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# Functional Analysis of Chimeric Genes Obtained by Exchanging Homologous Domains of the Mouse *mdr1* and *mdr2* Genes

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**A full-length cDNA clone for the mouse *mdr1* gene can confer multidrug resistance when introduced by transfection into otherwise drug-sensitive cells. In the same assay, a full-length cDNA clone for a closely related member of the mouse *mdr* gene family, *mdr2*, fails to confer multidrug resistance. To identify the domains of *mdr1* which are essential for multidrug resistance and which may be functionally distinct in *mdr2*, we have constructed chimeric cDNA molecules in which discrete domains of *mdr2* have been introduced into the homologous region of *mdr1* and analyzed these chimeras for their capacity to transfer drug resistance. The two predicted ATP-binding domains of *mdr2* were found to be functional, as either could complement the biological activity of *mdr1*. Likewise, a chimeric molecule in which the highly sequence divergent linker domain of *mdr2* had been introduced in *mdr1* could also confer drug resistance. However, the replacement of either the amino- or carboxy-terminus transmembrane (TM) domain regions of *mdr1* by the homologous segments of *mdr2* resulted in inactive chimeras. The replacement of as few as two TM domains from either the amino (TM5-6) or the carboxy (TM7-8) half of *mdr1* by the homologous *mdr2* regions was sufficient to destroy the activity of *mdr1*. These results suggest that the functional differences detected between *mdr1* and *mdr2* in our transfection assay reside within the predicted TM domains.**

The emergence of multidrug resistance in cultured cells is linked to the overexpression of a membrane phosphoglycoprotein termed P-glycoprotein, believed to act as an ATP-dependent efflux pump to reduce the intracellular accumulation of antitumor drugs. P-glycoprotein has been shown to interact directly with a remarkably broad range of substrates; most are small hydrophobic lipid-soluble molecules of natural origin such as anthracyclines, vinca alkaloids, and colchicine (14, 22). P-glycoproteins are encoded by a small gene family, termed *mdr* or *pgp*, which comprises three members in rodents and two members in humans (40). Analysis of the nucleotide and predicted amino acid sequences of full-length cDNA clones corresponding to the three mouse (13, 24, 25, 29) and two human (7, 53) *mdr* genes have revealed that the predicted *mdr* polypeptides are highly homologous, sharing the same predicted structural features, which include 12 putative transmembrane (TM) domains and two ATP-binding sites encoded by two sequence-homologous halves. The three mouse *mdr* genes appear to have originated from a common ancestor by two successive gene duplication events, the most recent one producing *mdr1* and *mdr3* (13). Amino acid sequence comparisons indicate that the human *MDR1* gene is the counterpart of mouse *mdr1* and *mdr3*, while human *MDR2* (*MDR3*; 53) appears to be the homolog of mouse *mdr2* (30).

Despite a high degree of sequence homology, striking functional differences have been detected between individual *mdr* genes. In humans, *MDR1* is overexpressed in most multidrug-resistant cell lines (34, 43, 45) and in certain tumor specimens refractory to chemotherapy (21) and can confer multidrug resistance when transfected and overexpressed in otherwise drug-sensitive cells (52). Human *MDR2* is expressed only at marginally detectable levels if at all in drug-resistant cells (8, 45) and does not show biological activity in transfection experiments (53). In the mouse, *mdr1* and *mdr3* but not *mdr2* are found independently overexpressed in multidrug-resistant cell lines of fibroblastic, lymphoid (42), and reticuloendothelial (30) origins. Both *mdr1* and *mdr3* can confer drug resistance in transfection experiments, but the two proteins appear to have overlapping but distinct substrate specificities (13). In transfection experiments, mouse *mdr2* fails to confer multidrug resistance (25).

Analysis of the mouse *mdr* gene transcripts in normal tissues shows that expression of the three genes is controlled in a tissue-specific manner (11). *mdr1* is constitutively expressed in adrenal glands and kidney and is induced at high levels in the endometrial glands of the pregnant uterus (1, 2). *mdr2* is primarily expressed in liver and muscle, and *mdr3* is primarily expressed in intestine and lung. The structural similarities and functional differences detected between mouse *mdr1*, *mdr2*, and *mdr3* in transfection experiments together with the specific tissue distribution of their mRNAs suggest that *mdr* genes code for membrane proteins which may participate in similar transport functions of perhaps distinct substrates. The discrete protein domains responsible for substrate specificity have not yet been identified. We have taken advantage of the strong sequence homology and differential capacity of *mdr1* and *mdr2* to confer multidrug resistance to study, in chimeric molecules, protein domains of *mdr1* that are essential for its capacity to confer drug resistance and may be functionally distinct in *mdr2*.

## MATERIALS AND METHODS

**Construction of chimeric cDNA clones.** Full-length cDNA clones for mouse *mdr1* (24) and *mdr2* (25) were initially introduced in the unique *Eco*RI and *Kpn*I sites, respectively, of plasmid vector pGEM7Zf (Promega, Madison, Wis.) to create clones pGEMK4 (*mdr1*) and pGEM2.3 (*mdr2*). All chimeric cDNAs were obtained by exchanging restriction fragments between these two plasmids and were propagated

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into *Escherichia coli* JM83 or NM1255 (*dam dcm*). The positions of restriction sites in *mdr1* and *mdr2* used for cloning are given below, with nucleotide 1 being the adenosine residue within the predicted initiator ATG in each cDNA. Chimeras A and C were produced by introducing a 0.7-kb *Pfl*mI (position [pst] 3182)-to-*Sph*I (polylinker) fragment and a 0.5 kb-*Pst*I (pst 3365)-to-*Sph*I (polylinker) fragment of pGEM2.3 into the homologous and unique sites of pGEMK4, respectively. Chimera I was constructed by inserting the 2.0-kb *Hin*dIII (polylinker)-to-*Acc*I (pst 1762) fragment of pGEM2.3 into the corresponding sites of pGEMK4. Chimeras G and H were produced by reciprocal exchange of the 5′-terminal 1.1-kb *Nsi*I (polylinker)-to-*Nsi*I (pst 916) fragment of each gene. Chimeras D and E were also produced by reciprocal exchange of a 1.5-kb *Acc*I (pst 1762)-to-*Pfl*mI (pst 3183) internal fragment. To obtain chimera L, the 5′-terminal 1.1-kb *Nsi*I (polylinker)-to-*Nsi*I (pst 916) fragment of pGEMK4 was introduced in the corresponding sites of chimera E. For chimera M, the 0.4-kb *Eco*0109 (pst 1228)-to-*Eco*0109 (pst 1624) fragment of pGEM2.3 was used to replace the corresponding segment of pGEMK4. The integrity of all restriction enzyme sites used for cloning was verified for all chimeras, and restriction enzymes generating diagnostic fragments specific for pGEMK4 and pGEM2.3 were used to distinguish each chimera from the parental plasmids used in their construction. Full-length wild-type and chimeric *mdr* cDNA inserts were excised from the respective plasmids by digestion with *Hin*dIII and *Sph*I (polylinker), the cohesive ends were repaired with T4 DNA polymerase, and full-length cDNA inserts were cloned in the sense and antisense orientations into the mammalian expression vector pMT2 (gift of R. Kaufman, Genetics Institute, Cambridge Mass.), a derivative of plasmid p91023 (55). All enzymes were obtained from Pharmacia/LKB (Montreal, Quebec, Canada) and were used under conditions specified by the supplier.

**Site-directed mutagenesis.** To construct chimeras N and O, new restriction sites were introduced in *mdr1* and *mdr2* by site-directed mutagenesis. For this, a 1.4-kb internal *Bgl*II *mdr1* fragment (nucleotides 1219 to 2665) and a 1.7-kb internal *Pst*I *mdr2* fragment (nucleotides 1314 to 2998) were cloned into the unique *Bam*HI and *Pst*I sites of a bacteriophage M13mp8 cloning vector. Single-stranded DNA templates were purified from these clones and mutagenized in vitro with single primers, using commercially available reagents (Amersham Corp., Arlington Heights, Ill.). For introducing novel restriction sites in the *mdr1* gene, the following oligonucleotides bearing mismatched bases were used: 5′-CTGTGTCATGACTAGTTTGAA-3′ (pst 1884 to 1863; *Spe*I site), 5′-CCGCCAAAAGCTTACCAGAGGCAC-3′ (pst 2090 to 2067; *Hin*dIII site), and 5′-ATGTAACAAAGCTTATCAGCCCCA-3′ (pst 2301 to 2278; *Hin*dIII site). For introducing the homologous mutations in *mdr2*, the following mutant oligonucleotides were used: 5′-CATGTTAACTAGTCTGAA-3′ (pst 1881 to 1864; *Spe*I site), 5′-CTTCAGAAAGCTTACTGGTGGCAC-3′ (pst 2088 to 2065; *Hin*dIII site), and 5′-AGTAAAGAAGCTTAGGACTCCT-3′ (pst 2295 to 2274; *Hin*dIII site). The wild-type *mdr1* and *mdr2* templates were first mutagenized to introduce the *Spe*I sites. These mutants were then used to prepare single-stranded DNA templates into which the two *Hin*dIII mutant sites were independently introduced to produce a total of four separate mutants. The replicative double-stranded forms of these clones were used to introduce the newly created 0.2- and 0.4-kb *Spe*I-to-*Hin*dIII fragments (see Fig. 2) of *mdr2* into *mdr1*. To produce the full-length chimeric cDNA clones N and O, the 0.7-kb *Acc*I (pst 1762)-to-*Nar*I (pst 2481) *mdr1* fragment overlapping the exchanged *mdr2* domains was introduced in the corresponding sites of pGEMK4. The entire nucleotide sequence of the DNA fragments mutagenized in M13mp8 and used to reconstruct chimeras N and O was determined by the chain termination method of Sanger et al. (48). The integrity of all restriction sites used for cloning was also verified prior to cloning in the expression vector pMT2.

**Cell culture and DNA transfection.** Chinese hamster ovary LR73 cells (41) were used in all transfection experiments and were grown in alpha-minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Chimeric and wild-type (sense and antisense orientations) *mdr* cDNAs cloned in pMT2 were introduced as calcium phosphate coprecipitates (54) in LR73 cells by cotransfection with the dominant selectable marker *neo* (Tn*5*) gene contained in the mammalian expression vector $pSV_2Neo$ (50). A 1:10 molar ratio of $pSV_2Neo$ to test plasmid DNA was used for transfection, and $Neo^r$ colonies were selected and maintained in medium containing Geneticin (G418) at 0.5 mg/ml. Mass populations of $G418^r$ colonies were harvested 10 days after transfection and expanded in culture, and several aliquots were frozen at −90°C in complete medium containing 10% dimethyl sulfoxide. The drug survival characteristics of mass populations of $G418^r$ cells cotransfected with chimeric or wild-type *mdr* cDNA clones were determined as follows. A total of $10^5$ cells were plated in 60-mm tissue culture dishes in medium containing increasing concentrations of either Adriamycin (0, 10, 25, 50, and 100 ng/ml) or colchicine (0, 25, 50, 100, and 200 ng/ml). One week later, the cells were fixed in 0.4% formaldehyde and stained with 1% methylene blue. Adriamycin was purchased from Adria Laboratories, colchicine was obtained from Sigma Chemical Co. (St. Louis, Mo.), and Geneticin was purchased from GIBCO Laboratories (Grand Island, N.Y.).

**Southern hybridization.** Genomic DNA was isolated from LR73 control cells and $G418^r$ mass populations of transfected cells by using proteinase K (GIBCO) treatment and sequential phenol and chloroform extractions. Genomic DNA was digested to completion with *Hin*dIII, electrophoresed in a 1% agarose gel containing 40 mM Tris-acetate, 20 mM sodium acetate, and 20 mM disodium EDTA (pH 7.6), and DNA fragments were transferred to a nylon hybridization membrane (Hybond-N; Amersham Corp.). The blot was probed with a $^{32}P$-labeled 1.1-kb *Eco*RI fragment overlapping the C-terminal portion of the *mdr2* gene, including one of the two ATP-binding domains. Prehybridization was for 16 h at 42°C in a solution containing 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.15 M sodium citrate), 0.1% sodium dodecyl sulfate, 10% dextran sulfate, 50% formamide, 5× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.2% polyvinylpyrrolidone), and denatured salmon sperm DNA (100 μg/ml). Hybridization was conducted for 48 h at 42°C in the same solution containing $10^6$ cpm of radiolabeled probe per ml. The probe was labeled with [α-$^{32}P$]dATP (Dupont, NEN Research Products, Boston, Mass.) by random primer extension (15) to a specific activity of $10^9$ cpm/μg of DNA. The blot was washed to a final stringency of 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C and exposed to Kodak XAR films for 48 h at −80°C with an intensifying screen.

## RESULTS

The mouse *mdr1* and *mdr2* genes encode highly homologous polypeptides which share the predicted structural features shown in Fig. 1A. These include two series of six predicted TM segments (TM1 to -6 and TM7 to -12; 85% homology), two predicted nucleotide-binding (NB) sites (NB1 and NB2; 93% homology), and the highly sequence divergent linker domain (40% homology). Despite this high sequence homology, the two genes are functionally distinct in that only *mdr1* is capable of conferring multidrug resistance when transfected into otherwise drug-sensitive LR73 hamster cells (Fig. 1). Six strategically positioned restriction sites, precisely conserved in *mdr1* and *mdr2*, *Nsi*I, *Eco*0109 (two sites), *Acc*I, *Pfl*mI, and *Pst*I, were initially used to insert discrete *mdr2* domains into the homologous regions of *mdr1*. The capacity of wild-type and chimeric *mdr* cDNA clones to confer multidrug resistance was tested after insertion of each cDNA into the mammalian expression vector pMT2. These pMT2 constructs were then introduced in LR73 hamster cells by cotransfection with $pSV_2Neo$, and the drug survival characteristics of mass populations of $G418^r$ cotransfected clones were measured in medium containing Adriamycin (50 ng/ml) and colchicine (100 ng/ml). Under these experimental conditions, *mdr1* could induce the formation of drug-resistant colonies, while *mdr2* could not (Fig. 1C).

Initially, the predicted NB domains of *mdr2* were tested for the ability to complement *mdr1* in three chimeric cDNAs (Fig. 1B; C, A, and M). The NB domains are the most homologous segments of *mdr1* and *mdr2* but are also highly conserved among all cloned eucaryotic *mdr* and *mdr*-like genes. Each predicted NB domain is formed by two consensus motifs, a $G\text{-}(X)_4\text{-}G\text{-}K\text{-}(T)\text{-}(X)_6\text{-}I/N$ (motif A, pst 419 and 1068) and a hydrophobic pocket of sequence $R/K\text{-}(X)_3\text{-}G\text{-}(X)_3\text{-}L\text{-}(\text{hydrophobic})_4\text{-}D$ (motif B, pst 542 and 1186). Chimera C was constructed by using *Pst*I, which transfers motif B of NB2, the C-terminus protein, and the 3′ untranslated region of *mdr2* into the homologous region of *mdr1*. This chimera was biologically active and conferred multidrug resistance. Likewise, chimera A, in which *Pfl*mI was used to transfer in *mdr1* the entire NB2 segment and the C terminus of *mdr2*, retained biological activity. Chimera M, in which *Eco*0109 was used to transfer in *mdr1* a 133-residue segment of *mdr2* overlapping both motifs A and B of NB1, was also functional and conveyed drug resistance. Taken together, these results indicate that both ATP-binding domains and the C-terminal segment of *mdr2* are functional, can reconstitute a biologically active *mdr1* polypeptide, and are not responsible for the functional differences detected between *mdr1* and *mdr2*.

In a second series of chimeras, *mdr2* segments overlapping the membrane-associated TM domains, which include the predicted hydrophobic TM segments and the predicted intracellular and extracellular loops, were used to replace the corresponding portions of *mdr1*. By using *Acc*I, the amino-terminal half of *mdr2* was replaced in *mdr1* to create chimera I. This chimera could not confer multidrug resistance. Given that chimera M is biologically active and that *mdr1* and *mdr2* differ only by a single residue between the *Eco*0109(544) and *Acc*I(590) sites, these results suggest that *mdr2* segments overlapping the amino terminus or TM1–6 regions are not compatible with and cannot complement the biological activity of *mdr1*. To further analyze these regions, three additional chimeras (G, H, and L) were constructed. Chimera G contains the amino terminus and the first four TM domains of *mdr2*, up to the *Nsi*I site located within TM5, inserted in *mdr1*. This chimeric cDNA could not confer drug resistance, suggesting functional differences between *mdr1* and *mdr2* in these regions. However, results obtained with the reverse construct, chimera H, indicate that these regions are not solely responsible for the different drug resistance phenotypes of *mdr1* and *mdr2* and suggest that additional protein segments located elsewhere in *mdr2* are also functionally distinct from their *mdr1* counterparts. Chimera L, which was generated by inserting into chimera E the 1.1-kb 5′-end *Nsi*I fragment of *mdr1*, including the TM4 region, also failed to confer drug resistance. Considering that chimeras A and M were biologically active, these results indicate that the two discrete segments spanning the *Nsi*I(307)-to-*Eco*0109(411) sites and *Eco*0109(544)-to-*Acc*I(590) sites are functionally distinct in *mdr2*. Only six (Cys-350, Val-363, Asp-366, Lys-373, Ser-394, and Lys-407) and one (Glu-567) residues in these segments, respectively, represent nonconservative substitutions which are unique to *mdr2* and are not conserved in either of the two other biologically active genes, *mdr1* and *mdr3*. Therefore, the combined analysis of chimeras G, H, and L indicates that the amino-terminus segment of *mdr2* including TM1–6 region is functionally distinct from the corresponding region of *mdr1* but also suggests that functional differences within the region are not restricted to a unique discrete segment or specific residue but involve domains on either side of the *Nsi*I site. Two additional chimeras (D and E) were constructed to analyze the *Acc*I-to-*Pfl*mI segment, which encodes the highly divergent linker domains and the second group of membrane-associated domains (TM7 to -12). Introduction of this segment of *mdr2* into *mdr1* (chimera D) resulted in an inactive chimera, suggesting that functional differences between *mdr1* and *mdr2* also include determinants on the 3′ side. As expected from previous results obtained with chimeras I and G, chimera E was also found to be inactive.

The functional role of the highly sequence divergent linker domain and the predicted membrane-associated domains from the carboxy half of the protein was further investigated. To exchange these segments, discrete restriction enzymes sites were first introduced in *mdr1* and *mdr2* by site-directed mutagenesis (Fig. 2). A *Spe*I site was introduced immediately upstream the L domain (pst 624), and two *Hin*dIII sites were independently engineered immediately downstream of the linker domain (pst 693) and near the end of TM8 (pst 764). The predicted linker domain of *mdr2* was substituted in *mdr1* to create chimera N. This chimeric cDNA was found capable of conferring drug resistance, indicating that although highly sequence divergent, the linker domain does not account for the distinct drug resistance properties of the two genes. However, the addition to chimera N of the TM7–8 region of *mdr2* to produce chimera O resulted in an inactive cDNA, suggesting that the segment spanning the two *Hin*dIII sites which overlaps the TM7–8 region is functionally distinct in *mdr1* and *mdr2*. Comparison of the predicted amino acid sequences of *mdr2* with that of the two biologically active *mdr1* and *mdr3* genes in this 72-residue segment revealed that only 7 residues represent nonconservative substitutions unique to *mdr2* (Leu-695, Thr-712, Ala-717, Leu-729, Glu-731, Val-743, and Gly-757).

To establish that the functional differences detected between the various wild-type and chimeric cDNAs were not linked to varying degrees of transfection efficiency of the cDNA constructs, total genomic DNA from mass populations of $G418^r$ cotransfected clones was digested with *Hin*dIII and analyzed by Southern blotting for the presence of

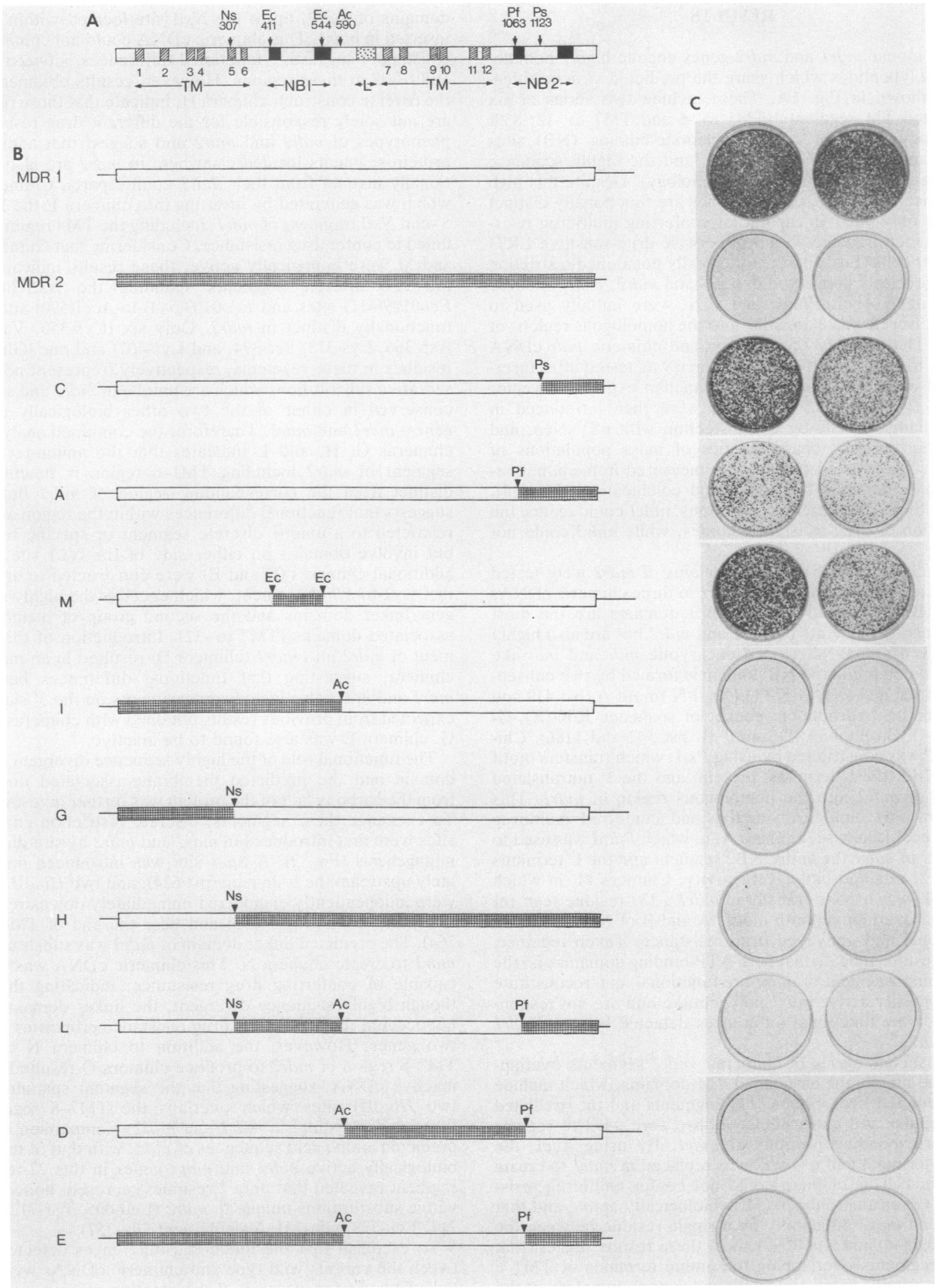


FIG. 1. Analysis of *mdr1-mdr2* chimeric cDNA clones constructed by using conserved restriction enzyme sites. (A) Schematic representation of a wild-type *mdr* cDNA and some of the predicted structural features of the protein. Symbols: ▨, TM domains, numbered 1 to 12; ■, predicted nucleotide-binding folds (NB1 and NB2); ▩, linker domain (L). Restriction enzyme sites (Ns, *Nsi*I; Ec, *Eco*0109; Ac,

*Acc*I; Pf, *Pfl*mI; Ps, *Pst*I) used to construct the chimeras are identified by arrows above the cDNA. The positions of amino acids immediately proximal to these sites are indicated. (B) Schematic representation of wild-type *mdr1* (▭), *mdr2* (▦), and the different chimeric cDNAs (identified by letters on the left). Thin lines identify untranslated regions, and the parental origin of each segment of the chimeras is indicated. (C) Capacity of wild-type and chimeric *mdr* cDNA clones to confer multidrug resistance. Mass populations of G418$^r$-resistant clones cotransfected with $pSV_2Neo$ and each *mdr* clone inserted into pMT2 were plated in culture medium containing either colchicine at 100 ng/ml (left) or Adriamycin at 50 ng/ml (right). Dishes were stained 7 days later.

cotransfected plasmids, using as a hybridization probe a cDNA subclone overlapping NB2 which cross-hybridizes to all *mdr* genes. (Fig. 3). *Hin*dIII cleaves on either side of the cloning site in the expression vector pMT2 and does not cleave within either wild-type or chimeric cDNAs except for N and O, which contained the introduced *Hin*dIII sites. The cDNA probe detected six *Hin*dIII fragments present at single-copy level in LR73 cells and in each of the cotransfected mass populations representing the endogenous copy of the *mdr* genes. It also detected an additional fragment of 9.4 to 9.7 kb in cell populations cotransfected with either wild-type or chimeric cDNAs which corresponded to the introduced *mdr* constructs. The slight variation in molecular size of this hybridizing fragment originated from the parental cDNA which provided the 3′ untranslated region in each construct, being 400 and 75 bp for *mdr1* and *mdr2*, respectively. The hybridizing *Hin*dIII genomic DNA fragment was smaller in populations transfected with N and O because of the introduction of the internal *Hin*dIII sites. The signal intensity of the plasmid-specific *mdr* fragment was similar in all lanes except that for the *mdr2* control, which contained an apparently higher copy number of the transfected plasmids. These results indicate that all expression cDNA constructs were successfully introduced to equivalent degrees in LR73 cells.

## DISCUSSION

Biochemical characterization of intact cells (12, 17, 34, 35) and membrane vesicles (28, 38) from multidrug-resistant cells overexpressing P-glycoprotein strongly suggests that this protein functions as an ATP-driven efflux pump which reduces the net intracellular accumulation of drugs. P-glycoprotein has been shown to interact directly with ATP (10, 49) and drug molecules (46, 47) and has been shown to possess ATPase activity (26). It has been proposed that the evolu-

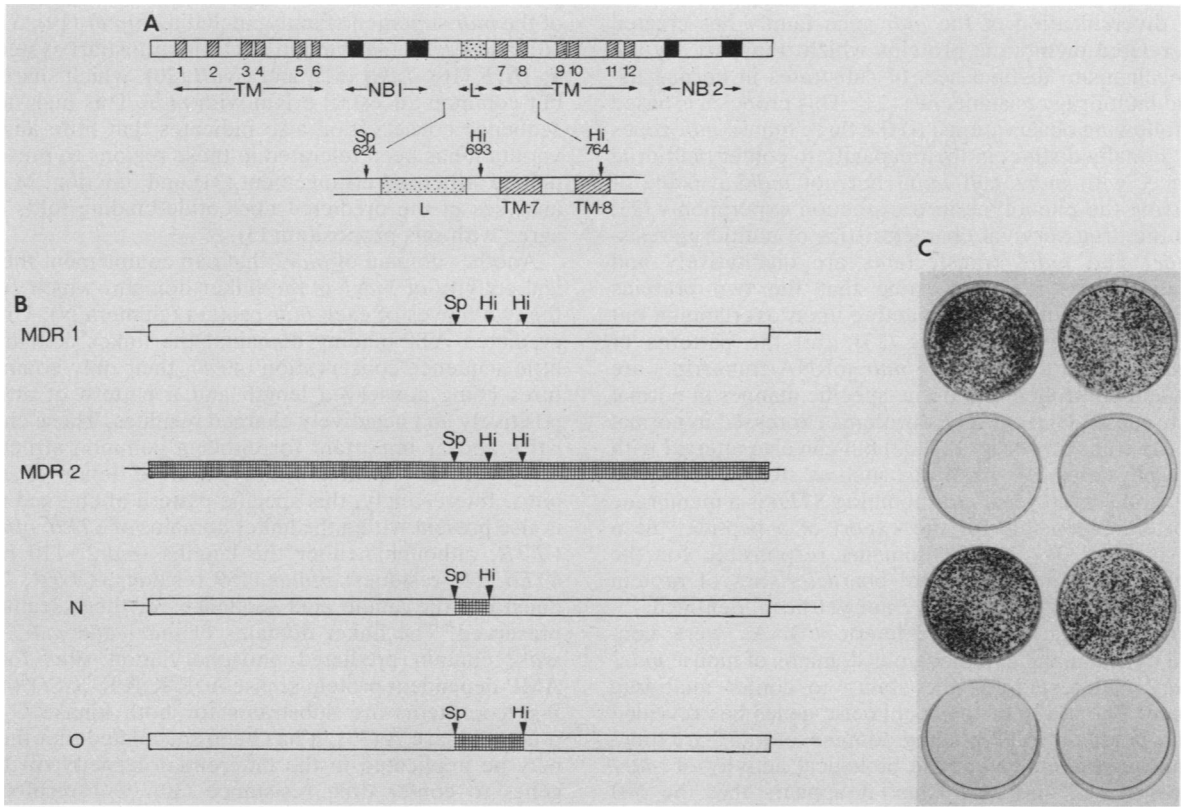


FIG. 2. Analysis of *mdr1-mdr2* chimeric cDNA clones constructed by site-directed mutagenesis. (A) Schematic representation of a wild-type *mdr* cDNA and the predicted structural features of the corresponding protein; symbols are as given in the legend to Fig. 1. The middle segment of the cDNA has been magnified to emphasize the linker region (L), TM7, and TM8, in which one *Spe*I (Sp) and two *Hin*dIII (Hi) restriction enzyme sites have been independently introduced in *mdr1* and *mdr2* by site-directed mutagenesis. (B) Positions of restriction enzyme sites used to construct chimeras and parental origin of each segment of the chimeras. (C) Biological activities of the chimeric cDNAs; for details, see the legend to Fig. 1.

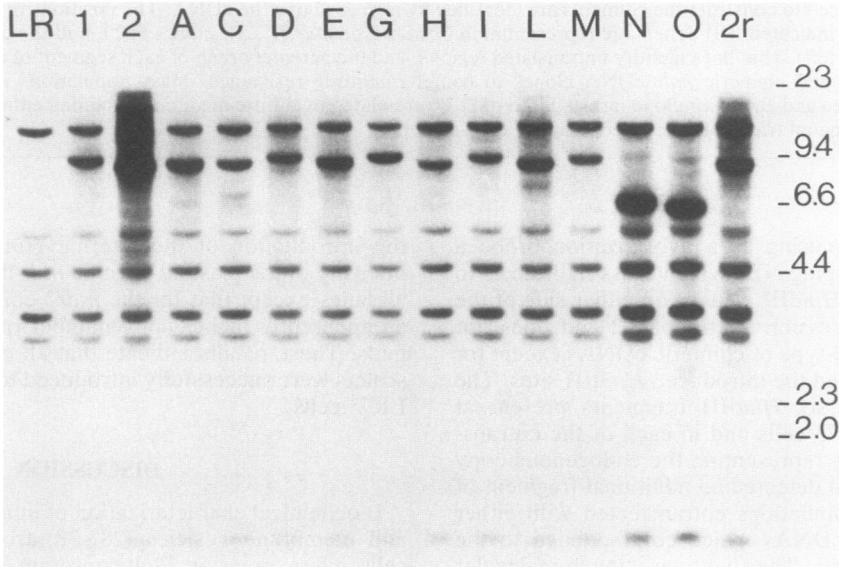


FIG. 3. Southern analysis of genomic DNA of cells cotransfected with $pSV_2Neo$ and wild-type or chimeric *mdr* cDNA constructed in pMT2. Ten micrograms of genomic DNA from either LR73 Chinese hamster control cells or $G418^r$ mass populations of cells cotransfected with either *mdr1* (lane 1), *mdr2* (lane 2), *mdr2* in the antisense orientation (lane 2r), or *mdr1-mdr2* chimeras (lanes A to O) was digested and analyzed by Southern blotting. The blot was hybridized with a $^{32}P$-labeled *mdr* cDNA subclone overlapping the highly conserved 3′ NB domain. This probe identifies the transfected *mdr* construct on the background of endogenous hamster *mdr* genes. The size markers (shown in kilobases on the right) are the *Hin*dIII fragments of bacteriophage lambda.

tionary diversification of the *mdr* gene family has created closely related membrane proteins which transport, by the same mechanism, distinct sets of substrates in normal tissues and multidrug resistant cells (13). This proposal is based on the following observations: (i) the three mouse *mdr* genes are functionally distinct in their capacity to confer multidrug resistance, with *mdr1* and *mdr3* but not *mdr2* capable of transferring the phenotype in transfection experiments (23, 25); (ii) the drug survival characteristics of multidrug-resistant *mdr1* and *mdr3* transfectants are qualitatively and quantitatively distinct, suggesting that the two proteins function as drug efflux pumps acting upon overlapping but yet distinct sets of substrates (13); (iii) the patterns of expression of the three mouse *mdr* mRNA transcripts are distinct and controlled in a tissue-specific manner in normal mouse tissues (11); (iv) P-glycoproteins expressed in normal tissues can transport drugs (27, 32) but can also interact with putative physiological substrates such as steroid hormones (39, 56); and (v) the yeast *mdr* homolog *STE6* is a membrane transporter responsible for the export of a peptide, the **a** mating factor (33). Protein domains responsible for the common and distinct functional characteristics of protein encoded by the *mdr* family have not yet been identified.

To initiate this analysis, chimeric cDNAs were constructed by exchanging homologous domains of mouse *mdr1* and *mdr2* and tested for the ability to confer multidrug resistance. The study of these chimeric genes has revealed that both predicted ATP-binding domains of *mdr2* are functional and can complement the biological activity of *mdr1* (chimeras A, C, and M). Thus, it appears that the NB domains are involved in the common mechanistic basis of action of the two encoded proteins, most likely the energy coupling component of the transport system. Indeed, these domains show a remarkable degree of amino acid sequence conservation among the different members of the mouse *mdr* gene family and are also highly conserved in other members of the *mdr* supergene family, including *pfmdr* (19), *STE6* (33, 36), and *CFTR* (44), and in bacterial transport systems such as *HlyB* (16), *LtkB* (51), and *CyaB* (20), which share distant but common ancestral origin with *mdr*. This high degree of sequence conservation also indicates that little amino acid variation has been tolerated in these regions to preserve the unique structural arrangement (31) and function. Mutational analyses of the predicted nucleotide-binding folds of *mdr1* agree with this proposition (3).

Another domain of *mdr2* that can complement the biological activity of *mdr1* is the linker domain, which separates the two halves of each *mdr* protein (chimera N). Unlike the predicted ATP-binding domains, the linker domains show little sequence conservation (40%), their only common features being conserved length and a pattern of alternating positively and negatively charged residues. These characteristics appear important for either a common structural arrangement or a common mechanism of action of *mdr* proteins. Interestingly, this specific pattern of charged residues is also present within the linker domains of *STE6*, *pfmdr*, and *CFTR*, although neither the lengths (*mdr2*, 110 residues; *STE6*, 143 residues; *pfmdr*, 159 residues; *CFTR*, 245 residues) nor the amino acid sequences of these segments are preserved. The linker domains of *mdr1* and *mdr3* but not *mdr2* contain predicted phosphorylation sites for cyclic AMP-dependent protein kinase A, R/K-R/K-X-S/T (4). Since P-glycoproteins are substrates for both kinase C (6) and protein kinase A (37), it has been speculated that these sites may be implicated in the differential capacity of the three genes to confer drug resistance (30). Our results do not support this hypothesis.

The analysis of chimeric cDNAs in which portions of the TM domains (TM segment and intra- and extracellular loops) of *mdr2* have been substituted in *mdr1* suggest that these sites are functionally distinct in the two proteins. The introduction of any segment of *mdr2* overlapping the TM

domains in *mdr1* destroyed its biological activity in all chimeras tested. Moreover, the introduction of a segment overlapping as few as two TM domains from either the amino (TM5 and -6, chimera L) or carboxy (TM7 and -8, chimera O) halves of *mdr2* into *mdr1* was sufficient to cause a loss of activity. These results strongly suggest that the TM domains of *mdr1* are essential for its capacity to cause drug resistance and are functionally distinct in *mdr2*. The data also suggest that the functional differences between the two proteins are not encoded by a single TM segment but rather involve protein domains present in both homologous halves of each protein. These findings are compatible with the hypothesis that TM domains in either half of *mdr1* may cooperate to form a three-dimensional structural arrangement that is essential for drug transport and can be disrupted by substituting small corresponding segments of *mdr2* (insertion mutagenesis). Sequence comparison between *mdr1*, *mdr3*, and *mdr2* in the TM5–6 and TM7–8 regions (chimeras L and O) identifies only a few nonconserved residues specific to *mdr2* that may be important for the functional differences detected among the three genes. The role of these specific residues is now being addressed more directly by site-directed mutagenesis.

The importance of TM domains for the specific drug efflux property of *mdr1* suggested by our results is in good agreement with the results of independent analyses of other members of the *mdr* supergene family. A single Val-to-Gly substitution near TM3 (pst 185) in the human *MDR1* gene strongly affects the degree of colchicine resistance conferred by this gene in transfected cells (9). Recently, two polymorphic variants of the *pfmdr1* gene were found to be predominantly associated with chloroquine resistance in 36 independent isolates of *Plasmodium falciparum* (18). One of the two alleles encodes a single nonconservative Asn-to-Asp substitution at pst 1042 of the protein, within the predicted TM11. The *mdr* yeast homolog *STE6* is a membrane-associated protein responsible for the export of the pheromone **a** mating factor, while the *STE3* gene is the **a** mating factor receptor expressed on α cells. Two regions of sequence homology between *STE3* and *STE6*, possibly denoting common sequences implicated in the **a**-factor recognition and binding, are found immediately upstream TM7 and downstream TM12 in *STE6* (33). Finally, our results are also in agreement with recent findings suggesting that both halves of P-glycoprotein may be implicated in substrate binding: two independent studies (5, 57) have identified discrete tryptic fragments mapping to either half of the protein, which can be specifically photolabeled by the drug analog azidopine in cross-linking experiments.

In conclusion, our analysis of *mdr1-mdr2* chimeras indicates that both predicted ATP-binding sites and the linker domain of *mdr2* can complement the drug resistance phenotype encoded by *mdr1*, therefore suggesting that these regions encode common structural or functional aspects of these two proteins. Segments corresponding to the membrane-associated domains either in the carboxy- or amino-terminus side of *mdr2* could not, when inserted at the homologous position of *mdr1*, sustain the drug resistance phenotype of *mdr1*. These findings suggest that membrane-associated domains are critical for the drug efflux function of *mdr1* and are functionally distinct in *mdr2*. Since membrane-associated domains of biologically active *mdr* proteins have been implicated in drug interactions (5, 9, 57), our findings suggest that the corresponding *mdr2* domains may not be structurally or functionally capable of similar interactions.

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