Coexpression of a Multidrug-resistance Gene (*MDR*1) and Herpes Simplex Virus Thymidine Kinase Gene as Part of a Bicistronic Messenger RNA in a Retrovirus Vector Allows Selective Killing of *MDR*1-transduced Cells[1]

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ABSTRACT

A new retroviral vector, pSXLC/pHa, was constructed to coexpress drug-selectable markers with a second gene of interest as a part of a bicistronic mRNA in a retroviral vector using an internal ribosome entry site (IRES) from encephalomyocarditis virus. This system was used to develop a new retroviral vector pHa-MDR-IRES-TK which expresses a single mRNA from which translation of the *MDR*1 gene is cap dependent and translation of the herpes simplex virus thymidine kinase gene is IRES dependent. The pHa-MDR-IRES-TK transfectants showed high levels of P-glycoprotein expression and multidrug resistance. More than 95% of the vincristine-resistant cells transfected or transduced with pHa-MDR-IRES-TK showed hypersensitivity to ganciclovir, which selects against cells expressing herpes simplex virus thymidine kinase. An amphotropic retrovirus titer of 7.8 × 10^4/ml was obtained with this vector. This safety-modified vector should be useful for introducing the *MDR*1 gene into bone marrow cells to protect normal cells from the toxic effects of cancer chemotherapy because this vector allows the elimination of cancer cells that have been unintentionally transduced with the *MDR*1 vector.

INTRODUCTION

In cancer chemotherapy, there are two major problems to be overcome. One is the innate or acquired resistance of cancer cells to anticancer drugs, and the other is the toxicity of the chemotherapeutic drugs to certain normal tissues, such as bone marrow. The study of the mechanisms of drug resistance in cancer cells has led to the identification of some of the genes and gene products that confer drug resistance. In particular, cell lines showing resistance to multiple drugs such as *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and actinomycin D have been studied intensively, and one gene responsible for this form of multidrug resistance, termed *MDR*1,[5] has been identified and a full-length cDNA of the gene has been cloned and sequenced (1, 2). The *MDR*1 gene encodes the plasma membrane P-glycoprotein with a molecular mass of 170 kDa. P-glycoprotein acts as an ATP-dependent efflux pump for various structurally unrelated natural product antitumor agents (reviewed in Ref. 3). The *MDR*1 cDNA has been shown to confer multidrug resistance when introduced into drug-sensitive cells (4, 5).

The *MDR*1 gene is normally expressed on the biliary surfaces of hepatocytes, the brush border of the proximal tubules of kidney, the lumen of small and large intestine, and the capillary endothelial cells of the brain and testes (3). However, it is not widely expressed in bone marrow cells with the possible exception of some CD34-positive cells (6). Lack of protection by an endogenous transporter may be one of the reasons for the severe suppression of bone marrow cells by many chemotherapeutic drugs, which is a major dose-limiting factor in cancer chemotherapy. Retrovirus-mediated expression of the *MDR*1 cDNA has been shown to confer multidrug resistance *in vivo* when the *MDR*1-carrying vector is introduced into bone marrow cells of mice (7, 8). Studies using *MDR*1-transgenic mice suggest that the expression of the human *MDR*1 cDNA in bone marrow cells does not affect the normal function of the bone marrow cells (9, 10). Therefore, in principle, the *MDR*1 gene can be used to protect bone marrow cells from intensive chemotherapy.

There still remain, however, some potential problems associated with *MDR*1 gene transfer into normal bone marrow cells. One problem is to optimize the efficiency of *MDR*1 gene transfer and expression. Using an *MDR*1 retrovirus, relatively efficient *MDR*1 gene transfer is possible, but development of higher titer retroviruses and an optimized protocol for selection of the transduced cells *in vivo* are still required. Another prob-

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[5] The abbreviations used are: MDR, multidrug-resistance gene; HSV-TK, herpes simplex virus thymidine kinase; IRES, internal ribosome entry site; TK, thymidine kinase; LTR, long terminal repeat; IC_{50}, concentration of drug that inhibits cell growth by 50%; FACS, fluorescence-activated cell sorting; HAT, hypoxanthine-aminopterin-thymidine; MCS, multicloning site.

lem is that it is possible to transduce other cells contained within bone marrow preparations in which the expression of the transduced gene might cause undesirable side effects. In transducing bone marrow cells of cancer patients with the *MDR*1 retrovirus, if the bone marrow contains contaminating cancer cells and the cancer cells receive the *MDR*1 retrovirus, then multidrug-resistant cancer cells will result. In such cases, treatment protocols involving drugs related to multidrug resistance will be ineffective. To eliminate such unintentionally transduced cells, introduction of a negative drug-selectable marker (a "suicide" gene) that confers hypersensitivity to a certain drug would be valuable.

The HSV-TK gene acts as a suicide gene both *in vitro* and *in vivo*. Cells that express HSV-TK are hypersensitive to the nucleoside analogue ganciclovir because HSV-TK can phosphorylate ganciclovir more efficiently than the endogenous TK of mammalian cells. Ganciclovir treatment was reported to be effective *in vivo* against rat cerebral gliomas transduced with the retrovirus carrying HSV-TK (11, 12). Multidrug-resistant cells do not show cross-resistance to ganciclovir. Therefore, the HSV-TK gene would appear to be a suitable gene to coexpress with the *MDR*1 gene to eliminate cancer cells transduced with the *MDR*1 vector. HSV-TK can also confer HAT resistance *in vitro* when it is introduced into TK-deficient cells such as Ltk^-.

There are three possible strategies for coexpression of the *MDR*1 gene with the HSV-TK gene. The first strategy is to utilize two independent promoters (*e.g.*, retroviral LTR and another promoter). However, this does not guarantee the expression of both genes. Most transduced cells express only one gene because expression of one gene may suppress the activity of the second promoter (13). The second strategy is to engineer a chimeric bifunctional protein between P-glycoprotein and HSV-TK. This approach has been shown to be successful when a second gene product, such as adenosine deaminase, is connected to the carboxyl terminus of P-glycoprotein (14, 15). This strategy guarantees the coexpression of the two gene products; however, the activity of chimeric P-glycoprotein as a drug transporter is less than the intact P-glycoprotein and some gene products may not function when tethered to the inside of the plasma membrane. The third strategy is to use an IRES isolated from a picornavirus such as encephalomyocarditis virus (16–18). In this construct, a single mRNA is transcribed under the control of an upstream promoter, and two gene products are translated independently from the mRNA. The first open reading frame is translated in a cap-dependent fashion, and the second is translated under control of the IRES.

We have developed a new retroviral vector system, pSXLC/pHa, in which the drug-selectable genes such as the *MDR*1 gene or the HSV-TK gene is translated under control of the IRES (19). To express the *MDR*1 gene and the HSV-TK gene together, we constructed a new *MDR*1-retrovirus vector pHa-MDR-IRES-TK that allows simultaneous translation of the cap-dependent *MDR*1 gene and the IRES-dependent HSV-TK gene.

MATERIALS AND METHODS

Cell Culture and Drug Sensitivity Assay. The ecotropic retrovirus packaging cell line Ψ-cre, the amphotropic retrovirus packaging cell line Ψ-crip (20), and the mouse fibroblast cell line NIH3T3 were cultured in DMEM supplemented with 10% calf serum. The murine TK-deficient cell line Ltk^- and the amphotropic retrovirus packaging cell line PA317 (21) were grown in DMEM supplemented with 10% fetal bovine serum. The sensitivities of the cultured cell lines to drugs were evaluated by the inhibition of cell growth after incubation at 37°C for 6 days in the presence of various concentrations of drugs as described previously (22). Cell numbers were determined in a Coulter counter and the IC_{50} was calculated.

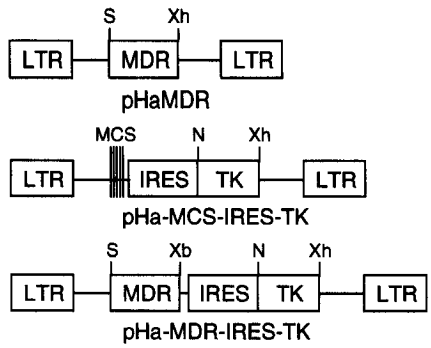

Fig. 1 Structure of pHaMDR, pHa-MCS-IRES-TK, and pHa-MDR-IRES-TK retrovirus. Drawing is not to scale. LTR, LTR of Harvey murine sarcoma virus; MDR, human multidrug resistance gene *MDR*1; TK, herpes simplex virus thymidine kinase gene; *S*, *Sac*II; *Xh*, *Xho*I; MCS, *Sac*II-*Bam*HI-*Bgl*II-*Sac*I-*Xba*I-*Sal*I; *N*, *Nco*I; *Xb*, *Xba*I.

Construction of Vectors. The construction of the pSXLC/pHa retrovirus system was described previously (19). The plasmid pSXLC-TK has the entire open reading frame of HSV-TK DNA downstream from the IRES sequence and 6 unique sites (*Sac*II, *Bam*HI, *Bgl*II, *Sac*I, *Xba*I, and *Sal*I) for the cloning of another gene upstream from the IRES. To insert the *MDR*1 cDNA into pSXLC-TK, we made pSXbaMDR, which contains *MDR*1 cDNA with 5′-*Sac*II and 3′-*Xba*I sites in a pGEM2-derived vector, then subcloned the *Sac*II-*Xba*I-digested *MDR*1 cDNA between the *Sac*II and *Xba*I sites of pSXLC-TK (pSXLC-MDR-TK). The pSXLC-MDR-TK insert was isolated after *Sac*II-*Xho*I digestion and transferred into the pHa retroviral vector (pHa-MDR-IRES-TK). pHaMDR, which carries the wild-type *MDR*1 cDNA in the pHa retrovirus vector (5, 23), was used as a control *MDR*1 vector. pHa-MCS-IRES-TK, which carries the insert of pSXLC-TK, was also used as a control (19). Structures of retrovirus constructs used in this study are summarized in Fig. 1.

DNA Transfection. Transfection was carried out using the calcium phosphate coprecipitation method (24). Recipient cells were plated at 5×10^5 cells/100-mm dish on day 1 and transfected with 20 μg of the expression plasmid DNA on day 2. Cells were exposed to the DNA precipitate until day 3 when the medium was aspirated and fresh medium was added. On day 4, the cells were split at 1:10 or 1:100. The cells were selected either in vincristine (25 ng/ml for Ψ-cre, Ψ-crip, and NIH3T3; 30 ng/ml for PA317 and 35 ng/ml for Ltk^-) or HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) on day 5, if necessary.

Retrovirus Transduction. To examine the production of retrovirus, retrovirus-producing cells were plated on day 1 at

2×10^6 cells/100-mm dish. On day 2, the medium of the packaging cell culture was changed, and the recipient cells were plated at 3×10^4 cells/100-mm dish in medium containing 2 μg/ml polybrene (Aldrich, Milwaukee, WI). On day 3, the retrovirus-containing supernatant was collected, passed through a 0.45-μm pore filter to remove cells and debris, and added to each dish of recipient cells. On day 5, the medium was removed, and fresh medium containing drugs was added at appropriate concentrations as needed. To estimate the retrovirus titer, the medium was removed and the colonies were stained with 0.5% methylene blue dissolved in 50% methanol on days 11–13.

Protein Expression Analysis. To examine the expression of human P-glycoprotein on the cell surface of mouse transfectants, FACS analysis was used in which cells were reacted with a human P-glycoprotein-specific mAb MRK16 (25, 26). Cells (10^6) harvested after trypsinization were washed and incubated with MRK16 (5 μg/10^6 cells), washed twice, and incubated with fluorescein-conjugated goat anti-mouse IgG (1:10 diluted; Jaxon Immunoresearch Lab., West Grove, PA). The cells were washed twice and the fluorescence staining level was analyzed using a FACSort (Becton Dickinson FACS System, San Jose, CA).

Nucleic Acid Isolation and Analysis. Total RNA was isolated from cultured cells by acid-guanidinium-thiocyanate-phenol-chloroform extraction (Stratagene, Inc., La Jolla, CA; Ref. 27). Cells were first homogenized by passage through a 20-gauge needle after removal from the dish by direct addition of the guanidinium thiocyanate. For Northern blot hybridization, RNA was resolved on a 1% formaldehyde-agarose gel and subsequently transferred to nitrocellulose (BA83; Schleicher & Schuell) (28). The blot was probed with a random-primed 0.8-kilobase *MDR*1 probe obtained from an *Eco*RI-*Hin*dIII digestion of *MDR*1 cDNA (29). The blot was subsequently stripped and reprobed with a random-primed 1.2-kilobase *Nco*I-*Xho*I fragment from pSXLC-TK containing the entire open reading frame for HSV-TK. Hybridization and washing conditions were as described previously (30).

RESULTS

We have been using the pHaMDR retroviral vector which uses the promoter of the Harvey murine sarcoma virus LTR to drive expression of the *MDR*1 gene in mammalian cells (5, 31). In a previous study we reported the generation of a retroviral vector system pSXLC/pHa that uses the IRES sequence to coexpress drug-selectable genes with the gene of interest (19). To coexpress the HSV-TK gene as a suicide gene in the pHa vector, we made pSXLC-TK with the entire open reading frame of the HSV-TK gene downstream from the IRES and six unique sites (*Sac*II, *Bam*HI, *Bgl*II, *Sac*I, *Xba*I, and *Sal*I) for the cloning of another gene (*MDR*1) upstream from the IRES. This IRES-TK construct was shown to be effective when the insert of pSXLC was transferred into the pHa vector (pHa-MCS-IRES-MDR) and introduced to Ltk$^-$ cells (19). In the current study, we inserted the *MDR*1 cDNA into pSXLC-TK upstream from the IRES (pSXLC-MDR-TK) and transferred the whole insert into the pHa vector. The resulting construct was termed pHa-MDR-IRES-TK (Fig. 1).

Table 1 Transfection efficiency of pHa-MDR-IRES-TK and pHaMDR

Cell	Vector	Transfection efficiency[a]	
		Vincristine[b]	HAT[c]
Ψ-cre	pHa-MDR-IRES-TK	3.2×10^{-3}	nd[d]
	pHaMDR	8.4×10^{-3}	nd
	pMC1neopolyA	0	nd
Ltk$^-$	pHa-MDR-IRES-TK	2.0×10^{-3}	7.6×10^{-3}
	pHaMDR	3.2×10^{-3}	0
	pMC1neopolyA	0	0

[a] Calculated probability of the emergence of drug-resistant colonies.
[b] The transfected cells were selected with vincristine at 25 ng/ml for Ψ-cre cells and 35 ng/ml for Ltk$^-$ cells.
[c] The transfected cells were selected in HAT medium.
[d] nd, not determined.

Both *MDR*1 and HSV-TK Genes Are Expressed in Cells Transfected with pHa-MDR-IRES-TK. The ecotropic retrovirus-packaging cells Ψ-cre and the murine TK-deficient cells Ltk$^-$ were transfected with the retroviral expression constructs, pHa-MDR-IRES-TK and pHaMDR, and selected with vincristine as described in "Materials and Methods." Ten days after transfection, approximately 100 vincristine-resistant colonies per dish were observed in pHa-MDR-IRES-TK-transfected Ψ-cre cells when transfected cells were split 1:100 on day 4, giving a transfection efficiency of 3.2×10^{-3}. Transfection efficiencies of pHa-MDR-IRES-TK and pHaMDR are summarized in Table 1. As shown in Table 1, both the pHa-MDR-IRES-TK and pHaMDR vectors efficiently transformed Ψ-cre and Ltk$^-$ cells to vincristine resistance. No vincristine-resistant colonies were found in pMC1 neopoly(A) (a G418-resistant plasmid obtained from Stratagene)-transfected cells. This result clearly shows that the *MDR*1 gene is expressed in pHa-MDR-IRES-TK-transfected cells. Transfection efficiencies of pHa-MDR-IRES-TK were 2–3-fold less than those of pHaMDR (Table 1). Higher numbers of vincristine-resistant colonies were obtained in Ψ-cre cells than in Ltk$^-$ cells. The Ltk$^-$ cells transfected with the *MDR*1 vectors were also selected with HAT medium (Table 1). When the pHa-MDR-IRES-TK-transfected Ltk$^-$ cells were selected with HAT medium, approximately 190 HAT-resistant colonies/dish were observed (transfection efficiency, 7.6×10^{-3}). No HAT-resistant colonies were found among pHaMDR-transfected cells or pMC1 neopoly(A)-transfected cells. These results indicate that pHa-MDR-IRES-TK confers both vincristine resistance and HAT resistance in Ltk$^-$ cells. Three to four times as many colonies were obtained by HAT selection than by vincristine selection.

Expression of a single bicistronic mRNA species encoding both P-glycoprotein and HSV-TK was confirmed by Northern blot analysis performed on RNA isolated from HAT-resistant transfectants using *MDR*1-and *HSV-TK*-specific cDNA fragments (Fig. 2). In the RNA from Ltk$^-$ cells transfected with the pHa-MDR-IRES-TK construct, both probes revealed a single transcript of approximately 10–10.5 kilobases corresponding to the predicted size (Fig. 2, *Lanes 4*). Using the *MDR*1 probe, no signal was detected in the RNA from Ltk$^-$ cells transfected with pHa-MCS-IRES-TK (Fig. 2*A*, *Lane 2*), but the

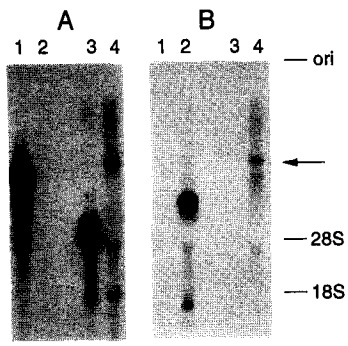

Fig. 2 Northern blot analysis of KB-V1 cells and transfectants with pHa-MCS-IRES-TK, pHaMDR, or pHa-MDR-IRES-TK. In *A*, total RNA was isolated from cells, fractionated on a 1% formaldehyde-agarose gel (20 μg/lane), and transferred to a nitrocellulose membrane as described in "Materials and Methods." The membrane was then probed with a 0.8-kilobase *MDR*1 probe. In *B*, the membrane was subsequently stripped and reprobed with a 1.2-kilobase probe for HSV-TK. *A* and *B*, *Lane 1*, NIH3T3 cells transfected with pHaMDR and selected in 60 ng/ml colchicine; *Lane 2*, Ltk$^-$ cells transfected with pHa-MCS-IRES-TK and selected in HAT medium; *Lane 3*, multidrug-resistant KB-V1 cells grown in 1 μg/ml vinblastine; *Lane 4*, Ltk$^-$ cells transfected with pHa-MDR-IRES-TK and selected in HAT medium. *Right*, positions of the origin (*ori*) and 18S and 28S species. *Arrow*, expected position of the pHa-MDR-IRES-TK transcript.

correct size species of approximately 7 kilobases was detected in these same cells using the *HSV-TK* probe (Fig. 2*B*, *Lane 2*). As additional controls, the *MDR*1 probe revealed the expected size transcripts of approximately 8 kilobases and 4.4 kilobases in NIH3T3 cells transfected with pHaMDR (5) and in KB-V1 cells (32), respectively (Fig. 2*A*, *Lanes 1* and *3*), but as predicted no signal was observed using the *HSV-TK* probe (Fig. 2*B*, *Lanes 1* and *3*).

Expression of P-glycoprotein on the cell surface was examined by FACS with a human P-glycoprotein-specific mAb MRK16 (25). Mixed populations of vincristine-resistant Ψ-cre cells transfected with pHa-MDR-IRES-TK and selected with 25 ng/ml vincristine showed significant expression of the human P-glycoprotein (Fig. 3*A*). The complete shift of the fluorescence peak of the transfectants suggests that all of the transfectants selected with vincristine express human P-glycoprotein. The pHa-MDR-IRES-TK transfected Ψ-cre cells showed slightly lower expression of P-glycoprotein than the pHaMDR-transfected cells (Fig. 3*B*). Mixed populations of the pHa-MDR-IRES-TK-transfected Ltk$^-$ cells selected with vincristine (Fig. 3*C*) or HAT medium (Fig. 3*E*) also showed significant expression of human P-glycoprotein. The expression of P-glycoprotein in the vincristine-resistant cells was higher than the expression in the HAT-resistant cells. The shift in the fluorescence peak of the HAT-resistant transfectants suggests that all of the transfectants selected with HAT medium express human P-glycoprotein. Again, the pHa-MDR-IRES-TK-transfected Ltk$^-$ cells showed slightly lower expression of P-glycoprotein than the pHaMDR-transfected cells (Fig. 3, *C* and *D*).

To determine whether the two genes are coexpressed in clonal cell lines, we randomly isolated 20 vincristine-resistant clones and 20 HAT-resistant clones from pHa-MDR-IRES-TK-transfected Ltk$^-$ cells. Growth of each clone was examined in the presence of vincristine (15 ng/ml, V15 or 30 ng/ml, V30) or HAT medium. The results shown in Table 2 clearly demonstrate that both the *MDR*1 and HSV-TK gene are expressed together in most of the transfectants. All of the HAT-resistant clones (20/20) showed more resistance to vincristine than the parental Ltk$^-$, although some clones showed only marginal levels of resistance. On the other hand, 19 of 20 vincristine-resistant clones showed HAT resistance. Only one clone (termed L39) showed HAT sensitivity. The pHa-MDR-IRES-TK clones selected with vincristine appeared to have higher levels of vincristine resistance than the transfectants selected with HAT medium.

Sensitivities to vincristine, ganciclovir, and HAT medium of pHa-MDR-IRES-TK-transfected Ltk$^-$ cell clones were determined and are shown in Table 3. In HAT medium, aminopterin acts as a toxic substance and hypoxanthine and thymidine are needed to protect TK-expressing cells from the toxicity of aminopterin. Therefore, we determined the IC_{50} values of aminopterin in the presence of hypoxanthine (100 μM) and thymidine (16 μM) as a measure of sensitivity to HAT medium. Growth of most pHa-MDR-IRES-TK transfectant clones was not inhibited at 400 nM aminopterin, which is the concentration of this drug in HAT medium. The IC_{50} values for ganciclovir in the pHa-MDR-IRES-TK-transfected clones (except for clone L39) were 10–40 nM, whereas those of the parental Ltk$^-$ cells or control *MDR*1 transfectants were 16–20 μM. The IC_{50} value for ganciclovir in clone L39 was 8.6 μM. This result indicates that ganciclovir shows very high selective toxicity (approximately 10^3-fold) against cells expressing the HSV-TK gene. Ganciclovir has been shown to be effective *in vivo* against HSV-TK-transduced cells (11, 12). Therefore this strategy should be useful to eliminate *MDR*1-expressing tumor cells that have been unintentionally transduced with the pHa-MDR-IRES-TK vector.

To examine the probability that cells expressing the *MDR*1 gene would not express the HSV-TK gene, we determined the proportion of ganciclovir-resistant cells in the pHa-MDR-IRES-TK-transfected, vincristine-resistant cell population. Ψ-cre cells transfected with pHa-MDR-IRES-TK were treated with vincristine (25 ng/ml) for 7 days and subsequently cultured in drug-free medium for 2 days. The cells were plated onto 100-mm dishes at various concentrations of the viable cells and treated with 5 μM ganciclovir. When 10^4 cells were plated, approximately 160 ganciclovir-resistant colonies were observed. This result shows that pHa-MDR-IRES-TK can confer hypersensitivity to ganciclovir although the recipient cells express endogenous TK. Since the plating efficiency was 65% (130 colonies from 200 cells in drug-free medium), the frequency of ganciclovir-resistant cells in the pHa-MDR-IRES-TK-transfected Ψ-cre cells was $160/0.65 \times 10^4 = 0.025$ (Table 4). These ganciclovir-resistant clones were pooled after trypsinization and tested for *MDR*1 expression.

As shown in Fig. 4, *A* and *B*, almost 100% of the ganciclovir-resistant cells were shown to express human P-glycoprotein. We also did a similar experiment using Ltk$^-$ cells. The pHa-MDR-IRES-TK-transfected Ltk$^-$ cells were selected with vincristine (35 ng/ml), plated, and treated with 5 μM ganciclovir as described above. Approximately 120 ganciclovir-resistant colonies were found from 10^4 cells (Table 4). Since the plating

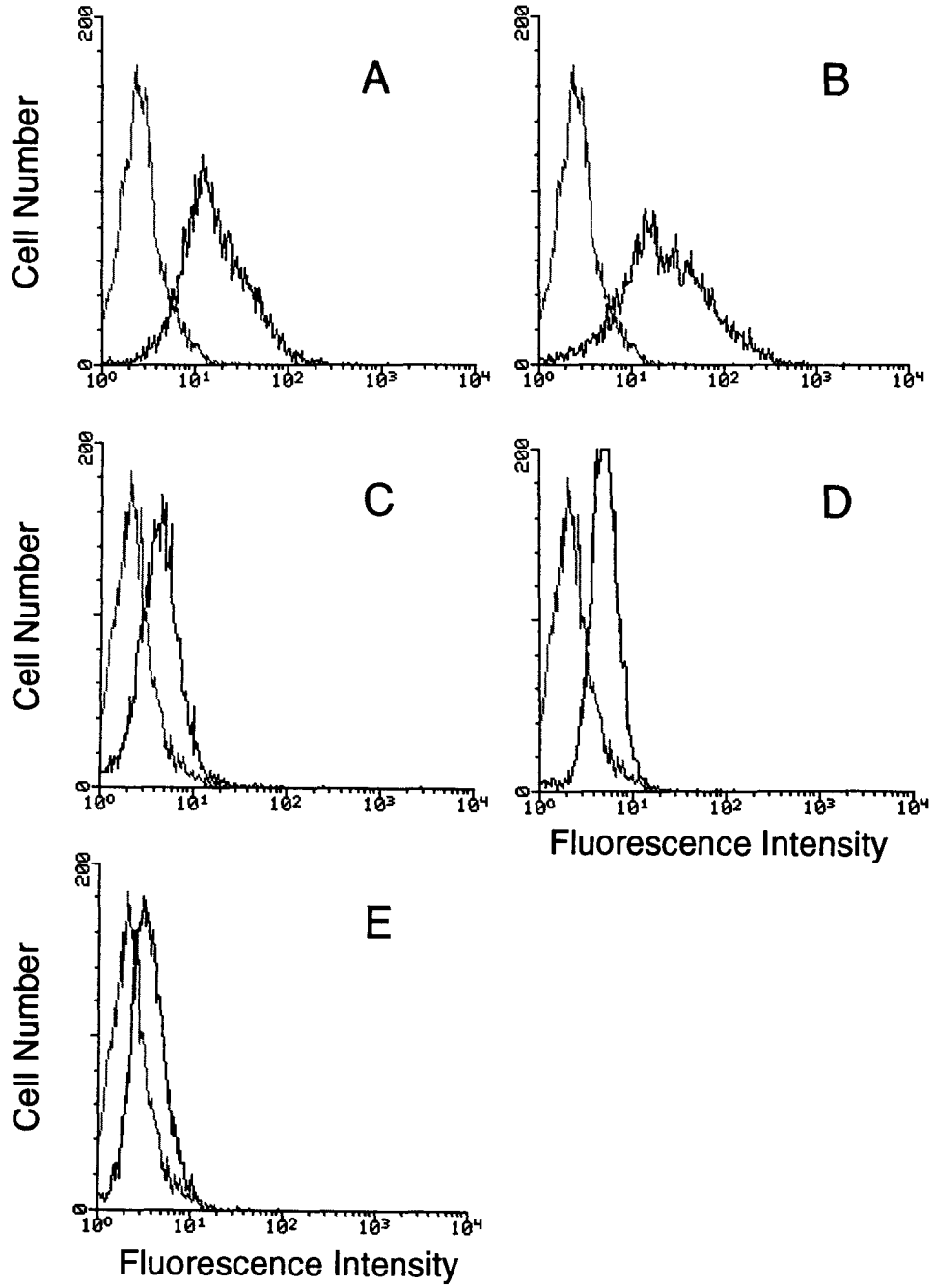

Fig. 3 FACS analysis of the transfectants with pHa-MDR-IRES-TK or pHaMDR. Cells (10^6) were harvested after trypsinization, stained with the anti-P-glycoprotein mAb MRK16, washed, and stained with fluorescein-conjugated antimouse IgG. The fluorescence was analyzed by FACS. *A*, Ψ-cre cells (*left*) and the pHa-MDR-IRES-TK-transfected Ψ-cre population selected with vincristine (*right*); *B*, Ψ-cre cells (*left*) and the pHaMDR-transfected Ψ-cre population selected with vincristine (*right*); *C*, Ltk^- cells (*left*) and the pHa-MDR-IRES-TK-transfected Ltk^- population selected with vincristine (*right*); *D*, Ltk^- cells (*left*) and the pHaMDR-transfected Ltk^- population selected with vincristine (*right*). *E*, Ltk^- cells (*left*) and the pHa-MDR-IRES-TK-transfected Ltk^- population selected with HAT medium (*right*).

efficiency was 85%, the frequency of ganciclovir-resistant cells in the pHa-MDR-IRES-TK-transfected Ltk^- cells was $120/0.85 \times 10^4 = 0.014$ (Table 4). These ganciclovir-resistant Ltk^- clones were pooled after trypsinization and tested for HAT sensitivity and the *MDR*1 expression. When 5×10^4 cells of the pHa-MDR-IRES-TK-transfected, ganciclovir-resistant population was plated and selected with HAT medium, no HAT-resistant colonies appeared, suggesting that these ganciclovir-resistant cells do not express the foreign HSV-TK gene. On the other hand, almost 100% of the ganciclovir-resistant cells were

Table 2 Growth of pHa-MDR-IRES-TK-transfected Ltk$^-$ clones in the presence of vincristine or HAT

	Cell growth (% of control)					
	HAT-selected clone			Vincristine-selected clone		
Clone	V15[a]	V30[b]	HAT[c]	Clone	V15[a]	V30[b]	HAT[c]
L01	94	85	96	L21	93	80	100
L02	90	60	95	L22	103	78	101
L03	74	57	101	L23	100	74	100
L04	78	54	102	L24	98	68	98
L05	80	49	109	L25	90	62	103
L06	88	45	97	L26	85	55	98
L07	77	40	97	L27	70	55	98
L08	63	35	98	L28	84	49	101
L09	80	22	98	L29	80	48	102
L10	57	22	100	L30	72	41	95
L11	48	22	102	L31	77	40	101
L12	71	19	100	L32	88	37	102
L13	56	19	105	L33	76	36	103
L14	43	19	95	L34	70	35	97
L15	38	17	101	L35	61	35	92
L16	43	16	103	L36	67	32	105
L17	51	13	97	L37	69	24	100
L18	30	12	93	L38	58	21	95
L19	55	9	88	L39	52	15	0
L20	33	9	101	L40	53	10	85
Parental Ltk$^-$					10	4	1
pHaMDR transfectant					105	90	0
pHa-MCS-IRES-TK transfectant					12	5	95

[a] Cell growth (percentage of control) in the presence of 15 ng/ml vincristine.
[b] Cell growth (percentage of control) in the presence of 30 ng/ml vincristine.
[c] Cell growth (percentage of control) in HAT medium.

shown to express human P-glycoprotein (Fig. 4, *C* and *D*), suggesting that these cells did not have functional HSV-TK, but the *MDR*1 gene was expressed. As a control experiment, the pHa-MDR-IRES-TK-transfected cells were selected with HAT medium. When the HAT-resistant population was treated with 5 μM ganciclovir, no colonies were observed as expected. These results suggest that 1–3% of the pHa-MDR-IRES-TK-transfected cells may express the *MDR*1 gene alone and show resistance to ganciclovir.

Retroviral Transduction of pHa-MDR-IRES-TK. To test the ability of pHa-MDR-IRES-TK to be packaged as retrovirus, culture supernatants of vincristine-resistant, pHa-MDR-IRES-TK-transfected Ψ-cre cells (mixed population) were added to the culture of amphotropic retrovirus-packaging lines PA317 or Ψ-crip. After retrovirus transduction and subsequent vincristine selection, many drug-resistant colonies were obtained, indicating that retrovirus carrying the *MDR*1 gene existed in the supernatants. The retrovirus titer produced by mixed populations of Ψ-cre cells transfected with pHa-MDR-IRES-TK was 1.8×10^3/ml when PA317 cells were used as recipient cells, whereas the retrovirus titer produced by a mixed population of Ψ-cre cells transfected with pHaMDR was 7.1×10^3/ml.

The retrovirus titers of mixed populations of PA317 or Ψ-crip cells transduced with pHa-MDR-IRES-TK were examined using Ltk$^-$ and NIH3T3 cells as recipients (Table 5). PA317 cells produced higher titers of retrovirus on the average than Ψ-crip cells. Higher numbers of vincristine-resistant colonies were obtained in NIH3T3 cells than in Ltk$^-$ cells. Retrovirus titers of pHa-MDR-IRES-TK were 2–3-fold less than

Table 3 Sensitivity of pHa-MDR-IRES-TK-transfected Ltk$^-$ clones to vincristine, ganciclovir, and aminopterin

	IC_{50} values to		
Cell lines	Vincristine (ng/ml)	Ganciclovir (nM)	Aminopterin (nM)
Ltk$^-$	5.8	17,000	6.0
pHaMDR			
Vincristine-selected clones			
LM1	71	16,000	7.2
LM2	62	20,000	5.5
LM3	21	16,000	6.3
pHa-MCS-IRES-TK			
HAT-selected clones			
LIT1	5.7	26	>6,400
LIT2	5.3	13	>6,400
LIT3	5.1	40	>6,400
pHa-MDR-IRES-TK			
HAT-selected clones			
L01	45	13	>6,400
L02	39	27	>6,400
L03	35	13	>6,400
L07	25	13	>6,400
L10	21	36	>6,400
L14	16	11	>6,400
L17	12	17	>6,400
L19	11	40	>6,400
L20	11	15	>6,400
Vincristine-selected clones			
L21	43	26	>6,400
L25	36	9	>6,400
L33	23	32	>6,400
L39	17	8,600	5.2
L40	15	31	>6,400

Table 4 Ratio of ganciclovir-resistant cells in the populations of vincristine-selected pHa-MDR-IRES-TK-transfectants

Cell	Total colonies[a]	Ganciclovir-resistant colonies	
		No.	%
Ltk$^-$	1.7×10^4	250	1.5
	8.5×10^3	120	1.4
	4.3×10^3	75	1.7
Ψ-cre	1.3×10^4	280	2.2
	6.5×10^3	160	2.5
	3.2×10^3	100	3.1

[a] Calculated number of colonies obtained in drug-free medium.

those of pHaMDR. To obtain retrovirus-producing cells with higher titers, we isolated PA317 or Ψ-crip clones transduced with pHa-MDR-IRES-TK retrovirus. The highest titer we obtained from 17 clones of PA317 was 7.8×10^4/ml, and that from 14 clones of Ψ-crip was 2.9×10^4/ml. In general, PA317 cells produced 2–5-fold higher titers of retrovirus than Ψ-crip. The retroviral titers of pHa-MDR-IRES-TK were a few-fold less than those of pHaMDR. According to previous results, however, retroviral titers close to 10^5/ml may be enough to transduce mouse bone marrow cells for animal experiments (7, 8). It seems likely that further screening could result in clones with higher supernatant retrovirus titers.

As described above, we observed a few-fold difference in the number of pHa-MDR-IRES-TK transfectant colonies between vincristine selection and HAT selection. This result may suggest that many transduced cells with low expression of P-glycoprotein were killed by the vincristine selection. To estimate more accurately the transduction efficiency of the *MDR*1 retroviruses, we determined the percentages of MRK16-positive cells in the transduced cell population without drug selection. Ltk$^-$ cells were plated at 3×10^4 cells/100-mm plate on day 1. The culture supernatants (1 ml or 2.5 ml) of the retrovirus-producing PA317 cells (mixed population) were added to the Ltk$^-$ cells on day 2. Two independently transduced populations of PA317 cells producing pHa-MDR-IRES-TK retrovirus were used in this experiment. On day 4, the medium was removed, and drug-free medium or HAT medium was added to the culture. On day 8, cells in the drug-free medium were analyzed by FACS (Fig. 5, *A* and *B*). On the same day, cells selected in HAT medium were trypsinized and counted in a Coulter counter. The percentages of MRK16-positive cells and percentages of HAT-resistant cells (number of cells grown in HAT medium compared to number of cells in drug-free medium) are summarized in Table 6. As shown in Table 6, the percentages of MRK16-positive cells were similar to the percentages of cells that can grow in HAT medium. As shown in Table 2, HAT medium does not affect the growth of the pHa-MDR-IRES-TK-transduced cells. Therefore, the percentages shown in Table 6 represent the actual transduction efficiency of pHa-MDR-IRES-TK. Since the number of transfectant colonies obtained by HAT selection were 2–3-fold larger than the number obtained by vincristine selection (Table 1), the actual titer of pHa-MDR-IRES-TK retrovirus may be 2–3-fold higher than the titers observed after vincristine selection.

Next we compared the transduction efficiency in Ltk$^-$ cells with the efficiency in NIH3T3 cells. As described above, the transfection efficiency of Ltk$^-$ cells was somewhat lower than that of Ψ-cre cells (a subline of NIH3T3), and the transduction efficiency of Ltk$^-$ cells was lower than that of NIH3T3 (Table 5). The transduction efficiencies of Ltk$^-$ and NIH3T3 were obtained from selections using different concentrations of vincristine. Therefore it is not clear what percentage of transduced cells actually survived during each selection. To compare the transduction efficiency without drug selection, we examined the percentages of MRK16-positive cells in the nonselected populations of transduced cells. This experiment was done at the same time as the experiment shown in Table 6 as described above. Ltk$^-$ and NIH3T3 cells were plated at 3×10^4 cells/100-mm plate on day 1. The culture supernatants (1 ml or 2.5 ml) of the retrovirus-producing PA317 cells (mixed population) were added to the cells on day 2. On day 4, the medium was removed, and drug-free medium was added to the culture. On day 8, cells in drug-free medium were analyzed by FACS (Fig. 5). The percentages of MRK16-positive cells are summarized in Table 7. As shown in Table 7, the percentages of the cells which were MRK16 positive were slightly (20–80%) higher in NIH3T3 cells than in Ltk$^-$ cells; however, the observed difference was smaller than the difference in transduction efficiencies after vincristine selection (2–3-fold, Table 5). These results suggest that a higher percentage of pHa-MDR-IRES-TK-transduced Ltk$^-$ cells were killed by the vincristine selection than that of NIH3T3 cells.

DISCUSSION

In this article we show that it is possible to create a bicistronic retroviral vector system for coexpression of the human multidrug resistance gene (*MDR*1) and a second gene (HSV-TK). A high percentage of cells express both of these genes. Since the HSV-TK gene confers sensitivity to ganciclovir, cells transduced with pHa-MDR-IRES-TK can be selectively killed with this drug. In essence, pHa-MDR-IRES-TK is a safety-modified retroviral vector system in which cells unintentionally selected for multidrug resistance can be killed. Such a situation could occur with cancer cells during gene therapy intended to confer drug resistance on normal bone marrow during chemotherapy. Relatively high titer retroviral supernatants (close to 10^5/ml) can be prepared using the pHa-MDR-IRES-TK vector.

We have chosen the pHaMDR retrovirus backbone for these studies since we have had good success in the past using the promoter of Harvey murine sarcoma virus LTR for the expression of the *MDR*1 gene in mammalian cells (5, 31). Since the pHa vector has only two cloning sites, we made a new subcloning vector pSXLC to introduce various genes into the retrovirus vector. The pSXLC/pHa system should also prove useful for cloning other genes upstream from the IRES-TK sequence because this vector has six unique sites for cloning. In another study we have demonstrated that this IRES-TK construct was active when the insert of pSXLC-TK was transferred into the pHa vector (pHa-MCS-IRES-TK) and introduced into Ltk$^-$ cells. In this study we introduced the *MDR*1 gene between the *Sac*II and *Xba*I sites of pSXLC-TK (pSXLC-MDR-TK, Fig. 1). The whole insert of pSXLC-MDR-TK was isolated after

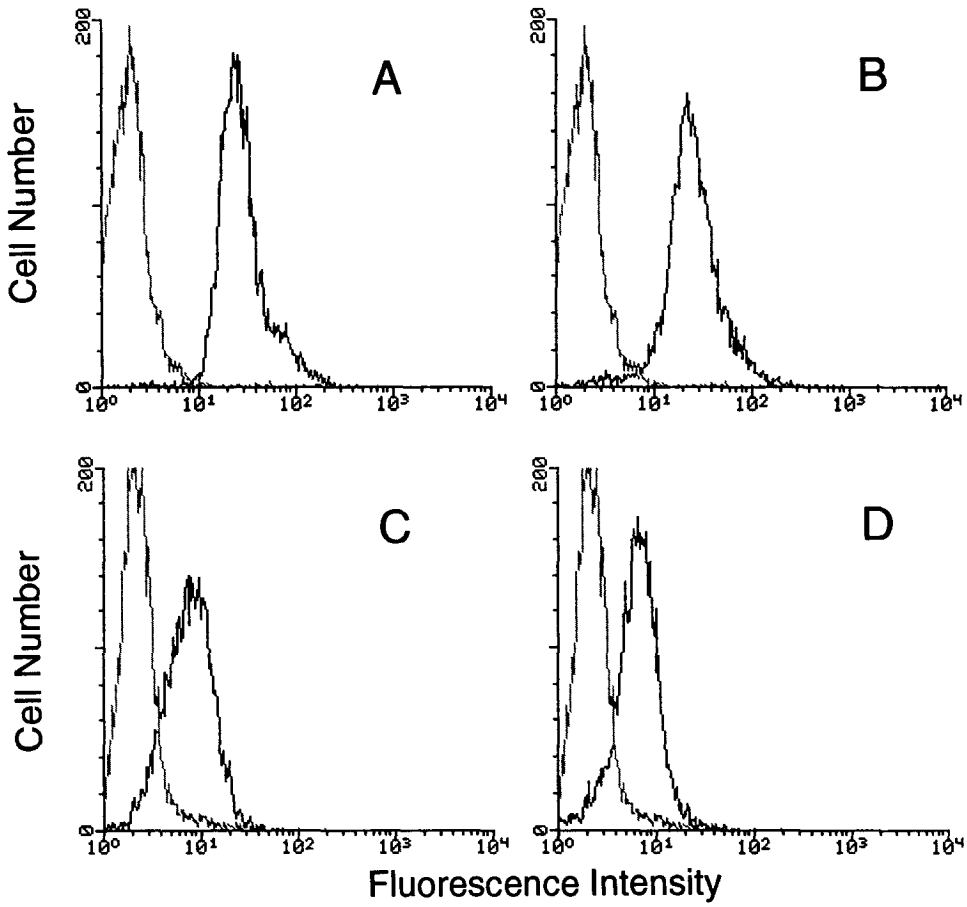

Fig. 4 FACS analysis of the pHa-MDR-IRES-TK transfectants showing resistance to ganciclovir. Cells (10^6) were harvested after trypsinization, stained with the anti-P-glycoprotein mAb MRK16, washed, and stained with fluorescein-conjugated antimouse IgG. The fluorescence was analyzed by FACS. *A*, Ψ-cre cells (*left*) and the pHa-MDR-IRES-TK-transfected Ψ-cre population selected with vincristine (*right*); *B*, Ψ-cre cells (*left*) and the pHa-MDR-IRES-TK-transfected Ψ-cre population selected with vincristine and subsequently selected with ganciclovir (*right*); *C*, Ltk^- cells (*left*) and the pHa-MDR-IRES-TK-transfected Ltk^- population selected with vincristine (*right*); *D*, Ltk^- cells (*left*) and the pHa-MDR-IRES-TK-transfected Ltk^- population selected with vincristine and subsequently selected with ganciclovir (*right*).

Table 5 Titers of pHa-MDR-IRES-TK and pHaMDR retrovirus produced by PA317 or Ψ-crip cells determined using Ltk^- and NIH3T3 cells as recipient cells

	Retrovirus titer[a]	
Producer cells/retrovirus	Ltk^-	NIH3T3
PA317/pHa-MDR-IRES-TK	1.5×10^4	3.6×10^4
PA317/pHaMDR	3.1×10^4	8.5×10^4
Ψ-crip/pHa-MDR-IRES-TK	0.7×10^4	2.0×10^4
Ψ-crip/pHaMDR	1.5×10^4	2.8×10^4

[a] Calculated number of vincristine-resistant colonies when NIH3T3 cells transduced with 1-ml supernatants from mixed populations of retrovirus-producing cells were selected with 25 ng/ml vincristine.

*Sac*II-*Xho*I digestion and transferred into the pHa retroviral vector (pHa-MDR-IRES-TK, Fig. 1). Northern blot analysis demonstrated that this vector allows for expression of a single bicistronic mRNA species when introduced into cells (Fig. 2). Additionally, the transfection and transduction experiments using pHa-MDR-IRES-TK clearly show that the *MDR*1 gene and the HSV-TK gene are efficiently expressed in each cell containing this construct. In a related construction in which the *MDR*1 gene was placed upstream from an IRES that allowed coexpression of a human glucocerebrosidase cDNA, efficient expression of both genes was also seen (29).

The pHa-MDR-IRES-TK construct is able to confer multidrug resistance when it is transfected into drug-sensitive cells or transduced as a retrovirus. However, in both transfection and transduction experiments, numbers of drug-resistant colonies derived from the pHa-MDR-IRES-TK were a few-fold lower than those derived from pHaMDR. One reason for these lower frequencies may be that pHa-MDR-IRES-TK has an insert 1.8 kilobase longer than that of pHaMDR. Another reason may be that the downstream sequences (IRES-TK) affect the transcription of the bicistronic mRNA and/or the translation of the P-glycoprotein.

We examined the expression level of human P-glycoprotein in pHa-MDR-IRES-TK-transduced, nonselected cells (Fig. 5). As

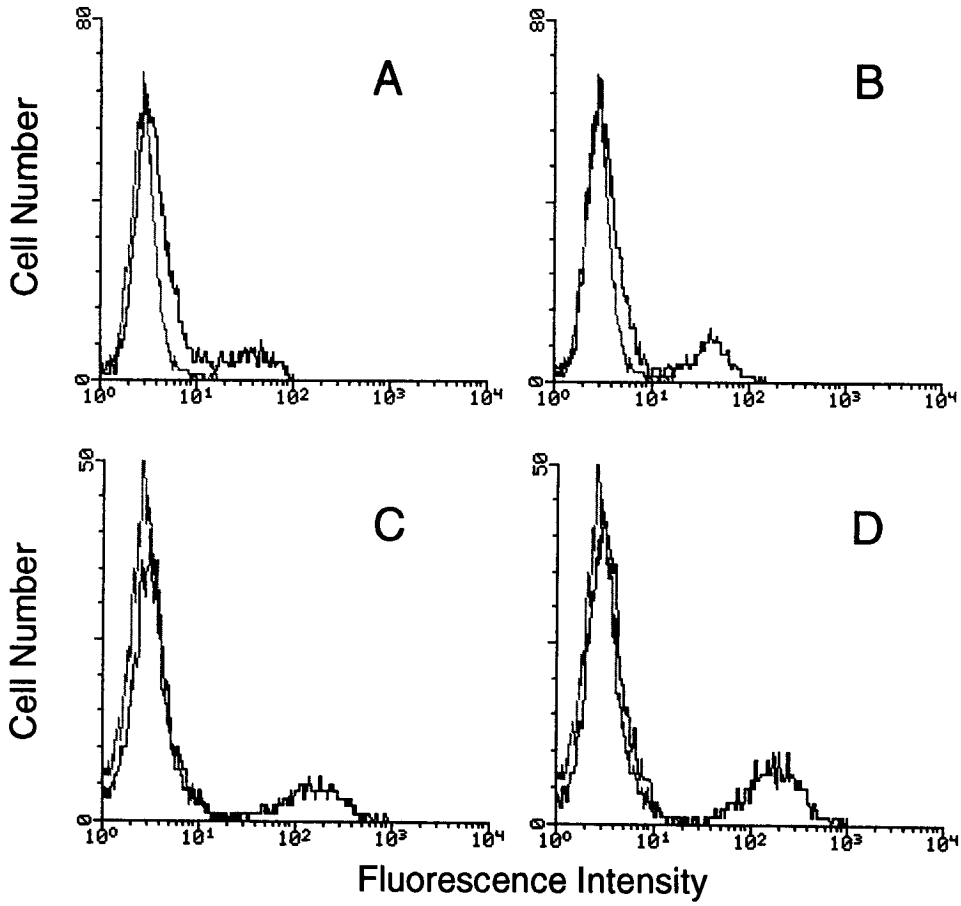

Fig. 5 FACS analysis of nonselected cells transduced with pHa-MDR-IRES-TK or pHaMDR. The supernatant (2.5 ml) of retrovirus-producing cells was used for transduction. Cells (10^6) were harvested after trypsinization, stained with the anti-P-glycoprotein mAb MRK16, washed, and stained with fluorescein-conjugated antimouse IgG. The fluorescence was analyzed by FACS. In each fluorogram, the single fluorescence peak of the parental cells (*left*) was superimposed. MRK16-positive cells are shown as a small fluorescence-positive peak (*right*). *A*, Ltk$^-$ cells transduced with pHa-MDR-IRES-TK (supernatant 1); *B*, Ltk$^-$ cells transduced with pHaMDR; *C*, NIH3T3 cells transduced with pHa-MDR-IRES-TK (supernatant 1); *D*, NIH3T3 cells transduced with pHaMDR.

Table 6 Correlation of P-glycoprotein-positive cells and HAT-resistant cells in pHa-MDR-IRES-TK-transduced Ltk$^-$ populations

Retrovirus supernatant		P-glycoprotein-positive cells[a] (%)	HAT-resistant cells[b] (%)
pHa-MDR-IRES-TK (ml)			
Supernatant 1	1	7.1	6.1
	2.5	14.6	12.6
Supernatant 2	1	4.5	4.8
	2.5	7.5	11.4
pHaMDR (ml)	1	8.4	0
	2.5	18.2	0

[a] Percentage of MRK16-positive cells determined by FACS in *MDR*1 retrovirus-transduced, nonselected population.

[b] Percentage of HAT-resistant cells in *MDR*1 retrovirus-transduced population.

Table 7 Transduction efficiency of pHa-MDR-IRES-TK and pHaMDR in NIH3T3 and Ltk$^-$ cells

Retrovirus supernatant		P-glycoprotein-positive cells[a] (%)	
		Ltk$^-$	NIH3T3
pHa-MDR-IRES-TK (ml)			
Supernatant 1	1	7.1	8.9
	2.5	14.6	18.7
Supernatant 2	1	4.5	6.0
	2.5	7.5	13.2
pHaMDR (ml)	1	8.4	12.8
	2.5	18.2	24.9

[a] Percentage of MRK16-positive cells determined by FACS.

shown in Fig. 5, the mean fluorescence intensities of the pHa-MDR-IRES-TK-transduced cells was almost the same as those of the pHaMDR transfectants. Because these cells have not been treated with any drugs, the expression level of P-glycoprotein directly reflects the ability of the retroviral constructs to drive the expression. Therefore, we can conclude from this result that pHa-MDR-IRES-TK confers similar levels of multidrug resistance as pHaMDR when the vectors are introduced into drug-sensitive cells.

This result along with the observation that the pHa-MDR-IRES-TK construct produces somewhat fewer vincristine-resistant colonies than pHaMDR indicate that the true difference between the vectors is relatively small.

The HAT-selected pHa-MDR-IRES-TK transfectants showed significant levels of resistance to vincristine although the cells had not been treated with vincristine, and all of the transfectants expressed human P-glycoprotein by FACS analysis using MRK16. When we establish *MDR*1-transfected cells, we usually use antitumor agents such as vincristine or Adriamycin to select the *MDR*1-expressing cells. It is possible that certain mechanisms of drug resistance could be activated or selected for in recipient cells during drug treatment. In this IRES-TK system, we can establish *MDR*1-expressing cells with high efficiency without treating cells with multidrug resistance-related drugs. This strategy is useful to express *MDR*1 mutants whose activity to confer drug resistance is unknown or to analyze the cross-resistance patterns of *MDR*1 mutants. Such experiments are now ongoing in our laboratory. Apart from studies on P-glycoprotein itself, the pHa-MCS-IRES-TK construct should prove useful for the expression of many different genes in TK-deficient cells to analyze the function of these genes without needing to select directly for them.

When we compared drug resistance among the HAT-selected clones and vincristine-selected clones (Tables 2 and 3), the vincristine-resistant clones seemed to have a higher resistance to vincristine than the HAT-selected clones, when 35 ng/ml vincristine was used for the selection. This stringent selection condition could inhibit the growth of some transfectants (Table 3). Low level vincristine-resistant clones, such as might be selected in HAT, would not grow in this concentration of vincristine. Therefore, the transfectant clones obtained after vincristine selection might have higher vincristine resistance on the average than HAT-selected cells. On the other hand, there was no difference in HAT resistance between the HAT-selected clones and vincristine-selected clones. HAT medium has no effect on the growth of most pHa-MDR-IRES-TK transfectant clones (Table 3). This result suggests that the selection is less stringent for HAT resistance than for vincristine resistance, *i.e.*, that some of the cells express relatively low amounts of the bicistronic message and P-glycoprotein, but express enough HSV-TK to allow growth in HAT medium.

One of the main goals of this study was to kill the *MDR*1-transduced cells selectively using ganciclovir. For this purpose, it is desirable that all of the *MDR*1-transduced cells express the HSV-TK gene. To clarify this point, we examined the probability that expression of P-glycoprotein would not be accompanied by expression of HSV-TK. When we analyzed the growth of 20 randomly isolated vincristine-resistant clones and 20 HAT-resistant clones from pHa-MDR-IRES-TK-transfected Ltk^- cells, all of the HAT-resistant clones (20/20) showed more resistance to vincristine than the parental Ltk^- and 19 vincristine-resistant clones of 20 (19/20) showed HAT resistance. Only one clone (termed L39) showed HAT sensitivity (Table 2). The overall frequencies of ganciclovir-resistant cells in a larger vincristine-resistant, pHa-MDR-IRES-TK-transfected cell population were 2.2–3.1% for Ψ-cre cells and 1.4–1.7% for Ltk^- cells (Table 4). Not surprisingly, the ganciclovir-resistant cells were shown to express human P-glycoprotein (Fig. 4). These results suggest that 1–5% of the pHa-MDR-IRES-TK-transfected cells may express the *MDR*1 gene alone and be resistant to ganciclovir. Therefore, it is possible that there will remain a small but potentially troublesome population of multidrug-resistant tumor cells that will not be killed by this strategy. In a previous report, treatment with ganciclovir *in vivo* against s.c. injected tumor in mice produced complete tumor regression when 50% of the tumor cells were transduced with the HSV-TK retrovirus (11). The mechanism mediating the killing of bystander untransduced tumor cells is not fully understood; however, this result suggests that the minor population of the pHa-MDR-IRES-TK-transfected cells expressing the *MDR*1 gene alone can also be killed by ganciclovir treatment *in vivo*. Animal studies would be needed to test this hypothesis.

Another possible problem with this strategy is that ganciclovir will kill normal bone marrow cells intentionally transduced with pHa-MDR-IRES-TK as well. In clinical situations, patients will be treated with ganciclovir only when MDR-transduced tumors present as disease. Since this may occur several months to years after the gene therapy and subsequent cancer chemotherapy, normal bone marrow cells that were not transduced with the retrovirus may occupy the bone marrow of the patients. Clinical studies examining the expression of the *MDR*1 gene as well as the HSV-TK gene in bone marrow cells for long periods of time would resolve this issue.

These preliminary studies suggest that the pHa-MDR-IRES-TK safety-modified vector may be useful for gene therapy of human cancer to protect bone marrow cells during intensive chemotherapy. Further preclinical studies in various animal model systems are needed to prove this hypothesis.

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