Expression of Retroviral Vectors Containing the Human Multidrug Resistance 1 cDNA in Hematopoietic Cells of Transplanted Mice

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Transfer of the human multidrug resistance 1 (MDR1) gene to hematopoietic stem cells offers an approach to overcome the myelosuppression caused by a number of antineoplastic drugs. This study was designed to determine the effect of MDR1 gene transfer on overall P-glycoprotein (P-gp) expression in murine hematopoietic cells. Mice were transplanted with bone marrow cells infected with either of two different MDR1 retroviral vectors. A reverse-transcriptase polymerase chain reaction-based assay was used to quantify expression levels of both endogenous and vector-derived P-gp encoding transcripts in hematopoietic cells of transplanted mice. Expression of both a truncated and full-length MDR1 mRNA species was noted in bone marrow and spleen colony cells. The truncated message resulted from cryptic mRNA splice sites within the MDR1 cDNA and was detected with both vectors. Full-length message levels exceeded those from the endogenous genes in all but one case and roughly approximated that seen in the modestly drug-resistant cell line SW620. We conclude that transfer of MDR1 retroviral vectors resulted in a significant increase in P-gp expression in most cases; however, aberrant splicing of MDR1 transcripts can result in reduced expression of vector-derived P-gp.

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Knowledge of the molecular events leading to cellular drug resistance has allowed investigators to develop gene transfer strategies designed to confer drug resistance in normal host cells. Antineoplastic drugs commonly exhibit a narrow therapeutic index, reflected by frequent patient toxicity that limits the amount of drug that can be administered. This dose attenuation may lead to decreased tumor responses in some malignancies.1-3 For most antineoplastic drugs, the dose-limiting side effect is suppression of hematopoesis. Transfer of drug-resistance genes to hematopoietic cells offers an attractive strategy to overcome this current limitation in cancer treatment. Drug-resistance genes may also be useful as dominant selectable markers for genetically altered hematopoietic stem cells. Patients and animals that are transplanted with hematopoietic cells exposed to retroviral vectors contain only a minority of genetically altered cells after hematopoietic reconstitution.4-6 Experiments using a mouse model have shown that a drug-resistance gene can be used to enrich for vector-transduced cells by administering drug to the animal after bone marrow transplant.7,8 This approach may offer a means to overcome the inefficiency in hematopoietic stem cell gene transfer seen with current human gene transfer protocols.

The first eukaryotic drug-resistance gene to be transferred to reconstituting bone marrow cells was a methotrexate-resistant rodent dihydrofolate reductase (mDHFR) gene. These studies showed that mice transplanted with cells transduced with a mDHFR retroviral vector were protected from methotrexate-induced myelosuppression and increased survival after methotrexate administration.8,9 Transfer of the human multidrug resistance 1 (MDR1) gene to hematopoietic cells has also been described. P-glycoprotein (P-gp), the product of the MDR1 gene, functions as a drug efflux pump and confers resistance to a wide variety of naturally occurring chemotherapeutics.10 The feasibility of using the MDR1 gene to protect hematopoietic cells has been shown by transgenic mouse experiments.11,12 In addition, retroviral transfer of MDR1 to murine clonogenic progenitors resulted in drug resistance in vitro.13 In murine transplant experiments, mice transplanted with MDR1-transduced cells showed attenuation of taxol-induced myelosuppression.6,14 In taxol-treated animals, the number of circulating leukocytes transduced with the MDR1 virus increased, showing that cells expressing the transferred MDR1 gene can be dominantly selected in vivo with taxol.6,7 Recent work has suggested that the MDR1 gene can be used to select for other therapeutic genes when the second gene is linked to the MDR1 cDNA in bicistronic retroviral vectors.15,16

Because hematopoietic cells express endogenous P-gp,17 one goal of this study was to clarify if MDR1 gene transfer resulted in an increase in P-gp expression above basal levels. Our prior observation that MDR1 vectors allowed selection of transduced hematopoietic cells with taxol suggested that MDR1 gene transfer led to increased overall P-gp expression in transduced primary hematopoietic cells. To test this hypothesis, we compared levels of vector-derived MDR1 mRNA with endogenous murine mdrl and 3 mRNA levels in hematopoietic tissues from transplanted mice and with MDR1 mRNA levels seen in P-gp-expressing human tumor cell lines. Vector expression was assayed in a highly enriched stem cell population to determine if MDR1 gene transfer was causing increased P-gp expression in primitive hematopoietic cells.

We report here that transfer of MDR1 vectors resulted in full-length MDR1 mRNA levels that exceeded that from the endogenous mdrl and 3 genes in 11 of 12 cases. Full-length
MDR1 transcript levels in mouse hematopoietic tissues approximated that seen in the drug-resistant human tumor cell line SW620. Expression of an MDR1 vector in a primitive population of bone marrow cells highly enriched for repopulating stem cells was documented 8 months after transplant. Expression of a truncated MDR1 transcript was detected and shown to be caused by cryptic splice donor and acceptor sites within the MDR1 cDNA. This aberrant splicing likely reduces the overall expression of P-gp in transduced cells and may in part explain the animal to animal variability in bone marrow chemoprotection and in vivo selection of transduced cells.

MATERIALS AND METHODS

Vector construction and retroviral producer clone isolation. Construction of the Harvey murine sarcoma-based MDR1 vector and isolation of a ecotropic producer line has been previously described. The MDR1 cDNA used in the Harvey-based vector contains a point mutation resulting in a substitution of valine for glycine at codon 185. This mutation results in decreased resistance to taxol and increased resistance to colchicine. This mutant cDNA was modified by site-directed mutagenesis to restore codon 185 to the glycine residue found in the wild-type MDR1 cDNA. This wild-type MDR1 cDNA was further modified by truncations of the 5' and 3' untranslated regions that preserved 12 bp upstream of the initiation codon and 8 bp downstream of the termination codon. This shortened wild-type MDR1 cDNA was inserted into the Moloney murine retroviral backbone pG1 (Genetic Therapy, Inc, Gaithersburg, MD) to yield G1MD vector (see Fig 2). This vector was introduced by calcium phosphate transfection into the amphotropic packaging line PA317 and supernatant was collected in 48 hours. From candidate producer clones to 3T3 cells and analyzing the 3T3 transduced cells. Then plated at limiting dilution and 30 clones were isolated for further analysis. Expression of a truncated MDR1 transcript was detected and increased resistance to colchicine. This wild-type MDR1 cDNA was modified by site-directed mutagenesis to restore codon 185 to the glycine residue found in the wild-type MDR1 cDNA. This aberrant splicing likely reduces the overall expression of P-gp in transduced cells and may in part explain the animal to animal variability in bone marrow chemoprotection and in vivo selection of transduced cells.

RNA isolation. Total RNA was isolated from bone marrow or spleen cells using the RNazol B method according to the manufacturer's recommendations (Tel Test Inc, Friendswood, TX). Cell sorting. Bone marrow cells were incubated with the following rat monoclonal antibodies (MoAbs) directed against murine hematopoietic cell lineage-specific surface markers: B lymphocytes (B220, CD4 + T lymphocytes (L3T4), CD68 + T lymphocytes (LY2), granulocytes (Gr-1), and myelomonocytic cells (Mac-1) (Caltag Laboratories, Inc, San Francisco, CA). Antibody-labeled cells were subcontracted using magnetic immunobeads (Advanced Magnetics, Cambridge, MA). Cell populations depleted of these lineage-positive cells were then stained with a biotin-conjugated anti-c-kit MoAb, Ack-2 (a gift from Dr S.I. Nishikawa, Department of Pathology, Kumamoto University Medical School, Kumamoto, Japan), as previously described, c-kit-positive cells were isolated using magnetic immunobeads.

Polymerase chain reaction (PCR) primers. PCR primers that amplify a 610-bp fragment from the murine mdrl and 3 cDNAs but do not amplify murine mdrl or human MDR1 cDNA were 5' GAGTCGTGAACCTGGTGATG 3' (MM4.3) and 5' TCCGCTCTCGGCTTACTTG 3' (MM4.5B). PCR primers that amplify a 497-bp fragment from both full-length and spliced forms of the MDR1 cDNA were 5' GCCACATCATCATGATC 3' (P13) and 5' CATTGTGATGGACTCCGGAGACGG 3' (5'BM). PCR primers specific for the full-length MDR1 cDNA that amplify a 504-bp fragment were 5' GTCAAACTGACAGACGGAGCAGG 3' (P15) and 5' CGGAAGACCCATTATGGGAGACGGC 3' (5'BM). PCR primers specific for the full-length MDR1 cDNA that amplify a 504-bp fragment were 5' GTCAAACTGACAGACGGAGCAGG 3' (P15) and 5' CGGAAGACCCATTATGGGAGACGGC 3' (5'BM). PCR primers that amplify a 258-bp fragment from the murine β-2 microglobulin cDNA were 5' TGCATCAAGAAAAAC1CTGTCAATCCGAGCC 3' (P15) and 5' CTTGACAGCATCATTGGCCG 3' (P16). PCR primers that amplify a 258-bp fragment from the murine β-2 microglobulin cDNA were 5' TGCATCAAGAAAAAC1CTGTCAATCCGAGCC 3' (P15) and 5' CTTGACAGCATCATTGGCCG 3' (P16). PCR primers that amplify a 258-bp fragment from both murine and human β-actin cDNA were 5' CTTGTCGATGATGACTCCGAGGCCGCCG 3' (5'BA) and 5' CATCCTGTGCTCGAGCTTGAGCCG 3' (3'BA).

Quantitation of murine mdrl and 3 and vector-derived human MDR1 transcripts by RT-PCR analysis. Fifteen nanograms of total cellular RNA was used for a first-strand cDNA synthesis. The reverse transcription reaction was performed in a 40 µL volume using the oligo dT primer and other reagents, provided in a commercially available Taq-PCR kit (Perkin Cetus Elnor, Norwalk, CT) according to the manufacturer's protocol. Ten-microliter aliquots of the cDNA reaction mix were then used in separate PCR reactions to amplify either vector-derived MDR1 cDNAs (primers P7 and P8), murine mdrl and 3 cDNAs (primers 4.3 and 4.5B), or internal control cDNAs (ß-actin or β-2 microglobulin primers). All PCR reactions were performed in a 50 µL volume with 80 ng of each PCR primer.
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Fig 1. Expression of mdr1 and 3 transcripts in hematopoietic cells of C57Bl/6J mice. (A) Relative expression of mdr 1 and 3 genes in hematopoietic tissues of mice. RT-PCR was used to amplify RNA isolated from kidney, bone marrow, thymus, intestine, and adrenal tissue of a single C57Bl/6J mouse. PCR products were digested with either SpeI or StuI as indicated to differentiate between products amplified from mdr1 or mdr3 transcripts. Schematics of the mdr1 and 3 cDNAs are shown below and indicate the location of unique restriction sites and the PCR primers used. Molecular weight markers are shown in the first lane on the left. (B) Quantitative RT-PCR assay of mdr1 and 3 transcripts in hematopoietic tissues and other organs. RNAs from bone marrow, spleen, thymus, peripheral blood, kidney, adrenal, and intestine were assayed for mdr1 and 3 transcripts. Primers for β-2-microglobulin (B2M) and β-actin served as internal controls for RNA loading. (C) Quantitative RT-PCR from sorted bone marrow cells. RNA from CD4+ and CD8+ bone marrow-derived T lymphocytes, B220+ B cells, MAC1+ macrophages, and whole bone marrow cells was assayed for mdr1 and 3 or β-2 microglobulin mRNA levels. (D) Assay of serial fivefold dilutions of whole bone marrow RNA (BM 0.4, 2x, and 10x representing 3.0, 15.0, and 75 ng of RNA, respectively). Primers for β-2 microglobulin and β-actin were used as internal controls. RNA samples used in each of the three experiments shown in (B), (C), and (D) were prepared from different individual mice.

and 0.2 μL of P32-labeled CTP at 800 μCi/mmL (Amersham, Arlington Heights, IL). Other buffer conditions were as specified in the manufacturer’s protocol. Human MDR1 and murine mdr1 and 3 amplifications were performed for 32 cycles. β-2 microglobulin and β-actin amplification were performed for 22 cycles. Each cycle consisted of denaturation at 94°C for 1 minute, reannealing at 56°C for 1 minute, and extension at 72°C for 1 minute. After amplification, 20 μL of the reaction mix was electrophoresed on a 5% nondenaturing polyacrylamide gel. Dried gels were analyzed using a Molecular Dynamics (Sunnyvale, CA) phosphoimager to quantify band intensity.

Determination of the proportion of truncated MDR1 transcripts by RT-PCR analysis. Two hundred nanograms of total cellular RNA was used for a first-strand cDNA synthesis in a total reaction volume of 40 μL using random hexamers as primers. Ten-microliter aliquots of the cDNA reaction mix were then used in separate PCR reactions to amplify either full-length MDR1 cDNAs (primers P13 and P14), truncated MDR1 cDNAs (primers P15 and P16), or internal control cDNAs. Reaction conditions were otherwise as outlined above. MDR amplifications were performed for 25 cycles and internal control reactions for 20 cycles using the following thermal profile: 94°C for 1.5 minutes, 55°C for 1.0 minute, and 72°C for 1.0
RESULTS

Expression of endogenous multidrug resistance genes in murine hematopoietic cells. Mice contain three genes that code for membrane P-gps designated mdr1, 2, and 3. Previous studies have shown that mdr1 and 3, but not mdr2, can confer the multidrug resistance phenotype. To evaluate expression of both transcripts in a RT-PCR assay (Fig 1A). The relative amounts of each transcript expressed in a given sample was determined by subsequent digestion of the amplification products with the restriction enzymes Spe I and Stu I. Spe I is unique in the mdr1 cDNA and Stu I is unique for the mdr3 cDNA. For example, only the mdr3 allele is expressed in intestine. Digestion of PCR product from intestine derived RNA shows complete digestion with Stu I and no digestion with Spe I, as expected (Fig 1A). Conversely, adrenal RNA shows a pattern consistent with exclusive expression of mdr1. Samples from bone marrow and thymus show that both the mdr1 and 3 genes are expressed in hematopoietic tissue in roughly a 2:1 ratio (Fig 1A).

A semiquantitative RT-PCR assay was used to measure levels of mdr1 and 3 transcripts in hematopoietic tissues from unmanipulated C57Bl/6J mice. A standard curve showed a near linear signal response for β-actin, β-2 microglobulin, and murine mdr1 and 3 when between 3.0 and 75 ng of RNA was assayed (Fig 1D and data not shown). This assay was used to compare endogenous mdr1 and 3 expression in hematopoietic tissues with that in other tissues known to express relatively large amounts of these transcripts, namely adrenal, kidney, and intestine. mdr1 and 3 mRNAs were expressed in bone marrow, in the spleen, at lower levels in the thymus, and at barely detectable levels in peripheral blood cells (Fig 1B). The overall expression level of mdr1 and 3 in hematopoietic tissues was much less than that seen in adrenal and kidney. To test if expression of endogenous mdr genes differed in various hematopoietic lineages, bone marrow cells expressing lineage-specific markers were isolated using MoAbs and immunobeads. The highest levels of mdr1 and 3 expression were noted in CD4 and CD8 expressing T cells, intermediate amounts in B cells, and the lowest levels in macrophages (Fig 1C). Very low levels were detected in purified granulocytes (data not shown). This differential expression profile observed in divergent hematopoietic lineages is similar to what has been reported for human hematopoietic cells. Differing amounts of mdr1 and 3 mRNA were noted in whole bone marrow samples from different individual mice (compare BM10X in Fig 1D with BM-whole in Fig 1C). The basis for these differences is uncertain, but could be due to variability in the proportion of cells from each hematopoietic lineage within the bone marrows of individual mice.

Expression of retroviral vectors containing the human MDR1 gene in transduced mouse hematopoietic cells. To determine if retroviral-mediated transfer of the human MDR1 gene resulted in significantly increased levels of P-gp encoding transcripts in murine hematopoietic tissues, mice were transplanted with bone marrow cells transduced with either a Harvey murine sarcoma-based MDR1 vector (HaMDR1) or a Moloney-based MDR1 vector (G1MD) (Fig 2). The main features that distinguish G1MD from HaMDR1 are (1) HaMDR1 is over 3 kb larger, mostly due to rat VL30 gene sequences present in the vector and located downstream from the MDR1 cDNA; (2) the MDR1 cDNA in G1MD has less 5' and 3' untranslated sequences; (3) the MDR1 cDNA in G1MD has been corrected to the wild-type sequence by restoring codon 185 to code for a glycine residue. This change was made in the G1MD vector based on the demonstration that the valine 185 substitution results in decreased resistance to taxol. Ecotropic producer clones were generated using the GP+E86 packaging line. The titers of the HaMDR1 and G1MD producer clones used for subsequent transplant experiments were 2.2 x 10⁶ and 4.0 x 10⁶ particles/ml, respectively. For both of these producer lines, there was no evidence of either amphotropic or ecotropic replication competent retrovirus when 3T3 cells exposed to viral supernatants were analyzed for viral DNA sequences by a PCR assay.

A semiquantitative PCR assay was used to compare levels of vector-derived MDR1 mRNA with endogenous murine mdr1 and 3 mRNA levels and with MDR1 mRNA levels in
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Fig 3. Quantitative RT-PCR for MDR1 vector derived transcripts in hematopoietic cells from transplanted mice. (A) Linearized plasmid DNAs containing either murine mdr1 or human MDR1 sequences were amplified using semiquantitative conditions. The amount of DNA in femtograms is shown above each lane and the primers used for each reaction are shown on the right. Assays of RNA samples from transduced murine bone marrow are shown in the last two lanes. (B) Tissue RNA from animals transplanted with cells transduced with the HaMDR1 (left section), G1MD (middle section), or controls (right section) was amplified with primers specific for the murine mdr1 and 3 transcripts (top row), human MDR1 transcripts (middle row), or β-actin transcripts (bottom row). Primers P7 and P8 were used to amplify total vector derived transcripts. Bone marrow RNA was prepared from individual mice (M3, M8, and M7) or from marrow pooled from 3, 4, or 6 mice. RNAs from individual 14-day spleen colonies were also analyzed. Controls show RNAs from nontransplanted mice at serial fivefold dilutions and from cultured human tumor cell lines (Hct15 and SW620). Vector-transduced bone marrow was obtained from taxol-treated mice. Bone marrow from untransplanted controls was not exposed to taxol selection.

Two P-gp-expressing human tumor cell lines. To compare the relative amplification efficiencies for murine and human cDNA sequences, equimolar amounts of human MDR1 and murine mdr1 plasmid DNA were assayed (Fig 3A). In the range of signal intensities seen with RNA samples from transduced mice, equivalent amounts of murine mdr1 plasmid yielded a signal intensity four times greater than that seen with the human MDR1 plasmid. Based on this standard curve, signal intensities for murine mdr1 and 3 were divided by four when calculating the ratio of human MDR1 to murine mdr1 and 3 mRNA.

Fourteen-day spleen colonies were isolated from irradiated recipients and whole bone marrow was isolated from mice 14 days after taxol treatment and 4 to 8 months after transplant. MDR1 vector expression was seen in hematopoietic tissues from transplanted mice using both vectors (Fig 3). Endogenous mdr1 and 3 levels varied between samples but were greatest in bone marrow of taxol-treated animals, less in marrow from nontransplanted control animals not receiving taxol, and least in 14 day spleen colonies. Within individual samples, the level of retroviral MDR1 transcripts exceeded that from the endogenous murine mdr1 and 3 genes in all but two cases (bone marrow M3 and spleen colony 3). The G1MD vector conferred a higher average level of MDR1 mRNA expression when compared with the HaMDR1 vector. No differences in vector expression were detected in bone marrow versus spleen colony cells.

MDR1 mRNA levels in transduced mouse tissues were compared with that from the human tumor cell lines Hct15 and SW620. Hct15 cells express a high level of MDR1
mRNA and stain brightly with MoAbs against P-gp. SW620 cells express 15-fold less MDR1 mRNA and P-gp is at the lower limit of detection using MoAbs. Despite this, SW620 cells exhibit significant P-gp-mediated drug resistance as shown by a 2- to 11-fold increase in drug sensitivity seen with P-gp reversing agent verapamil. Our semiquantitative RT-PCR assay indicated that SW620 cells express 20-fold less MDR1 mRNA than Hct15 (Fig 3B), closely approximating the value reported using a RNA slot blot assay.

Expression levels of the MDR1 vectors in tissues from transplanted mice were intermediate between that observed in SW620 and Hct cells (Fig 3B).

Expression of the HaMDR1 vector in hematopoietic stem cells. To determine the level of expression of the HaMDR1 vector in hematopoietic stem cells, RNA was analyzed from a sorted population of bone marrow cells known to be highly enriched for repopulating hematopoietic stem cells. Four mice known to be positive for DNA proviral sequences in circulating leukocytes 8 months after transplant were killed 10 days after taxol administration and bone marrow cells were pooled. Mature lineage-specific cells were subtracted using an MoAb cocktail as previously described. Cells expressing c-kit on the cell surface were then isolated from the lineage-negative population. These c-kit-positive, lineage-negative (c-kit+, lin-) cells have been shown to be highly enriched for repopulating stem cells. The stem cell enriched population showed an approximately threefold increase in expression of the endogenous murine mdr1 and 3 genes compared with whole bone marrow, paralleling what has been described in human hematopoietic cells (Fig 4). Vector-derived human MDR1 transcripts were detected in all populations. The MDR1 mRNA level in the c-kit+, lin- population was calculated to be four times greater the level of the endogenous mdr1 and 3 transcripts, after correcting the signal intensity using the standard curve shown in Fig 3A. These data show significant expression of the MDR1 vector in an enriched stem cell population at an extended period after gene transfer.

Aberrant mRNA splicing in MDR1 vectors. Southern blot analysis of genomic DNA from cultured fibroblasts transduced with the MDR1 vectors showed the expected full-length provirus as well as a shortened proviral form (data not shown). Mapping of the short form showed a junction region arising from the joining of a cryptic splice donor site (CMTATGC, codon 113 underlined) and a cryptic splice acceptor site (ACATITTTCCTTWG, codon 773 underlined) within the MDR1 cDNA (K. McDonagh, manuscript in preparation). Aberrant splicing of vector-derived genomic RNA within the producer cells results in passage of this truncated proviral form to target cells. Sequence analysis of the truncated mRNA predicts a nonfunctional protein based on the large deletion of coding sequence and a downstream frameshift mutation resulting in a premature termination codon.

The PCR primers used to measure transcript levels in the previously described experiments (P7 and P8 in Fig 5) amplify both full-length and spliced MDR1 transcripts. To determine the prevalence of spliced MDR1 mRNA in trans-
duced hematopoietic tissues, primer sets were designed to discriminate between full-length and aberrantly spliced MDR1 transcripts. P13 and P14 are specific for full-length MDR1 message given that sequences complementary to P13 are deleted in the spliced form (Fig 5). Primers P15 and P16 span the splice junction and produce a 490-bp amplification product specific to the spliced form.

Figure 6 shows the results of a RT-PCR assay for spliced and full-length MDR1 transcripts. Plasmid DNA containing the MDR1 cDNA shows amplification product only with the full-length primers as expected. RNA from the Hct15 cell line shows that less than 1% of the total MDR1 transcripts are truncated spliced forms. In contrast, cytoplasmic RNA from the HaMDR1 producer clone shows equivalent amounts of both full-length and spliced message. cDNA prepared from hematopoietic tissues of transplanted mice showed variable proportions of spliced transcripts. Some samples expressed predominantly truncated nonfunctional message (Spleen Colony 5 and Bone Marrow M16 in Fig 6). This variability in the degree of truncated transcript is explained in part by variable degrees of passage of truncated provirus. The ratio of truncated to full-length provirus correlated with the ratio of truncated to full-length MDR1 transcripts (data not shown). In the primary mouse hematopoietic
Fig 7. Comparison of overall levels of specific P-gp-encoding transcripts in tissues from transplanted mice. The levels of MDR1 vector-derived transcripts and of endogenous murine mdr1 and 3 transcripts for various samples were calculated as signal intensity ratios of MDR1 (mdr1 and 3) to β-actin bands from the experiment shown in Fig 3. Endogenous mdr1 and 3 levels were divided by four to correct for amplification efficiency and to allow direct comparison with vector-derived MDR1 levels. The proportion of spliced and full-length MDR1 transcripts was calculated from the experiment shown in Fig 6. Overall expression levels in various samples are shown for endogenous murine mdr1 and 3 transcripts (■), spliced MDR1 vector transcripts (□), and full length MDR1 vectors (□). Samples are designated as in prior figures. HaMDR1-transduced samples (Harvey) are shown on the left, G1MD-transduced samples (Moloney) in the middle, and control samples on the right. C57Bl shows RNA data for bone marrow from an untransplanted control mouse.

Overall expression levels in various samples are shown for endogenous murine mdr1 and 3 transcripts (W), spliced MDR1 vector transcripts (0), and full length MDR1 vectors (B). Samples are designated as in prior figures. HaMDR1-transduced samples (Harvey) are shown on the left, G1MD-transduced samples (Moloney) in the middle, and control samples on the right. C57Bl shows RNA data for bone marrow from an untransplanted control mouse.

cell populations analyzed, the spliced transcript averaged about 60% of total vector-derived message. No significant difference in the degree of aberrant mRNA splicing was noted between the two vectors, showing that aberrant splicing is independent of the vector backbone.

To quantify the level of expression of endogenous and vector-derived P-gp-encoding transcripts, intensities of appropriate bands on gels were measured using a Molecular Dynamics phosphoimager. For a given sample, the level of vector-derived transcripts was calculated as the ratio of MDR1 product to β-actin product. The proportion of vector-derived transcripts that were full-length or spliced was calculated as the ratio of full length or spliced product to the sum of full length plus spliced product. The signal intensity ratio of murine mdr1 and 3 to β-actin was divided by four to allow comparison with the MDR1 to β-actin ratio. Figure 7 shows the relative levels of expression of endogenous mdr1 and 3 transcripts, full-length MDR1, and spliced MDR1 transcripts in various samples. The level of full-length MDR1 transcripts exceeded that from the endogenous genes in 11 of 12 cases. In most cases, the level of full-length MDR1 mRNA expression approximated that seen in the modestly drug-resistant cell line SW620. The G1MD vector resulted in somewhat higher levels of full-length MDR1 expression due to increased expression of both full-length and spliced forms. Taken together, these results indicate that MDR1 gene transfer resulted in significant augmentation of P-gp-encoding mRNA levels in transplanted murine hematopoietic cells.

These experiments measured MDR1 mRNA levels in populations of cells. Because not all cells in bone marrow-derived populations necessarily contain the transferred gene, it is possible that the overall MDR1 mRNA levels in the subset of cells that were actually transduced were higher than the calculated values. For example, the average proviral DNA copy number in the whole bone marrow cells used for stem cell purification was 1.2 copies per cell (data not shown). If 100% of the bone marrow cells contained one copy of the vector, then the mRNA levels measured for the population would represent the average expression seen in each transduced cell. An average copy number of 1.2 is also consistent with 50% of the cells containing two copies of the vector. In contrast to bone marrow samples, mRNA ratios calculated for CFU-S-derived colonies can be extrapolated to each cell in the population because these colonies are derived from a single transduced cell; therefore, all cells contain the transferred provirus.

Protein expression. Attempts to show P-gp expression in mouse bone marrow and peripheral blood cells using the
MoAb MRK-16 showed little to no detectable surface staining (data not shown). This finding is consistent with the observation that overall full-length MDR1 transcript levels in transduced cells approximated that seen in the SW620 cell line, which expresses P-gp at the threshold of detection using available MoAbs.35

**Aberrant splicing in producer clones.** The demonstration of truncated provirus in target cells transduced with MDR1 vectors suggested that aberrantly spliced genomic transcripts were packaged and transferred by producer cell clones. To confirm this finding and to determine if the degree of aberrant splicing varied among individual producer clones, a number of isolated producer clones were analyzed for the prevalence of spliced transcripts in the cell cytoplasm. Ecotropic producer clones were isolated after screening supernatants for the ability to confer rhodamine dull phenotype on 3T3 cell populations. RNAs prepared from six individual G1MD producer clones and the one HaMDR1 producer clone were analyzed. These results showed similar levels of spliced and full-length transcripts in all clones analyzed (Fig 8). This experiment established that aberrant splicing of the MDR1 cDNA is not specific to individual producer clones.

**DISCUSSION**

Several groups have shown that retroviral-mediated MDR1 gene transfer to murine hematopoietic cells results in drug-resistant hematopoietic cells in transplanted animals.5,7,14 This work provides the preclinical rationale for testing this strategy as a therapeutic modality in cancer patients. This approach may allow reduction of chemotherapy related toxicity and enable drug dose intensification. Another potential application is the use of MDR1 as a dominant selectable marker in vivo. Chimeric vectors containing both the MDR1 cDNA and other nonselectable therapeutic genes may allow selection of transduced cells after transplantation as a means to overcome current limitations in hematopoietic stem cell transduction efficiencies.

Our prior observation that taxol treatment can lead to stable increases in provirally marked murine hematopoietic cells suggested that selection may occur at the stem cell level in some instances. This interpretation implies that MDR1 gene transfer should increase P-gp expression in primitive murine hematopoietic cells. It has been shown that primitive human hematopoietic cells express relatively high levels of endogenous P-glycoprotein compared with more differentiated hematopoietic cells.7 Given these considerations, we directly tested if MDR1 gene transfer was resulting in a increase in P-gp expression in several populations of hematopoietic cells. The experiments reported here show that MDR1 gene transfer resulted in a several fold increase in the overall level of P-gp encoding transcripts in whole bone marrow, differentiated spleen colonies, and a population of primitive bone marrow cells. Full-length MDR1 mRNA levels were equivalent to that seen in the modestly drug-resistant cell line SW620. These data provide support for the interpretation that the transferred MDR1 gene is expressed at physiologically significant levels in proliferative murine hematopoietic cells. In agreement with these conclu-
T-lymphocyte activation and maturation. We have also shown that murine hematopoietic cells endogenously express both mdrl and mdr3 transcripts. The overall levels of mdrl and 3 expression in hematopoietic tissues was much lower than that seen in adrenal and kidney, tissues known to endogenously express high levels of P-gp. This modest endogenous P-gp expression in hematopoietic cells is consistent with the known sensitivity of hematopoietic cells to P-gp–influenced antineoplastic drugs. Analogous to what has been reported for human hematopoietic cells, the endogenous murine mdrl and 3 genes were differentially expressed in divergent hematopoietic lineages and were expressed at relatively high levels in primitive undifferentiated cells. The physiologic significance of these differences are not understood. Our data show relatively high levels of mdrl and 3 expression in bone marrow derived T cells, intermediate levels in thymic cells, and very low levels in peripheral blood leukocytes (which contain 50% to 70% lymphocytes). Similar variability in P-gp expression in specific human lymphocyte subsets has been reported. It may be that P-gp expression in T lymphocytes is a function of T-lymphocyte activation and maturation. 

Unexpectedly, we found that all cells transduced with MDR1 vectors contained a shortened provirus and expressed a truncated MDR1 mRNA. Aberrant splicing of vector derived transcripts was shown to be mediated by cryptic splice donor and acceptor sites within the wild-type MDR1 cDNA. It is likely that aberrant MDR1 mRNA processing results in attenuation of P-gp expression. Both the transfer of truncated provirus and aberrant splicing of transcripts derived from full-length provirus could result in expression of truncated transcripts. It is uncertain to what degree each mechanism contributes to expression of truncated MDR1 in transduced hematopoietic cells. The variable degree to which full-length MDR1 transcripts are expressed in different samples likely contributes to variability in the degree of in vivo selection observed among individual mice and in the difficulty we have had in showing consistent protection from myelosuppression after MDR1 gene transfer. Animals repopulated with a cell stem clone expressing predominantly truncated MDR1 mRNA would not expected to be protected from drug-induced myelosuppression.

Several forms of spliced P-gp transcripts have been detected in drug-resistant hamster cells. Unlike the truncated transcript arising from MDR1 retroviral vectors, these endogenously occurring spliced mRNAs maintain the open reading frame and naturally occurring stop codon present in the full-length mRNA. It is not known if the spliced rodent transcripts encode proteins capable of conferring functional drug resistance. It is interesting that the sequence and location of the splice acceptor site noted in one of these transcripts is very similar to that seen in the human MDR1 cDNA. Our observation that very small amounts of truncated MDR1 transcripts are present in the P-gp–expressing human tumor cell line Hct 15 suggests that the cryptic splice sites can be activated at a very low frequency with transcription of genomic MDR1 sequences. Increased usage of these cryptic splice sites within the context of retroviral vectors may be due to the lack of intron retention by splicing machinery, as has been seen in β-thalassemia mutations that abolish normal RNA splicing. It is also possible that secondary structure of the genomic viral RNA may result in increased aberrant mRNA processing.

We are in the process of engineering MDR1 vectors that do not splice at these cryptic sites to test if expression can be augmented and if the level of drug resistance in bone marrow can be increased. Testing of these second-generation MDR1 vectors in mice may allow identification of an improved MDR1 vector for clinical use.

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Expression of retroviral vectors containing the human multidrug resistance 1 cDNA in hematopoietic cells of transplanted mice

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