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Genetics

Retroviral transfer of a murine cDNA for multidrug resistance confers pleiotropic drug resistance to cells without prior drug selection

(retrovirus expression vector/human histone H4 promoter)

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ABSTRACT **We have constructed a retrovirus expression vector that carries the murine mdr cDNA transcribed under the control of the human H4 histone promoter to examine the feasibility of efficiently transferring a multidrug resistance phenotype to cells without requiring drug selection. This approach will facilitate the transfer of mdr cDNA to hematopoietic progenitor cells for the study of multidrug resistance *in vivo*. The retrovirus vector pHmdr has been used for transmission and expression of the mdr cDNA in initially drug-sensitive NIH 3T3 fibroblasts. Selection of pHmdr infectants in the cytotoxic agents colchicine or doxorubicin gave rise to highly multidrug-resistant colonies containing a single gene copy of the vector. Moreover, in the analysis of 12 cloned unselected NIH 3T3 cell infectants, a multidrug resistance phenotype was conferred by as few as two copies of the pHmdr vector. Overexpression of the mdr cDNA in drug-selected and unselected pHmdr infectants was directly related to cell survival in three cytotoxic agents tested. These results hold significant implications for the study of multidrug resistance *in vivo*.**

The treatment of human tumors with chemotherapeutic agents often results in the emergence and expansion of multidrug-resistant cells (1). In a similar fashion, outgrowths of drug-resistant cells arise when cultured cells are exposed to a selective regimen of cytotoxic agents. Drug-resistant cells initially selected in one drug usually display a broad spectrum of resistance to a variety of cytotoxic agents (2–5). Acquisition of a multidrug resistance phenotype is associated with amplified domains of DNA and overexpression of sequences from a small *mdr* gene family (6–9). Both murine (10) and human (11) cDNA clones for one member of this *mdr* gene family have been isolated, sequenced, and found to encode a protein characteristic of a 170-kDa cell-surface glycoprotein that bears striking homology to bacterial transport proteins. This homology suggests a role for *mdr* in active cellular transport processes (10) that could result in a decreased intracellular accumulation of cytotoxic agents (3, 12–14) thus accounting for the mdr phenotype. The actual mechanism by which *mdr* achieves a net decrease of drug accumulation in drug-resistant cell lines remains unresolved.

We have shown that the introduction by DNA-mediated gene transfer of multiple copies of a cDNA encoding a mouse *mdr* gene can confer the multidrug resistance phenotype on Chinese hamster ovary cells (15). The requirements in those experiments for the transfer of multiple copies of the mdr cDNA and the inherent inefficiency of DNA-mediated gene transfer led us to develop a more efficient method for transferring and expressing the *mdr* gene. To accomplish these objectives we have introduced the mdr cDNA into the retroviral vector pH, which permits high-efficiency transfer of a transcriptionally active mdr cDNA (B.C.G., M. Finer, D.E.H., and R.C.M., unpublished observations).

MATERIALS AND METHODS

Plasmid Construction, Transfection of ψ-2 Packaging Cells, and Selection of pHmdr Virus Producer Cells. The murine cDNA for *mdr*, with its polyadenylylation sequence deleted, was cloned into the retrovirus expression vector pH (B.C.G., M. Finer, D.E.H., and R.C.M., unpublished observations) by the following method. Digestion of λDR11 DNA (10) with *Eco*RI released a 4.3-kilobase (kb) fragment containing the entire mdr cDNA, which was then apportioned into two aliquots. The insert contained in one portion was adapted with *Bcl* I linkers and digested with *Acc* I, yielding a 1.9-kb *Bcl* I–*Acc* I subfragment. The insert of the second aliquot was digested to completion with *Dra* I (thereby removing the polyadenylylation sequence), adapted with *Bcl* I linkers, and digested with *Acc* I, yielding a 2.1-kb *Acc* I–*Bcl* I subfragment. The combined subfragments were cloned into the *Bam*HI site of the retrovirus expression vector pH (B.C.G., M. Finer, D.E.H., and R.C.M., unpublished observations), reconstituting an intact mdr cDNA within the transcriptional unit of the recombinant provirus. The expression vector pHmdr is contained within mouse genomic DNA flanking sequences of pZIPNeo on a backbone of pBR322 extending from the *Hin*dIII site at nucleotide 29 to the *Eco*RI site at nucleotide 4361.

The retrovirus expression vector pHmdr was stably introduced into ψ-2 packaging cells (16) by cotransfection with pSV2Neo (at a pHmdr/pSV2Neo weight ratio of 10:1), and transfected cells were selected in G418 (0.7 mg/ml). Single colonies of G418-resistant ψ-2 transfectants were isolated and tested for transmission of pHmdr by infecting NIH 3T3 cells (16). Three days later, DNA was extracted and digested with *Kpn* I, which releases the provirus in two fragments of 1.5 and 4.1 kb as determined by Southern gel analysis (17). The producer cell line ψ2Hmdr7, a high-titer producer of the virus, was used to infect all NIH 3T3 cells described herein.

Cell Culture, Viral Infection, and Selection in Cytotoxic Agents. NIH 3T3 cells and ψ-2 packaging cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum. Viral supernatant from the pHmdr producer cell line ψ2Hmdr7 was prepared by harvesting 10 ml of normal medium, applied 18 hr earlier, from a confluent 10-cm^2 monolayer and then filtering the medium through a 0.45-μm (pore size) filter. NIH 3T3 cells

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Abbreviation: LTR, long terminal repeat.

(5×10^5 cells) were infected with 2 ml of viral supernatant in the presence of Polybrene as described (16). Two days after infection, cultures were divided into selective medium containing either colchicine (0.1 or 0.2 μg/ml) or doxorubicin (0.05 or 0.1 μg/ml). After a 2-week selection in colchicine or a 3- to 4-week selection in doxorubicin, with changes of medium at 3-day intervals, drug-resistant colonies were picked and expanded.

Nineteen unselected pHmdr-infected NIH 3T3 cell clones (US-1 to -19) were isolated by single-cell cloning of infected-cell populations 2 days after infection with pHmdr. Single-cell cloning was done without drug selection. All drug-selected and unselected pHmdr infectants were maintained in normal medium until tested.

Cells from pHmdr infectants or transfectants (500 cells from each clone) and control NIH 3T3 cells were plated in medium containing doxorubicin, colchicine (0, 0.025, 0.050, 0.075, 0.10, and 0.20 μg/ml), or vinblastine (0, 0.003, 0.009, 0.015, 0.030, and 0.060 μg/ml). Medium was changed 4 days after culturing. At day 9 colonies were stained with crystal violet, and colonies were counted. The relative plating efficiency of each clone was determined as the percentage of the plating efficiency of NIH 3T3 control cells grown in the absence of drugs versus the logarithm of the drug concentration. D_{50} is the drug dose necessary to produce 50% plating efficiency.

Southern Hybridization Analysis. Genomic DNA was prepared from confluent 10-cm^2 plates of cells, digested to completion with either *Kpn* I or *Eco*RI, subjected to electrophoresis in 1% agarose gels, and transferred to nylon hybridization membranes (Zetabind, Cuno) as described (17). Southern blots were probed with ^{32}P-labeled insert from λDR11 prepared by oligonucleotide labeling (18).

RNA Gel Blot Hybridization Analysis. Total cellular RNA was prepared from plates of 5×10^6 confluent cells as described (19). RNA was subjected to electrophoresis in 1% agarose gels containing 20 mM Mops (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA, and 7% (vol/vol) formaldehyde for 24 hr. RNA was transferred to nitrocellulose filters and probed with ^{32}P-labeled insert DNA from λDR11 or with a U3 probe taken from the long terminal repeat (LTR) of a Moloney murine leukemia virus.

RESULTS

Construction and Transmission of Retrovirus Expression Vector pHmdr. The insertion of λDR11 into the retrovirus expression vector pH is outlined in Fig. 1. We have shown (B.C.G., M. Finer, D.E.H., and R.C.M., unpublished results) that the human histone H4 promoter is a strong constitutive promoter in NIH 3T3 fibroblasts, in F9 embryonal carcinoma cells, and in the murine hematopoietic system *in vivo*. The vector backbone contains a mutation at the normal 5′-splice site to reduce the possibility of aberrant splicing events that might interfere with expression of inserts. In addition, the viral enhancer sequences have been removed from the 3′-LTR to eliminate transcription from the LTRs after integration of the provirus into a recipient genome.

To assess transmission of the pHmdr vector and to determine whether the mdr cDNA retained its biological activity, drug-sensitive NIH 3T3 fibroblasts were infected with pHmdr and cultures were either divided into selective medium containing the drug colchicine or single-cell-cloned in the absence of drug selection. Colonies (1–7 mm in diameter) appeared within 8 days of selection in colchicine 0.1 μg/ml in infected target cell populations. Approximately one cell in 10^3 formed a large colony at this drug concentration, whereas no colonies appeared on NIH 3T3 cell control plates. Analysis of *Kpn* I digestion products of genomic DNA isolated from drug-resistant cells indicated that vector DNA sequences were transmitted intact as judged by release of 1.5- and 4.1-kb proviral fragments (Fig. 2*A*). No amplification of endogenous *mdr*-hybridizing bands was observed in any of the clones examined, which suggested that acquisition of the drug resistance phenotype occurred as a result of transfer of pHmdr to cells rather than as a consequence of an increase in the number of endogenous copies of the *mdr* gene. Identical results were obtained for NIH 3T3 cells selected in doxorubicin (0.05 and 0.1 μg/ml) (data not shown). In unselected infected fibroblasts transmission of pHmdr was examined in 19 individual isolates (12 of which are shown in Fig. 2*B*); 16 clones had the expected proviral structure after integration, 2 clones (US-6 and -9) had aberrant integration of the vector, and 1 clone (US-14) contained no proviral sequences. Clone US-4 contains less than a single gene copy of pHmdr, which suggests that it may not be clonal, whereas US-19 contains a single gene copy of the vector more evident on longer exposures of the blot. These data indicated that pHmdr could be efficiently and stably introduced into drug-sensitive fibroblasts without drug selection.

Analysis of Genomic DNA for Sites of Vector Integration in pHmdr-Infected NIH 3T3 Cells. To determine the number of vector copies that integrated into the genome of either selected or unselected clones, genomic DNA was isolated from each clone and digested with *Eco*RI. This restriction enzyme does not cleave within proviral sequences and thus releases a different-sized fragment for each integration site. Southern blot analysis of colchicine-resistant clones was performed, and the resulting autoradiograph was examined (Fig. 3). The appearance of distinctive bands revealed the number of integration events per clone. This analysis revealed that 5 of 5 clones selected at a dose of 0.1 μg/ml contained a single copy of the pHmdr vector and that, of two clones selected at a higher dose of colchicine (0.2 μg/ml), one contained a single copy of pHmdr, and the other con-

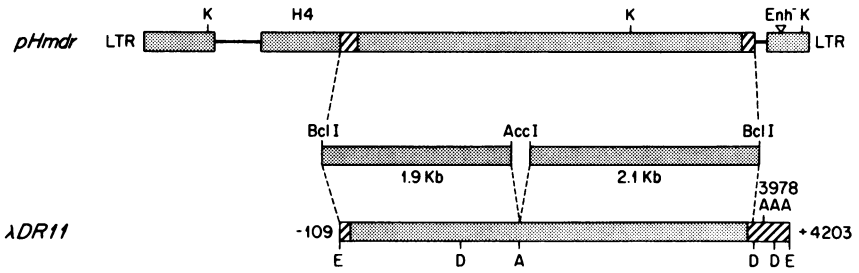

FIG. 1. Retrovirus expression vector pHmdr containing a biologically active murine cDNA for multidrug resistance. The murine mdr cDNA taken from λDR11 (ref. 10) was deleted of its polyadenylylation sequence and cloned into retrovirus expression vector pH (B.C.G., M. Finer, D.E.H., and R.C.M., unpublished observations). Elimination of viral enhancer sequences from the 3′ LTR results in transcriptional inactivation of the viral 5′ LTR after transfer and integration of the provirus into a recipient genome. In infected cells, transcription of the inserted mdr cDNA is initiated at the internal human histone promoter and terminates in the viral 3′ LTR. A, *Acc* I; D, *Dra* I; E, *Eco*RI; and K, *Kpn* I.

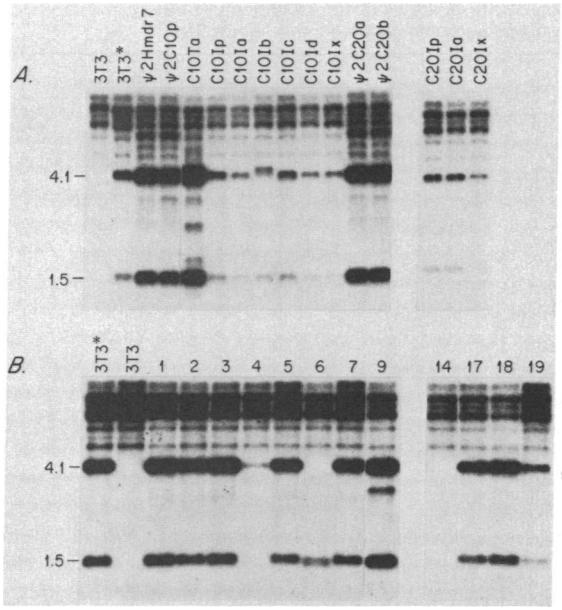

FIG. 2. Southern blot analysis of genomic DNA from NIH 3T3 fibroblasts infected with pHmdr, digested with *Kpn* I, and hybridized to radioactive insert DNA from λDR11. (*A*) Colchicine-selected pHmdr infectants. Lanes: 3T3 and 3T3*, control cells that are uninfected NIH 3T3 cells and NIH 3T3* cells plus pHmdr plasmid DNA marker (10 pg), respectively; ψ2Hmdr7, a pHmdr ψ-2 producer cell line; ψ2C10p, a ψ2Hmdr7 cell population selected with colchicine at 0.1 μg/ml; ψ2C20-a and -b, clones selected with colchicine at 0.2 μg/ml; C10Ta, an NIH 3T3 clone transfected and selected with colchicine at 0.1 μg/ml; C10Ip, an NIH 3T3 cell population infected and selected with colchicine at 0.1 μg/ml; C10I-a, -b, -c, -d, and -x, cell clones derived from C10Ip cells; C20Ip, an NIH 3T3 cell population infected and selected with colchicine at 0.2 μg/ml; and C20I-a and -x, cell clones derived from C20Ip cells. (*B*) Cloned unselected pHmdr infectants (lanes 1–7, 9, 14, and 17–19; lanes correspond to names of clones). Controls (lanes 3T3* and 3T3) are as in *A*.

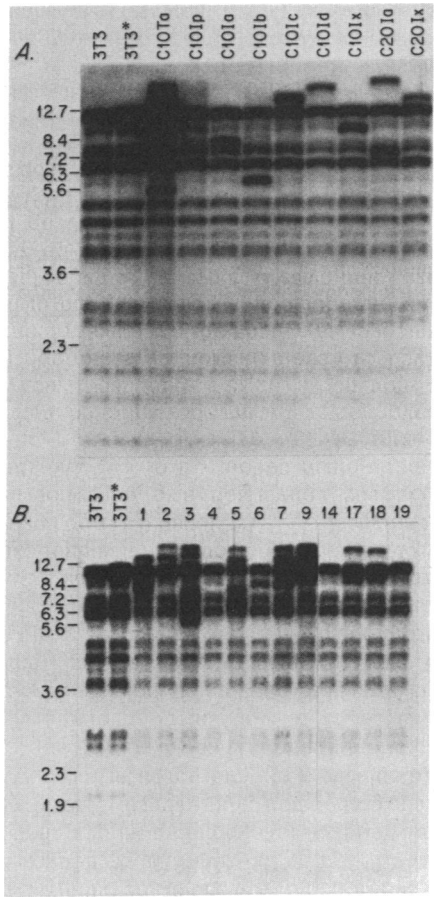

FIG. 3. Southern blot of integration events in genomic DNA of pHmdr infectants. Genomic DNA isolated from each infectant was digested to completion with *Eco*RI and probed with radioactive insert from λDR11. (*A*) Colchicine-selected NIH 3T3 cell infectants (for doses and lane assignments, see Fig. 2). Note, the 12.7-kb pHmdr marker comigrates with an endogenous *mdr*-hybridizing band. (*B*) Unselected NIH 3T3 cell clones infected with pHmdr. Quantitation of vector integrations is presented in Table 1 for each clonal cell line (except C20Ix, which contains one copy of the provirus). Lanes are as in Fig. 2.

tained two copies (Fig. 3*A*). These observations indicated that transfer of a single copy of the mdr cDNA was sufficient to confer a high-level drug-resistance phenotype to fibroblasts. Unselected pHmdr-infected NIH 3T3 fibroblasts were also examined for site-specific integration events (Fig. 3*B*). Unselected pHmdr infectants contained from one to four copies of the vector, with the exceptions of US-14 that contained no pHmdr sequences and US-4 that may not be clonal as it contains less than a single gene copy of the vector.

Analysis of *mdr* Expression in pHmdr-Infected Cells. When a 4.3-kb mdr cDNA insert from λDR11 was used as a hybridization probe to screen RNA gel blots of either pHmdr-infected cells selected in colchicine (Fig. 4*A*) or unselected cells (Fig. 4*C*), one 4.5-kb band was detected, and, in some lanes, a 6.0-kb band was found. The 4.5-kb mdr transcript represents endogenous and vector-derived mRNAs, which are superimposed on these blots. The 6.0-kb band, most prominent in the ψ2Hmdr7-packaging cell line and in cells transfected with pHmdr, represents transcript initiated in the LTR of the recombinant pHmdr provirus (Fig. 4*A*). A detectable level of the 6.0-kb transcript (initiated in the viral 5′ LTR) was found in some pHmdr infectants despite the deletion of viral enhancer sequences in that LTR after proviral integration. Transcriptional enhancers present in the internal human histone H4 promoter of pHmdr may account for this low-level expression of the 6.0-kb transcript initiated in the enhancer-deleted 5′ LTR of the provirus.

To distinguish the endogenous 4.5-kb mdr cellular transcript from the vector-derived 4.5-kb mdr transcript initiated at the histone H4 promoter, it was necessary to use a 0.5-kb hybridization probe containing a portion of the U3 region obtained from a Moloney murine leukemia virus LTR (Fig. 4 *B* and *D*). The U3 probe detects those transcripts that utilize the polyadenylylation sequence in the 3′ LTR of the recombinant provirus and, in the ψ-2 packaging cell line, this probe detects the genomic and spliced transcripts of the ψ-Moloney murine leukemia virus packaging genome. There was no detection of mdr transcripts in uninfected NIH 3T3 cells even after prolonged autoradiographic exposures of the blots. High levels of vector-derived 4.5-kb mdr mRNA were present in all drug-resistant infectants and transfectants tested as compared with control, NIH 3T3, and ψ-2 cells (Fig. 4 *A* and *B*). Detectable levels of pHmdr-derived mdr mRNA were found in unselected infectants containing correctly integrated copies of the provirus, whereas US-4 and -19 (which contain one vector copy each), US-6 (aberrant pHmdr integration), and US-14 (no integration of pHmdr) showed no detectable amounts of the 4.5-kb transcript when the U3 probe was used. Quantitation of mRNA levels performed on linearly exposed RNA gel blots containing identical quantities (5 μg) of mRNA probed simultaneously with the same ^{32}P-labeled U3 probe revealed 2- to 20-fold greater expression of *mdr* in preselected versus unselected pHmdr infectants. Both the U3 and mdr hybridization

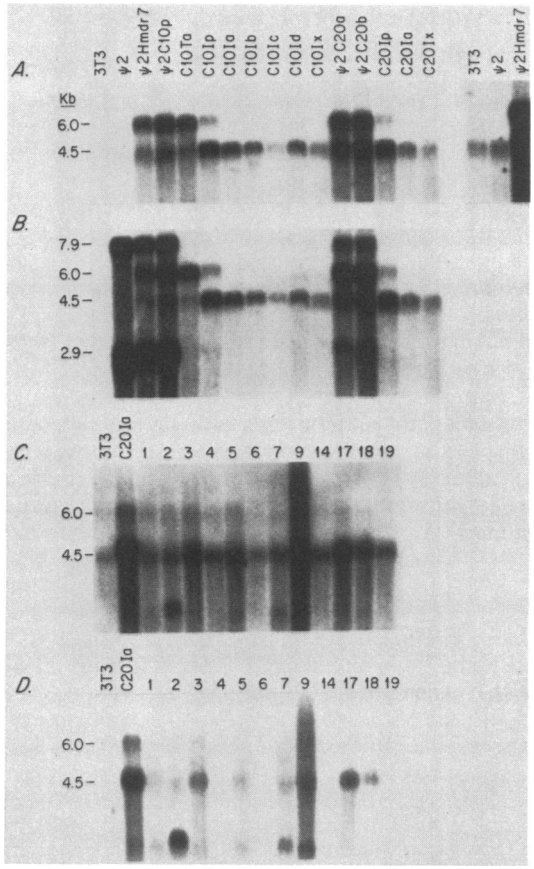

FIG. 4. Transcription analysis of NIH 3T3 cells infected or transfected with pHmdr. RNA gel blot of total cellular RNA (*A* and *B*, 5 μg from preselected infectants; *C* and *D*, 10 μg from unselected infectants) probed with radioactive cDNA insert of λDR11 (*A* and *C*) or with a U3 probe derived from the 3′ LTR of pHmdr (*B* and *D*). Exposure times: *A* and *C*, 18 hr; *B* and *D*, 48 hr. Lanes are as in Fig. 2 except that controls are unselected NIH 3T3 cells (lane 3T3), ψ-2 packaging cells (lane ψ2), and unselected ψ2Hmdr7 (lane ψ2Hmdr7). Longer exposures are presented for lanes indicated at the right side of *A*. Size markers 7.9 and 2.9 kb (*B*) indicate the full length and spliced forms, respectively, of RNA transcripts from the ψ genome in ψ-2 packaging cells.

probes also detected a smaller RNA species, most prominent in unselected clones, which may represent a truncated mRNA derived from the vector.

Analysis of Multidrug Resistance in pHmdr-Infected Cells. To characterize the spectrum of drug resistance in both the unselected and colchicine-selected cells, individual clones were tested in an inhibition of cell growth assay in the presence of three cytotoxic agents, colchicine, vinblastine, or doxorubicin (Table 1). All preselected colonies tested displayed a high level of drug resistance for all three drugs. A pleiotropic drug resistance phenotype was also shared by unselected pHmdr infectants, US-1, -2, -3, -5, -7, -9, -17, and -18, although they were less resistant to high doses of cytotoxic agents than were preselected clones. Unselected and preselected pHmdr infectants were further tested for their ability to survive prolonged exposure to colchicine at a dose of 0.1 μg/ml (Fig. 5). After a 1-week selection all of the cloned preselected infectants were stained, and each showed substantial growth in the presence of the drug when compared with six cloned uninfected NIH 3T3 cell controls. Unselected infectants grew more slowly than preselected colonies and were stained after 17 days revealing growth only from clones US-3, -7, -9, -17, and -18. A comparison of mRNA levels (Fig. 4*D*) to cell growth of unselected infectants (Fig. 5) revealed a direct relationship between the expression levels of exogenous *mdr* sequences and the rate

Table 1. Spectrum of drug resistance in individual drug-selected and unselected NIH 3T3 cells infected with pHmdr

Cell line	*mdr* copies, no. per cell	% drug resistance		
		Doxorubicin	Vinblastine	Colchicine
C10Ia	1	376	740	>700
C10Ib	1	238	830	350
C10Ic	1	250	560	460
C10Id	1	470	620	450
C10Ta	Many	580	>700	420
C20Ia	2	410	660	450
ψ2C20a	Many	500	>700	>700
ψ2C20b	Many	430	>700	560
US-1	3	140	240	172
US-2	2	150	170	180
US-3	4	170	170	150
US-4	1	105	110	75
US-5	3	120	160	150
US-6	*	100	130	90
US-7	3	132	190	190
US-9	3*	117	130	120
US-14	*	94	90	84
US-17	2	173	260	210
US-18	2	150	270	200
US-19	1	103	120	100

Results are presented as the ratio of the D_{50} for individual clones to the D_{50} of NIH 3T3 control cells expressed as a percentage. An asterisk indicates anomalous or no integration of pHmdr in those genomes (Fig. 2*B*).

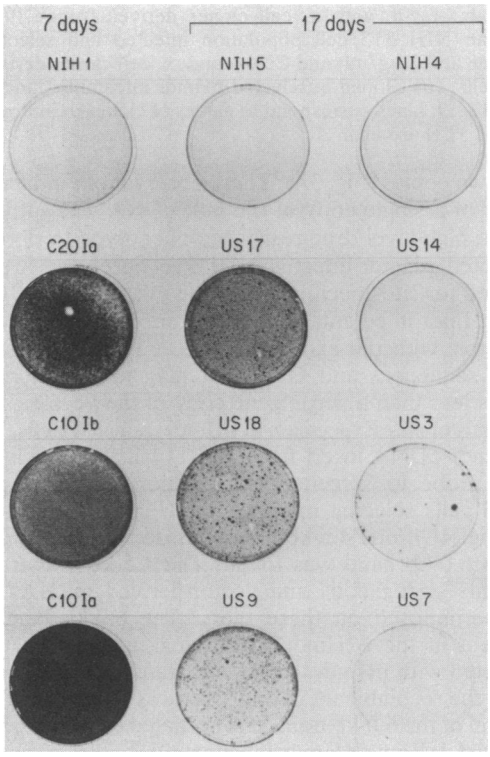

FIG. 5. Prolonged exposure to colchicine of cloned unselected and preselected pHmdr infectants. Clones were maintained in the absence of drug prior to being cultured in 10-cm dishes in medium containing colchicine (0.1 μg/ml). Cells were given fresh medium containing drug at 3-day intervals. Plates were maintained in colchicine for the times indicated and were then stained with crystal violet.

of drug-resistant cell growth. No growth was observed in other unselected infectants or in any of six cloned uninfected NIH 3T3 cell controls. No growth was observed for clones US-2 and -5 even though detectable amounts of exogenous mdr transcript were observed by using the U3 hybridization probe (Fig. 4*D*).

DISCUSSION

The experimental results obtained from retroviral transfer of *mdr* to unselected cloned cells demonstrate that without prior drug selection a complete multidrug resistance phenotype can be conferred by as few as two copies of pHmdr to initially drug-sensitive fibroblasts. Transfer of multidrug resistance previously has only been observed in drug-selected cells expressing high levels of *mdr* from multiply transfected copies of *mdr*-expression plasmids (15, 20, 21). The pHmdr vector was efficiently transferred to NIH 3T3 cells as was determined by analysis of cloned unselected infectants; however, high-dose drug selection of infected cells gave rise to drug-resistant colonies at a frequency of one cell in 10^3 cells. One explanation for this may be that colonies that are highly drug-resistant arise from those cells where sufficient overexpression of the mdr cDNA is favored by vector position within the recipient genome. Alternatively, a few cells within any apparently uniform cell population may undergo epigenetic changes at some low frequency that in conjunction with exogenous expression of *mdr* may confer a drug resistance phenotype. Colchicine selection of pHmdr-infected NIH 3T3 cell populations gave rise to drug-resistant colonies at a frequency of one cell in 10^3 cells when those cells were initially exposed to a high dose of colchicine (0.2 μg/ml). Unselected pHmdr infectants, when initially exposed to a lower dose of colchicine (0.1 μg/ml), gave rise to drug-resistant cell growth in five out of eight clones that express exogenous mdr transcripts, a frequency that is too high to be explained by epigenetic changes. Moreover, these five unselected infectants displayed growth rates in colchicine (0.1 μg/ml) that were directly related to their *mdr* expression levels. These data favor the hypothesis that vector position within the genome influences the level of *mdr* expression and that the level of *mdr* expression with time is a primary factor determining cell survival in selective medium.

The drug resistance phenomenon is difficult to study *in vivo*, as tumor samples obtained from patients are often composed of both drug-resistant and drug-sensitive cells in unknown proportions and are often contaminated with cells from the immune and reticuloendothelial systems. A more direct *in vivo* experimental approach is needed to elucidate the parameters of drug resistance in whole animals and to analyze the kinetics of response of drug-resistant cells during experimental chemotherapy. This paper describes the development and characterization of a model system for retroviral transfer of *mdr* to study drug resistance *in vivo*.

The pHmdr expression vector can be used to genetically modify hematopoietic stem cells for reintroduction into the murine hematopoietic system. Cells of the hematopoietic system may be more sensitive to cytotoxic agents *in vivo* than are tissue culture cells *in vitro*; therefore, low-level expression of exogenous *mdr* may provide suitable protection for genetically modified reconstituting blood cells during chemotherapeutic treatment of mice. The mdr cDNA can be used as a dominant selectable marker (15, 20, 21); therefore, drug selection of pHmdr-infected bone marrow cultures prior to transplantation may give rise to outgrowths of multidrug-resistant hematopoietic progenitor cells. A functional multidrug-resistant hematopoietic compartment would provide a model system useful for the development and screening of new chemotherapeutic agents within the organism. An animal model of this kind would provide the opportunity to study the pharmacology and toxicity of antineoplastines in relation to the kinetics of bone marrow repopulation. Additionally, exogenous expression of *mdr* during hematopoiesis will provide the opportunity to study this gene in a developmental context. The vector described in this report should prove useful in the functional analysis of multidrug resistance *in vivo*.

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