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Multidrug Resistance in Cells Transfected with Human Genes Encoding a Variant P-Glycoprotein and Glutathione S-Transferase-π

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SUMMARY

The nucleotide sequence of the *mdr1* gene encoding a putative drug efflux pump (P-glycoprotein) is homologous to a class of bacterial membrane-associated transport proteins. These bacterial proteins are part of a multicomponent system that includes soluble periplasmic proteins that bind substrates, channeling them through the membrane in an energy-dependent manner. We have investigated the possibility that a similar multicomponent transport system exists in a multidrug-resistant human MCF-7 breast cancer cell line that was initially selected for resistance to doxorubicin (AdrR MCF-7). AdrR MCF-7 cells overexpress both the *mdr1* gene and the π class isozyme of glutathione *S*-transferase (GST-π) (EC 2.5.1.18). The latter is one of several isozymes known to have a ligand-binding function in addition to drug-metabolizing capabilities. Although we have recently shown that transfection of a functional GST-π expression vector is insufficient to confer resistance to doxorubicin in cells that lack P-glycoprotein expression [*Mol. Pharmacol.* 36:22–28 (1989)], we examined the possibility that GST-π interacts with P-glycoprotein to alter multidrug resistance. To do this, we have cloned cDNAs encoding these proteins from AdrR MCF-7 cells, constructed expression vectors containing these two genes, and transfected these vectors sequentially into drug-sensitive MCF-7 cells. The human *mdr1* cDNA isolated from AdrR MCF-7 is a variant gene whose sequence differs from that isolated previously from vinblastine-resistant KB cells [*Cell* 53:519–529 (1989)], resulting in an amino acid substitution of alanine to serine at position 893 (*mdr1*/893ala). Transfection of eukaryotic expression vectors containing the *mdr1* gene isolated from AdrR MCF-7 cells produced a multidrug-resistant phenotype in recipient cells, with a cross-resistance pattern similar to that in the AdrR MCF-7 cells. To determine whether GST-π expression could augment resistance provided by *mdr1*, two clones transfected with *mdr1*, one with high levels (153% of *mdr1* RNA in AdR MCF-7 cells) and one with low levels (10% of *mdr1* RNA in AdrR MCF-7 cells), were subsequently cotransfected with a GST-π expression vector and pSVNeo and selected for resistance to G418. Six of these clones contained levels of GST-π that were 8- to 18-fold greater than GST levels found in *mdr1*-expressing clones transfected with nonspecific DNA. We found no difference in the degree of resistance to doxorubicin, actinomycin D, and vinblastine between the clones expressing *mdr1* only and the clones expressing both *mdr1* and GST-π. Therefore, under these conditions, GST-π does not appear to act in conjunction with P-glycoprotein to alter the pattern or level of multidrug resistance in MCF-7 cells.

The role of P-glycoprotein in the development of MDR *in vitro* has been firmly established (reviewed in Refs. 1 and 2). This membrane glycoprotein is overproduced in tissue culture cell lines that display a broad spectrum of resistance to a wide variety of cytotoxic agents (3, 4). These cell lines also exhibit overexpression and frequently amplification of *mdr* gene sequences, which encode P-glycoprotein (5–8). P-glycoprotein apparently enhances cellular resistance to toxins by acting as a drug efflux pump that decreases intracellular drug accumulation in an energy-dependent process (9–12). Previous reports have demonstrated that transfection of *mdr* genes encoding P-glycoprotein is sufficient to cause the phenotype of MDR (13, 14). However, the observation that each cell line selected for MDR displays a different pattern of resistance and cross-resistance suggests that additional intracellular changes may be involved in the development of the full expression of MDR.

This diversity cannot be explained simply by the expression of different *mdr* genes. Although multiple *mdr* genes exist in mouse, hamster, and human cells, only a limited number (one in human, *mdr1*, and two in mouse and hamster) have actually been implicated in the development of antineoplastic drug resistance (13–19). Studies by Choi and co-workers (20) indicate that alterations in the P-glycoprotein amino acid sequence may provide additional phenotypic diversity. They demonstrated that a single point mutation in the *mdr1* gene isolated from KB cells selected for primary resistance to colchicine

ABBREVIATIONS: MDR, multidrug resistance; AdrR, doxorubicin (Adriamycin)-resistant; WT, wild type; GST, glutathione *S*-transferase; CMV, cytomegalovirus; SSC, standard saline citrate; IC_{50}, drug concentration required to inhibit colony formation of the cells by 50%; kb, kilobase, bp, base pair, MOPS, 3-(*N*-morpholino)propanesulfonic acid.

apparently encodes a P-glycoprotein that produces a different pattern of resistance, one with higher levels of colchicine resistance relative to other agents.

Changes in the intracellular milieu may also effect the function of the P-glycoprotein and, thus, contribute to the diversity in patterns of MDR. Nucleotide sequence analysis of genes encoding the mammalian P-glycoprotein have revealed that these proteins have regions of homology with several bacterial membrane transport proteins (21–23). These bacterial proteins belong to a multicomponent system that transports substrates such as amino acids and sugars. In this system, a periplasmic protein binds the substrate and the resulting protein-ligand complex is transported through a membrane protein in an energy-dependent fashion (24, 25). Another bacterial transport system involves export of the protein hemolysin directly through the membrane pore (26). It has been suggested that a similar multicomponent system exists for toxin transport in mammalian cells expressing the phenotype of MDR (23, 24).

We have previously reported the isolation of a multidrug-resistant human breast cancer cell line that was initially selected for resistance to doxorubicin, AdrR MCF-7 (27, 28). This cell line has amplified and overexpressed *mdr1* gene sequences (29). Other biochemical changes have also been described in the AdrR MCF-7 cells, including increased activity of the π class isozyme of GST (GST-π) (27, 30), which is associated with increased expression, but not amplification, of the GST-π gene.

Concurrent elevation of Pi class GST activity with overexpression of P-glycoprotein has been described in two other models of drug resistance. In a rat model of hepatocellular carcinogenesis, preneoplastic hepatocytes become resistant to xenobiotics (31) and to antineoplastic agents (32). In these cells, resistance is also associated with increased expression of both P-glycoprotein and the rat Pi class GST (GST-P) (33–35). In addition, v-H-*ras*-transformed rat liver epithelial cells demonstrate a multidrug-resistant phenotype along with increased *mdr1* and GST-P gene expression (36). Because of the parallel overexpression of P-glycoprotein and Pi class GST in these models of MDR, and the observation that GSTs are potent binders of lipophilic toxins (37–39), it is possible that this cytosolic protein may act in conjunction with P-glycoprotein by presenting protein-bound toxins to the membrane-bound multidrug transporter.

To test this hypothesis, we cloned complete cDNA sequences for both the *mdr1* and GST-π genes from AdrR MCF-7 cells and developed expression vectors containing these sequences. We have recently reported that transfection of the GST-π gene results in low levels of resistance to the carcinogens benzo[*a*]pyrene and benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide but does not appear, by itself, to alter cellular sensitivity to doxorubicin (40). In this study, we first transfected the *mdr1* expression vectors into drug-sensitive WT MCF-7 cells and analyzed the pattern of resistance produced by the expression of this gene. Two *mdr1*-transfected clones were then transfected with a GST-π expression vector, in order to determine whether GST-π expression can alter the pattern or level of MDR produced by P-glycoprotein alone.

Materials and Methods

Construction of plasmids. We previously described the isolation of a 3.08-kb cDNA (pADR-1) from the AdrR MCF-7 cell line, which contains the 3′ region of this gene including a polyadenylation site (29). This cDNA, pADR-1, recognizes a 4.8-kb mRNA that is overexpressed in the AdrR MCF-7 cells. We have obtained additional cDNA sequences by screening the same library with a portion of the mouse *mdr2* gene, which contains the 5′ region of this gene (kindly provided by P. Gros, McGill University) and which cross-hybridizes with the human *mdr1* gene (13). One of the isolated sequences (pADR-2) is 1295 bp in length and extends 5′ from the internal *Eco*R1 site. The complete nucleotide sequences of pADR-1 and pADR-2 were analyzed using the dideoxy sequencing procedure (Sequenase; United States Biochemical, Cleveland, OH) and specific oligonucleotide primers. Sequence analysis indicated that these two cDNA clones (pADR-1 and pADR-2) together contained the complete human *mdr1*-coding region, in addition to 119 bp of 5′ leader sequence and 402 bp of 3′ untranslated region.

A human *mdr1* expression vector was constructed by ligation of pADR-1 and pADR-2 into a vector, pMTP (generously provided by Drs. Dwight Kaufman and Neal Rosen, National Cancer Institute), (40), that contains a human metallothionein IIA promoter and an SV40 polyadenylation signal, and the expression vector is referred to as pMTAdr (see Fig. 2). Two additional expression vectors were constructed by ligation of the *Bam*HI fragment from pMTAdr, which contained the entire *mdr1* cDNA. The vector pHD1013, kindly provided by Drs. Michelle Davis and Eng-Shang Huang (University of North Carolina) (41), contained the CMV immediate-early promoter. The vector pBC12/CMV/IL2 (42), which contained a CMV promoter as well as an intron and polyadenylation signal from the rat preproinsulin gene, was generously provided by Dr. Brian Cullen (Roche Institute). The latter vector contained a cDNA for interleukin 2, which was removed and replaced with the *Bam*HI/*Hin*dIII portion of the multiple cloning site from pUC18, which allowed insertion of the *Bam*HI fragment from pMTAdr. These two expression vectors were designated as pHDAdr and pBCAdr, respectively.

The cDNA for human GST-π was also cloned from AdrR MCF-7 cells (30, 43, 44). This 725-bp full length cDNA, which contains the polyadenylation signal and extends 17 bp upstream from the start of translation, was inserted into the *Eco*RI site of the pMTP expression vector and is referred to as pMTG. The construction of this expression vector is described elsewhere (40).

Cell culture and transfection of expression vectors. The human breast cancer cell lines WT MCF-7 and AdrR MCF-7 were maintained in improved minimal essential medium containing 5% fetal bovine serum, as previously described (27). Transfections were performed using a standard DNA-calcium phosphate precipitate procedure (45). Briefly, 2.75×10^6 WT MCF-7 cells were plated onto 10-cm plates and, 24 hr later, 20 μg of plasmid DNA were precipitated with calcium phosphate and applied to the cells. The ratio of DNA from *mdr1* expression vectors (pMTAdr, pHDAdr, or pBCAdr) to pSVNeo was 10:1. Control cells were transfected with pUC8 and pSVNeo in a 10:1 ratio. After 16 hr, the DNA precipitate was removed and replaced with fresh medium. Twenty-four hours later, the cells were split 1:3 into medium containing G418 (0.75 mg/ml) or doxorubicin (40 or 100 nM). After 3 weeks of selection, colonies that developed were isolated and expanded for analysis.

For analysis of GST-π, MCF-7 cells were transfected with the *mdr1* expression vector pBCAdr and selected in doxorubicin. Colonies were isolated and studied for resistance as described above. Two of the clones that exhibited the MDR phenotype and contained the *mdr1* expression vector were then transfected with pMTG or pUC8 and pSVNeo (20 μg in a 10:1 ratio), in the same manner as described above, and selected in G418 (0.75 mg/ml). Colonies resistant to G418 were isolated and assayed for GST-π activity and drug resistance.

Southern and Northern blot analyses. DNA and RNA were isolated from cell lines using guanidinium isothiocyanate followed by cesium chloride centrifugation, as previously described (46, 47). DNA removed from the cesium chloride gradient was diluted at least 1:2 with distilled water and extracted twice with butanol/chloroform (4:1), twice with phenol, twice with phenol/chloroform (1:1), and twice with chlo-

roform. DNA was then dialyzed against 10 mM Tris (pH 7.4), 1 mM EDTA.

For Southern blot analysis, DNA was digested with the appropriate restriction enzyme, separated by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) using 10× SSC (1× SSC = 0.15 M NaCl, 0.15 M sodium citrate), according to the manufacturer's instructions. After baking for 2 hr at 80°, blots were prehybridized for 4 hr and hybridized overnight at 42° with a 347-bp fragment from the 5′ region of pADR-2 (position −119 to position 229), which was ^{32}P-labeled by nick translation. The prehybridization and hybridization buffers were those recommended by the manufacturer.

For Northern blot analysis, 20 μg of total cellular RNA were separated by electrophoresis on a 1% agarose, 0.6 M formaldehyde, 0.02 M MOPS gel, stained with ethidium bromide to check for equal loading, and transferred to nitrocellulose using 20× SSC (48). After baking for 2 hr at 80°, the filter was prehybridized at 42° for 4 hr and hybridized at the same temperature overnight with pADR-1, which was ^{32}P-labeled by nick translation as previously described (33). All autoradiography was done −70°.

For RNA slot blot analysis, total cellular RNA was blotted to nitrocellulose according to the manufacturer's instructions. Hybridization conditions were the same as for Northern analysis, using radiolabeled pADR-1 probe. The blot was stripped by boiling for 10 min in distilled H_2O and hybridized with radiolabeled β-actin cDNA (Oncor, Gaithersburg, MD) to determine equivalence of RNA loading. The autoradiographs were measured by densitometry and the resulting values were expressed as a percentage of *mdr1* RNA, relative to that in AdrR MCF-7 cells and normalized for β-actin RNA expression.

Primer extension analysis. A 20-bp primer was synthesized (Midland Certified Reagent Co., Midland, TX) that is complementary to a region 40 bp upstream from the start of translation of the *mdr1* RNA. This primer was end-labeled with [^{32}P]ATP and annealed to total cellular RNA isolated from these cell lines. Deoxynucleotides and reverse transcriptase were added and the labeled primer was extended to the end of the *mdr1* mRNA (44). Dideoxy sequencing reactions, using a genomic fragment containing the 5′ sequences of the human *mdr1* gene as a template, the same 20-bp primer, [^{32}P]dATP, and reverse transcriptase, provided convenient size markers to determine the length of the extension products. The extension products and the sequencing reactions were run on a 6% polyacrylamide, 7 M urea, sequencing gel for size determination.

Assays for drug resistance. Cytotoxicity was assessed in WT and AdrR MCF-7 cells and the transfected clones by colony-forming assay. Four hundred cells were plated in duplicate in 12-well Linbro dishes. The medium was replaced after 24 hr with fresh medium containing increasing concentrations of either doxorubicin, actinomycin D, vinblastine, or colchicine. The cells were exposed to drug continuously for 10 days, at which time the medium was removed and the colonies were stained with methylene blue/methanol (0.1 g/60 ml) for 10 min. The colonies were counted after the plates were rinsed with distilled water and allowed to air dry. The relative colony-forming efficiency is expressed as the percentage of the number of colonies formed in control wells containing no drug compared with colonies formed in the presence of drug. The results represent the mean of at least two separate experiments.

Assay for GST activity. Total GST activity was assayed in subconfluent cultures grown in 25-cm^2 flasks. Cells were harvested by scraping into 1× phosphate-buffered saline and pelleted by centrifugation at 500× *g* for 5 min. Cell pellets were resuspended in 3 to 4 volumes of 50 mM Tris·HCl, pH 7.5, containing 5 mM EDTA, and were sonicated at 4°. Total GST activity was assayed using 1-chloro-2,4-dinitrobenzene as substrate, as described (49). One unit of GST activity is defined as that which will conjugate 1 nmol of substrate/min with glutathione.

Western blot analysis. Subconfluent MCF-7 cells were harvested and washed with 1× phosphate-buffered saline. The cell pellet was resuspended in lysis buffer (50 mM Tris·HCl, pH 7.4, containing 10 mM EDTA). After sonication and centrifugation, 150 μg of cytosolic protein were electrophoresed on a 13.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose, using a semidry electroblotting apparatus (LKB) (30). The blots were blocked with 5% milk and incubated overnight in 5% milk containing a 1:500 dilution of rabbit antibodies directed against either the human α, μ, or π class GSTs. These antisera have been shown by Western blotting to be specific for each class of GST, with no cross-reactivity among classes. After several washes in 5% milk, the blot was incubated for 2 hr at room temperature in 5% milk containing a 1:500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel). The blots were developed in phosphate-buffered saline containing 0.5 mg/ml 4-chloro-1-napthol, 15% methanol, and 0.015% H_2O_2 (50).

Results

Variant *mdr1* cDNA in AdrR MCF-7 cells. We have isolated two human nonoverlapping *mdr1* cDNAs (pADR-1 and pADR-2), which span the entire gene. Sequence analysis of these two clones indicates that they contain the entire protein-coding region, as well as 119 bp of 5′ and 402 bp of 3′ untranslated sequences. There are two nucleotide changes in this *mdr1* cDNA isolated from AdrR MCF-7 cells, compared with that previously isolated from vinblastine-resistant KB cells (20, 22, 51). The first is a silent substitution of cytosine to thymidine at nucleotide 3435 (nucleotide 1 is assigned to the start of translation). The second change is a guanine to thymidine substitution at nucleotide 2677 (Fig. 1A), which results in a change of alanine to serine at amino acid 893 (Fig. 1B). These differences are apparently not an artifact of cloning, because an independent clone (pADR-3) containing this region, obtained from the same library, had precisely the same changes (Fig. 1A). Furthermore, the sequence of an *mdr1* cDNA isolated from normal adrenal cells has been reported that also contains these two sequence alterations (52). A comparison of the differences in nucleotide and amino acid sequences of the P-glycoprotein produced in the AdrR MCF-7 cells, vinblastine-selected KB cells (KB-V1), and colchicine-selected KB cells (KB-C2.5) is shown in Fig 1B.

Transfection of *mdr1* DNA. Human *mdr1* expression vectors were constructed as described in Materials and Methods (Fig. 2). MCF-7 cells were transfected with each of the *mdr1* expression vectors and were selected at low (40 nM) or high (100 nM) concentrations of doxorubicin. A total of 19 doxorubicin-resistant colonies were isolated and expanded for further study. No colonies were observed in WT cells transfected with pUC8 and pSVNeo and selected in either doxorubicin concentration. Of the 19 resistant colonies, 14 had been transfected with the pBCAdr vector, 3 with the pHDAdr vector, and 2 with the pMTAdr vector. Fifteen of the 19 resistant clones were selected in medium containing 40 nM doxorubicin (frequency of 51 colonies/10^6 cells), whereas the other 4 colonies were selected at 100 nM doxorubicin (frequency of 15 colonies/10^6 cells).

Genomic DNA isolated from each of the resistant clones was analyzed by hybridization to a 347-bp fragment of the 5′ end of the *mdr1* gene. This probe hybridized to two fragments (6.4 and 3.0 kb) of *Eco*RI-digested genomic DNA representing the endogenous *mdr1* gene in WT MCF-7 cells (Fig. 3). These sequences were present in single copy level in WT MCF-7 cells and in each of the transfected clones and were highly amplified in the AdrR MCF-7 cells. This probe also hybridized to a 1.35-

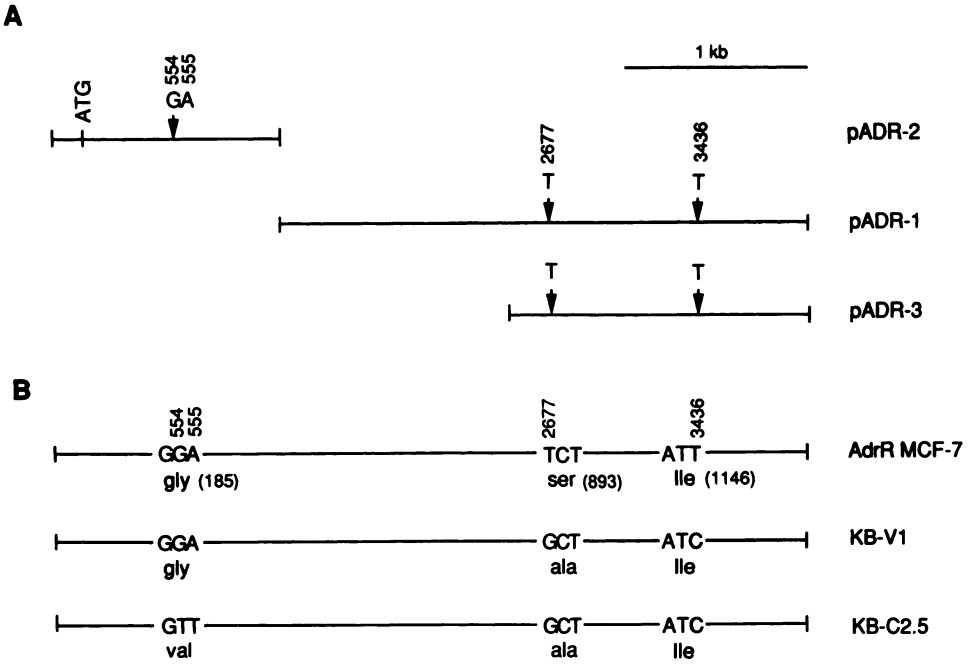

Fig. 1. *mdr1* cDNA clones isolated from AdrR MCF-7 cells. A, Maps for three *mdr1* cDNAs (pADR-1, pADR-2, and pADR-3) isolated from AdrR MCF-7 cells. pADR-1 and pADR-2 are nonoverlapping *mdr1* cDNA clones that together contain the complete coding sequence. The nucleotide sequence in these clones that differs from the sequence of the *mdr1* cDNA isolated from KB-C2.5 cells (22) is shown. The relative position of these base changes is indicated above each map. Position 1 is the start of translation. B, Amino acid sequence comparison of three *mdr1* cDNAs isolated from different multidrug-resistant cell lines. The position of the nucleotide changes in *mdr1* cDNAs isolated from AdrR MCF-7 cells, KB-V1 cells (20), and KB-C2.5 cells (22) are shown above each nucleotide, relative to the start of translation. The corresponding amino acid and its position (in parenthesis) are indicated.

kb *Eco*RI fragment (Fig. 3, *arrow*) in the transfected cell lines, which is the expected size fragment obtained from *Eco*RI digestion of the expression vectors. This size fragment was not detected in WT and AdrR MCF-7 cells. Some of the transfected clones contained multiple copies of the expression vectors, whereas others contained only a single copy. The level of selective pressure did not correlate with the relative number of copies of expression vector. For example, BCAdr-21 was selected at the low concentration of doxorubicin (40 nM) yet has multiple copies of vector present in its genome, whereas BCAdr-19, a clone selected at the higher concentration of doxorubicin (100 nM), has a single copy of expression vector. Under the conditions of these experiments, selection in 40 or 100 nM doxorubicin did not result in amplification of the endogenous *mdr1* gene.

Levels of *mdr1* RNA in transfected clones. The level of *mdr1* RNA expression in transfected clones was examined by Northern blot analysis (Fig. 4). These studies indicated that the RNA species produced in the transfected cells was approximately equal in size (4.8 kb) to that of the endogenous gene. In order to determine the origin of the *mdr1* RNA in the transfected cells, primer extension studies were performed. Because the *mdr1* cDNA in the expression vectors pBCAdr and pHDAdr was linked to the CMV promoter, the length of a primer extension product of the RNA transcribed from either of these expression vectors would be significantly longer than an extension product made from the *mdr1* mRNA transcribed from the downstream promoter of the endogenous *mdr1* gene. A schematic representation of the expected extension products is shown in Fig. 5A. In the AdrR MCF-7 cells, a major product of 97 bp was observed, which is the expected size for a transcript using the downstream *mdr1* promoter (51). There is no evidence in these cells of transcription from the upstream *mdr1* promoter (51). In pHDAdr- and pBCAdr-transfected cells, the sizes of the primer extension products were 241 and 214 bp, respectively, which are the expected sizes for transcription from the expression vectors (Fig. 5B). The 97-bp species was not seen in any of the transfected clones, indicating that the elevated levels of *mdr1* mRNA in these clones resulted from the transcription of the transfected expression vectors and not from the endogenous *mdr1* gene.

Slot blot analysis was performed to quantitate the level of RNA in each of the transfected clones. These studies demonstrated that the transfected cell lines contained *mdr1* RNA levels greater than those in WT MCF-7 cells, ranging from 10 to 177% of the *mdr1* RNA content of AdrR MCF-7 cells (Table 1). Several of the *mdr1*-transfected clones were examined for cell surface P-glycoprotein expression by fluorescence-activated cell sorting, using the MRK16 antibody (53). The results indicate that protein expression generally corresponded to RNA expression in both the transfectants and AdrR MCF-7 cells.[1] RNA levels for *mdr1* were variable in the different clones and unrelated either to the number of copies of expression vector contained in the genome or to the selective pressure (40 versus 100 nM doxorubicin).

Analysis of MDR in the transfected cell lines. Six of the transfected cell lines that exhibited either low or high *mdr1* RNA levels, compared with AdrR MCF-7, were examined by colony-forming assay for their patterns of cross-resistance to agents that produce the phenotype of MDR (Table 1). Sensitivity to four of the drugs involved in MDR were examined, doxorubicin, actinomycin D, vinblastine, and colchicine. All of the transfected clones were resistant to doxorubicin, although the level of resistance varied from 2.8- to 17-fold. Furthermore, each clone developed the phenotype of MDR. In general, these cell lines displayed higher resistance to actinomycin D and vinblastine than they did to doxorubicin and colchicine. This pattern of resistance (actinomycin D ≈ vinblastine > doxorubicin ≈ colchicine) is similar to the pattern of resistance in the AdrR MCF-7 cells, the cell line from which this *mdr1* cDNA was isolated. Except for clone HDAdr-1, the cell lines with relatively high *mdr1* RNA tended to be more resistant to the

[1] J. Trepel, L. Neckers, and C. R. Fairchild, unpublished data.

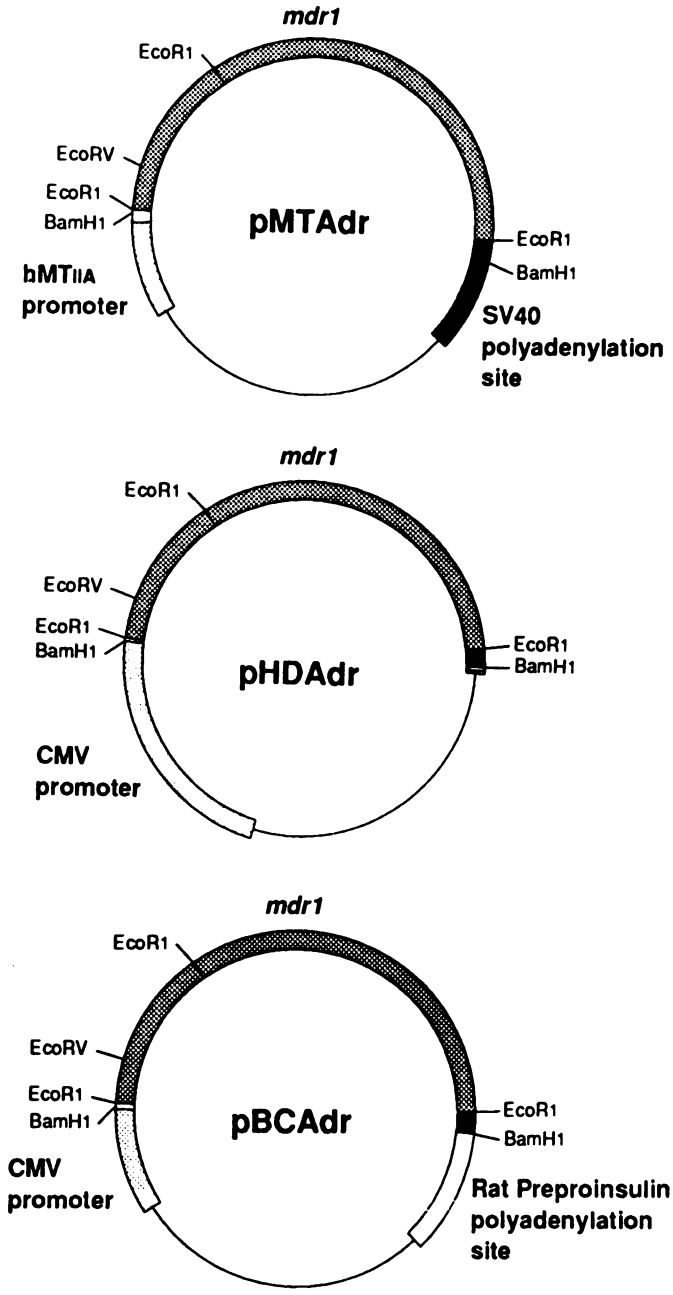

Fig. 2. Maps of *mdr1* expression vectors. The expression vector pMTAdr consists of the human metallothionein promoter, hMTIIA, the cDNAs pADR-1 and pADR-2 (joined at the internal *Eco*RI site), which together make the full length *mdr1* gene, and polyadenylation sequences from SV40. *mdr1* was removed from pMTAdr by *Bam*H, digestion and inserted into the expression vectors pHDAdr and pBCAdr. Therefore, both plasmids contain small regions of SV40 sequences. The vector pHDAdr contains a CMV immediate early gene promoter. The vector pBCAdr also uses CMV immediate early gene promoter sequences but has a polyadenylation signal from the rat preproinsulin gene. Selected restriction enzymes sites are shown.

various drugs than BCAdr-21, a cell line with a lower level of *mdr1* RNA. However, all of the clones displayed less resistance than observed in AdrR MCF-7 cells. The relatively high levels of *mdr1* RNA exhibited by some of the transfected clones, despite only modest levels of resistance, are similar to the findings in other reports (13, 14, 54).

Transfection of GST-π *mdr1*-transfected cell lines. In order to determine whether GST-π is able to act in concert with P-glycoprotein, two of the *mdr1*-transfected cell lines that expressed the phenotype of MDR were transfected with an expression vector containing the cDNA for GST-π. These two *mdr1*-transfected clones, BCAdr-19 an BCAdr-21, express high and low levels of *mdr1* RNA, respectively, and display the phenotype of MDR (Table 1). These two cell lines were subsequently transfected with pMTG and pSVNeo and selected in G418, as described in Materials and Methods. Colonies from each transfected cell line were expanded and analyzed for GST activity. The level of GST activity in WT MCF-7 cells is approximately 5 units/mg of protein and was similar in control transfected BCAdr-19 and BCAdr-21, which were transfected with pUC8 and pSVNeo and selected for G418 resistance in parallel. In comparison, the activity of GST in AdrR MCF-7 cells is 120 units/mg of protein. Three clones isolated from BCAdr-19 cells transfected with GST-π expression vector and pSVNeo had GST activities ranging from 40 to 78 units/mg of protein and two clones from transfected BCAdr-21 cells had activities of 44 and 50 units/mg of protein.

Northern blot hybridization using the GST-π cDNA as a probe showed strong hybridization to a GST-π mRNA in the transfected clones that was slightly longer than the endogenous message that was overexpressed in the AdrR MCF-7 cells (Fig. 6A). The difference in GST-π mRNA size in the transfected cells results from the increased size of the 5′ leader sequence of the metallothionein IIA promoter in the pMTG constructs and has been described previously (40).

That the GST activity in these transfected cells was due to increased expression of the π class isozyme was further shown by Western blot, using antibody to the π class GST isozyme (Fig. 6B). There was no reactivity in any of these transfected cells with either the human α or μ class antibodies (data not shown). Thus, the level of GST activity in these stably transfected clones results from increased expression of the π isozyme. Total GST activity was 8- to 18-fold higher than that for the WT MCF-7 cells and 30 to 65% of that present in the AdrR MCF-7 cells.

Analysis of the effect of GST-π expression on *mdr1* transfectants. The relative resistance of multidrug-resistant subclones transfected with GST-π expression vector was compared with the resistance of cells transfected with *mdr1* only (Table 2). As controls, the two clones BCAdr-19 and BCAdr-21 were transfected with pSV2Neo and selected for G418 resistance. The resulting cells, BCAdr-19(Neo) and BCAdr-21(Neo), had levels of resistance essentially identical to that of the original BCAdr-19 and BCAdr-21 clones, indicating that they had not changed during the time required for selection in G418. Furthermore, *mdr1* RNA levels did not vary significantly during the time for subsequent transfection and selection (data not shown). As shown in Table 2, although minor differences in resistance were seen, in general subclones that expressed both *mdr1* and GST-π have no consistent differences in drug resistance to any of the three drugs (doxorubicin, actinomycin D, or vinblastine), compared with the clones expressing only *mdr1*. Therefore, we conclude that GST-π, under the conditions of these studies, does not significantly alter the level of resistance conferred by *mdr1* alone.

Discussion

Previous studies have indicated that P-glycoprotein alone is sufficient to produce the phenotype of MDR (13, 14). In the

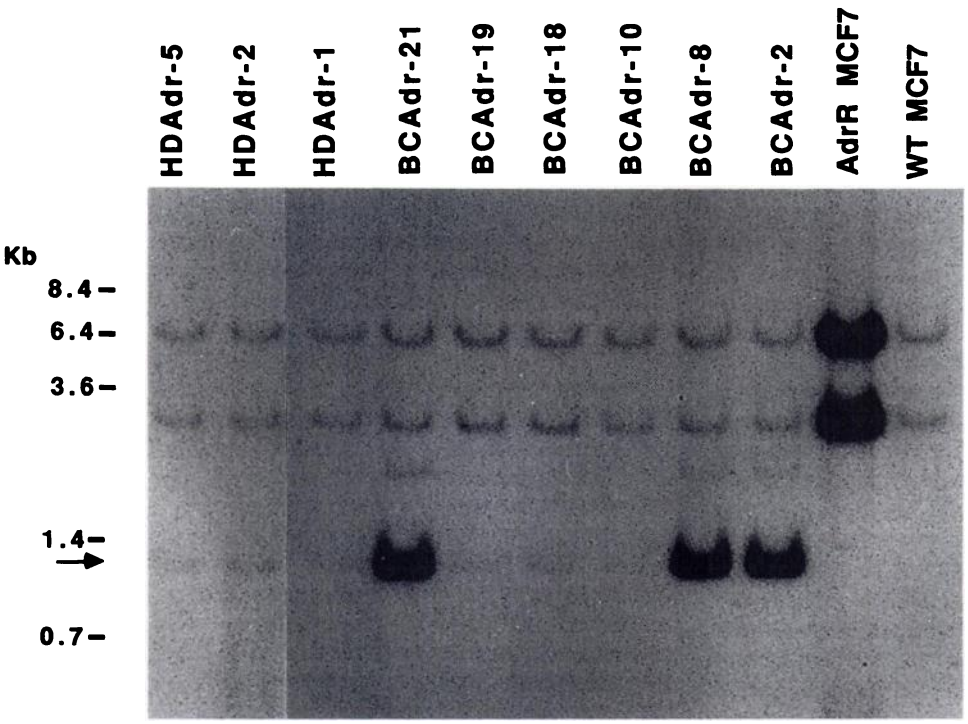

Fig. 3. Southern analysis of *mdr1*-transfected clones. Each lane contains 20 μg of *Eco*RI-digested genomic DNA isolated from *mdr1*-transfected WT MCF-7 cells. The probe used was a 347-bp fragment from the 5′ end of pADR-2 (residues −119 to 229). The DNA fragment resulting from the transfected *mdr1* gene is marked with an *arrow*. Molecular weight markers are from λ DNA digested with *Bst*EII.

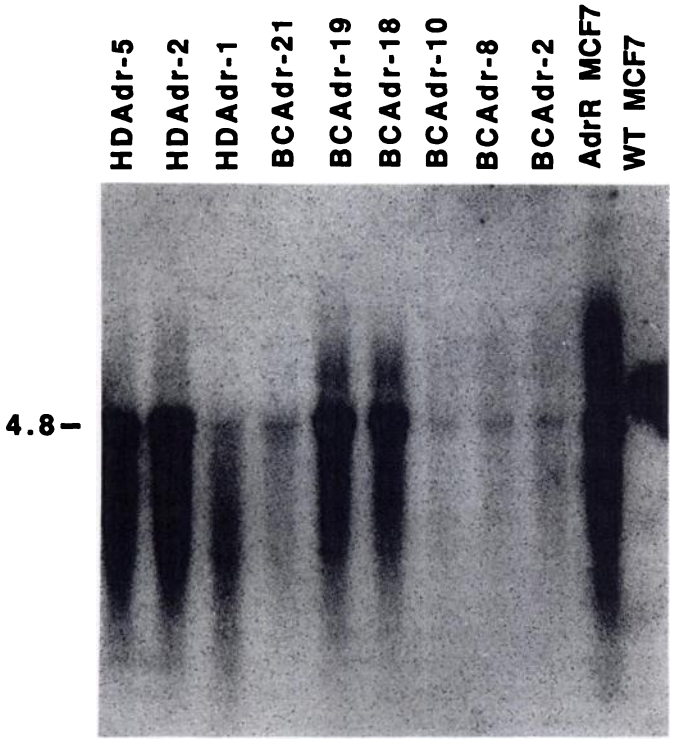

Fig. 4. Analysis of *mdr1* RNA levels in transfected cells. Total cellular RNA (20 μg) isolated from the *mdr1*-transfected cell lines was hybridized with a ^{32}P-labeled probe (pADR-1). Ethidium bromide staining indicated equal loading in all lanes.

studies presented here, we also demonstrate that a full length cDNA isolated from AdrR MCF-7 breast cancer cells was able to confer MDR upon drug-sensitive WT MCF-7 cells. Moreover, the pattern of resistance produced in the transfected clones in this study, in which resistance to vinblastine and actinomycin D was greater than to doxorubicin and colchicine, was similar to that observed in the AdrR MCF-7 cells. Interestingly, some of the *mdr*-transfected clones that had *mdr1* RNA and protein expression that was equal to or greater than that observed in AdrR MCF-7 cells exhibited levels of resistance that were significantly less than observed for the AdrR MCF-7 cells. This discrepancy between P-glycoprotein expression and resistance in the transfected cells could be due to alterations in posttranslational modifications (e.g., phosphorylation) of the protein in different clones. Another possibility is that other mechanisms, such as alterations in topoisomerase activity, may play a role in resistance in the AdrR MCF-7 cells and account for part of the difference.

The *mdr1* gene expressed in AdrR MCF-7 cells differs from the cDNAs isolated from multidrug-resistant KB cells (20, 22), resulting in a substitution of an alanine for a serine at position 893 within the cytoplasmic portion of a transmembrane domain of the P-glycoprotein. Analysis by polymerase chain reaction of the *mdr1* genomic DNA in WT MCF-7 cells indicates that this gene (alanine at 893) is present in the WT cells and, therefore, does not arise in the AdrR MCF-7 cells by mutation but rather through amplification and expression of the normal allele.[2] This same amino acid change has also been recently observed in human adrenal cells (52). Thus, the human *mdr1* gene is polymorphic and this raises the possibility that the pattern of resistance in different tumors may vary depending on the presence of different *mdr1* alleles (55, 56). Studies by Choi and co-workers (20) have shown that a single amino acid substitution of serine to glycine at amino acid position 185 in the cytoplasmic portion of another transmembrane domain of the P-glycoprotein results in a higher level of colchicine resistance relative to other agents. The substitution of an alanine for a serine at position 893 in the *mdr1* gene in AdrR MCF-7 cells, which is also within an intracytoplasmic region, further

[2] C. R. Fairchild and K. H. Cowan, unpublished data.

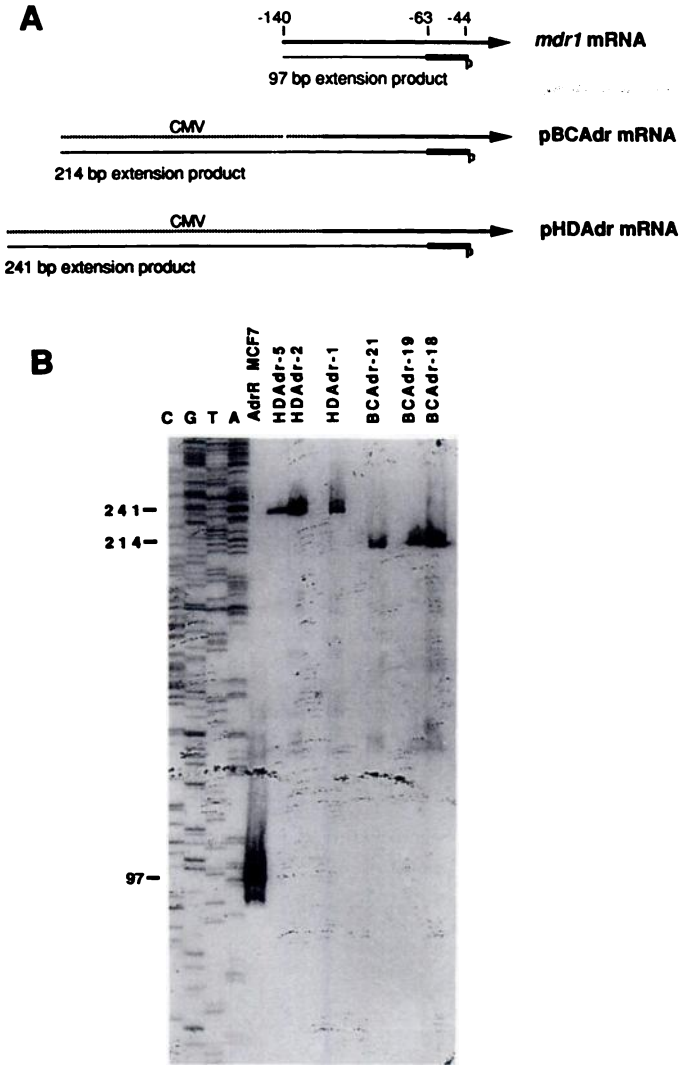

Fig. 5. Primer extension analysis of *mdr1*-transfected cells. A, Schematic representation of a primer extension strategy to differentiate endogenous *mdr1* RNA transcription from transcription of the transfected fusion gene. *Solid bar with P* represents ^{32}P-end-labeled oligonucleotide primer. After hybridization of the primer to the mRNA, reverse transcriptase was used to extend the primer to the end of the DNA. B, RNA (60 μg) isolated from the *mdr1*-transfected MCF-7 cell lines was hybridized to a ^{32}P-end-labeled 20-bp oligonucleotide. The primer extension products were analyzed on a sequencing gel. Markers for size determination were obtained by using the same 20-bp primer to sequence the 5′ portion of a genomic clone of the *mdr1* gene.

suggests that the variant *mdr1* expressed in these cells may result in a resistance pattern that differs from that in human cell lines expressing other *mdr1* alleles.

Changes in expression of genes other than *mdr1* have been found in multidrug-resistant cell lines. These changes include alterations in expression of several Phase I and Phase II drug-metabolizing enzymes, including increased expression of GST-π (27), glutathione peroxidase (27), and UDP-glucuronyltransferase (28) and decreasee expression of cytochrome P-450IA1 (28, 57). Other changes have included increased activity of protein kinase C (58) and changes in both cytosolic and membrane-bound receptors (30, 59). In addition, other laboratories have found increased expression of genes that have been coamplified with the *mdr1* gene in multidrug-resistant cells. These include the calcium-binding protein sorcin, which is elevated

TABLE 1

P-glycoprotein RNA and drug resistance in *mdr1*-transfected clones

Levels of *mdr1* RNA were determined by slot blot analysis of total cellular RNA isolated from the transfected cell lines. The level of RNA in AdrR MCF-7 cells was arbitrarily assigned a value of 100%. Cytotoxicity was determined by colony-forming assay. IC_{50} values were determined and the relative resistance was calculated as the ratio of the IC_{50} for transfected cells to the IC_{50} for WT MCF-7 cells. Values are the average of at least two separate experiments.

Cell line	Relative *mdr1* RNA	Relative resistance*			
		DOX	ACTD	VBL	COL
	%				
WT MCF7	<1	1	1	1	1
AdrR MCF7	100	77	98	362	65
BCAdr-18	105	16	24	64	9
BCAdr-19	153	14	71	24	12
BCAdr-21	14	3.0	16	10	3.3
HDAdr-1	22	16	51	21	7
HDAdr-2	157	10	89	16	6
HDAdr-5	177	16	92	93	10

* IC_{50} values for WT MCF-7 cells for doxorubicin (DOX), actinomycin D (ACTD), vinblastine (VBL), and colchicine (COL) are 6.4, 0.17, 0.55, and 5.6 nM, respectively.

in some multidrug-resistant cell lines (60). Although some of these changes have been implicated in the development of antineoplastic drug resistance, direct evidence for the role of these genes in the production of resistance to particular antineoplastic agents is still not available.

The increased GST-π activity and P-glycoprotein expression in AdrR MCF-7 cells parallels the overexpression of both P-glycoprotein and π class GST in carcinogen-induced rat hyperplastic nodules (33, 34) and in v-H-*ras*-transformed rat liver epithelial cells (36). In recent studies, we have shown that transfection of the GST-π gene into drug-sensitive MCF-7 cells alters the cell sensitivity to the carcinogens benzo[*a*]pyrene and benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide but produces few if any alterations in sensitivity to doxorubicin, cisplatin, or melphalan (40).

The gene encoding P-glycoprotein displays marked homology with bacterial membrane transport genes. This raised the possibility that mammalian multidrug transport may include a cytosolic drug-binding protein similar to that involved in the bacterial transport system, which channels a variety of substrates across the membrane (24). Because GSTs are known to bind toxins and GST-π is overexpressed in several models of MDR, this isozyme appeared to be a likely candidate as a binding protein that could interact with the multidrug transport protein P-glycoprotein. We, therefore, examined the ability of GST-π to alter the pattern of MDR in two clones of cells transfected with a P-glycoprotein expression vector. Even with relatively high GST-π expression in the resulting *mdr1*/GST-expressing clones, the drug resistance pattern was not consistently altered, compared with the corresponding clones expressing *mdr1* alone. Thus, under the conditions with which we have expressed GST-π in the MCF-7 system, this protein does not appear to play an accessory role in conjunction with P-glycoprotein.

These findings leave many questions unanswered. It is still possible that the P-glycoprotein works in conjunction with cytosolic transport proteins to pump drugs out of the cell, although such accessory proteins have not yet been identified. Furthermore, because the GST-π and *mdr1* genes are both overexpressed in several models of resistance, it is apparent that there may be factors common to the regulation of these two genes. There are cell lines that do not coexpress both genes.

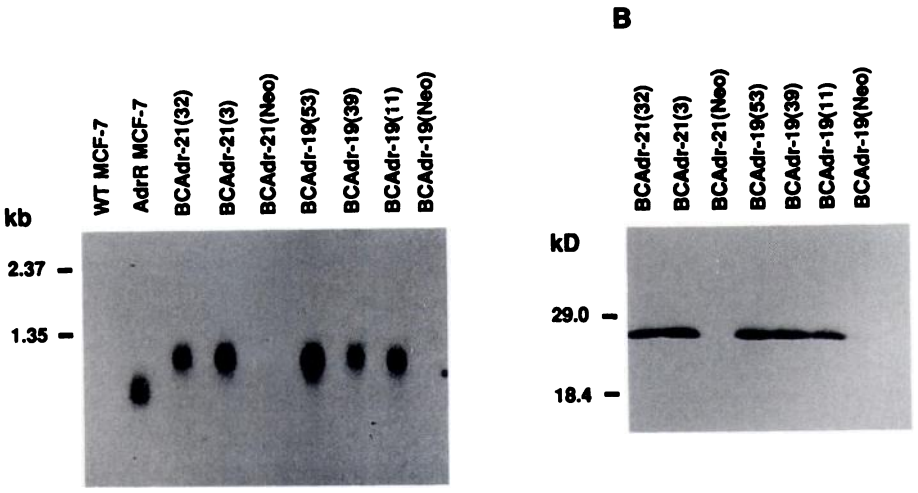

Fig. 6. Characterization of the GST-π RNA and protein in *mdr1*/GST-π transfectants. A, Northern hybridization of RNA from *mdr1*/Neo- and *mdr1*/GST-π-transfected cell lines to a GST-π-specific probe. Each lane contains 20 μg of total cellular RNA. Ethidium bromide staining indicated equal loading in all lanes. B, Western hybridization for GST-π protein in *mdr1*/Neo- and *mdr1*/GST-π-transfected cell lines. Each lane contains 150 μg of cytosolic protein. Standard molecular weight markers were used for size determination. The band present in each lane is the same size as purified GST-π.

TABLE 2

GST activity and relative resistance of WT MCF-7 cells transfected with pBCAdr alone or cotransfected with pBCAdr and pMTG

Subconfluent transfected clones were measured for GST activity, as described in Materials and Methods. Drug resistance was measured by colony-forming assay and IC_{50} values were determined. Relative resistance was expressed as the ratio of the IC_{50} for a transfected clone to the IC_{50} for BCAdr-19 (Neo) or BCAdr-21 (Neo) cells. Values are the average of two separate experiments.

Cell Line	GST activity	Relative resistance[a]		
		DOX	ACTD	VBL
	units/mg of protein			
BCAdr-19(Neo)	4	1	1	1
BCAdr-19(11)	40	1.4	1.2	ND[b]
BCAdr-19(39)	78	1.1	2.5	2.1
BCAdr-19(53)	63	0.8	1.6	1.6
BCAdr-21(Neo)	3	1	1	1
BCAdr-21(3)	50	0.6	0.7	1.7
BCAdr-21(32)	44	0.8	1.2	1.0

[a] Change in resistance relative to the BCAdr(Neo)-transfected clones, which are resistant to doxorubicin (DOX), actinomycin D (ACTD), and vinblastine (VBL), and express *mdr1* only.
[b] ND, not determined.

However, it is likely that the regulation of these genes is complex. Therefore, although the *mdr1* and GST-π genes may share some common regulatory mechanisms, each gene may be regulated by different mechanisms as well. Comparison of the regulatory regions of the GST-π and *mdr1* genes (44, 61–63) may determine whether similar factors are involved in the regulation of these two genes in different models of transformation and resistance. Finally, although GST-π may not have a direct role in producing MDR, evidence that this enzyme is found to be elevated in some tumors, compared with normal tissue (43, 64, 65), suggests that it may be a clinical marker for carcinogen-induced tumors or *de novo* drug resistance.

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