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Expression of a Human Multidrug Resistance cDNA (*MDR*1) in the Bone Marrow of Transgenic Mice: Resistance to Daunomycin-Induced Leukopenia

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The human multidrug resistance gene (*MDR*1) encodes a drug efflux pump glycoprotein (P-glycoprotein) responsible for resistance to multiple cytotoxic drugs. A plasmid carrying a human *MDR*1 cDNA under the control of a chicken β-actin promoter was used to generate transgenic mice in which the transgene was mainly expressed in bone marrow and spleen. Immunofluorescence localization studies showed that P-glycoprotein was present on bone marrow cells. Furthermore, leukocyte counts of the transgenic mice treated with daunomycin did not fall, indicating that their bone marrow was resistant to the cytotoxic effect of the drug. Since bone marrow suppression is a major limitation to chemotherapy, these transgenic mice should serve as a model to determine whether higher doses of drugs can cure previously unresponsive cancers.

Intrinsic and acquired resistance to multiple chemotherapeutic agents is a major clinical problem in the treatment of cancer. Cell lines resistant to multiple drugs such as vinca alkaloids, doxorubicin (adriamycin), colchicine, and actinomycin D have been studied (2, 3, 9, 25); among these are lines derived from a human KB carcinoma cell line after selection in culture for resistance to a single agent (1, 37). A human gene responsible for multidrug resistance, termed *MDR*1, has been identified and shown to encode a 4.5-kilobase-pair (kb) mRNA that is present in multidrug-resistant cell lines (35, 36, 38, 45), in large and small intestine, kidney, liver, and adrenal gland, and in many tumors (12). Tumors with elevated *MDR*1 RNA levels include intrinsically drug-resistant cancers of the colon, kidney, adrenal gland, and liver as well as tumors with acquired drug resistance (12, 13, 15). Full-length cloned human *MDR*1 or mouse *mdr* cDNA can confer multidrug resistance on drug-sensitive cells after transfection or infection with retroviral vectors (17, 19, 30, 42). The protein product of the *MDR*1 gene is a 170-kilodalton membrane glycoprotein (P-glycoprotein) (44) that is overexpressed in multidrug-resistant cell lines (2, 25, 35–38, 45) and acts as a multidrug transporter to pump drugs out of resistant cells (9, 11, 23). P-glycoprotein is located in the plasma membrane of resistant cells (47) and binds both cytotoxic drugs (7) and ATP (8). Sequence analysis has shown homology to bacterial transport proteins (5, 18).

One of the major side effects of various forms of cancer chemotherapy is bone marrow suppression. Higher doses of drugs could be used for treatment if the bone marrow were protected. Currently, this is done in an experimental setting by removing bone marrow before treatment and replacing it after treatment. To develop an animal model in which larger doses of chemotherapeutic agents can be given, we introduced into mice a transgene containing a human *MDR*1 cDNA sequence under the control of the chicken β-actin promoter. Since β-actin is abundantly expressed in a wide range of eucaryotic cells and is evolutionarily conserved (11, 28, 31), we reasoned that the β-actin promoter may satisfy our requirements for high-level expression and activity of the *MDR*1 transgene in bone marrow and other cell types.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* DH5 was used for transformation. pMDR2000XS, which carries the human *MDR*1 cDNA with a unique *Xho*I site at the 3′ end, was constructed in this laboratory (30). pHaMDR is a retroviral vector containing Harvey murine sarcoma virus long terminal repeats (42). pβA-CAT, which carries the bacterial chloramphenicol acetyltransferase gene with a chicken β-actin promoter in pUC18 (plasmid p8CAT), was a gift from B. Paterson, National Cancer Institute, Bethesda, Md.

Plasmid construction. A plasmid placing the full-length cDNA coding region of *MDR*1 under chicken β-actin promoter control was constructed as follows. The 330-base-pair (bp) chicken β-actin promoter region was removed from pβA-CAT (32) with *Sal*I. The fragment was inserted into the unique *Sal*I site of pMDR2000XS, which carries 4,380 bp of the *MDR*1 cDNA extending from position −140 (*Sac*I site) to +4240 (*Eco*RI site), downstream of the T7 promoter in a pGEM2 vector (30). The resulting plasmid is termed pHG1 (pβAP-MDR).

A second plasmid, termed pHG2 (ptβA-MDR), was constructed in which 60 bp of the β-actin promoter (*Sal*I-*Xho*I) was removed. pHG1 was digested with *Xho*I, and the resulting 4.8- and 2.9-kb fragments were eluted after separation on an agarose gel. The 2.9-kb fragment containing 60 bp of the 5′ region of the β-actin promoter was cleaved with *Sal*I to remove the 60-bp promoter sequences, dephosphorylated with alkaline phosphatase, and religated to the 4.8-kb fragment containing the *MDR*1 cDNA downstream of the *Xho*I-*Sal*I (270-bp) truncated promoter.

Cell culture and transfection. NIH 3T3 mouse cells and human KB-3-1 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum and fetal bovine serum, respectively. Transfection was by the calcium phosphate precipitation method (39).

DNA preparation for microinjection. pHG1 was cut with *Xho*I, and a 4.7-kb fragment containing the chicken β-actin promoter–*MDR*1 cDNA was separated from the vector

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fragment on a low-melting-point agarose gel (Sea Plaque) and recovered by standard techniques (26). The final DNA pellet was dissolved in 0.2 M NaCl and Tris-EDTA (TE) and then purified on a NACS column (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as recommended by the supplier except that the DNA was eluted with 1.0 M NaCl–TE–1% caffeine. The DNA sample in sterile H_2O was microdialyzed on a membrane filter (no. VMWPO1300; Millipore Corp., Bedford, Mass.) against sterile H_2O for 30 min. The concentration of the DNA sample was determined by gel electrophoresis, using ethidium bromide-stained DNA standards (λ DNA-*Hin*dIII fragments). The DNA was diluted before microinjection with 7.3 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.4)–0.1 mM EDTA–5 mM NaCl.

Microinjection into fertilized mouse eggs. CR NIH and C57BL/6 × SJL F_1 mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Fertilized eggs were flushed from oviducts of C57BL/6 × SJL F_1 females and microinjected with 1 to 2 ng of DNA. The microinjected embryos were transferred to CR NIH surrogate pseudopregnant females. Manipulation of mice and eggs and microinjection techniques were performed as described previously (22).

Preparation and analysis of mouse genomic DNA. High-molecular-weight genomic DNA was isolated from 1- to 2-cm tail samples (22). DNA samples (20 μg each) were digested with *Eco*RI, electrophoresed on 1% agarose in 1× TBE buffer, and transferred to nitrocellulose paper (40). For semiquantitative analysis, DNA samples were applied to filters by using a slot blot apparatus. The 1.4-kb *Eco*RI *MDR*1 probe was obtained from pMDR5A (42). Hybridization was performed with nick-translated (34) probes at 42°C in 50% formamide–5× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2× Denhardt solution (0.04% bovine serum albumin, 0.04% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], 0.04% polyvinylpyrrolidone)–20 mM sodium phosphate (pH 6.8)–10% dextran sulfate–100 μg of sonicated salmon sperm DNA per ml, followed by washing with 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 65°C (high stringency) or 1× SSC–0.1% SDS at 50°C (low stringency).

RNA isolation and analysis. All samples were kept frozen at −70°C. Solid tissues were pulverized on a metal surface placed on a bed of dry ice before RNA extraction. Marrow was flushed from long bones with phosphate-buffered saline, using a syringe with a 25-gauge needle. Bone marrow cells were separated from the matrix core by manual pipetting and washed twice with phosphate-buffered saline. The 1,000 × *g* (10 min) cell pellet was dissolved in guanidine isothiocyanate. Total cellular RNA was prepared by homogenization in guanidine isothiocyanate (6) and electrophoresed in 1% agarose–6% formaldehyde gels (38). A 10-μg sample of total RNA was loaded per lane and transferred to nitrocellulose paper or Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) as described elsewhere (26). For semiquantitative analysis, RNA samples were applied to filters by using a slot blot apparatus (12). Hybridization conditions were identical to those used for DNA hybridization. Filters were washed in 0.2× SSC–0.1% SDS at 50°C (low stringency) or in 0.1× SSC–0.1% SDS at 70°C (high stringency). The primer extension analysis was carried out with a synthetic oligonucleotide of 35 bases (43).

Immunofluorescence localization. Bone marrow cells were smeared onto glass slides, air dried, and fixed in 3.7% formaldehyde in phosphate-buffered saline for 5 min at 23°C. The samples were incubated with monoclonal antibody MRK16 followed by rhodamine-conjugated affinity-purified goat anti-mouse immunoglobulin G (47).

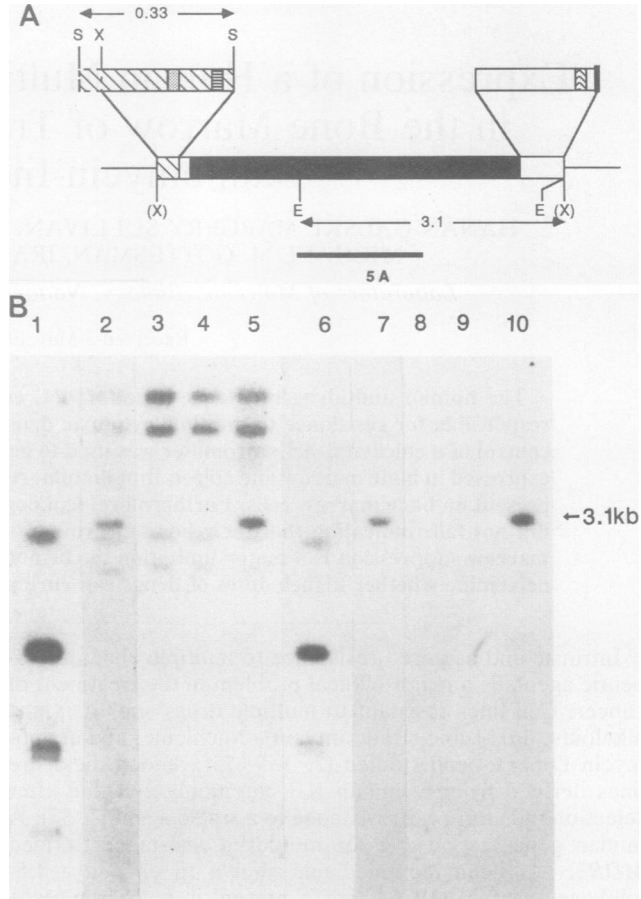

FIG. 1. Southern hybridization of tail genomic DNA from mice. (A) Diagrammatic representation of the β-actin promoter–*MDR*1 fusion transgene. A 3.1-kb labeled fragment is expected after *Eco*RI digestion and hybridization with probe 5A, derived from the middle part of *MDR*1 cDNA. Abbreviations and symbols: E, *Eco*RI; S, *Sal*I; X, *Xho*I; ▨, β-actin promoter; ▯, CAAT box; ▤, TATA box; □, untranslated regions of *MDR*; ■, *MDR* translated region; ▨, polyadenylation site; ▮, *Xho* linker. (B) Southern blot analysis of genomic DNA (20 μg) digested with *Eco*RI and hybridized with probe 5A at low (lanes 1 to 5) and high (lanes 6 to 10) stringency as described in Materials and Methods. Genomic DNA was from KB-3-1 cells (lanes 1 and 6), normal mouse cells mixed with 10 pg of injected DNA fragment (lanes 2 and 7), normal mouse cells (lanes 3 and 8), negative mouse cells from a litter produced after pronuclear injection (lanes 4 and 9), and cells of a transgenic mouse from the same litter (lanes 5 and 10).

WBC measurements in peripheral blood. Peripheral blood was collected by eye bleeding with heparinized capillary tubes and diluted 1:20 (vol/vol) in 3% acetic acid solution for erythrocyte lysis. The leukocytes (WBC) were counted on a standard hemacytometer. Only the refractile viable WBC were counted.

RESULTS

Activity of β-actin gene promoters in transfected cells. To produce a fragment for microinjection into embryos, a plasmid (pHG1) was made in which a 330-bp chicken β-actin gene promoter fragment (32) was inserted 5′ to the *MDR*1

TABLE 1. Frequency of drug-resistant colonies[a]

Plasmid	Colonies/dish with colchicine at (ng/ml):		
	5	6	8
pHaMDR	620	130	25
pHG1 (pβAP-MDR)	680	32	1
pHG2 (ptβAP-MDR)	890	46	7
No DNA	0	0	0

[a] KB-3-1 cells (10^6/10-cm-diameter dish) were transfected with 10 μg of DNA; 48 h later, cells were divided among four dishes and cultured with colchicine. On day 12, cells were stained and colonies were counted. pHaMDR, a retroviral expression vector containing Harvey murine sarcoma virus long terminal repeats (42), was used as a positive control.

cDNA. Digestion with *Xho*I produced a fragment containing 270 bp of the promoter and the *MDR*1 cDNA without vector sequences (Fig. 1A).

To determine whether this truncated promoter was still active, a second β-actin promoter–*MDR*1 plasmid (pHG2) was constructed that contains the 3'-most 270-bp promoter sequences but is otherwise identical to pHG1. Both promoter-*MDR*1 constructions were tested by transfection into drug-sensitive KB-3-1 cells. The truncated 270-bp β-actin promoter (in pHG2) and the 330-bp promoter (in pHG1) were equally active (Table 1). Similar results were obtained with mouse NIH 3T3 cells (data not shown).

Production of β-actin–*MDR* transgenic mice. The fragment containing the chicken β-actin promoter–*MDR*1 cDNA (βAP-MDR; Fig. 1A) was microinjected into fertilized eggs to generate *MDR*1 transgenic mice. The mice were screened by Southern blot analysis of tail genomic DNA digested with *Eco*RI. An example of the identification of an *MDR*1 transgenic mouse is shown in Fig. 1B. Hybridization with *MDR*1 probe 5A showed the expected unique 3.1-kb internal fragment from the human *MDR*1 cDNA. The mouse endogenous fragments were detected under low-stringency (lanes 1 to 5) but not under high-stringency (lanes 6 to 10) conditions. A diluted sample of the 4.7-kb microinjection fragment was mixed with negative mouse genomic DNA, digested with *Eco*RI, and used to estimate the *MDR*1 copy number in each transgenic mouse (Fig. 1B, lanes 2 and 7). Copy numbers were also determined by slot blot analyses (data not shown).

Five founder mice containing integrated human *MDR*1 sequences were generated. Only founder M39 (female), containing one to three copies of the microinjected fragment per genome, will be described here. M39 was mated with a C57B6 × SJL F_1 mouse to generate an *MDR*1 heterozygous mouse line, termed line MDR-39. Restriction fragment pattern analyses of genomic DNAs and pedigree analyses of over 400 mice indicated that the transgene was transmitted through the germ line, integrated into a single chromosome, and inherited in an autosomal fashion. No variations in the number or structure of the acquired DNA sequences could be detected in F_1 and later generations (data not shown).

Expression studies in *MDR*1 transgenic mice. Total RNA was prepared from 18 tissues (brain, liver, kidney, spleen, heart, lung, stomach, small intestine, colon, skin, tail, bone, bone marrow, skeletal muscle, ovary, uterus, oviduct, and testes) of 7 F_2 generation MDR-39 transgenic mice and 10 negative sibling mice. These were analyzed by slot blot hybridization with *MDR*1 probe 5A. For comparison, RNAs from drug-sensitive and multidrug-resistant KB cells with different levels of *MDR*1 mRNA were included in each experiment. Relative to the drug-sensitive line KB-3-1, line KB-8-5 has a 40-fold increase and line KB-V-1 (Vb1) has a 500-fold increase in *MDR*1 mRNA (12). All blots were rehybridized with a γ-actin probe to control for RNA loading and transfer.

The human *MDR*1 RNA was expressed in bone marrow and spleen at levels equal to or greater than those in KB-8-5 cells (Fig. 2). Slot blot analyses of RNA from transgenic and control sibling mice under low-stringency hybridization conditions revealed that the levels of the human *MDR*1 RNA in bone marrow were at least 20-fold higher than those of the mouse *mdr* RNA (data not shown).

Of seven MDR-39 mice tested, six showed significant expression in bone marrow and spleen. The remaining showed expression only in spleen (Fig. 2, mouse D). The transgene RNA was detected at lower levels in the skeletal muscle and ovaries of all MDR-39 mice. One male mouse showed expression in kidney and liver as well. Northern blot analysis (data not shown) showed a diffuse RNA band ranging from 4.5 (the expected full-length *MDR*1 message) up to approximately 11 kb, suggesting that the endogenous polyadenylation signal at the 3' end of the *MDR*1 cDNA functioned poorly in the transgenic mice. Primer extension analysis with a human-specific synthetic oligonucleotide revealed that human *MDR*1 RNA transcription initiated at the correct site 27 bp downstream of the TATA box in the chicken β-actin promoter (data not shown).

Detection of human P-glycoprotein by immunofluorescence. To determine whether the transgene *MDR*1 RNA was translated into protein, immunofluorescence with a monoclonal antibody directed against the human P-glycoprotein (MRK16) (21) was performed. Surface expression of human

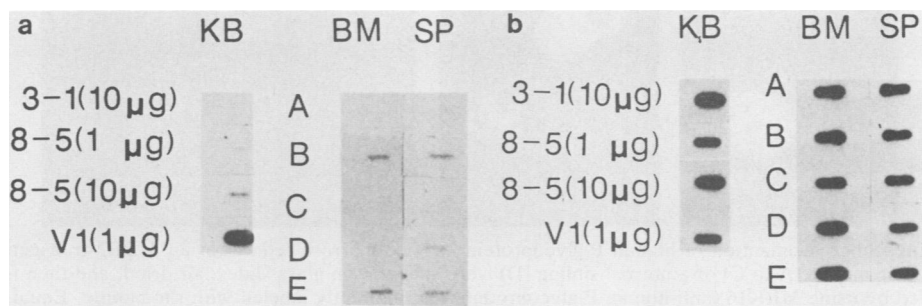

FIG. 2. RNA expression analysis of transgenic and normal mice. Shown is a slot blot of total RNA samples (10 μg) extracted from bone marrow and spleen cells of three *MDR*1 transgenic mice (B, D, and E) or two normal sibling mice (A and C) and hybridized with either *MDR*1 probe 5A (a) or a human γ-actin probe (b) at high and low stringency, respectively, as described in Materials and Methods. 3-1, Drug-sensitive KB cell; 8-5 and V-1, multidrug-resistant cells; BM, bone marrow; SP, spleen.

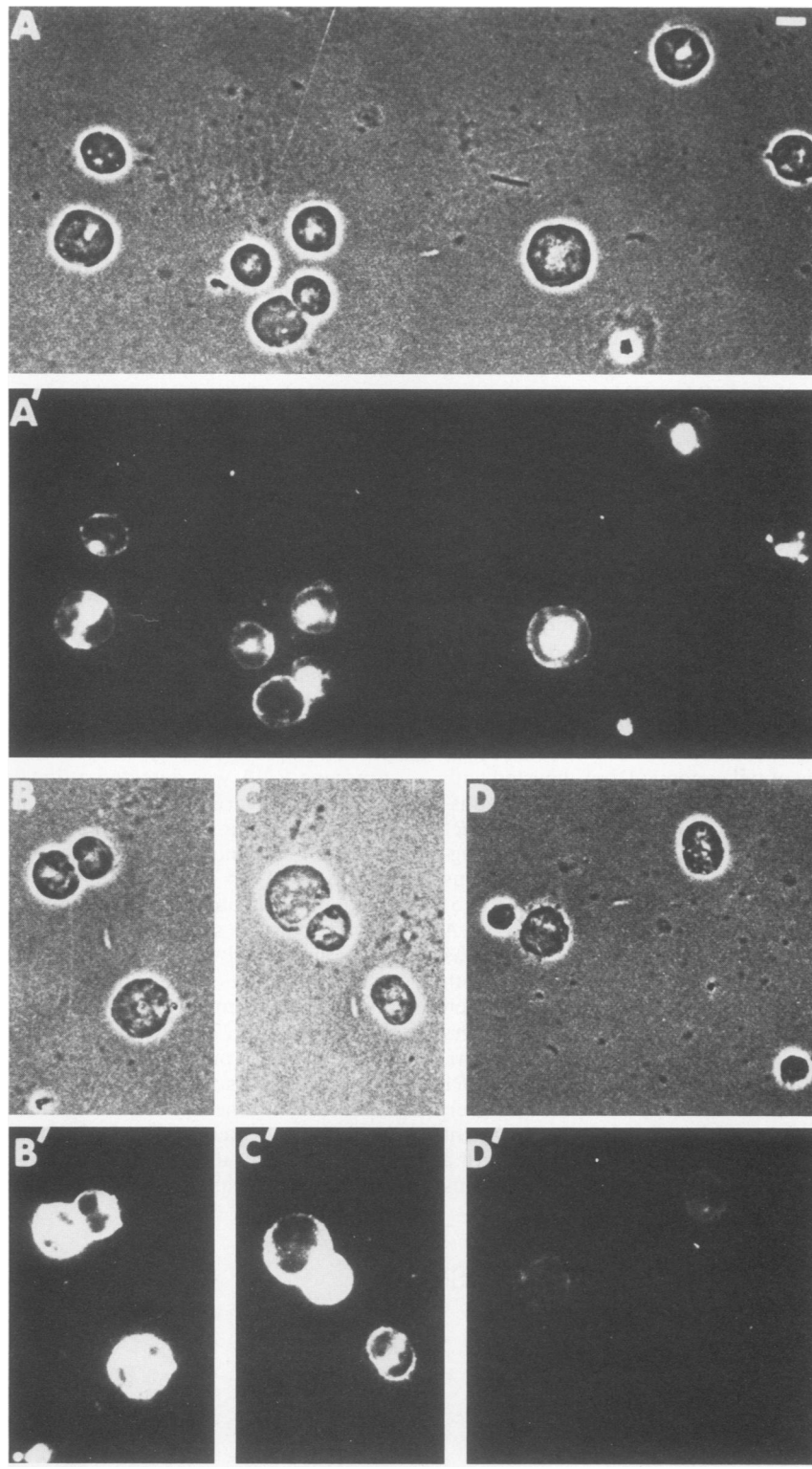

FIG. 3. Immunofluorescence localization of human P-glycoprotein in bone marrow cells from an *MDR*1 transgenic mouse. Cells from either an *MDR*1 transgenic mouse (A to C) or a normal sibling (D) were smeared on glass slides, air dried, and then fixed in formaldehyde. The smears were labeled by using MRK16 (anti-human P-glycoprotein) and indirectly labeled with rhodamine. Equal time exposures show bright expression of P-glycoprotein in all cells from the *MDR* mouse (A′ to C′) but not in cells from the control mouse (D′). The fluorescence that appears to be in the central region of some cells is actually surface fluorescence aggregated into a large patch and does not overlie the nucleus. Panels A to D are phase-contrast images of the cells shown in panels A′ to D′. Magnification, ×630; bar, 6.5 μm.

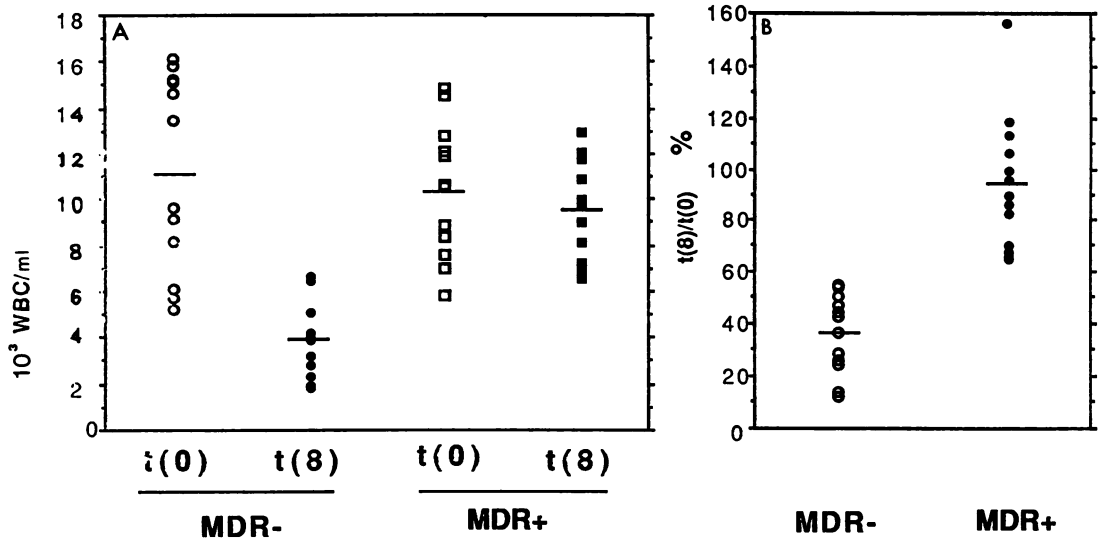

FIG. 4. Daunomycin effects on WBC of normal and *MDR*1 transgenic mice in peripheral blood. Groups of transgenic (n = 13) and nontransgenic (n = 13) sibling mice (6-month-old males, F_2 generation) were injected intraperitoneally with 8.5 mg of daunomycin per kg. The WBC in peripheral blood were counted just before treatment [t(0)] and 8 days after injection [t(8)]. (A) Scattergram of total WBC counts of individual mice; (B) scattergram of WBC counts 8 days after injection as percentage of initial counts. Bars indicate group means.

P-glycoprotein was detected in most of the bone marrow cells (>95%) of two transgenic MDR-39 mice examined (Fig. 3), demonstrating that the *MDR*1 RNA is functional. The type of bone marrow cells expressing the *MDR*1 gene has not been determined.

Drug resistance in *MDR*1 transgenic mice. To test whether *MDR*1 expression confers drug resistance in the bone marrow of transgenic mice and prevents bone marrow suppression, groups of *MDR*1-carrier and -noncarrier sibling mice were injected intraperitoneally with a single dose of the cytotoxic drug daunomycin (8.5 mg/kg of body weight). This dose is twice the 50% lethal dose of normal mice (data not shown) and is highly toxic to bone marrow of mice and humans, mostly affecting the WBC population and resulting in a reduced level of leukocytes (leukopenia) in bone marrow and peripheral blood (14, 27). The number of WBC in the peripheral blood was measured before and 8 days after injection of daunomycin (Fig. 4). The WBC counts of control mice dropped on the average by threefold. In contrast, the WBC counts of the *MDR*1-positive mice did not fall (Fig. 4). In some of the treated *MDR*1 mice, the WBC level was actually elevated after the drug treatment; this elevation was not detected in five saline-injected control mice (data not shown). WBC elevation could result from an in vivo selection and overproliferation of bone marrow cells, which are highly resistant to the drug. The hematocrits of both groups of mice were not changed after treatment (data not shown). The platelet levels were not measured, but none of the mice exhibited bleeding during the experiments.

DISCUSSION

We have generated transgenic mice carrying and expressing the human *MDR*1 cDNA under the control of a chicken β-actin promoter. RNA studies revealed that the *MDR*1 transgene is expressed mainly in bone marrow and spleen, which are hemopoietic tissues. Lower expression was detected in skeletal muscle and ovary. Immunofluorescence studies confirmed that the *MDR*1 RNA was translated into P-glycoprotein in the majority of the bone marrow cells and that the protein was located on the surface of bone marrow cells. Surface expression of the *MDR*1 protein product is required for its function as a transporter to pump chemotherapeutic drugs out of the cells (47) and to prevent bone marrow suppression caused by chemotherapy. In vivo studies showed that the marrow of the transgenic mice was resistant to leukopenia induced by the anticancer drug daunomycin. This finding proves that the human P-glycoprotein is expressed in mouse bone marrow cells as a functional protein. To our knowledge, this study supplies the first direct evidence that acquired drug resistance can result from expression of the human *MDR*1 gene in vivo, as was previously suggested by the correlation of drug resistance and *MDR*1 expression in tumors (15). Thus, these results support the model (16) of P-glycoprotein as a multidrug transporter whose presence in a normal or tumor cell results in drug resistance.

Histological analysis of tissues expressing the human *MDR*1 cDNA as well as of those with no detectable expression did not reveal any abnormalities (data not shown), and no obvious phenotypic changes have been detected in animals expressing the transgene. These data suggest that the de novo expression of the human *MDR*1 cDNA in the expressing mouse tissues does not overtly affect the normal function of these tissues.

In this study, the chicken β-actin promoter was used as a heterologous promoter to express the human *MDR*1 cDNA. It was chosen because β-actin is abundant in all animal tissues, and therefore the promoter should be active in most cell types. The β-actin gene contains a constitutive promoter that can be inhibited by a region 3′ to the promoter sequences and by a small region located 5′ to the polyadenylation signal of the β-actin gene (10, 11). Neither of these inhibitory regions was contained in our constructions. A study using the human β-actin promoter fused to a chloramphenicol acetyltransferase gene has shown that the human β-actin promoter is equal to or stronger than the simian virus 40 early promoter in a wide range of cell types (20). When the *MDR*1 cDNA was put under the control of the chicken

β-actin gene promoter and transfected into human KB cells and mouse 3T3 cells, the cells became drug resistant (Table 1).

In the MDR-39 line, the transgene is expressed in bone marrow, spleen, skeletal muscle, and ovary. However, there is no detectable expression in many other tissues. Our failure to find more widespread expression is probably explained by different requirements for expression in transfected somatic cells compared with developmentally regulated expression in transgenic mice (4, 29). In this study, differential transcription activity of the introduced β-actin promoter–*MDR*1 transgene could be caused by one or a combination of the following: the absence of the 5′ sequences of the truncated β-actin promoter; the presence of intrinsic control elements in the *MDR*1 cDNA itself; the presence of mouse endogenous control elements adjacent to the integration site of the transgene; tissue-specific transcriptional inactivation caused by de novo methylation of the transgene (24, 33); and tissue-preferential mRNA instability or splicing (41).

Quantitative measurements of RNA expression levels in bone marrow of MDR-39 transgenic mice showed relatively high expression of *MDR*1 RNA. The expression level is comparable to, or up to threefold greater than, expression levels of *MDR*1 mRNA in KB-8-5 drug-resistant cells (Fig. 2), which have *MDR*1 RNA levels comparable to those of many multidrug-resistant human tumors (12, 13, 15).

*MDR*1 transgenic mice can serve as a model to study *MDR*1-dependent multidrug resistance in vivo as well as the normal function of this gene product. These mice may also be useful for testing high-dose chemotherapy regimens and for the development of new chemotherapeutic agents and treatment protocols that might overcome the obstacle of multidrug resistance. Finally, since the major expression site of *MDR*1 in the MDR-39 transgenic line is bone marrow, these mice might aid in the evaluation of somatic gene therapy in which expression vectors carrying the *MDR*1 gene and a nonselectable gene are introduced into bone marrow and selected in vivo.

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