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Expression of a full-length cDNA for the human *"MDR1"* gene confers resistance to colchicine, doxorubicin, and vinblastine

(P-glycoprotein/actinomycin D/chemotherapy/multidrug resistance/retrovirus)

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ABSTRACT **Intrinsic and acquired multidrug resistance (MDR) is an important problem in cancer therapy. MDR in human KB carcinoma cells selected for resistance to colchicine, vinblastine, or doxorubicin (former generic name adriamycin) is associated with overexpression of the *"MDR1"* gene, which encodes P-glycoprotein. We previously have isolated an overlapping set of cDNA clones for the human *MDR1* gene from multidrug-resistant KB cells. Here we report the construction of a full-length cDNA for the human *MDR1* gene and show that this reconstructed cDNA, when inserted into a retroviral expression vector containing the long terminal repeats of Moloney leukemia virus or Harvey sarcoma virus, functions in mouse NIH 3T3 and human KB cells to confer the complete multidrug-resistance phenotype. These results suggest that the human *MDR1* gene may be used as a positive selectable marker to introduce genes into human cells and to transform human cells to multidrug resistance without introducing nonhuman antigens.**

The development of multidrug resistance (MDR), which is an important clinical problem in cancer therapy, has been studied in tissue culture (1, 2). Characteristics of MDR mammalian cell lines include the following: (*i*) cross-resistance to many naturally occurring lipophilic cytotoxic drugs, including various plant alkaloids and antitumor antibiotics (3); (*ii*) decreased accumulation of these drugs resulting from an energy-dependent increase in drug efflux (4–6); (*iii*) phenotypic reversal of MDR by several agents including verapamil (4, 6, 7); (*iv*) increased amounts of a membrane glycoprotein of $M_r \approx 170{,}000$ (P-glycoprotein) (8–10); (*v*) amplification and overexpression of *"MDR1,"* the gene conferring MDR, or the gene for P-glycoprotein (11–18). Transfer of the human, mouse, and hamster *MDR1* or P-glycoprotein gene sequences has been linked to the transfer of the MDR phenotype (19–21). In this work we show that a full-length cloned human *MDR1* cDNA sequence can confer multidrug resistance in sensitive cells. Similar results using the mouse *MDR* gene have been recently reported (22).

MATERIALS AND METHODS

Transfection. DNA transfection of NIH 3T3 cells and KB3-1 cells was performed by a calcium phosphate precipitation method as described (20).

DNA and RNA Blot Hybridization. DNA was extracted from transfectants (13) and used for Southern blots (23). Total cellular RNA was isolated from the transfectants (20) and used for RNA blots (23). The 3.4-kilobase (kb) cDNA probe was labeled with ^{32}P to a specific activity of $1–2 \times 10^9$ dpm/μg by nick translation. Hybridization was done at 42°C in 50% formamide containing 5× NaCl/Cit (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% $NaDodSO_4$, 50 mM Tris·HCl, and 100 μg of salmon sperm DNA per ml, followed by washing with 0.2× NaCl/Cit containing 0.2% $NaDodSO_4$ at 60°C for DNA hybridization and with 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$ at 70°C for RNA hybridization at high stringency.

Ribonuclease Protection Assay. Human *MDR1* mRNA transcribed from the retroviral promoter was detected with a ribonuclease protection assay using a uniformly labeled SP6 antisense RNA probe (785 nucleotides) derived from a 1-kb genomic fragment containing the *MDR1* transcription initiation site and the first intron of the *MDR1* gene cloned in pGEM3 (Promega Biotec, Madison, WI). Total cellular RNA (10 μg) was hybridized with 6×10^5 cpm of probe, and ribonuclease digestion was performed as described (24).

RESULTS

Construction of a Full-Length cDNA for the Human *MDR1* Gene. Using a genomic *MDR1* probe (14), we isolated an overlapping set of cDNA clones for the *MDR1* gene from a phage λgt11 cDNA library made from mRNA from the MDR human KB carcinoma mutant line KB-C2.5 (25). Sequence analysis (26) has indicated that this cDNA contains an open reading frame for a protein of 1280 amino acids (141,475 Da), consistent with the estimated size of the unglycosylated form of P-glycoprotein. The sequence indicates the presence of 12 membrane-spanning regions and two nucleotide-binding sites. These findings, together with direct evidence that P-glycoprotein is concentrated in the plasma membrane (M. C. Willingham, N. D. Richert, M. M. Cornwell, T. Tsuruo, H. Hamada, M.M.G., and I.P., unpublished results), indicate that it is an energy-dependent drug-efflux pump (26).

To examine more directly the role of the *MDR1* gene, we constructed a full-length cDNA for the human *MDR1* gene as shown in Fig. 1. Three inserts from phages λHDR5, 10, and 104 were subcloned into the *Eco*RI site of a pGEM4 vector. The insert from λHDR5 was subcloned as two fragments (A and B) after *Eco*RI digestion. Fragment 5A was the fragment 3′ to fragment 5B. Insert 10, prepared from pMDR10-2, was introduced into pMDR5A-2, which was partially digested with *Eco*RI. Then the *Sst* I–*Hin*dIII fragment from pMDR10+5A and the *Hin*dIII–*Eco*RI fragment from pMDR104-2 were inserted into the *Sst* I–*Eco*RI sites of pGEM2. This step removed the 5′-end *Eco*RI–*Sst* I noncoding region from fragment 10. The human *MDR1* gene that is expressed in colchicine-selected KB cells has two promoters (25). But most cell lines, including vinblastine- or doxorubicin (former generic name adriamycin)-selected MDR human KB cells, human hepatoma cells (HepG2), normal human kidney cells (25), and normal human liver, adrenal, and colon cells

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Abbreviations: MDR, multidrug resistance; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; Ha-MSV, Harvey murine sarcoma virus.

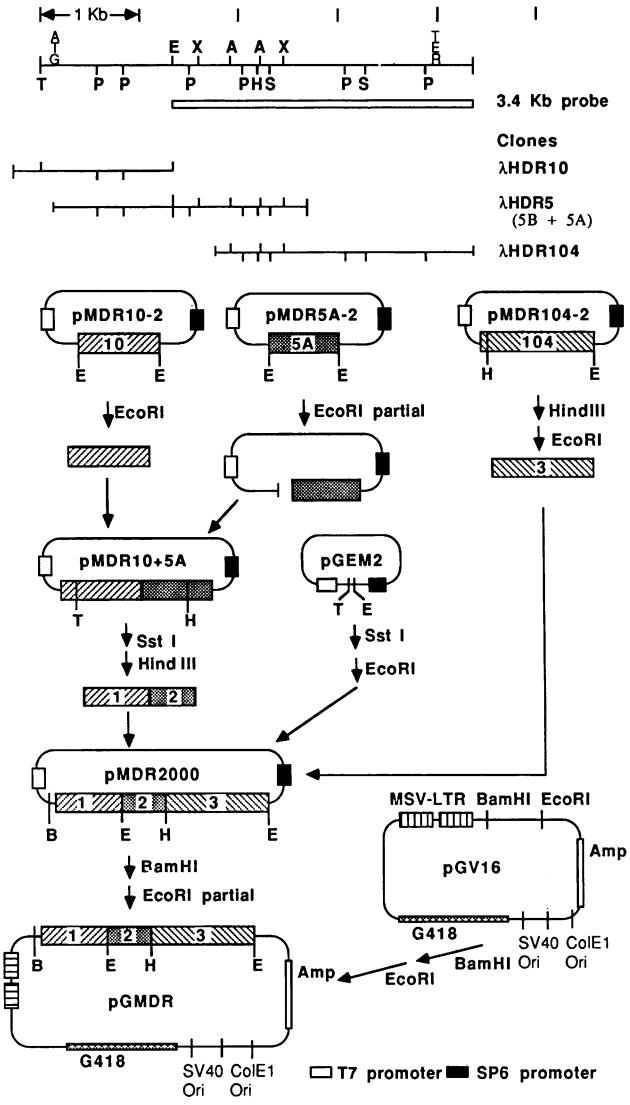

FIG. 1. Construction of a full-length cDNA for the human *MDR1* gene. At the top is the restriction map of the full-length cDNA for the *MDR1* gene and three cDNA overlapping clones isolated from a phage λgt11 cDNA library made from mRNA from the MDR human KB carcinoma mutant line KB-C2.5 (9), which was selected in medium containing colchicine at 2.5 μg/ml. The bottom shows the reconstruction of a full-length cDNA and its introduction into the mammalian expression vector pGV16. Restriction sites: A, *Acc* I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pvu* II; S, *Stu* I; T, *Sst* I; X, *Xmn* I.

(K.U., M.M.G., and I.P., unpublished results) use mainly the downstream promoter and transcription starts from the *Sst* I site. Therefore, the 4396-nucleotide insert in pMDR2000, containing a 23-nucleotide poly(A) stretch at its 3′ end, is a full-length cDNA for the human *MDR1* gene.

The Human *MDR1* Gene Confers MDR. To test the function of the isolated *MDR1* gene, the 4.4-kb insert of pMDR2000 was introduced into the eukaryotic expression vector pGV16, a gift of Terry Robins [Frederick Cancer Research Center (27)] and designated pGMDR (Fig. 1). The pGV16 vector has the long terminal repeats (LTRs) of Moloney murine leukemia virus (Mo-MuLV) as a promoter just before the cloning sites, and it also contains a G418-resistance gene (*neo*) under control of the simian virus 40 promoter. The expression plasmid pGMDR was transfected into drug-sensitive NIH 3T3 cells by using a two-step protocol previously described for the transfer of the human genomic *MDR1* gene into NIH cells, which was designed to enrich for transfected cells and to reduce the background of spontaneous drug-resistant NIH 3T3 cells (20). Transfected cells were first enriched by selection in medium containing G418 at 0.8 mg/ml (20); then pooled G418-resistant cells were selected in medium containing colchicine at 60 ng/ml, which is 3–5 times as high as the LD_{50} of colchicine for the parental NIH 3T3 cells. Six dishes of NIH 3T3 cells (3×10^5 cells per 10-cm dish) were transfected with 10 μg of pGMDR DNA per dish. After selection in medium with G418 for 9 days, about 2000 G418-resistant colonies were pooled. Two dishes of G418-resistant cells (1×10^4 cells per 10-cm dish) were then selected in medium containing colchicine at 60 ng/ml, and 5 resistant colonies were obtained from one dish and 7 from the other (frequency, 6×10^{-4} per G418-resistant cell). No nontransfected control NIH 3T3 cells and no G418-resistant NIH 3T3 cells transfected with the vector without the *MDR1* insert were found to be resistant to colchicine at 60 ng/ml. Therefore, the emergence of colchicine-resistant colonies appeared to result from transfection of the human *MDR1* gene. To examine the role of the human *MDR1* gene in the MDR phenotype, five colonies were isolated and their levels of cross-resistance to colchicine, vinblastine, and doxorubicin were examined (Fig. 2 and Table 1). All five colonies were cross-resistant to colchicine, vinblastine, and doxorubicin. They showed 6- to 15-fold, 3- to 10-fold, and 1.5- to 3-fold increases in resistance to colchicine, vinblastine, and doxorubicin, respectively, when compared with control NIH 3T3 cells. The pattern of relative resistance was not identical in each cell line. One transfectant was analyzed in greater detail and found to be 4-fold resistant to actinomycin D and 2-fold resistant to puromycin.

To demonstrate the presence of the human *MDR1* gene in the transfected cells, genomic DNAs were digested with *Eco*RI and analyzed by Southern hybridization (Fig. 3). The 3.4-kb cDNA probe was obtained from the 3′ region of the full-length human *MDR1* cDNA by digestion with *Eco*RI; it cross-hybridized to the endogenous mouse *mdr* gene of the parental NIH 3T3 cells and of each transfectant. The intensity of these bands clearly shows that each transfectant contains the same number of copies of the endogenous mouse *mdr* gene. This probe also strongly hybridized to a 3.4-kb *Eco*RI fragment (shown by an arrow) in each transfectant that was not detected in the parental NIH 3T3 cells and, therefore, represents the intact *Eco*RI fragment of pGMDR. Various copy numbers of pGMDR were found: low copy numbers in clones NT-15 and -16 (not shown), intermediate copy numbers in clones NT-11 and -13, and high copy numbers in clone NT-12. In the transfectants, no obvious rearrangement was

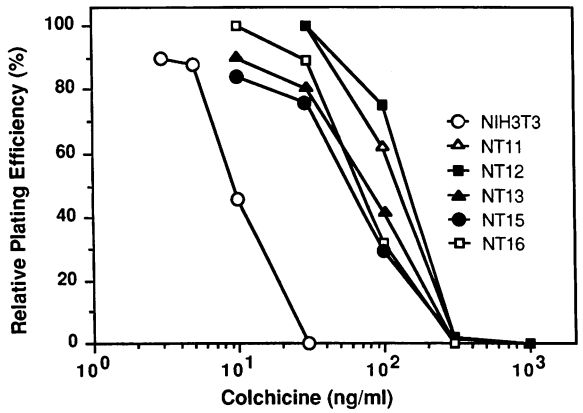

FIG. 2. Colony-forming ability of parental NIH 3T3 and transfectants as a function of colchicine concentration. NIH 3T3 cells (300 per dish) and the five independently isolated transfectants were plated in 60-mm dishes in medium containing various amounts of colchicine. On day 10, colonies were stained and counted.

Table 1. Relative resistance of transfectants

Cell line	Relative resistance*			Human *MDR1* copy number
	Colchicine	Vinblastine	Doxorubicin	
NIH 3T3	1	1	1	0
NT-11	13	9	3	Intermediate
NT-12	15	3	2.5	High
NT-13	8	10	2	Intermediate
NT-15	6	4	1.6	Low
NT-16	7	3	1.5	Low

*Relative resistance is expressed as the LD_{50} of the transfectants divided by the LD_{50} of NIH 3T3 cells.

detected except in NT-12. The relative drug resistance correlates to some extent with the copy number of *MDR1* cDNA (see Table 1).

The 3.4-kb cDNA probe was also used to analyze transcripts in transfectants by RNA hybridization (Fig. 4). This probe detected two major RNAs of 11 and 6 kb in all of the transfectants (shown by arrows), probably representing two transcripts initiated from the Mo-MuLV LTR. The 11-kb RNA probably terminates in the second LTR, and the 6-kb RNA uses the polyadenylylation signal at the 3′ end of the *MDR1* cDNA. This result suggests that the polyadenylylation signal at the end of the *MDR1* cDNA worked weakly in most of the transfectants. In addition, monoclonal antibody MRK-16, which recognizes human P-glycoprotein (28), detected overexpressed human P-glycoprotein on the cell surface of the transfectants (data not shown).

Transfer of Drug Resistance to Human KB Cells. To confirm that the vector pGMDR carried a *MDR1* cDNA that functions in human cells, we also introduced this vector into KB cells and isolated colchicine-resistant clones (selected in medium containing colchicine at 10 ng/ml) by the same procedure used to isolate NIH 3T3 transfectants. The frequency of drug resistance was approximately the same as for the NIH 3T3 transfectants. One tested transfectant (KB-T10) was 6 times more resistant to colchicine, 2 times more resistant to vinblastine, and 2 times more resistant to doxorubicin than the parent NIH 3T3. We detected no amplification of the endogenous *MDR1* gene but did see amplification of the 3.4-kb transferred *Eco*RI fragment, which was not detected in parental KB3-1 cells by Southern hybridization (Fig. 3). Transcription of exogenous *MDR1* cDNA from the Mo-MuLV LTR was demonstrated by the ribonuclease protection assay in one MDR KB transfectant (Fig. 5). A 1-kb genomic fragment of the human *MDR1* gene containing the transcription initiation site of the downstream promoter and the first intron was used as a template to produce an RNA probe. Total cellular RNA from KB-C4 protected two RNA species from ribonuclease digestion: a 134-nucleotide region of the RNA probe was protected by the *MDR1* transcript from the downstream promoter, and a 323-nucleotide region of the RNA probe was protected by the *MDR1* transcript from the upstream promoter. Total cellular RNA from human normal tissues protected only a 134-nucleotide RNA (unpublished result). The exogenous *MDR1* transcript from the Mo-MuLV LTR protected a 138-nucleotide region of the RNA probe because of the plasmid sequence (shown by an arrow in Fig. 5), so it can be distinguished from the endogenous *MDR1* transcript.

We also inserted the *MDR1* full-length cDNA into a retroviral expression vector, pCO1, containing two Ha-MSV (Harvey murine sarcoma virus) LTRs (ref. 29; D. R. Lowy, personal communication), and used this DNA from this plasmid, pHaMDR, to directly transfect NIH 3T3 cells and KB cells. The experiment in Table 2 shows that this construction also produced MDR clones. In these experiments,

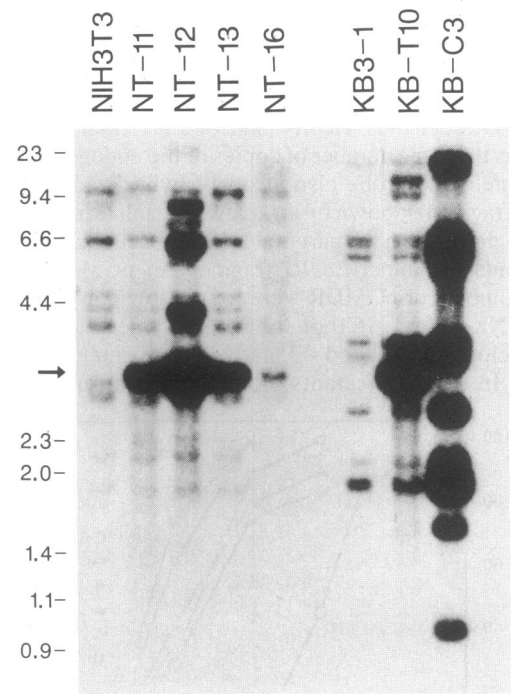

FIG. 3. Southern hybridization of genomic DNA from transfectants. Genomic DNA (7 μg) from transfectants, NIH 3T3, KB3-1, and KB-C3 were digested with *Eco*RI. After electrophoresis, DNA was transferred to a nitrocellulose filter and hybridized with the 3.4-kb *Eco*RI-digested cDNA fragment shown in Fig. 1. The band intensity seen in the KB-C3 lane represents about 20 copies of the *MDR1* gene. The arrow represents the position of the 3.4-kb *Eco*RI-digested cDNA fragment in pGMDR. Size markers are *Hin*dIII–digested phage λ DNA and *Hae* III-digested phage φX174 DNA.

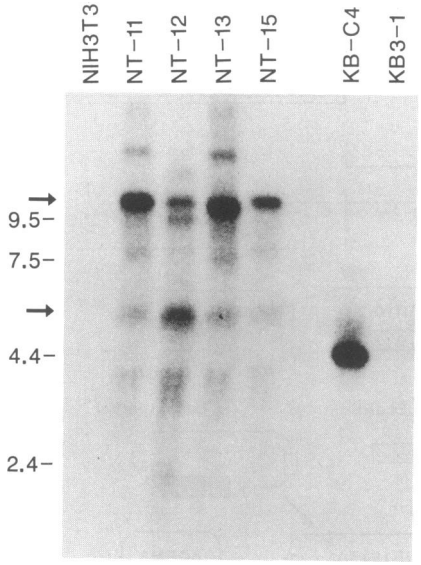

FIG. 4. Blot hybridization of RNA from NIH 3T3 transfectants. Total RNA (7 μg) from transfectants, and NIH 3T3, KB3-1, and KB-C4 (colchicine-selected MDR KB line) cells was transferred to a nitrocellulose filter after electrophoresis. The blot was hybridized with the 3.4-kb *Eco*RI-digested cDNA fragment (shown in Fig. 1) and washed at 70°C in 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$. Under these conditions the probe hybridized to an RNA of approximately 6 and 11 kb from the transfectants and an RNA of 4.5 kb from KB-C4 but not to mouse *mdr* mRNA (20). The size markers are an RNA ladder (Bethesda Research Laboratories).

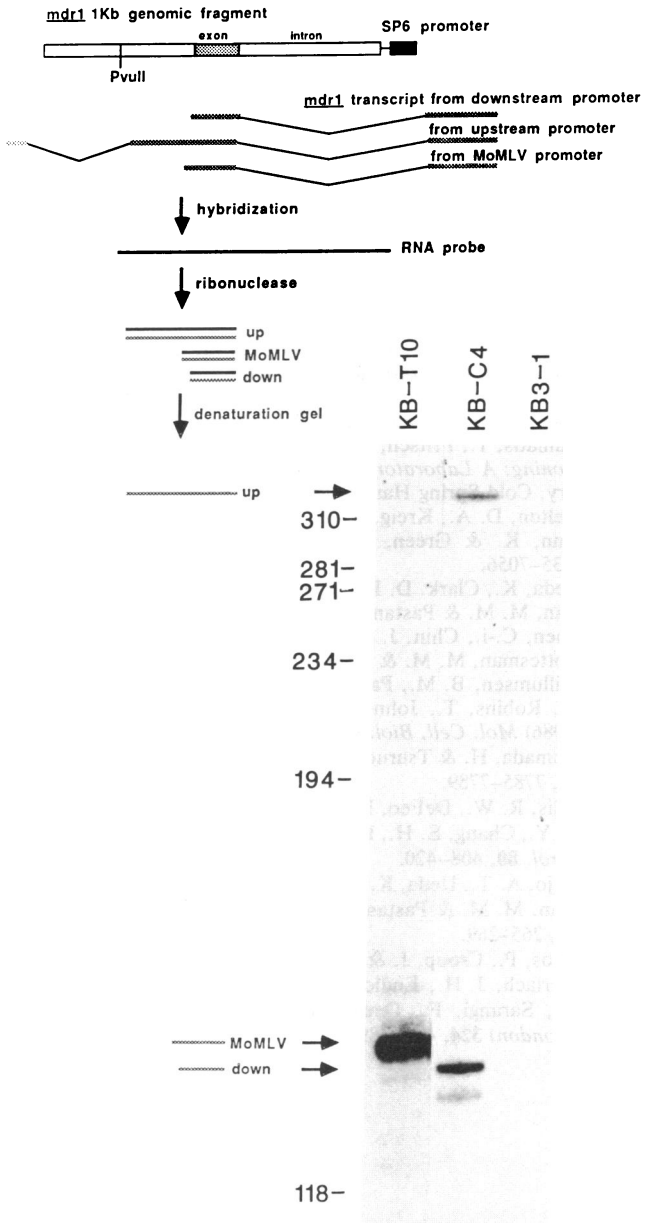

FIG. 5. Ribonuclease protection assay of RNA from the KB transfectant KB-T10. Human *MDR1* transcripts from the upstream promoter and the downstream promoter in KB-C4 cells and from the Mo-MuLV promoter in transfectant KB-T10 were detected by using a uniformly labeled nucleotide antisense RNA probe. The RNA probe was produced by *in vitro* transcription from the SP6 promoter in pGEM3 containing a 1-kb genomic fragment that encodes the downstream transcription initiation site and the first intron of the human *MDR1* gene. Total cellular RNA (10 μg) from KB3-1, KB-T10, and KB-C4 was hybridized to the probe. The numbers indicate size markers in nucleotides. MoMLV, Moloney murine leukemia virus.

instead of first selecting for G418 resistance and then screening the surviving cells for resistance to colchicine, vinblastine, or doxorubicin, we directly selected transfected cells with colchicine, vinblastine, or doxorubicin. Colchicine-, vinblastine-, or doxorubicin-resistant colonies could be selected directly with a frequency of about 1 in 1000–5000 (Table 2). The frequency of colchicine-resistant colonies was similar to the frequency of G418-resistant colonies when using 3 μg of pSV2neo DNA or 10 μg of pGV16 DNA with the same transfection protocol. No drug-resistant clones

Table 2. Frequency of drug-resistant colonies

Plasmid	Colonies per dish		
	Colchicine	Vinblastine	Doxorubicin
pHaMDR	110	20	30
pCO1 (p21)	0	0	0
pCO12 (EGFR)	0	0	0

KB3-1 (3×10^5 cells per 10-cm dish) were transfected with 10 μg of DNA; 48 hr after transfection, the cells were split into three dishes and cultured in the presence of colchicine (10 ng/ml), vinblastine (4 ng/ml), or doxorubicin (30 ng/ml). On day 9, the cells were stained and colonies counted.

resulted from transfecting KB cells with pCO1 itself, which contains a functional p21 gene or a similar vector in which a cDNA from the human epidermal growth factor receptor replaced p21 (T. J. Velu, D. R. Lowy, and I.P., unpublished results).

DISCUSSION

These results show that a reconstructed full-length cDNA for the *MDR1* gene produces a functional P-glycoprotein. High expression of this *MDR1* cDNA appears to be sufficient to confer the MDR phenotype. Although we cannot eliminate the possibility of the involvement of some host cell proteins or cofactors in expression of the MDR phenotype, for example in "trapping" the drug and "delivering" it to P-glycoprotein, the limiting step in MDR appears to be the amount of the product of the *MDR1* gene (P-glycoprotein). Our results also indicate that high copy numbers of the *MDR1* gene are not necessary for resistance to low concentrations of drugs. We have previously shown that in MDR cell lines, activation of transcription can precede gene amplification (15). In clinical situations, a low degree of resistance resulting from *MDR1* gene activation appears to be sufficient to cause MDR in some human tumors (30).

Previous studies have indicated that the relative degree of resistance to individual drugs in the MDR phenotype is highly variable (9, 15). In this work, using a transfected cDNA for the *MDR1* gene, we also observed considerable variation in relative drug-resistances (compare colchicine/vinblastine relative resistances in Table 1). These results suggest that host factors, such as expression of other proteins and posttranslational modification of the P-glycoprotein, may play a role in modulating the MDR phenotype.

In the two-step protocol, using the Mo-MuLV LTR containing vector pGV16, the frequency of MDR colonies among G418-selected transfectants was approximately 6×10^{-4} to 1×10^{-3}, to give an overall frequency of MDR transfectants of between 6×10^{-7} and 1×10^{-6}. This is lower than the frequency of 2×10^{-4} observed by Gros *et al.* (22), who selected directly for doxorubicin-resistance with a cloned mouse *mdr* cDNA. It seemed likely that the relatively low transfection efficiency was related to inefficient expression of the Mo-MuLV LTR *MDR1* construction, so we prepared another construction using a Ha-MSV LTR and found that we could directly select MDR colonies at a frequency of approximately 10^{-3}. These results suggest that when introduced into bone marrow, the human *MDR1* cDNA clone might be useful as a positive selectable marker for the introduction of other genes in gene therapy or to make human bone marrow cells resistant to drugs used in chemotherapy so that cancers can be treated more aggressively (30).

As already noted, sequence analysis of the human (26), mouse (31), and hamster (32) *mdr* gene products indicates that they have features consistent with the predicted function of P-glycoprotein as an energy-dependent efflux pump responsible for decreased drug accumulation in MDR cells. The functional full-length human cDNA under the control of an

active retroviral promoter constructed for this work will facilitate the biochemical study of this human drug efflux pump and may be useful for introduction of the human *MDR* gene into bone marrow and other tissues.

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