions between the two homologous halves of the respective P-gp.

Multidrug resistance (MDR)[1] is the phenomenon by which tumor cells in vivo and cultured cells in vitro acquire simultaneous resistance to a large group of structurally and functionally unrelated cytotoxic drugs (Moscow & Cowan, 1988). MDR is caused by the amplification and/or overexpression of a small family of closely related genes termed *mdr* or *pgp*, which code for a group of membrane glycoproteins termed P-glycoproteins (P-gps) (Endicott & Ling, 1989; Roninson, 1991). It has been shown that P-gp is a membrane phosphoglycoprotein capable of combining photoactivatable analogs of ATP (Cornwell et al., 1987; Schurr et al. 1989) and cytotoxic drugs (Cornwell et al., 1986; Safa et al., 1986, 1989) and possesses ATPase activity (Hamada et al., 1988). It is generally accepted that P-gp functions as a drug efflux pump to reduce the intracellular accumulation of cytotoxic drugs in MDR cells. Full-length cDNA clones corresponding to two human *MDR* genes, *MDR1* and *MDR2* (Chen et al., 1986; Van Der Bliek et al., 1987), and three rodent *mdr* genes, *mdr1*, *mdr2*, and *mdr3* (Gros et al., 1986a, 1988; Devault & Gros, 1990; Hsu et al., 1990), have been isolated and sequenced. Analysis of the predicted amino acid sequences of P-gps indicates that they share a high degree of amino acid sequence homology and identical hydropathy profiles. These common structural features include two sequence-homologous halves, each encoding a series of six predicted membrane-associated (TM) domains, and a consensus sequence for nucleotide binding (NB). Each of these homologous segments also shares considerable sequence similarity and possibly common ancestral origin with a group of bacterial genes participating in the extrusion of specific peptide and carbohydrate substrates in Gram-negative bacteria, such as *HlyB* (Felmlee et al., 1985), *LktB* (Stanfield et al., 1988), and *CyaB* (Glaser et al., 1988). The *mdr* gene family has been recently found to be a member of a larger family of sequence-related genes which include the *Saccharomyces cerevisiae* STE6 gene implicated in transport of the **a** mating pheromone (McGrath et al., 1989), the malarial *Plasmodium falciparum pfmdr1* gene associated with chloroquine resistance (Foote et al., 1989), the *CFTR* chloride channel in which mutations cause cystic fibrosis in humans (Riordan et al., 1989), and the *HAM/PSF/RING/mtp* peptide transporters participating in antigen presentation in lymphocytes (Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990; Deverson et al., 1990).

† This work was supported by grants to P.G. from the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Howard Hughes Medical Institute. P.G. is supported by a scientist award from the Fonds de Recherche en Santé du Québec and R.D. by a studentship from the Fonds Concerté d'Aide à la Recherche.
* To whom correspondence should be addressed.

[1] Abbreviations: VBL, vinblastine; COL, colchicine; ACT, actinomycin D; MDR, multidrug resistance; P-gp, P-glycoprotein; TM, transmembrane; LK, linker; BSA, bovine serum albumin; PCR, polymerase chain reaction; G418, genetycin; DMSO, dimethyl sulfoxide.

0006-2960/92/0431-6103$03.00/0

One of the major unresolved aspects of P-gp function is how a single transport protein can recognize a large group of structurally and functionally unrelated substrates such as the MDR drugs. In particular, the specific protein domains and amino acid residues implicated in substrate recognition and binding have not been identified. Energy-transfer experiments have suggested that hydrophobic drugs forming the MDR spectrum may be recognized by P-gp in association with the lipid bilayer (Raviv et al., 1990). Biochemical (Yoshimura et al., 1989; Bruggemann et al., 1989) and genetic analyses (Choi et al., 1988; Gros et al., 1991; Devine et al., 1991) of P-gp mutants strongly suggest that the predicted membrane-associated domains play an important role in this process. Striking functional differences have been detected in transfection experiments between members of the human and mouse *mdr* gene families. Human *MDR1* but not *MDR2* is overexpressed in drug-resistant cell lines (Chin et al., 1989) and in tumor specimens refractory to chemotherapy (Chan et al., 1990) and can directly confer MDR when transfected and overexpressed in otherwise drug-sensitive cells (Ueda et al., 1987; Schinkel et al., 1991). Mouse *mdr1* and *mdr3* but not *mdr2* are found overexpressed independently in MDR cell lines (Raymond et al., 1990), and can confer MDR in transfection experiments (Gros et al., 1986b, 1988; Devault & Gros, 1990). We have used the strong sequence homology and striking functional differences between members of the mouse *mdr* family to identify protein segments implicated in drug recognition. We have recently determined that the wild-type mouse *mdr1* gene and a mutant form of the *mdr3* (Phe^{939},*mdr3F*) gene confer similar degrees of vinblastine resistance in transfection experiments while *mdr1* confers preferential resistance to colchicine and *mdr3F* confers preferential resistance to actinomycin D (Devault & Gros, 1990; Gros et al., 1991). To map discrete segments responsible for the preferential drug resistance profiles expressed by each *mdr* parent, we have used restriction enzyme sites conserved between the two sequences or introduced by site-directed mutagenesis, to exchange in *mdr1*/*mdr3F* chimeric cDNA constructs homologous protein domains. The specific drug resistance profiles encoded by each chimera were then established in stably transfected cells clones expressing similar amounts of individual chimeric protein.

Materials and Methods

Construction of Chimeric cDNAs. The construction of chimeric *mdr* cDNA clones was performed according to a protocol that we have previously described (Buschman & Gros, 1991). Briefly, full-length cDNAs for the mouse *mdr1* and *mdr3* genes were previously isolated and inserted into the polylinker region of the plasmid pGEM-7Zf(+) (Promega, Madison, WN). cDNA subfragments to be exchanged were generated by restriction digestion of the respective *mdr1* and *mdr3* plasmids, using restriction enzyme sites conserved between the two clones. The fragments were separated by agarose (0.8–1.0%) gel electrophoresis and recovered from the gel matrix by extraction with hexadecyltrimethylammonium bromide (Langridge et al., 1980). Following purification, the *mdr1* and *mdr3* fragments were swapped, religated in the parental cDNA clone with T4 DNA ligase, and transformed into *Escherichia coli* strain JM 83. Chimeric constructs were screened for proper religation of the sites used for cloning and were distinguished from the parental *mdr1* and *mdr3* cDNAs by restriction mapping with diagnostic enzymes. Full-length hybrid cDNA inserts were excised from the polylinker of pGEM-7Zf(+) by digestion with *Eco*RI(*mdr1*) or *Sph*I/*Cla*I(*mdr3*) and purified by agarose gel electrophoresis, and their cohesive ends were repaired with T4 DNA polymerase. For expression studies, hybrid cDNAs were cloned into the mammalian expression vector pMT2 (gift of R. Kaufman, Genetics Institute, Cambridge, MA). All enzymes used for cloning were obtained from Pharmacia (Montreal, Canada) and were used under conditions recommended by the supplier.

Site-Directed Mutagenesis. A novel *Sal*I restriction site was introduced at the homologous position in *mdr1* (nt 412) and *mdr3* (nt 403) by mutagenesis using a two-step polymerase chain reaction (PCR) method. Oligonucleotides were as follows: 5′-TAGCATGCGTGATGGAGTTTGAAGAG-AAC-3′ (*mdr1*; nt −11 to +18) and 5′-TAGCATGCGTGATGGAACTTGAAGAG-3′ (*mdr3*; nt −11 to 15). They, in addition to priming synthesis, added an *Sph*I site (underlined) three nucleotides proximal to the ATG initiation codon. Two complementary mutant oligonucleotides were used independently to introduce the *Sal*I site. The first *Sal*I mutant oligo (5′-GTATCTGTGCACCAGCTGCC-3′; nt + 429 to +410 in *mdr1* and nt +420 to +401 in *mdr3*) was used in conjunction with the *Sph*I site oligonucleotide to generate 0.4-kb *Sph*I to *Sal*I fragments. The second and complementary *Sal*I oligo (5′-CTGGCAGCTGGTCGAC-AGATACACAAG-3′; nt +408 to +434 in *mdr1* and nt +399 to +425 in *mdr3*) was used in conjunction with a downstream oligonucleotide (5′-CCAGTGCATATGATGCAT-3′; nt +933 to +916 in *mdr1* and nt +924 to +907 in *mdr3*) overlapping an *Nde*I site to generate 0.5-kb *Sal*I to *Nde*I fragments. The two generated fragments were annealed and amplified in a second round of PCR using only the *Sph*I and *Nde*I oligonucleotides. The resulting 0.9-kb fragments were gel-purified, digested with *Sph*I and *Nde*I to create cohesive ends, and recloned into the full-length *mdr1* and *mdr3* cDNAs. The PCR reaction was performed with Taq DNA polymerase (BIO-CAN, Montreal, Canada) in a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 200 μg/mL bovine serum albumin (BSA), and deoxyribonucleotide triphosphates (200 μM each). Reaction cycles (25) for PCR were 94 °C for 1 min, 47 °C for 2 min, and 70 °C for 5 min.

DNA Transfection and Drug Selection Protocols. The biological activity of each chimeric cDNA cloned in pMT2 was tested by transfection into drug-sensitive Chinese hamster ovary LR 73 cells. Briefly, LR 73 cells were grown to subconfluency in 75 cm^2 tissue culture flasks (Nunclon) in alpha MEM medium supplemented with 10% fetal calf serum, 1 mM glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL). The cells were washed with phosphate-buffered saline (PBS)–citrate, harvested by trypsinization, and seeded at a density of 1 × 10^6 cells per 100-mm tissue culture plate, 16 h prior to transfection. Individual pMT2 construct DNAs (18 μg) were coprecipitated with the dominant selectable marker neomycin resistance gene (*Tn5*) of pSV2neo (Southern & Berg, 1982) (2.0 μg) and introduced into LR 73 cells as calcium phosphate coprecipitates, as described (Devault & Gros, 1990). Transfected cells were subcultured after 48 h in medium containing G418 (0.5 mg/mL), and G418^R clones were selected for 10–14 days. Mass populations of G418^R clones were harvested by trypsinization and expanded in culture, and frozen aliquots were prepared in 10% dimethyl sulfoxide and 90% serum and kept frozen at −70 °C. Independent drug-resistant cell clones were further isolated from these mass populations of G418^R clones by selection with the drug vinblastine (VBL). For this, approximately 1 × 10^6 cells from each mass population of cotransfected clones were plated in medium containing G418 and 50 ng/mL VBL. Cultures were fed every 3–4 days for up to 2 weeks or until individual

drug-resistant cell clones appeared. Mass populations yielding no clones at 50 ng/mL VBL were plated in medium containing lower VBL concentrations (10–15–25 ng/mL). Drug-resistant clones appearing at these concentrations were picked and subsequently grown in gradually increasing increments up to 50 ng/mL VBL. Independent cell clones resistant to 50 ng/mL VBL were expanded in culture, and several aliquots were frozen at −70 °C in 10% DMSO and 90% serum.

Detection of Chimeric P-gps. Wild-type and mutant P-gps were detected by Western blotting in independent cell clones transfected with chimeric *mdr* cDNA clones and selected for resistance to VBL. In preparation for immunoblotting, individual cell clones were grown to confluency in 175 cm^2 flasks containing 50 ng/mL VBL and harvested by trypsinization, and a crude membrane extract was prepared as described previously (Schurr et al., 1989). For further purification, membranes were centrifuged on a discontinuous sucrose gradient (Schurr et al., 1989). Proteins were determined using an amido black based assay (Bio-Rad), and the preparations were stored at −70 °C in 10 mM Tris, pH 8, and 40% glycerol. Membrane proteins from either crude extracts (20 μg) or purified sucrose gradients (5 μg) were separated by sodium dodecyl sulfate–polyacrylamide (7.5%) gel electrophoresis (SDS–PAGE) and transferred by Western blotting to nitrocellulose membranes. The blots were treated at 4 °C for 16 h with 1% bovine serum albumin (fraction V) and TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.02% Tween 20) to reduce nonspecific binding. P-gp was detected by incubating the blots (1 h at 20 °C) with the mouse anti-Pgp monoclonal antibody C219 (Centocor Inc., Philadelphia, PA) used at a dilution of 1:300. After the blots were washed with TBST, they were further incubated with a goat anti-mouse IgG antiserum linked to alkaline phosphatase (1:3000 dilution). The blots were washed and developed in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine (0.17 ng/mL) and nitroblue tetrazolium chloride (0.33 ng/mL) substrates. Blots were dried and photographed.

Drug Survival Measurements. For drug survival analyses, three individual cell clones expressing equivalent quantities of parental or chimeric P-gps were selected. Cells were thawed, grown overnight in selective medium, trypsinized, and diluted to 500 cells/mL. Five hundred cells of each VBL resistant clone along with *mdr1* and *mdr3* controls and drug-sensitive LR73 cells were plated (500 cells per 60-mm culture dishes) in increasing concentrations of VBL, COL, and ACT. The cells were grown for 9 days, plates were fixed with 4.0% formaldehyde and stained with 1% methylene blue, and colonies containing more than 50 cells were scored. The relative plating efficiency of each clone was calculated by dividing the mean number of colonies observed at a given drug concentration by the mean number of colonies formed by the same clone in control medium lacking drug, and is expressed as a percentage. The drug survival characteristics of certain cell clones were also measured using a microwell titer plate assay based on sulforhodamine B staining of total cellular proteins (Skehan et al., 1989).

Results

Construction of Hybrid cDNA Clones. Figure 1 illustrates the postulated structural domains of P-glycoproteins (P-gps) encoded by the *mdr1* and *mdr3F* cDNA clones, together with the key restriction enzyme sites used to construct the hybrids. The predicted ATP binding sites (ATP1 and ATP2), the linker domain (LK) separating the two homologous halves of each protein, and the two series of membrane-associated (TM)

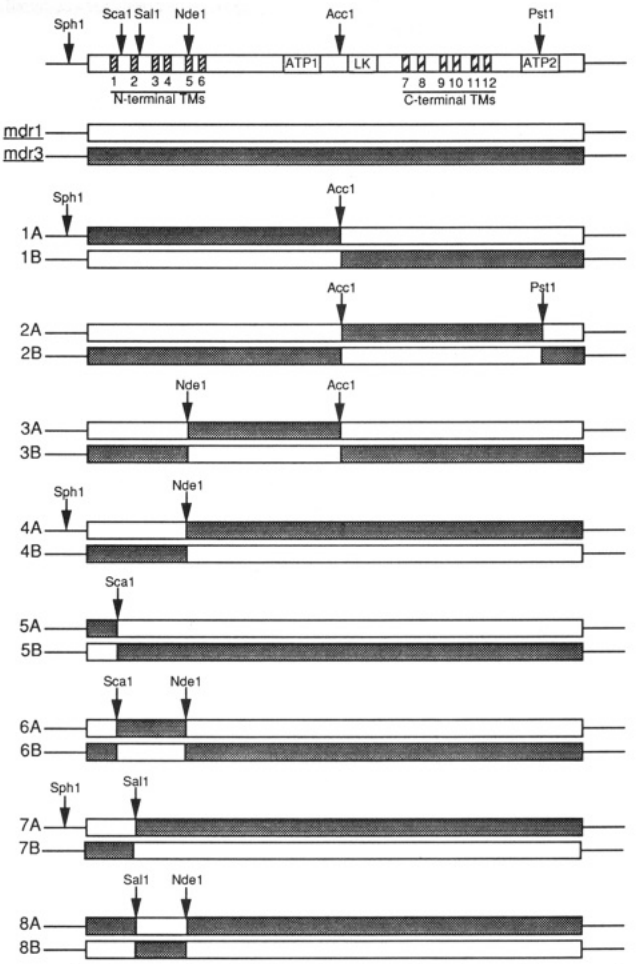

FIGURE 1: Construction of chimeric *mdr* cDNA clones. A schematic representation of a wild-type *mdr* cDNA and some of the predicted structural features of the protein are shown at the top. Hatched bars, TM domains numbered 1–12; ATP1 and ATP2, predicted nucleotide binding folds; LK, linker domain. Restriction enzyme sites used to construct the chimeras are identified by arrows above the cDNA. The *Sph*I site was from the polylinker of plasmid pGEM7Zf in which both parental *mdr* cDNAs were cloned. A schematic representation of *mdr1* (open bars) and *mdr3F* (shaded bars) cDNAs and the various chimeras (identified on the left) is shown. Thin lines identify untranslated regions, and the parental origin of each cDNA fragment is shown.

domains from the amino-terminal (TM1–6) and carboxy-terminal (TM7–12) halves are identified. Chimeric cDNA clones were constructed by exchanging homologous domains of *mdr1* and *mdr3F*, using restriction enzymes sites conserved in the nucleotide sequence of the two clones. The conserved sites used in these constructions included *Sph*I (within the polylinker segment of plasmid vector pGEM7Zf, in which both cDNAs were originally cloned), *Sca*I (pst 264 in *mdr3F* and pst 291 in *mdr1* with respect to the proposed initiator AUG codon), *Nde*I (pst 915 in *mdr3F* and pst 924 in *mdr1*), *Acc*I (pst 1756 in *mdr3F* and pst 1765 in *mdr1*), and *Pst*I (pst 3364 in *mdr3F* and pst 3371 in *mdr1*). Although polypeptides encoded by the *mdr1* and *mdr3F* cDNAs are predicted to contain 1276 amino acid residues, it is necessary to introduce 3 small gaps to optimize sequence alignments between them (Devault & Gros, 1990). Consequently, the first proposed extracellular loop of *mdr1* is predicted to be three residues longer than that of *mdr3F*, while the linker domain and extreme C-terminal segments of *mdr3F* are one and two residues longer than their counterparts in *mdr1*, respectively. Therefore, some of the chimeric proteins constructed here are predicted to differ in length from the respective *mdr1* and *mdr3F*

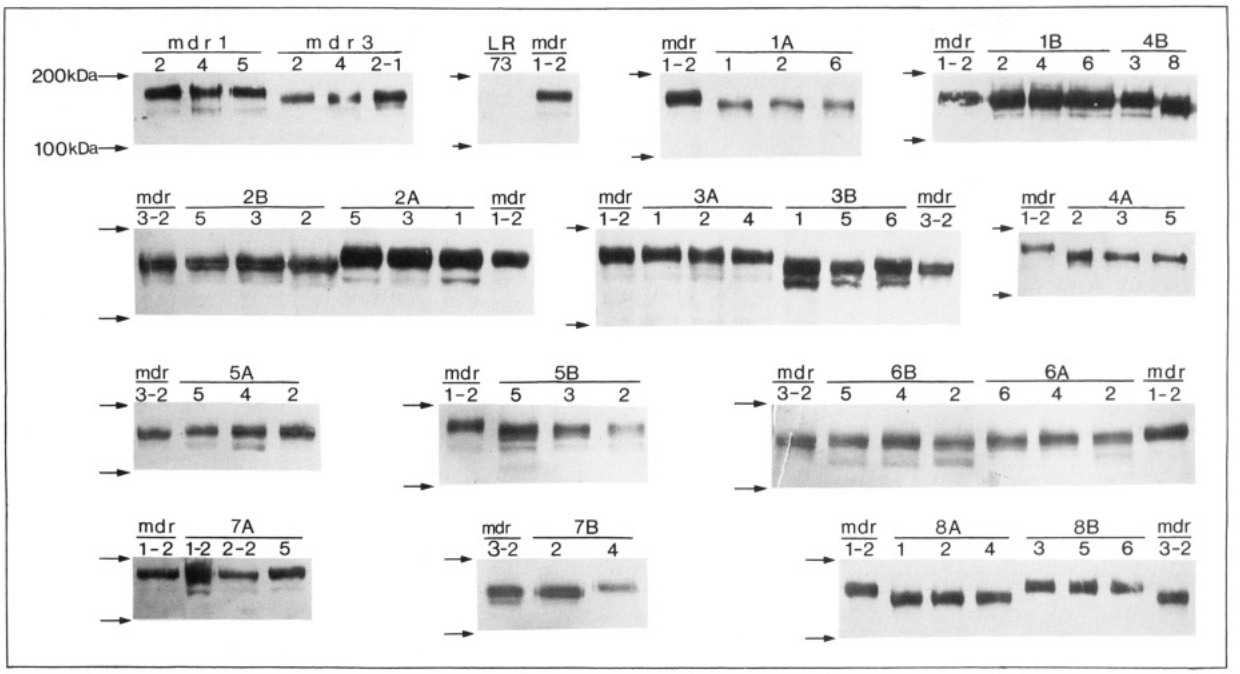

FIGURE 2: Detection of wild-type and chimeric P-gps in stably transfected cell clones. Two or three independent cell clones transfected with pSV2neo and either wild-type *mdr1* (1–2), or *mdr3F* (3–2), or chimeric *mdr* cDNAs, and selected in medium containing VBL (50 ng/mL), were analyzed for the presence of P-gps. Twenty (crude membrane extract) or five micrograms (sucrose-gradient-purified) of proteins was used for Western analysis with the mouse anti-P-gp monoclonal antibody C219. The molecular mass markers were myosin (200 kDa) and phosphorylase *b* (97 kDa).

parents by a few amino acid residues. All restriction sites used are located at the homologous position within the predicted domains of each parental cDNA except for *Sca*I; although this enzyme cuts within the first predicted extracellular loop in both parents, the actual site is not conserved and maps in *mdr3F* nine amino acids proximal to its position in *mdr1*. Therefore, hybrid cDNAs constructed using this enzyme (5 and 6 series) produce chimeric proteins with significant differences in the length of this predicted loop (see below).

The first constructs, 1A (1273 aa) and 1B (1279 aa), utilize the *Acc*I site to exchange the amino- and carboxy-terminal halves of each cDNA. This pair of constructs was created to determine if the preferential drug resistance profiles detected in the parental cDNAs could be segregated with either half of the encoded proteins. In the second set of constructs, 2A (1277 aa) and 2B (1275 aa), the highly divergent linker segment, the complete set of TM domains from the C-terminal half, and part of the second nucleotide binding site (ATP2) were exchanged, using *Pst*I and *Acc*I. In a third set of hybrids (3A and 3B), part of TM5 and TM6, together with the first predicted ATP binding site (ATP1), was exchanged, using *Nde*I and *Acc*I. The 4A (1279 aa) and 4B (1273 aa) hybrids were created using *Sph*I and exchange the amino-terminal segment of the two proteins, up to the predicted TM5. The predicted amino-terminal domains of *mdr1* and *mdr3F* were further studied in a series of additional constructs. This was prompted by the findings that mutations in this region of the human *MDR1* gene and the protozoan homolog *pfmdr1* were found to modulate the specificity of the drug efflux pumps encoded by these genes (Choi et al., 1988; Foote et al., 1990). First, the enzyme *Sca*I was used to exchange the extreme amino-terminal segment (N-terminus and TM1) in constructs 5A and 5B. Second, the segments overlapping TM2 to TM5 were exchanged in hybrids 6A and 6B, using *Sca*I and *Nde*I. As stated above, the *Sca*I constructs having an *mdr1* N-terminus, 5B (1285 aa) and 6A (1282 aa), encode for nine additional *mdr3F* residues (ITNOSGPNS) within the first predicted extracellular domain, possibly coding for a new N-linked glycosylation site (underlined). On the other hand, the *Sca*I constructs having an *mdr3F* N-terminus, 5A (1267 aa) and 6B (1270 aa), encode a shorter predicted extracellular loop, where the *mdr1* segment TNMSEADKLR has been deleted, removing one putative N-linked glycosylation site. A last series of constructs was created using a *Sal*I site genetically engineered in *mdr1* and *mdr3F*, immediately downstream of TM2. The N-terminal segment of each protein up to and including TM2 was exchanged in constructs 7A (1279 aa) and 7B (1273 aa). The predicted TM3 to TM5 segment included in the *Sal*I to *Nde*I fragment was also exchanged in constructs 8A and 8B.

Detection of Chimeric P-gp Molecules in Stably Transfected Cell Clones. Full-length wild-type and chimeric *mdr* cDNA clones were inserted in the mammalian expression vector pMT2, which utilizes viral regulatory elements to direct high levels of expression of cloned genes. These constructs were introduced in drug-sensitive Chinese hamster ovary LR73 cells by cotransfection with a dominant selectable marker (*Tn5*), included in the plasmid pSV2neo. Mass populations of stably cotransfected cells were isolated after selection in genetycin (G418). Independent cell clones expressing individual chimeric *mdr* constructs were then isolated from these mass populations of $G418^R$ cells by selection in medium containing VBL (see Materials and Methods). Membrane-enriched fractions were prepared from these clones, and the presence of corresponding wild-type or chimeric proteins was monitored by Western blotting, using the mouse anti-hamster P-glycoprotein antibody C219, which recognizes an identical epitope in *mdr1* and *mdr3F* (Figure 2). In some cases, chimeric proteins were also identified using *mdr1* and *mdr3F* isoform-specific antibodies (data not shown). At least two and sometimes three independent clones were tested for C219-reactive proteins. All VBL-resistant clones tested were found to express readily detectable P-gp. The levels of expression of P-gps among these various cell clones were similar, although not identical. These levels of expression also appeared similar to the levels of wild-type P-gps expressed in cell clones

Table I: Effects of Vinblastine on the Survival Characteristics of Stably Transfected Cell Clones Overexpressing Chimeric Mouse *mdr1* and *mdr3* Proteins[a]

	rel plating efficiency (%) for VBL concn (ng/mL) of								
construct	0	32	50	64	100	128	200	256	D_{50}
5A	100		100 ± 3		83 ± 6		13 ± 12		140 ± 13
2B	100		86 ± 10		80 ± 4		25 ± 8		135 ± 26
1A	100		90 ± 8		58 ± 26		18 ± 21		127 ± 48
mdr1	100		85 ± 8		43 ± 17		0		91 ± 22
mdr3	100		88 ± 12		37 ± 22		0		87 ± 23
3A	100		88 ± 6		28 ± 14		0.8 ± 1		79 ± 5
3B**	100	90 ± 19		53 ± 24		20 ± 11		6 ± 4	71 ± 28
4A	100		73 ± 3		22 ± 19		0.3 ± 0.6		71 ± 10
6B**	100		85 ± 6		4 ± 1		0		68 ± 2
5B	100		75 ± 8		12 ± 6		0		67 ± 1
6A**	100		67 ± 9		9 ± 2		0		61 ± 6
4B**	100		66 ± 18		7 ± 14		0		60 ± 11
8A	100		56 ± 4		10 ± 5		0		55 ± 3
1B**	100	78 ± 3		37 ± 3		13 ± 3		7 ± 3	52 ± 2
7B**	100		52 ± 15		1 ± 1		0		52 ± 8
2A*	100	78		18		5		0	50 ± 8
8B**	100	69 ± 1		38 ± 6		5		1.5 ± 0.7	50 ± 5
7A**	100		50 ± 5		7 ± 1		0		50 ± 4
LR 73									5.2 ± 0.4

[a] For each chimeric cDNA, three, two (two asterisks), or one (one asterisk) cell clone was plated in duplicate in increasing concentrations of VBL. The relative plating efficiency (average ± standard error) is shown for each drug concentration tested. The drug dose necessary to reduce the plating efficiency of cell clones expressing individual *mdr* cDNAs by 50% (D_{50}) is also shown (average ± standard error).

Table II: Effects of Colchicine on the Survival Characteristics of Stably Transfected Cell Clones Overexpressing Chimeric Mouse *mdr1* and *mdr3* Proteins[a]

	rel plating efficiency (%) for COL concn (ng/mL) of									
construct	0	30	60	100	200	300	400	600	1000	D_{50}
mdr1	100					82 ± 6		57 ± 13	5 ± 1	597 ± 93
3A	100					92 ± 19		42 ± 15	5 ± 2	503 ± 127
4B**	100			90 ± 1	92 ± 28	67 ± 5	55 ± 25	15 ± 21		405 ± 106
5A	100			101 ± 3	88 ± 7	59 ± 10	18 ± 9	0		310 ± 18
6B	100			98 ± 2	79 ± 11	21 ± 3	2.5 ± 0.8			238 ± 13
7A	100	87 ± 19	86 ± 11	61 ± 14	36 ± 38					167 ± 88
6A	100			88 ± 6	17 ± 10	2 ± 2	0			143 ± 8
8A	100			88 ± 6	73 ± 20	58 ± 23	11 ± 10			135 ± 7
7B**	100			62 ± 1	92 ± 28	67 ± 5	1 ± 2			132 ± 4
2B	100	93 ± 1	90 ± 1	76 ± 9	19 ± 13					129 ± 11
1B	100			69 ± 12	15 ± 2	1 ± 2	0			118 ± 13
5B	100	103 ± 3	91 ± 8	51 ± 19	2 ± 3					95 ± 17
2A	100	82 ± 8	79 ± 6	42 ± 14	0					90 ± 13
mdr3	100	102 ± 2	84 ± 11	26 ± 30	2 ± 2					86 ± 25
1A	100	97 ± 13	73 ± 9	37 ± 26	0					85 ± 21
4A	100	99 ± 10	85 ± 5	20 ± 23	0					77 ± 11
8B**	100	95 ± 9	73 ± 6	9 ± 5	0					69 ± 1
3B	100	91 ± 8	52 ± 16	1 ± 2	0					56 ± 11
LR 73										24 ± 4

[a] For each chimeric cDNA, three, two (two asterisks), or one (one asterisk) cell clone was plated in duplicate in increasing concentrations of COL. The relative plating efficiency (average ± standard error) is shown for each drug concentration tested. The drug dose necessary to reduce the plating efficiency of cell clones expressing individual *mdr* cDNAs by 50% (D_{50}) is also shown (average ± standard error).

transfected with the *mdr1* and *mdr3F* control cDNAs. The identification of P-gp in membrane fractions from all cell clones transfected with the various hybrid cDNA constructs suggests that individual chimeric proteins could be properly targeted to the cell membrane in these cells. These independent cell clones were then analyzed for their specific drug resistance phenotypes.

Drug Resistance Phenotypes Encoded by Chimeric P-gps. The drug resistance phenotypes of cell clones transfected and overexpressing individual chimeric *mdr* constructs were determined and compared for the drugs VBL (*mdr1* = *mdr3F*), COL (*mdr1* > *mdr3F*), and ACT (*mdr1* < *mdr3F*). For this, the plating efficiency of cell clones expressing equivalent amounts of individual chimeras was measured in medium containing increasing concentrations of these drugs, and is expressed as the D_{50}. The D_{50} is the drug concentration necessary to reduce the plating efficiency by 50%. Results of these analyses are summarized in Tables I (VBL), II (COL), and III (ACT).

For VBL, the levels of resistance conferred by control *mdr1* (D_{50} = 97 ng/mL) and *mdr3F* (D_{50} = 87 ng/mL) cDNAs were comparable, as previously established (Devault & Gros, 1990; Gros et al., 1991). All chimeric cDNA constructs appeared functional and were capable of conferring VBL resistance, as demonstrated by our ability to isolate VBL^r clones for all constructs after transfection and drug selection. The D_{50} values for VBL of cell clones expressing hybrid *mdr* cDNAs were determined and compared to those of *mdr1* and *mdr3F*. These D_{50} values fell into three overlapping groups. Constructs 5A (140 ng/mL), 2B (135 ng/mL), and 1A (127 ng/mL) appeared to confer levels of VBL resistance slightly superior to that expressed by *mdr1* or *mdr3F*. Constructs 3A (79 ng/mL), 3B (71 ng/mL), 4A (71 ng/mL), 6B (68 mg/mL), and 5B (67 ng/mL) were similar to wild-type *mdr1* and

Table III: Effects of Actinomycin D on the Survival Characteristics of Stably Transfected Cell Clones Overexpressing Chimeric Mouse *mdr1* and *mdr3* Proteins[a]

construct	rel plating efficiency (%) for ACT concn (ng/mL) of								D_{50}
	0	3	5	6	10	12	20	50	
5B	100		91 ± 13			89 ± 12	75 ± 7.0	5 ± 9	27 ± 2
mdr3	100		87 ± 8			81 ± 23	33 ± 10	0	16 ± 1
1A	100		77 ± 13			54 ± 24	5 ± 8	0.3 ± 0.6	12 ± 3
3B**	100	93 ± 2		78 ± 22		45 ± 30	14 ± 13		11 ± 4
2B**	100	99 ± 10		78 ± 7		52 ± 12	8 ± 6		11 ± 1
7A	100	85 ± 16		66 ± 9		28 ± 16	14 ± 18		8 ± 1
5A	100	96 ± 13		77 ± 26	27 ± 14		0		8 ± 2
mdr1	100	89 ± 8		66 ± 4	26 ± 17		0		8 ± 1
4A	100	85 ± 9		66 ± 8	33 ± 7		0		7.2 ± 0.3
2A	100	84 ± 6		65 ± 11		18 ± 12	0		7 ± 1
6A	100	95 ± 12		66 ± 12	19 ± 9		0		7.0 ± 0.9
7B	100	69 ± 9		61 ± 19		20 ± 19	0		7 ± 2
6B	100	95 ± 26		29 ± 12		1 ± 2	1 ± 2		5 ± 1
8B**	100	76 ± 1		24 ± 11		0	0		4.0 ± 0.2
8A	100	79 ± 11		21 ± 6		0	0		3.9 ± 0.4
3A	100	69 ± 11		14 ± 1	9 ± 11		3 ± 5		3.6 ± 0.3
1B	100	66 ± 2		25 ± 8		2 ± 1	0		3.6 ± 0.3
4B	100	70 ± 7		17 ± 11		1 ± 1.5	0		3.6 ± 0.1
LR 73									1.4 ± 0.8

[a] For each chimeric cDNA, three, two (two asterisks), or one (one asterisk) cell clone was plated in duplicate in increasing concentrations of ACT. The relative plating efficiency (average ± standard error) is shown for each drug concentration tested. The drug dose necessary to reduce the plating efficiency of cell clones expressing individual *mdr* cDNAs by 50% (D_{50}) is also shown (average ± standard error).

mdr3F, while constructs 6A (61 ng/mL), 4B (60 ng/mL), 8A (55 ng/mL), 1B (52 ng/mL), 7B (52 ng/mL), 2A (50 ng/mL), 8B (50 ng/mL), and 7A (50 ng/mL) appeared to express levels of VBL resistance somewhat lower than that of parental controls (Table I). The levels of VBL resistance expressed by individual chimeras were clearly superior to the control LR73 cells (5 ng/mL) and did not deviate from those of *mdr1* and *mdr3F* controls by a factor of more than 1.7. These results indicate that VBL resistance levels expressed by the various chimeras were similar but not identical to that of parental controls and that the capacity to confer resistance to this drug was retained in these constructs.

The COL resistance characteristics of cell clones expressing individual chimeras were determined and are tabulated in Table II. Cell clones expressing the two parental cDNAs *mdr1* (597 ng/mL) and *mdr3F* (86 ng/mL) show a 7-fold difference in the D_{50} values for this drug. The chimeric cDNAs tested were all found capable of conferring some degree of resistance to this drug, above the control LR 73 cells (24 ng/mL). The D_{50} values for COL for chimeric cDNAs fell into three major groups. Constructs 3A (503 ng/mL) and 4B (405 ng/mL) were similar to *mdr1* (597 ng/mL). On the other hand, constructs 1B (118 ng/mL), 5B (95 ng/mL), 2A (90 ng/mL), 1A (85 ng/mL), 4A (77 ng/mL), 8B (69 ng/mL), and 3B (56 ng/mL) appear to confer COL resistance levels similar to those detected in *mdr3F* (86 ng/mL), while constructs 5A (310 ng/mL), 6B (238 ng/mL), 7A (167 ng/mL), 6A (143 ng/mL), 8A (135 ng/mL), 7B (132 ng/mL), and 2B (129 ng/mL) appeared to confer COL resistance levels intermediate between those encoded by the two parental clones. No clear segregation of the high and low levels of COL resistance characteristics of *mdr1* and *mdr3F*, respectively, was observed with any specific segment of either parental cDNA clone. For example, constructs 1A/2B and 1B/2A which exchange the carboxy- and amino-terminal halves of each protein all exhibit low levels of COL resistance. On the other hand, the chimeric constructs found to confer the highest levels of COL resistance (3A, 4B, and 5A) all contained mostly *mdr1* sequences, in particular membrane-associated segments from either half of the protein.

Finally, the ACT resistance phenotype afforded by chimeric cDNAs was analyzed and compared to that conferred by wild-type *mdr1* and *mdr3F* (Table III). The D_{50} values for cell clones expressing the wild-type *mdr3F* (16 ng/mL) were found to be 2-fold higher than that of cell clones expressing *mdr1* (8 ng/mL). In all cases, hybrid *mdr* cDNAs conferred some level of ACT resistance above control values of drug-sensitive LR73 recipient cells (1.4 ng/mL). Construct 5B had a unique phenotype and conferred levels of ACT resistance (27 ng/mL) somewhat greater than that encoded by wild-type *mdr3F*. Results from other chimeras fell into three overlapping groups. Constructs 1A (12 ng/mL), 3B (11 ng/mL), and 2B (11 ng/mL) expressed phenotypes similar to that of *mdr3F* (16 ng/mL), while that of hybrids 7A (8 ng/mL), 5A (8 ng/mL), 4A (7 ng/mL), 2A (7 ng/mL), 6A (7 ng/mL), and 7B (7 ng/mL) was very similar to that of *mdr1* (8 ng/mL). A third group of chimeras, including 6B (5 ng/mL), 8B (4 ng/mL), 8A (4 ng/mL), 3A (4 ng/mL), 1B (4 ng/mL), and 4B (4 ng/mL), appeared to convey ACT resistance levels lower than *mdr1*. There was no clear segregation in *mdr* chimeras of the *mdr3F* preferential ACT resistance phenotype with any specific protein segment exchanged. As noted previously in hybrids 3A and 5A for the preferential COL resistance phenotype of *mdr1*, two of the *mdr* chimeras expressing ACT resistance levels comparable (3B, 11 ng/mL) or superior (5B, 27 ng/mL) to that of *mdr3F* retained mostly *mdr3F* sequences, including membrane-associated segments from either half of the protein.

Discussion

The goal of our study was to identify candidate protein segments and amino acid residues of P-glycoprotein that are important for substrate recognition and binding. In particular, we wished to delineate those segments responsible for the distinct drug resistance profiles encoded by two members of the mouse P-gp family. A comparison of the drug resistance profiles displayed by cell clones stably transfected and overexpressing cDNAs corresponding either to the wild-type mouse *mdr1* or to a mutant form of *mdr3F* (Ser to Phe, pst 939; Gros et al., 1991) shows that although the two P-gps encode similar levels of VBL resistance, *mdr1* encodes preferential COL resistance while *mdr3F* encodes increased levels of ACT resistance (Devault & Gros, 1990). We wished to identify in chimeric proteins produced by exchanging homologous do-

mains of the *mdr1* and *mdr3F* cDNAs the segments responsible for preferential COL and ACT resistance. For this, wild-type and chimeric cDNA constructs were introduced by transfection in drug-sensitive LR73 cells followed by selection of drug-resistant colonies in VBL. The specific drug resistance profiles of cell clones expressing comparable amounts of the various chimeras were then compared for the drugs COL and ACT.

Results from these experiments show that all chimeric proteins could be expressed in transfected cell clones and all conferred similar levels of VBL resistance. This is in agreement with the proposition that (i) both parental P-gps transport VBL by the same mechanism and (ii) VBL transport involves protein determinants that are conserved in both parents and that can be interchanged in chimeric proteins. On the other hand, the preferential COL resistance of *mdr1* and the preferential ACT resistance of *mdr3* could not be segregated in our series of chimeras with any specific protein domain. This is exemplified by results obtained with the 1A/1B and 2A/2B pairs of constructs which exchange large segments of the two homologous halves of P-gp, but where preferential COL and ACT resistance fails to segregate. Instead, several constructs produced levels of resistance intermediate between the two parents, this being most obvious in the case of COL resistance where the difference in resistance between the two parental clones is the greatest. Only a few constructs expressed parental levels of COL and ACT resistance. Of particular interest are the 3A/3B and 5A/5B pairs of constructs which express levels of COL resistance (3A, 5A) close to wild type, while the reciprocal constructs (3B, 5B) express near-wild-type ACT resistance levels. The only common characteristic of these constructs is that they contain large segments overlapping the majority of membrane-associated regions from both the amino- and carboxy-terminal halves of the respective parent. Taken together, these observations indicate that the preferential drug resistance profiles encoded by *mdr1* and *mdr3F* are not encoded by a single specific protein segment but rather involve several determinants from both homologous halves of P-gp, in general, and membrane-associated domains, in particular. These determinants may cooperate through complex interactions that seem to be disrupted in most chimeric constructs tested in our study.

The previous biochemical and genetic analyses of P-glycoprotein also suggest that drug recognition and binding involve the participation of membrane-associated domains from both homologous halves of P-gp. First, protease digestion of P-gp labeled with the photoaffinity probe azidopine reveals the presence of two labeled tryptic fragments of size 95 and 55 kDa. Epitope mapping of these fragments using site-specific antibodies indicates that these two fragments originate from the amino-terminal (95 kDa) and carboxy-terminal (55 kDa) halves of P-gp (Yoshimura et al., 1989). In an independent study, Bruggemann et al. (1989) have used four site-specific antibodies to further restrict the azidopine binding site(s) on P-gp to a 38-kDa tryptic fragment overlapping the TM 1–6 segment, and a 30-kDa segment overlapping the TM7–12 region. Second, while the mouse *mdr1* gene can confer drug resistance upon transfection into drug-sensitive LR 73 cells, the mouse *mdr2* cannot. The analysis of chimeric cDNA clones constructed between *mdr1* and *mdr2* identifies the membrane-associated domains as functionally distinct between the two proteins. Although both nucleotide binding sites of *mdr2* are functional and can complement the biological activity of *mdr1*, the introduction of as few as two TM domains from either half of *mdr2* into *mdr1* was enough to abrogate function (Buschman & Gros, 1991). Third, the functional characterization of independent P-gp mutants has revealed that mutations within the two large membrane-associated regions modulate the substrate specificity of P-gp. A single Val to Gly substitution near TM3 (pst 185) of the human *MDR1* strongly modulates the degree of colchicine resistance conferred by this gene in transfection experiments (Choi et al., 1988). In the mouse *mdr1* and *mdr3*, mutations within TM11 (pst 941 and 939, respectively) drastically modulate the overall activity and substrate specificities of the two encoded P-gps (Gros et al., 1991), while in the hamster *pgp-1* (*mdr3*) gene a Gly-Ala to Ala-Pro substitution within the predicted TM6 domain (pst 338–339) has been shown to cause increased resistance to actinomycin D (Devine et al., 1991). Finally, mutations in the TM1–2 interval (K1 allele, Asn to Tyr substitution, pst 86) and within TM11 (7G8 allele, Ser^{1034}/Asn^{1042} to Cys^{1034}/Asp^{1042} substitution) of the *Plasmodium falciparum mdr* homolog *pfmdr1* have been found to be associated with increased chloroquine efflux in drug-resistant isolates of the parasite (Foote et al., 1990), while mutating charged residues within TM1, TM6, and TM10 of the *CFTR* protein modulate the halide specificity of the channel (Anderson et al., 1991).

Taken together, our experimental results and the genetic and biochemical analyses of P-gp suggest that drug recognition and transport by P-gp involve complex interactions between structural elements encoded by the two homologous halves of P-gp. These elements interact to form a three-dimensional structure essential for transport. In the case of the *mdr1*/*mdr3* chimeras analyzed here, it appears that the spatial arrangement of specific determinants required for vinblastine transport was preserved in most chimeras tested. However, it appears that preferential COL and ACT resistance phenotypes encoded by each parent depend on a spatial structure that could not be recreated optimally in most chimeras tested. These results also suggest that transport of VBL by the two P-gps studied involves structural determinants distinct from those required for ACT/COL transport. This proposition is supported by the genetic analysis of P-gp mutants that retain the capacity to confer VBL resistance but have lost the ability to confer COL resistance (Gros et al., 1991). Identifying such structural determinants is an important prerequisite to the rational design of compounds capable of overcoming the transport activity of P-gp in drug resistant tumor cells.

Acknowledgments

We thank Drs. N. Sonenberg and R. Mackenzie (McGill University) for critical review of the manuscript and helpful discussions during the course of this work and Dr. R. Kaufman (Genetics Institute) for the gift of pMT2.

Registry No. VBL, 865-21-4; ACT, 50-76-0; COL, 64-86-8.

References

Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Sucharita, P., Mulligan, R. C., Smith, A. E., & Welsh, M. J. (1991) *Science 253*, 202–205.

Bruggemann, E. P., German, U. A., Gottesman, M. M., & Pastan, I. (1989) *J. Biol. Chem. 264*, 15483–15488.

Buschman, E., & Gros, P. (1991) *Mol. Cell. Biol. 11*, 595–603.

Chan, H. S. L., Thorner, P., Haddad, G., & Ling, V. (1990) *J. Clin. Oncol. 8*, 689–704.

Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., & Roninson, I. B. (1986) *Cell 47*, 381–389.

Chin, J. E., Soffir, R., Noonan, K. E., Choi, K., & Roninson, I. B. (1989) *Mol. Cell. Biol. 9*, 3808–3820.

Choi, K., Chen, C. J., Kriegler, M., & Roninson, I. B. (1988) *Cell 53*, 519–529.
Cornwell, M. M., Safa, A. R., Felsted, R. L., Gottesman, M. M., & Pastan, I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3847–3850.
Cornwell, M. M., Tsuruo, T., Gottesman, M. M., & Pastan, I. (1987) *FASEB J. 1*, 51–54.
Devault, A., & Gros, P. (1990) *Mol. Cell. Biol. 10*, 1652–1663.
Deverson, E. V., Gow, I. R., Coadwell, J., Monaco, J. J., Butcher, G. W., & Howard, J. C. (1990) *Nature 348*, 738–741.
Devine, S. E., Ling, V., & Melera, P. W. (1991) *Proc. Am. Assoc. Cancer Res. 32*, 363.
Endicott, J. A., & Ling, V. (1989) *Annu. Rev. Biochem. 58*, 137–171.
Felmlee, T., Pellet, S., & Welch, R. A. (1985) *J. Bacteriol. 163*, 94–105.
Foote, S., Thompson, J. K., Cowman, A. F., & Kemp, D. J. (1989) *Cell 57*, 921–930.
Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M. J., Forsyth, K., Kemp, D. J., & Cowman, A. F. (1990) *Nature 345*, 255–258.
Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A., & Danchin, A. (1988) *EMBO J. 7*, 3997–4004.
Gros, P., Croop, J., & Housman, D. E. (1986a) *Cell 47*, 371–380.
Gros, P., Ben Neriah, Y., Croop, J. C., & Housman, D. E. (1986b) *Nature 323*, 728–731.
Gros, P., Raymond, M., Bell, J., & Housman, D. E. (1988) *Mol. Cell. Biol. 8*, 2770–2778.
Gros, P., Dhir, R., Croop, J., & Talbot, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7289–7293.
Hamada, H., & Tsuruo, T. (1988) *J. Biol. Chem. 263*, 1454–1458.
Hsu, S. I. H., Cohen, D., Kirschner, L. S., Lothstein, L., Harstein, M., & Horwitz, S. B. (1990) *Mol. Cell. Biol. 10*, 3596–3606.
Langride, J., Langridge, P., & Berquist, P. L. (1980) *Anal. Biochem. 103*, 264–271.
McGrath, J. P., & Varshavsky, A. (1989) *Nature 340*, 400–404.
Monaco, J. J., Cho, S., & Attaya, M. (1990) *Science 250*, 1723–1726.
Moscow, J. A., & Cowan, K. H. (1988) *J. Natl. Cancer Inst. 80*, 14–16.
Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I., & Gottesman, M. M. (1990) *J. Biol. Chem. 265*, 3975–3980.
Raymond, M., Rose, E., Housman, D. E., & Gros, P. (1990) *Mol. Cell. Biol. 10*, 1642, 1651.
Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczack, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., & Tsui, L. C. (1989) *Science 245*, 1066–1073.
Roninson, I. B. (1991) *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*, Plenum Press, New York.
Safa, A. R., Glover, C. J., Meyers, M. B., Biedler, J. L., & Felsted, R. L. (1986) *J. Biol. Chem. 261*, 6137–6140.
Safa, A. R., Metha, N. D., & Agresti, M. (1989) *Biochem. Biophys. Res. Commun. 162*, 1402–1408.
Schinkel, A. H., Roelofs, M. E. M., & Borst, P. (1991) *Cancer Res. 51*, 2628–2635.
Schurr, E., Raymond, M., Bell, J. C., & Gros, P. (1989) *Cancer Res. 49*, 2729–2734.
Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J., Bokesch, H., Kenney, S., & Boyd, M. R. (1989) *Proc. Am. Assoc. Cancer Res. 30*, 612.
Southern, P. J., & Berg, P. (1982) *J. Mol. Appl. Genet. 1*, 327–341.
Spies, T., & De Mars, R. (1991) *Nature 351*, 323–324.
Stanfield, S. W., Ielpi, L., O'Brochta, D., Helinski, D. R., & Ditta, G. S. (1988) *J. Bacteriol. 170*, 3523–3530.
Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., & Kelly, A. (1990) *Nature 348*, 741–743.
Ueda, K., Cardarelli, C., Gottesman, M. M., & Pastan, I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3004–3008.
Van der Bliek, A. M., Baas, F., Ten Houte de Lange, T., Kooiman, P. M., Van der Velde-Koerts, T., & Borst, P. (1987) *EMBO J. 6*, 3325–3331.
Yoshimura, A., Kuwazuru, Y., Sumizawa, T., Ichikawa, M., Ikeda, S. I., Ueda, T., & Akiyama, S. I. (1989) *J. Biol. Chem. 264*, 16282–16291.