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Selectable Markers for Gene Therapy

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I. INTRODUCTION

A. The Use and Choice of Selectable Markers

One of the major problems with current approaches to gene therapy is the instability of expression of genes transferred into recipient cells. Although in theory, homologous recombination or use of artificial chromosomes can stabilize sequences with wild-type regulatory regions, such approaches to gene therapy are not yet feasible and may not be efficient for some time to come. In most high efficiency DNA transfer in current use in intact organisms, selectable markers must be used to maintain transferred sequences; in the absence of selection the transferred DNAs or their expression is rapidly lost.

There are several different selectable markers that might be used for in vivo selection, including genes whose expression has been associated with resistance of cancers to anticancer drugs. Examples include: (a) methotrexate resistance due to mutant dihydrofolate reductase [DHFR] (1); (b) alkylating agent resistance due to expression of methylguanine methyltransferase [MGMT] (2); and (c) the expression of the multidrug transporting proteins P-glycoprotein (P-gp, the product of the *MDR*1 gene) (3) and MRP (multidrug resistance associated protein) (4). In this chapter, we will detail our experience with the *MDR*1 gene.

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The resistance of many cancers to anticancer drugs is due, in many cases, to the overexpression of several different ATP-dependent transporters (ABC transporters), including the human multidrug resistance gene *MDR*1 (ABC B1) (3,5,6), MRP1 (ABC C1), the multidrug resistance-associated protein (7) and other MRP family members (8), and *MXR* (ABC G2) (9). *MDR*1 encodes the multidrug transporter, or P-glycoprotein (P-gp). P-gp is a 12 transmembrane domain glycoprotein composed of 2 homologous halves, each containing 6 transmembrane (TM) domains and one ATP binding/utilization site. P-gp recognizes a large number of structurally unrelated hydrophobic and amphipathic molecules, including many chemotherapeutic agents, and removes them from the cell via an ATP-dependent transport process (see Fig. 1).

MDR1 has many obvious advantages for use as a selectable marker in gene therapy. It is a cell surface protein that can be easily detected by FACS or immunohistochemistry. Cells expressing P-gp on their surfaces can be enriched using cell sorting or magnetic bead panning technologies. The very broad range of cytotoxic substrates recognized by P-gp makes it a pharmacologically flexible system, allowing the investigator to choose among many different selection regimens with differential toxicity for different tissues and different pharmacokinetic properties. Furthermore, as will be discussed in detail in this chapter, P-gp can be mutationally modified to increase resistance to specific substrates and alter inhibitor sensitivity. Hematopoietic cells initially appeared to tolerate relatively high levels of P-gp expression without major effects on differentiated function (10).

B. Lessons from Transgenic and Knockout Mice

Two lines of evidence support the concept of using *MDR*1 as a selectable marker in human gene therapy. Transgenic mice

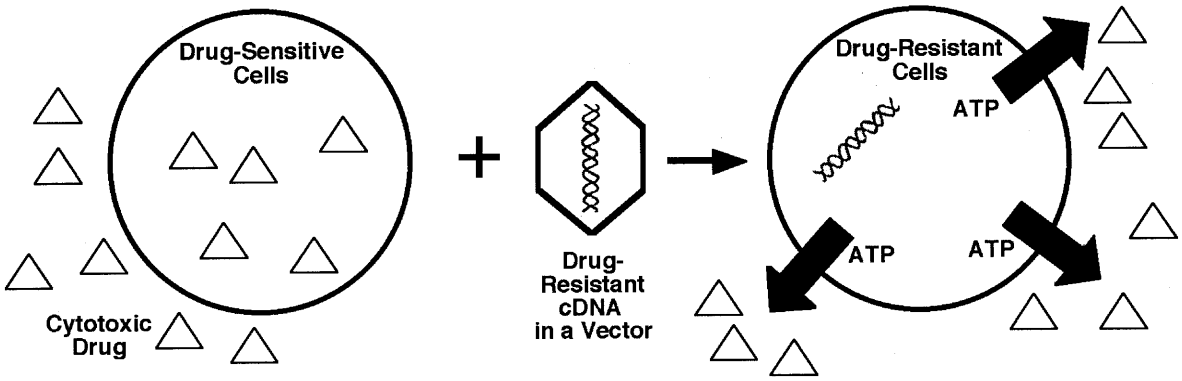

Figure 1 Multidrug transporters such as MDR1, MRP1, and MXR confer resistance on transduced cells. The triangles are cytotoxic drugs; the large, bold arrows are transporters.

expressing the *MDR*1 gene in their bone marrow are resistant to the cytotoxic effects of many different anticancer drugs (10–12). *MDR*1 transgenic bone marrow can be transplanted into drug sensitive mice, and the transplanted marrow is resistant to cytotoxic drugs (13). Mice transplanted with bone marrow transduced with the human *MDR*1 cDNA and exposed to taxol show specific enrichment of the *MDR*1-transduced cells (14–16), and this transduced marrow can be serially transplanted and remains drug resistant (16). Recently, this ability to select transduced bone marrow with taxol has been demonstrated in a canine bone marrow transplantation model (17).

The mouse *mdr*1a and *mdr*1b genes have been insertionally inactivated in mice (18–21). These animals, although otherwise normal, are hypersensitive to cytotoxic substrates of P-gp. This hypersensitivity is due in part to the abrogation of the *mdr*1a-based blood brain barrier (22), and to enhanced absorption and decreased excretion of *mdr*1 substrates (23). These studies demonstrate the critical role that P-gp plays in drug distribution and pharmacokinetics, and argue that specific targeting of P-gp to tissues that do not ordinarily express it (as in gene therapy), will protect such tissues from cytotoxic *mdr*1 substrates.

II. SELECTABLE MARKERS IN HEMATOPOIETIC SYSTEMS AND IN THE SKIN

As noted above, studies on mice transgenic for human *MDR*1 established that constitutive overexpression of this gene protects animals from antineoplastic agents. Drugs could be administered safely at dose-levels several-fold higher than to mice of the respective background strains (10,11). To demonstrate the specificity of this protection, verapamil, an inhibitor of P-glycoprotein, was coadministered, resulting in reversal of drug resistance (12). Similarly, mice transgenic for a mutated dihydrofolate reductase (DHFR) or an O^6-methylguanine DNA methyltransferase cDNA were protected from methotrexate or 1,3-bis (2-chloroethyl) nitrosourea (BCNU) toxicity, respectively (26–24).

Upon overexpression in target cells, drug resistance genes may also protect them from environmental toxins such as carcinogens in addition to amelioration of anticancer chemotherapy (27). For instance, transfer of O^6-methylguanine methyltransferase increases repair of DNA damage in sensitive cells. In vitro and in vivo studies confirmed this aspect of the function of drug resistance genes (28–29). Liu et al. (30) showed that rapid repair of O^6-methylguanine-DNA adducts in transgenic mice protected them from N-methyl-nitrosourea-induced thymic lymphomas. This protection from carcinogens can be targeted to other organs like liver or skin by suitable promoter systems (31,32).

Chemoprotection exerted by overexpression of chemoresistance genes in hematopoietic organs of transgenic animals could be transferred by transplantation of bone marrow to normal recipients (13,33). These experiments provided a basis for gene therapy approaches with drug resistance genes. Hence, drug resistance genes that were initially studied because of their association with failure of anticancer chemotherapy are expected to serve as useful tools for gene therapy of cancer by protecting patients from the toxic side effects of chemotherapy. Protection of chemosensitive cells from toxic compounds may be particularly helpful in the case of the hematopoietic system because most cells in blood and bone marrow are highly susceptible to antineoplastic compounds. $CD34^+$ hematopoietic progenitor cells do not express glutathione-S-transferases (34), and only very low levels of endogenous *MDR*1 gene are expressed in myeloid and erythroid progenitor cells (35,36). These low expression levels are not capable of providing protection from the cytotoxicity of anticancer drugs. Conversely, the high susceptibility of normal hematopoietic cells to cytotoxic agents allows selection strategies exploiting drug resistance genes if sufficient levels of resistance can be conferred.

A variety of different genes have been used to confer drug resistance on bone marrow cells (see Table 1). Retroviral transduction with a full-length *MDR*1 cDNA promoted by Long-Terminal Repeats (LTRs) of Harvey sarcoma virus protected normal, clonogenic hematopoietic precursors or erythroleukemia cells from anticancer drugs (37,38). Transduced cells were found to be resistant to multiple drugs including taxol, colchicine, and daunomycin. Murine hematopoietic stem cells originating from fetal liver (39), peripheral blood following mobilization with the use of growth factors (40), or from bone marrow (41), were efficiently transduced with retroviral *MDR*1 vectors. In the latter study, it was shown that transplantation of transduced hematopoietic stem cells results in efficient expression of functional human P-glycoprotein in recipient mice. In spite of generally lower transduction frequencies, $CD34^+$ human progenitor cells could also be transduced with retroviruses conveying the multidrug resistance gene (42,43). Similarly, vectors containing MRP1 (44) or mutated DHFR cDNAs are highly efficient in rendering bone marrow cells resistant to methotrexate or trimetrexate, respectively (45,46).

Pluripotent human hematopoietic stem cells or early progenitors, respectively, are difficult to transduce with amphotropic retroviruses (47). Fruehauf et al. (48) targeted immature, cobblestone area-forming progenitor cells. However, in this study significant vincristine resistance was achieved only in a small minority of the immature cell population. This might be due to endogenously high *MDR*1 expression in hematopoietic stem cells (49), which can make it difficult to analyze the function of the transgene. DHFR might be a better marker for selection at the level of long-term culture-initiating cells (50).

Transplantation of *MDR*1-transduced murine bone marrow cells into W/W^v mice (14) or lethally irradiated normal syngeneic mice (15) resulted in significant gene expression in the bone marrow of recipient animals. Both investigators detected elevated levels of *MDR*1 expression after treatment of recipient mice with taxol, favoring the idea of a selective advantage in vivo of hematopoietic cells overexpressing the *MDR*1 transgene. This observation was in marked contrast to previous studies with selectable markers such as genes conferring resistance to neomycin, puromycin, or hygromycin. Because of their pharmacology or pharmacokinetics such compounds cannot be used for selection in vivo.

Further support for the potential usefulness of drug resistance genes for selection in vivo was provided by experiments in which *MDR*1-transduced bone marrow was first transplanted into recipient mice (16). After taxol treatment of recipient mice, their bone marrow was then retransplanted into a second generation of recipient mice. In several cycles of retransplantation and taxol treatment of recipient animals, increasingly high levels of drug resistance were generated in vivo. Mice of the fifth and sixth generation survived doses of taxol that were lethal for mice that had not undergone bone marrow transplantation.

Bunting et al. (51) reported that transduction of murine bone marrow cells with pHaMDR1 retroviral vector enables *ex vivo* stem cell expansion, which might help account for the ability of transduced cells to survive multiple cycles of transplantation. However, the biological safety of expansion of transduced stem cells is currently under scrutiny. When *MDR*1-transduced progenitor cells are expanded with growth factors for extended periods (up to 12 days), uncontrolled proliferation occurs, as has been observed in one study (51). The authors concluded that the finding was related to the *MDR*1 transgene because the development of a myeloproliferative syndrome was not observed following transfer of DHFR. Other groups have not observed this adverse effect after *MDR*1 transfer to murine hematopoietic cells. More importantly, comparable studies in nonhuman primates did not reveal perturbations of myelopoiesis (52). It appears possible that these differences are related to the recent finding that the integration sites of retroviral vectors reveal predilections for certain chromosomes and are not randomly distributed in the genome of bone marrow repopulating cells (53).

These concerns have prompted recent investigations with vector systems other than retroviruses. For instance, SV40 pseudovirions allow for highly efficient *MDR*1 gene transfer to hematopoietic cells (54,55). Alternatively, Epstein-Barr virus-based vectors can be designed that contain the *MDR*1 cDNA episomally in target cells (56). With such vector sys-

Table 1 Use of Drug-Resistance Genes to Confer Resistance on Bone Marrow

Gene	Selection	Reference
Multidrug resistance gene 1 (MDR1)	Multiple cytotoxic natural product drugs	Gottesman et al., 1995 (3)
Multidrug associated protein 1 (MRP1)	Multiple cytotoxic natural product drugs	Omori et al., 1999 (44)
Dihydrofolate reductase (DHFR)	Methotrexate and trimethotrexate	Flasshove et al., 1998 (1) Warlick et al., 2002 (70)
Cytidine deaminase	Cytosine arabinoside	Momparler et al., 1996 (74)
Glutathione transferase Yc	Melphalan, mechlorethamine, chlorambucil	Letourneau et al., 1996 (75)
Aldehyde dehydrogenase	Cyclophosphamide	Magni et al., 1996 (76) Moreb et al., 1996 (77)
O^6-methylguanine methyltransferase (O^6-MGMT)	Nitrosourea (BCNU)	Allay et al., 1995 (78)

tems life-long expression cannot be achieved because they fail to integrate into the genome. Conversely, for protection of hematopoietic cells during a series of chemotherapy cycles, sustained expression may not be required. Loss of *MDR*1 expression after the period of chemotherapy might in fact increase the safety of *MDR*1 gene therapy.

With the use of retroviral vectors, functional drug resistance of hematopoietic progenitor cells transduced by *MDR*1 vectors was first demonstrated in tissue culture (37) and recently in mice transplanted with transduced human progenitor cells (57). As a result, recipient animals can be treated safely with intensified chemotherapy following reinfusion of *MDR*1-transduced cells (58).

The efficiency of chemoprotection during antineoplastic chemotherapy is questionable if the cancers themselves express high amounts of P-glycoprotein. To further enhance the efficiency of this approach a vector has been designed that contains a mutant *MDR*1 cDNA (59). A point mutation in amino acid position 938 by which phenylalanine is replaced by alanine has little effect on the substrate specificity of P-glycoprotein but abolishes the reversing effect of a chemosensitizing agent, trans(E)-flupenthixol (60). Such vectors can be useful to overcome multidrug resistance in cancer cells, which are protected by wild-type P-glycoprotein, whereas hematopoietic cells are protected by the mutant gene even in the presence of anticancer drug and chemosensitizer.

Treatment of *MDR*1-transduced hematopoietic cells with anticancer drugs in tissue culture increases expression of *MDR*1 mRNA and P-glycoprotein in the surviving cell population as a result of clonal selection (61). Moreover, *MDR*1 transduced hematopoietic cells exposed to high concentrations of anthracyclines or colchicine prior to transplantation maintain their ability to engraft and rescue recipient mice from otherwise lethal irradiation (62). These animal studies provide useful models for the optimization of conditions for clinical applications.

Several of the drug resistance genes have been used to protect hematopoietic cells from drugs used in anticancer treatment. As has been seen with *MDR*1, chemoprotection of hematopoietic progenitor cells and a selective advantage in vitro were demonstrated following transduction by mutated DHFR cDNAs that confer resistance to methotrexate and trimetrexate (1,63–66). Williams et al. (67), Cline et al. (68), and Vinh et al. (69) demonstrated protection of recipient animals from lethal doses of methotrexate. Human $CD34^+$ cells can be transduced efficiently with a DHFR vector (1).

Mice transplanted with DHFR-transduced hematopoietic cells display resistance against high levels of trimetrexate, which can be transferred to a second generation of transplant recipients (70). Interestingly, mice can be transplanted with low amounts of DHFR-transduced cells following mild total body irradiation at a reduced dose of 1_Gy and acquire methotrexate resistance by treatment with this drug for 60 days following transplantation (71). Retransplantation experiments performed with dihydrofolate reductase (72) gave results comparable to those obtained with *MDR*1 (16); both genes facilitate increased levels of resistance after several cycles of transplantation and drug treatment of recipient animals.

In vivo selection of retrovirally transduced hematopoietic cells has convincingly been demonstrated with the DHFR as a selectable marker (73). In this study mice were transplanted with DHFR-transduced bone marrow cells. Drug treatment resulted in significantly increased expression in granulocytes, erythrocytes, platelets, and T- and B-lymphocytes. Secondary recipients revealed that selection had occurred at the stem cell level.

Resistance to another antimetabolite drug, cytosine arabinoside, which is a major component of treatment for acute leukemias, is conferred by cytidine deaminase. Hematopoietic cells were rendered resistant to cytosine arabinoside by transfer of this gene (74).

Different patterns of chemoresistance can be attributed to various drug resistance genes. For instance, the *MRP*1 gene is genetically and functionally related to *MDR*1. Retroviral transfer of *MRP*1 resulted in resistance to doxorubicin, etoposide, and vincristine (4). However, since binding and transport of inhibitors to *MDR*1 may be different from *MRP*, transfer of this gene may be useful if naturally occurring resistance due to *MDR*1 overexpression in cancer cells has to be overcome to allow for effective chemotherapy of an *MDR*1-expressing cancer.

Resistance to alkylating agents is multicausative, and several genes may be useful as selectable markers. Retroviral transfer of a rat glutathione S-transferase Yc cDNA to hematopoietic cells conveyed moderate resistance to melphalan, mechlorethamine, and chlorambucil (75). Resistance to cyclophosphamide or 4-hydroperoxycyclophosphamide, respectively, could be conferred on hematopoietic cells by transfer of aldehyde dehydrogenase with the use of retroviral vectors (76,77). Leukemic or primary hematopoietic cells were rendered resistant to BCNU by retroviral transfer of a human O^6-alkylguanine-DNA alkyltransferase cDNA (2,78,79). Transplantation of transduced bone marrow cells rescued recipient animals from the toxicity of nitrosoureas (80). In particular, nitrosourea-induced severe immunodeficiency can be overcome by transduction of immature progenitor cells (80,81). Furthermore, resistance to nitrosoureas in combination with an inhibitor of O^6-alkylguanine-DNA alkyltransferase, a key enzyme involved in naturally occurring resistance to nitrosoureas, could be conferred by retroviral transfer of a mutated O^6-methylguanine DNA methyltransferase cDNA (26). This approach protected mice from lethal drug doses and allowed selection of transduced hematopoietic progenitor cells.

To widen the range of anticancer drugs to be inactivated by gene therapy, vectors have been constructed for coexpression of two different drug resistance genes. For instance, a vector containing *MDR*1 and O^6-alkylguanine-DNA-alkyltransferase rendered human erythroleukemia cells resistant to the MDR1 substrates, colchicine and doxorubicin, as well as to alkylating agents, N-Methyl-N-nitrosourea and temozolomide (82).

A different approach to utilize vectors that allow for coexpression of two genes is to include a second gene that may

enhance the efficacy of a selectable marker gene. This has been shown by construction of a vector that contained an MRP1-cDNA and a cDNA encoding γ-glutamyl-cysteine synthetase, the rate-limiting enzyme of glutathione biosynthesis (83). Resistance to substrates of MRP1 was thereby increased due to elevated glutathione levels in transduced cells. The MRP1 transporter extrudes glutathione-conjugated compounds from the cell; hence, elevated concentrations of glutathione increase the concentrations of potential substrates.

Based on experiments in tissue culture and animal models, early clinical trials on transfer of the *MDR*1 gene to hematopoietic progenitor cells have been conducted (84–86). Bone marrow or peripheral blood progenitor cells from patients suffering from advanced neoplastic diseases were retrovirally transduced and reinfused after high-dose chemotherapy (87–89). These studies revealed that transduction efficiencies using *MDR*1 vectors as detected in bone marrow or peripheral blood of patients tended to be low, and varied from one patient to another. Notably, in two recent studies enrichment of *MDR*1-transduced cells was observed following treatment with etoposide or paclitaxel, respectively (90,91). The studies confirm the concept that the human multidrug resistance gene can serve as a drug-selectable marker gene in vivo in the hematopoietic system. However, gene transfer procedures and selection strategies need to be improved to efficiently protect human hematopoietic cells from the cytotoxicity of drug treatment. In particular, clinical studies should be conducted with novel vector constructs and improved culture conditions that allow for increased transduction rates.

Improvements in vector design have been suggested by several groups. Using the multidrug resistance gene, Metz et al. (92) showed that retroviral vectors derived from Harvey viruses can be substantially shortened without reduction of gene transfer efficiency, thereby increasing the maximum size of the packaged gene of interest. By systematic analysis of the U3-region of various 5′-long-terminal repeats, Baum et al. (93) optimized *MDR*1 transfer to hematopoietic cells. Notably, transfer to immature hematopoietic progenitor cells, which are generally difficult to transduce, was improved (94). More recent improvements of posttranscriptional processing led to a vector that reliably ensured *MDR*1 expression and drug efflux in human hematopoietic cells following an in vivo passage in immunodeficient mice (95). Other vector systems used for chemoresistance gene transfer to hematopoietic cells include adeno-associated virus vectors (96) or liposomes (97) (see Sections IV and V.).

New vector constructs increase the efficiency of gene transfer to hematopoietic cells but do not necessarily ensure gene expression for sustained periods. A major obstacle to long-term gene expression is the limited lifespan of some transduced cell clones. Since only hematopoietic stem cells have the capability of self-renewal, the lifespan of progeny generated by more differentiated progenitor cells is limited. Berger et al. (98) have shown that expansion of cells with cytokines, particularly with interleukin-3 (IL-3), reduces the frequency of long-term culture-initiating cells (LTC-IC), which correlated with reduction of Rhodamine-123 efflux from immature progenitor cells. In accord with these findings, Schiedlmeier et al. (99) reported that IL-3-stimulated hematopoietic cells engrafted more poorly than cells grown in the presence of other growth factor combinations. Both studies resulted in efficient retroviral *MDR*1 transfer to primitive human progenitor/stem cells.

A different approach to improve the utility of selectable markers is to coexpress two drug resistance genes, thereby conferring resistance to a broad range of cytotoxic agents. To this end, mutated dihydrofolate reductase has been coexpressed with *MDR*1 or with thymidylate synthase (100,101), and *MDR*1 has also been expressed with O^6-methylguanine-DNA-methyltransferase (102,103). Coexpression of glutathione S-transferase and cytidine deaminase rendered cells resistant to cytosine arabinoside and alkylating agents such as melphalan and chlorambucil (104). These compounds are used in chemotherapy of malignant lymphomas.

Alternatively, a dominant-positive selectable marker gene can be coexpressed with a negative selectable marker such as thymidine kinase from Herpes simplex virus (HSV-TK) (105,106). The latter approach allows selective elimination of transduced cells. Such an approach may increase the safety of gene transfer if cancer cells contaminating hematopoietic cell preparations are inadvertently rendered drug-resistant, or if transduced cells become malignant (51,107). Selective killing of *MDR*1-HSV-TK transduced cells in vivo has been demonstrated (106). Thymidine kinase may not only facilitate selective killing of cancer cells but instead increase the efficacy of certain selectable marker genes. A bicistronic vector in which thymidine kinase was combined with dihydrofolate reductase displayed enhanced resistance as compared to a construct that contained a neomycin phosphotransferase instead of thymidine kinase (108). The authors concluded that thymidine kinase may be useful to salvage thymidine.

To increase the safety of gene therapy of cancer, drug resistance genes may be combined with cDNAs that specifically eliminate cancer cells. This has been demonstrated for chronic myeloid leukemia (CML), which is characterized by a specific molecular marker, the BCR/ABL gene fusion. A vector has been constructed that combined a methotrexate-resistant dihydrofolate reductase with an antiBCR/ABL antisense sequence (109). Transfer of this vector to CML cells led to the restoration of normal cellular function of BCR/ABL cDNA + cells due to reduced levels of transcripts while conferring drug resistance.

In addition to improvement of gene therapy of cancer, drug resistance genes may be helpful for gene therapy of nonmalignant diseases if increased gene expression is desired. In fact, there is considerable interest in using drug selectable marker genes to introduce and enrich otherwise nonselectable genes in target organs. Gene therapy, although thought to bear the potential of curing genetically determined diseases, is frequently hampered by low gene expression in target organs. This is particularly true for hematopoietic disorders because the efficiency of gene transfer is often limited, and stable expression of transgenes in bone marrow has been found difficult to accomplish.

For instance, Gaucher disease is characterized by accumulation of a glucosylceramide in glucocerebrosidase-deficient hematopoietic cells. These patients suffer from skeletal lesions, severe hepatosplenomegaly, anemia, and disorders of the central nervous system. While it is possible to efficiently transduce a glucocerebrosidase cDNA to hematopoietic progenitor cells (110,111), expression levels tend to decrease after several weeks or months in vivo because of silencing or limited lifespan of the transduced cells' progeny. To increase expression of glucocerebrosidase in vivo, Aran et al. (112) constructed a transcriptional fusion between *MDR*1 and the glucocerebrosidase gene. Increased expression of the latter gene was achieved by selection with cytotoxic substrates of P-glycoprotein. Appropriate selection strategies allowed complete restoration of the underlying genetic defect in cells from Gaucher patients (113). Transduction of such bicistronic vectors into hematopoietic stem cells might allow treatment of patients by chemotherapeutical elimination of non-transduced cells that continue to synthesize or store glucosylceramide. Moreover, following chemotherapy, the numbers of genetically corrected hematopoietic progenitor cells should increase in bone marrow to maintain physiological numbers of mature granulocytes, monocytes, and lymphocytes in peripheral blood. Recently, in vivo selection for cells expressing glucocerebrosidase was demonstrated with a vector containing the selectable marker gene, DHFR (114).

Similarly, bicistronic vectors that facilitate coexpression of *MDR*1 and α–galactosidase A have been engineered (115). Defects of α–galactosidase A are the cause of Fabry disease, a globotriaosylceramide storage disorder that affects the skin, kidneys, heart, and nervous system. Other applications for bicistronic fusions include immunological disorders such as chronic granulomatous disease and X-linked or adenosine deaminase (ADA) deficiency-related severe combined immunodeficiency (SCID) syndromes. For treatment of these diseases, vectors have been constructed that contain a gp91phox or an ADA cDNA (116–119). DHFR was used as a selectable marker gene in a bicistronic vector for correction of α_1-antitrypsin deficiency (120). Further discussion of the use of bicistronic vectors is found in Section III.

A different strategy to exploit the *MDR*1 gene as a drug-selectable marker for correction of ADA deficiency was described by Germann et al. (121). In this study, both genes were fused to a single cDNA encoding a bifunctional chimeric protein. This approach, however, cannot be used if the two proteins are physiologically located in different cellular compartments.

Another system in which selectable markers may be useful is in the skin. It is possible to grow keratinocytes in culture and introduce the *MDR*1 gene via retroviral vectors. Such keratinocytes are resistant to MDR drugs in vitro, and when transplanted on keratinocyte ''rafts'' to recipient animals, they remain resistant to colchicine, which can be applied as an ointment. If colchicine is withdrawn, transplanted keratinocytes are gradually replaced by nontransduced host skin; in the presence of selection, the transplanted keratinocyte graft is maintained. It should be possible in such a system to introduce other nonselectable genes via bicistronic vectors to serve as a source of protein to treat a genetic defect in the skin or elsewhere in the host (122).

While MDR vectors are well characterized in vitro, their usefulness in vivo has still to be established. We have recently demonstrated in a large-animal model that expression of a nonselectable gene that had been undetectable for more than one year can be recovered by coexpression with a drug-selectable marker, *MDR*1 (17). In this study, high levels of the nonselectable, therapeutic gene were achieved in peripheral blood and bone marrow following treatment of the animal with paclitaxel. The toxicity of this treatment, however, was considerable. Thus, further investigations in animal models are needed to optimize selection strategies in live animals.

Detection of the function of transferred genes may be difficult if normal animals are utilized because of the activity of the respective endogenous gene product. To circumvent this difficulty, ''knock-out'' animals whose gene has been inactivated by targeted disruption can serve as useful models. For instance, mice whose α–galactosidase gene has been disrupted may be helpful to characterize a bicistronic vector in which *MDR*1 is combined with the respective human gene for correction of Fabry disease (123). Another alternative is to use marking genes that are not physiologically expressed at high levels in normal tissues. To characterize bicistronic vectors containing *MDR*1, this gene has been coexpressed with a green fluorescent protein or β–galactosidase (124).

These model systems should help to improve protocols for efficient drug selection and to identify strategies for selection at limited systemic toxicity. For instance, addition of P-glycoprotein inhibitors at low concentration to cytotoxic drugs may increase the stringency of drug selection, thereby allowing use of anticancer drugs at low concentrations for selection (113).

III. BICISTRONIC VECTORS CONTAINING SELECTABLE MARKERS

Although coexpression of two proteins can be achieved through the use of separate promoters, the coexpression is frequently uncoupled due to promoter interference or shutoff of gene expression from one of the promoters, which causes the selected cells not to express the desired protein. To overcome this problem, the selectable marker may be expressed with the therapeutic gene as a translational or transcriptional fusion. A therapeutic protein can be directly linked to the carboxylterminus of the multidrug transporter P-glycoprotein (P-gp). The resulting fusion protein possesses functions of both P-gp and the target protein (125). Since P-gp is an integral membrane protein that functions on the cell plasma membrane, unless two proteins can be separated by a posttranslational proteolytic modification, the expressed target protein will be associated with the plasma membrane regardless of its normal cellular location. Thus, even though translational fusions guarantee protein coexpression, their potential is limited. On the other hand, transcriptional fusions, e.g., using

bicistronic or polycistronic mRNA to encode more than one cDNA, may prove to be more generally applicable.

A. *MDR*1 Bicistronic Vectors Containing Internal Ribosome Entry Sites (IRES)

A DNA segment corresponding to one polypeptide chain plus the translational start and stop signals for protein synthesis can be loosely defined as a cistron. An mRNA encoding only a single polypeptide is called monocistronic mRNA; if it encodes two or more polypeptide chains, it may be called bicistronic or polycistronic mRNA. Almost all eukaryotic mRNA molecules are monocistronic. Initiation of translation of eukaryotic mRNA is mediated by cap-binding protein that recognizes a methylated guanosine cap at the 5′ terminus of mRNA. However, some viral mRNA molecules transcribed in eukaryotic cells are polycistronic. They can use a cap-independent mechanism to initiate translation in the middle of mRNA molecules. For picornavirus, this cap-independent internal initiation of translation is mediated through a unique internal ribosome entry site (IRES) within the mRNA molecule (126,127).

Identification of IRES sequences has led to the development of bicistronic vectors that allow coexpression of two different polypeptides from a single mRNA molecule in eukaryotic cells (128,129). Using a bicistronic vector containing an IRES to coexpress a target gene and a selectable marker has several advantages. First, since two polypeptides are translated from the same mRNA molecule, the bicistronic vector guarantees coexpression of a selectable marker and a second protein. Secondly, bicistronic mRNA allows two polypeptides to be translated separately. Thus, this system does not compromise the correct intracellular trafficking of proteins directed to different subcellular compartments. In addition, using a bicistronic vector, expression of a target gene is proportionate to the expression of a selectable marker. Hence, expression of a target protein can be achieved quantitatively by applying selections of different stringencies.

To demonstrate coexpression of a dominant selectable marker with a therapeutic gene using a bicistronic vector, our laboratory has coexpressed P-gp with glucocerebrosidase (112,113), β-galactosidase (115), adenosine deaminase (118), a subunit of the NAPH oxidase complex (116,117), the shared gamma chain of the interleukin receptors (119), and a hammerhead ribozyme targeted to the U5 region of HIV-1 LTR (130). In those experiments, *MDR*1 served as a selectable marker linked to the target gene by an IRES from encephalomyocarditis virus (EMCV) and constructed in a retroviral vector containing Harvey sarcoma virus LTR (131). Two configurations, in which *MDR*1 is placed either before or after the IRES, have been examined in some cases. As demonstrated in those experiments, P-gp and the target gene are coexpressed in the cells selected using cytotoxic P-gp substrates, such as colchicine or vincristine; the expressed target proteins are functional as detected using in vitro, or ex vivo analysis. In one case, using subcellular fractionation, we have demonstrated that P-gp and glucocerebrosidase are translocated separately to the cell plasma membrane and lysosomes, indicating correct intracellular protein trafficking (112). The demonstration that a noncoding RNA, such as a hammerhead ribozyme, can function even though tethered to an mRNA encoding a functional *MDR*1 provides an additional powerful way to use bicistronic vectors (130).

Another approach to the use of *MDR*1-based bicistronic vectors is to develop ''suicide'' vectors for cancer gene therapy. Using *MDR*1 to protect bone marrow cells from cytotoxic drugs represents a promising approach to improve cancer chemotherapy. However, contaminating cancer cells may be inadvertently transduced with *MDR*1, or transduced bone marrow cells may accidentally develop new tumors. In those cases, overexpression of P-gp could cause multidrug resistance in inadvertently transduced tumor cells that contaminate bone marrow, or in any transduced cells that later become malignant. A bicistronic ''suicide'' vector developed in this laboratory links P-gp expression with herpes simplex virus thymidine kinase (TK) expression (105,106). Thus the cells containing this vector can be eliminated through ganciclovir treatment.

A third approach is to link two drug resistance genes together using a bicistronic vector system to extend the ability of the vector to confer drug resistance. Examples include the use of *MDR*1 with dihydrofolate reductase that confers methotrexate resistance (132), *MDR*1 plus methylguanine methyltransferase (MGMT) that confers resistance to certain alkylating agents (102,103,133), and *MRP*1 plus gamma-glutamylcysteine synthetase that confers resistance to alkylating agents as well (83).

Finally, bicistronic vectors can be used to introduce marker genes into selected cells. For example, *MDR*1 vectors containing green fluorescent protein or β-galactosidase have been constructed to determine the efficiency of expression of the target gene in transduced and *MDR*1 selected cells (124).

B. Efficiency of IRES-Dependent Translation

Using an IRES to generate a bicistronic mRNA ensures coexpression of two different proteins. However, IRES-dependent mRNA translation (or cap-independent translation) is less efficient than cap-dependent translation, so that the two proteins are not expressed in equal amounts. It has been shown that in a monocistronic vector, insertion of an IRES upstream from an open reading frame of either P-gp or dihydrofolate reductase (DHFR) reduces the translation efficiency by 2- to 10-fold (129,134). Using a bicistronic vector, expression of *neo* in the position downstream from the IRES is 25% to 50% of that observed when *neo* is in the upstream position (128). The asymmetric expression pattern of the bicistronic vector results in a significant difference in *MDR*1 transducing titer between a configuration with P-gp placed before the IRES and a configuration in which P-gp is placed after the IRES. We have found that the apparent titer of a bicistronic vector containing *ADA-IRES-MDR*1 was only 7% of the titer of a bicistronic vector containing *MDR*1-*IRES-ADA* (118). Similar reductions in *MDR*1 transducing titer and in expression of

the nonselected downstream gene was seen with *MDR*1-β-galactosidase bicistronic vectors too (115). The apparent *MDR*1 transducing titer of the retrovirus is based on the drug resistance conferred by expression of P-gp as the result of retroviral infection; thus the viral titer is proportional to the P-gp expression level. Insufficient expression of P-gp is unable to protect the cells from cytotoxic drug selection. To achieve P-gp expression at the same level, the lower efficiency of translation would have to be compensated for by a higher level of transcription, which can occur only in a minority of the cells in the transduced population. This may account for the apparent lower *MDR*1-transducing titer of bicistronic vectors with a configuration of P-gp placed after the IRES. On the other hand, when cells express P-gp at the same level (i.e., the cells survived vincristine or colchicine selection at the same concentration), ADA expressed from *ADA-IRES-MDR*1 is 15-fold higher than the ADA expressed from *MDR*1-*IRES-ADA*. This difference is probably due to a combination of the lower translation efficiency of ADA located downstream from the IRES and the high transcription level of *ADA-IRES-MDR*1 as the result of vincristine selection. A similar asymmetric expression of P-gp and human β−galactosidase A is also observed in NIH3T3 cells, where the difference is about 8-fold.

IRES-dependent translation is a complex process, in which mRNA containing IRES interacts with various cellular proteins, including IRES transacting factors [reviewed in Hellen and Sarnow (135)]. The efficiency of IRES-dependent translation can be affected by the cell type (136), IRES origin (137,138), and the size and structure of a particular mRNA molecule. We have found that the titer of retrovirus containing pHa-*MDR*1 was higher than pHa-*MDR*1-*IRES-ADA*, even though P-gp translation was cap-dependent in both cases. P-gp expressed from pHa-*MDR*1 was also at a higher level in a vincristine resistant cell population than the P-gp expressed from pHa-*MDR*1-*IRES-ADA*. A possible explanation for the relatively low retroviral titers observed is RNA instability or alternative splicing, since no DNA rearrangement was detected by Southern blot analysis of the transduced cells using an *MDR*1 probe.

In addition to IRESes derived from viruses, several IRES elements have been identified in human genes. Those IRESes play important roles in cell cycle-dependent or stress-response translation regulation [reviewed in Sachs (139)]. In contrast to viral IRESes, human IRESes are shorter and are complementary to 18s rRNA [reviewed in Mauro and Edelman (140)]. It has been found that a 9-nt sequence from the 5′-UTR of the mRNA encoding the Gtx homeodomain protein can function as an IRES. Ten linked copies of the 9-nt sequence are 3- to 63-fold more active than the classical EMCV IRES in all 11 cell lines tested (141). Similarly, an IRES isolated from the human EIF 4G gene also exhibits 100-fold more IRES activity than EMCV IRES in 4 different cell lines (142). In addition to higher efficiency and smaller size, translation from a human IRES can be regulated by cellular events (142), which may be advantageous for certain cancer gene therapies.

C. Flexibility Using Bicistronic Vectors in Coordinating Expression of Selectable Markers and a Therapeutic Gene

Selectable bicistronic vectors provide great flexibility in coordinating expression of a selectable marker, such as P-gp, and a therapeutic gene. The low translation efficiency of the IRES results in asymmetric expression of genes positioned before and after the IRES. This asymmetric expression pattern makes it possible to alter the relative expression level of a therapeutic gene and P-gp to achieve maximum therapeutic effects while applying minimal selective pressure using a cytotoxic drug. By choosing different configurations, i.e., placing *MDR*1 before or after the IRES, we can select cells expressing a therapeutic gene at either a low level (*MDR*1 before the IRES) or a high level (*MDR*1 after IRES).

In addition, expression of a therapeutic gene can also be achieved at a desired level by altering the selection conditions. The degree of multidrug resistance conferred by P-gp corresponds to the amount of P-gp expressed on the plasma membrane. Using a bicistronic vector, the expression of a target gene is proportional to the expression of P-gp, which is directly linked to the selection conditions. In a highly stringent selection, instead of increasing the concentration of cytotoxic drug, P-gp reversing agents can also be applied in combination with low concentrations of cytotoxic drugs (143). P-gp reversing agents, also known as chemosensitizers, are noncytotoxic hydrophobic compounds that interact with P-gp and cause a direct inhibition of P-gp function. In the presence of a P-gp reversing agent, most P-gp-expressing cells are killed by the cytotoxic drug unless they express a large amount of P-gp to overcome the inhibitory effects. Using a combination of cytotoxic drug and chemosensitizer allows selection of cells expressing the therapeutic gene at a high level without need for a high concentration of cytotoxic drug. This strategy is especially desirable for an in vivo selection in which avoiding systemic toxicity is essential.

High expression of the target gene can be selected using cytotoxic drugs, cytotoxic drugs combined with chemosensitizers, or the vector configured to place the target gene placed before the IRES. However, those approaches also reduce the overall number of cells that can survive the selection. Nevertheless, using a minimum concentration of drug, the selectable bicistronic vector provides options for selecting a large population of cells with low expression of the target gene, or a small population of cells with high expression of the target gene. Both options may be useful for gene therapy. For instance, ADA levels in normal individuals occur over a very broad range. Heterozygous carriers can be immunologically normal even with as little as 10% of the normal amount of ADA [reviewed by Blaese (144)]. Expression of ADA at a low level in a large number of cells may prove sufficient to treat SCID. On the other hand, high ADA-expressing lymphoid cells, even through present as a small percentage of total cells, are also able to correct the SCID syndrome due to a beneficial by-stander effect (145). In gene therapy applications, the choice of the approach depends on the thera-

peutic strategy for a specific disease. Experiments on animal models are essential to prove the concepts that underlie gene therapy using selectable markers such as *MDR*1.

IV. NONRETROVIRAL AND EPISOMAL VECTORS EXPRESSING SELECTABLE MARKERS: AAV, SV40, AND EBV

Efficient delivery of a therapeutic gene to the appropriate target cells and its subsequent maintenance and expression are important steps for successful gene therapy. Genes introduced into cells are rapidly lost unless there is a mechanism to retain these genes within the nucleus and to ensure that the genes are also replicated and partitioned into daughter cells during cell division. Long-term expression of the transgene within cells can be achieved either via the integration of the transferred DNA into the host genome or maintenance of the introduced DNA as an autonomously replicating extrachromosomal element or episome. In either case, inclusion of a drug-selectable marker, like the *MDR*1 gene, in the construct would ensure that rapidly dividing cells containing the transgene are given a selective growth advantage.

Delivery modalities can be viral or nonviral. Retroviral gene transfer, one of the most exploited systems for gene transfer into actively dividing cells, has been discussed earlier in this chapter while liposomal gene delivery will be discussed later in the chapter. In this section, nonretroviral and/or episomal vectors expressing selectable markers will be described.

A. AAV

In addition to retroviruses, adeno-associated virus (AAV) can also facilitate integration of the transgene into the host genome. Unlike retroviruses, AAV was found to integrate preferentially into a specific site on chromosome 19 (146). AAV is a naturally defective, nonpathogenic, single-strand human DNA parvovirus. For productive infection and viral replication, coinfection with helper viruses, e.g., adenovirus, herpesvirus, or vaccinia virus are required. In the absence of a helper virus, AAV establishes latency in the host by integrating itself into the host genome. AAV has a broad host range and is also able to infect both dividing and nondividing cells (147). Hence recombinant AAV (rAAV) vectors have been exploited as alternative vehicles for gene therapy.

AAV-based vectors (148) are simple to construct, requiring only that the viral inverted terminal repeat (ITR) (which are 145 nucleotides each) is upstream from the gene of interest. Other important viral genes like rep (involved in replication and integration) and cap (encoding structrual genes) can then be supplied in trans. One disadvantage with such rAAV vectors is that site-specific integration of the gene of interest into the host genome is not observed (96). This is probably because the rep gene, which is important for mediating site specific integration in the absence of helper viruses, is not included in the construct with the gene of interest. Nonetheless, rAAV has been successfully applied to the delivery of various genes into a variety of tissues and persistence of transgene expression in these nondividing tissues, was reported (149–154). Baudard et al. (96) demonstrated that in rapidly dividing cells, continuous selective pressure is necessary to sustain gene expression in cells. *MDR*1 was used as the selectable marker in this study. Being among the smallest DNA animal viruses (~20 nm in diameter), another disadvantage of the AAV system is its limited packaging capacity since it can accomodate only aproximately 4.7 kb of the gene of interest. As such, a small and efficient promoter would be required to drive the expression of large genes. One such promoter is the AAV p5 promoter, which, together with the ITR, forms a 263-base pair cassette capable of mediating efficient expression in a CF bronchial epithelial cell line (149,150). Baudard et al. further demonstrated that the reduction of the p5 promoter-ITR cassette to 234 bp was also able to promote efficient gene expression (96).

B. SV40

Vectors that facilitate extrachomosomal replication have some advantages. High gene expression is often observed in such vectors. This could be a result of vector amplification, promotion of nuclear localization and retention, as well as transcriptional activation by viral genes involved in episomal replication. Selective pressure using selectable markers like the *MDR*1 gene, however, is necessary to maintain these episomes in actively dividing cells. Thus, another potential advantage of using episomally replicating vectors is that since they are not integrated into the cells, one could potentially extinguish expression at will by withdrawing selective pressure to replicating cells. Episomally replicating vectors can be easily created by the inclusion into the vector design of replicons that can be derived from DNA viruses like the Simian Virus 40 (SV40) (155), Epstein-Barr virus (EBV) (156) and the (BK) virus (157–159). Such replicons usually comprise a viral origin of replication as well as a viral gene product that is important for maintaining extrachromosomal replication.

SV40 is a 5.2 kb DNA papovavirus that was discovered as a harmless contaminant in early preparations of the Salk polio vaccine (160,161). SV40 is a double-stranded circular 5.2 kb DNA simian virus. It is a nonenveloped virus that belongs to the papovavirus family. The SV40 capsid is composed of 72 pentamers of the major capsid protein VP1, which are tied together through their carboxy-termini. VP2 and VP3, which share 234 amino acids at their carboxy-termini, connect the minichromosome core to the axial cavities of VP1. It has been suggested that correct interpentamer bonding is facilitated by host chaperones (162,163). SV40 infection begins with the virus binding to its primary receptor, the major histocompatibility complex class I (MHC class I), without internalization of the receptors. The entry is mediated by caveolae and the virus is transported to the Golgi membranes. Its pathway extends to the endoplasmic reticulum (ER), where it is disassembled. The mechanism by which the virus reaches the nucleus is not yet known (164–167). The wild-type virus is unable to replicate its DNA in rodent cells; therefore no progeny

virions can be produced in these cells (168). Infection of SV40 wild-type virus in cells can result in the integration of viral DNA into the host chromosome, permitting transmission of expression to daughter cells (169). Some reports associate SV40 DNA (specifically the T-antigen) with human tumors, mainly based on the presence of sequences from SV40 wild-type in some brain tumors and melanomas (170,171).

The two major SV40 delivery systems are vectors that use SV40 sequences or the wild-type virus as a helper, and vectors that are packaged in vitro, with no SV40 sequences and in which the wild-type virus is not present. SV40 has numerous advantages as a gene-delivery vehicle (54,172,173): it is able to infect a wide variety of mammalian cells, including human cells, and to express its genes in these cells; the vector system has an ability to deliver untranslated RNA products; the gene expression may be transient or stable in cell lines, depending on the specific SV40 system that is used; and episomal replication in SV40 virus requires the SV40 replication origin as well as the large T antigen (T-Ag), which activates the replication origin . Such episomal replication can generate more than 105 copies per cell of recombinant plasmids (174). Using the SV40 delivery systems, no immune response is expected, as well as no inflammatory reaction.

Replacing the late or early region with a foreign gene can generate SV40 recombinant viral particles (169,172,175–181). These are then propagated using either wild-type, or a temperature-sensitive mutant of SV40 as helper, or via a viral producer cell line, COS7, that stably expresses an origin-defective SV40 mutant and is capable of supporting the lytic cycle of SV40. Multiple infections result in higher titers of the virus — up to 10^{10} infectious units/ml. It has been demonstrated that when the large T-antigen (T-Ag) gene is replaced with a reporter gene, replication-deficient recombinant SV40 viruses can be produced and can mediate gene transfer *in vivo*. Reporter gene expression was detectable for about 3 months without selection. Present SV40 vectors of the first type have most of the viral coding sequences removed, retaining only the packaging sequences, the polyadenylation signal, and the early promoter of the virus, thus increasing the capacity for DNA to ~5.3 kb. The DNA from these vectors integrates into the genome of the target cells.

Rund et al. (54) demonstrated very efficient delivery (> 95%) of the drug-selectable marker, *MDR*1, into various murine and human cell types including primary human bone marrow cells (54). SV40 vectors efficiently deliver HIV-1-inhibitory RNAs using pol II or III promoters. Other vectors, which encode a variable fragment antibody recognizing HIV-1 integrase, inhibited HIV-1 infection in SCID mice. This system may prove to be useful in antiHIV-1 therapeutics. Fang et al. (182) reported a different packaging system for SV40 vectors where the vector carrying the gene of interest contains only the SV40 origin of replication (182). Instead of using wild-type SV40 viruses as helpers to package the recombinant vector, recombinant adenoviruses expressing SV40 capsids were used in COS7 cells. The helper adenovirus can be effectively heat-inactivated without adverse effect on the infectivity of the recombinant SV40 viruses due to the differential heat sensitivity of these two viruses. Strayer et al. (169) found evidence for integration of the recombinant gene or parts of it, a few days after transduction in random sites, which might explain the long-term expression of this system.

Pseudovirions can transfer the gene of interest to a variety of cells (including hematopoietic cells) with high efficiency, but their clinical applicability is currently limited by the presence of wild-type SV40 sequences. The in vitro, method of preparing helper-free SV40 vectors utilizes the SV40 viral late proteins, VP1, VP2, VP3 and agno or VP1 only (183–186). Nuclear extracts of baculovirus-transduced *Spodopterafrugiperda* (Sf9) insect cells that include these proteins are incubated with supercoiled plasmid DNA in the presence of 8 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM ATP to form the SV40 in vitro, packaged vectors (55). Such in vitro, assembly allows larger DNA plasmids (up to 17.6 kb) to be packaged very efficiently, with no need for SV40 sequences. We have demonstrated very efficient delivery of the *MDR*1 (*ABC B1*), *MXR* (*ABC G2*), and *MRP*1 (*ABC C1*) genes, which can confer multidrug resistance on virtually all cell types (human, murine, and monkey cell lines), in addition to delivery of the *GFP* gene as a reporter (187). The expression of both *MDR*1 and *GFP* genes is dose dependent. The alteration in the level of expression suggests that MHC class I receptors play an important role in determining the efficiency of transduction. *MDR*1 constructs that carried a promoter with an intron demonstrated higher expression than those without the intron. In low-expressing MHC class I cell lines, the CMV promoter produced more P-gp expression compared with the SV40 promoter. In vitro packaged-*GFP* vectors that carried the CMV promoter consistently confirmed higher expression than those that carried the SV40 promoter. Expression was transient up to 21 days, but did last for 3 months under colchicine selection for MDR1 and was lost soon after selection was withdrawn (55).

The short-term expression of the SV40/*MDR*1 in vitro, vectors may be an advantage for use in chemoprotection. Long-term expression beyond the chemotherapy period is undesirable, and may put patients at risk for treatment-induced myelodysplasia or secondary leukemia. The SV40/*MDR*1 vectors that are prepared in vitro, may provide not only a safe vehicle for gene delivery but will also potentially avoid the problem of persistent bone marrow drug resistance in cancer patients.

C. EBV and Other Episomal Vectors

Episomal vectors based on EBV are also being developed for gene therapy purposes. EBV is a human B-lymphotropic herpesvirus that resides asymptomatically in more than 90% of the adult human population by establishing latency and maintaining its genome episomally (188). The life cycle of EBV comprises two phases, a lytic and a latent phase. During the lytic phase, EBV DNA replicates via a rolling circle intermediate to achieve a 1000-fold increase in copy number. The origin of replication, Ori Lyt, and the transacting element ZEBRA are required for the lytic replication. Rolling circle replication results in the formation of linear head-to-tail con-

catamers. The presence of the EBV terminal repeat (TR) sequence causes cleavage of the concatemerized DNA to molecules of about 150–200 kbp, which are then packaged into virions. Upon infection into a permissive cell, the viral DNA circularizes by ligation of TR. Latency is established in the cells by episomal replication of the circular DNA.

Episomal replication in EBV is maintained by two elements interacting to ensure that the viral genome is retained within the nucleus, efficiently replicated and partitioned into daughter cells. Although the copy numbers of episomal viral DNA varies from 1–800, only between 4–10 episomal copies per cell are usually observed using vectors containing EBV OriP and EBNA-1 (189). Unlike other episomal vector systems, very low rates of spontaneous mutation have been observed with EBV-based episomal vectors (190). The *cis*-acting element responsible for episomal replication is a 1.8 kb OriP while the transacting element is EBNA-1. OriP comprises two distinct sequence motifs, the dyad symmetry motif (DS) from which replication is initiated and the family of repeats (FR) that serves as a replication fork barrier. Interaction of EBNA-1 with DS initiates bidirectional replication, while binding of EBNA-1 to FR enhances transcription from the episome and terminates DNA replication. EBNA is reported not to be oncogenic nor immunogenic. It evades the host immune system via the presence of the repeat motif, Gly-Ala, which was found to interfere with antigen processing and MHC class I-restricted presentation (191). These EBV episomal vectors replicate once per cell cycle (192) and are capable of stably maintaining human genomic inserts of sizes between 60–330 kb for at least 60 generations (193).

Vos and colleagues (194) developed a helper-dependent infectious recombinant EBV to evaluate the feasibility of using such a vector system to correct hereditary syndromes in B-lymphocytes already harboring the EBV virus latently. The EBV-containing target B-lymphocytes will supply EBNA-1 *in trans* for the episomal maintenance of the transgene. Hence only minimal *cis*-EBV elements for episomal replication (OriP), viral amplification (Ori Lyt), and packaging (TR) are included in their construct. The hygromycin resistance gene was included as a selectable marker in their vector. Infectious virions are generated by the producer cell line HH514. They demonstrated successful transfer of such infectious virions carrying the therapeutic gene, Fanconi anemia group C (FA-C) cDNA, into HSC536, a FA-C patient cell line. Upon selection with hygromycin, long-term (at least 6 months) correction of the Fanconi phenotype in vitro, was observed, as determined by cellular resistance to the cross-linking agent, diepoxybutane. They also observed that in the absence of selective pressure, their episomal vector is retained in rapidly dividing cells at a rate of 98% per cell division translating to a half-life of 30 days in cells doubling every 20 hours.

Our laboratory has been exploring the use of EBV episomal vectors containing only the OriP and EBNA-1 and carrying the selectable marker *MDR*1 as potential gene therapy vectors. Using the liposome formulation, DOGS/DOPE (1:1) (195), we successfully delivered the vector to various cultured cells as well as human $CD34^+$ stem cells. *MDR*1 was found to be expressed at a higher level in the episomal vector compared to its nonepisomal counterpart and more drug colonies were obtained upon selection. Episomal plasmids could be recovered in drug selected cells for many weeks (56).

Other episomally replicating vectors can be derived from BPV viruses (196) or BK virus (158). Unfortunately, BPV vectors cannot be reliably maintained as episomes as they exhibit high spontaneous mutation rate (~1%), frequently undergoing integration, deletion, recombination, and rearrangements (197). Furthermore, BPV has a limited host range and BPV vectors cannot be efficiently maintained in human cells. Not too much is known about BK-virus-derived episomal vectors. Nonetheless, successful stable maintenance of episomal gene expression was reported in human transitional carcinoma cells using BK-based vectors but not EBV-based vectors, probably due to the differential tropism of BK and EBV viruses for human uroepithelial cells (157).

Various chimeric viruses have been developed to improve the efficiency of gene transfer as well as the maintenance of gene expression within target cells. These chimeric virus systems attempt to combine the favorable attributes of each vector system and overcome the limitations associated with each system. The episomal replication ability of EBV was exploited to produce both rapid and long-term high-titer recombinant retroviruses (up to 10^7 TU/ml) for efficient gene transfer into human hematopoietic progenitor cells (198,199). A novel adenoviral/retroviral chimeric vector was also reported in which an adenoviral delivery system was utilized to efficiently deliver both the retroviral vector and its packaging components, thereby inducing the target cells to function as transient retroviral producers capable of infecting neighboring cells. This system capitalizes on the superior efficiency of adenoviruses to deliver genes in vivo and the integrative ability of retroviruses to achieve stable gene expression (200). An EBV/HSV-1 amplicon vector system was also described that combines the efficiency of HSV-1 virus to transfer DNA into various mammalian cells, including the postmitotic neuronal cells and the ability of EBV to maintain genes episomally. This vector system contains the HSV-1 origin of DNA replication (oriS) and a packaging signal, which allow replication and packaging of the amplicon into HSV-1 virions in the presence of HSV-1 helper functions as well as EBV oriP and EBNA-1 (201). Another report describes the use of a similar HSV-1 amplicon system for efficient gene transfer, but AAV was included in their vector to achieve stable expression. This HSV/AAV hybrid vector contains OriS and packaging sequences from HSV-1, a transgene cassette that is flanked by AAV ITRs as well as an AAV rep gene residing outside the transgene cassette to mediate amplification and genomic integration of ITR-flanked sequences (202). An HVJ-liposome vector system reported by Dzau et al. (203) was utilized to improve the efficiency of liposome-mediated transfer of an EBV-episomally maintained transgene (204,205). This system exploits the fusigenic properties of the hemagglutinating virus of Japan (HVJ or Sendai virus) since envelope proteins of inactivated HVJ were found to mediate liposome-cell mem-

brane fusion and facilitate cellular uptake of packaged plasmid DNA, bypassing endocytosis and lysosomal degradation.

One of the limitations with using viral episomal systems is the limited host range of such vectors. Although EBV episomal vectors replicate well in various human and primate cells, they are unable to replicate in rodent cells, limiting their utility in gene therapy since testing of these vectors in rodent models is not easy. Nonetheless, it was found that large fragments of human genomic DNA (between 10–15 kb) can mediate autonomous replication if there is also a mechanism to retain them in the nucleus (206). Such vectors based on a human origin of replication were also found to be capable of replicating in rodent cells (207), probably due to the common host factors that drive their replication. A hybrid class of vectors was thus developed, which employs a human origin of replication to mediate vector replication as well as the EBV FR and EBNA-1 gene product to provide nuclear retention functions. [see Calos (208)]. EBNA-1 binding to the FR of the vector DNA causes the adherence of this complex to the chromosomal scaffold in a noncovalent fashion, thus retaining the vector DNA in the nucleus (209). These vectors were reported to replicate somewhat in synchrony with chromosomal DNA once per cell cycle. Maintenance of these vectors within cells is related to the frequency of cell division (208). Such vectors have been reported to persist in cells for at least 2 months under no selective pressure (206,210).

Ultimately, the development of a true mammalian artificial chromosome (MAC) without dependence on viral elements will be the key to obtaining stable episomal replication without dependence on selective pressure. Functional elements in mammalian cells important for maintaining DNA episomally as a minichromosome include a replication origin to promote autonomous replication, telomeres to protect ends of linear DNA and replicate DNA termini, and a centromere to facilitate correct segregation of the construct during mitotic division. Various mammalian chromosomal DNA replication initiation sites have been identified [reviewed in DePamphilis (211)] and found to comprise a 0.5–11 kb primary origin of bidirectional replication (OBR) flanked by an initiation zone of about 6–55 kb. These sequences show characteristics of DNA unwinding, a densely methylated island, attachment sites to the nuclear matrix, and some palindromic sequences.

Vectors utilizing human genomic sequences that promote extrachromosomal vector replication have already been successfully applied as mentioned above. Telomeres that are required for the stability and integrity of the eukaryotic chromosome have been well characterized. In mammalian cells, the telomeric tracts comprise 2–50 kb of tandem TTAGGG repeats. Human centromeres, necessary for proper chromosome segregation at mitosis and meiosis, have been localized cytogenetically as primary constrictions of the chromosomes. They are thought to consist of up to several megabases of highly repetitive DNA belonging to the alpha satellite DNA family (212) and are attached to microtubules (213). Until recently, the functional isolation of the centromere has been a great hurdle in the progress towards the construction of an MAC. The group of Willard et al. developed the first generation of human artificial microchromosomes (HAC) by creating synthetic alpha satellite arrays ~1 Mb in size (214). They found that such an HAC which is about 6–10 Mb is size is mitotically and cytogenetically stable for up to 6 months in culture in the absence of selective pressure. Nonetheless, the technical challenge of assembling a mammalian artificial chromosome is still formidable as cloning and manipulating such large constructs are not trivial using conventional bacterial cloning systems, and transfer to mammalian cells is difficult.

V. USE OF LIPOSOMES TO DELIVER VECTORS WITH SELECTABLE MARKERS

Liposome-mediated gene transfer appears to be a safe and noninvasive method of DNA delivery into cells. Since high efficiency and stable expression have not yet been achieved using liposomal methods, the use of the human *MDR*1 gene as a selectable marker may allow for the selection and enrichment of the recipient cells and may be useful in the future for the long-term maintenance of the cationic liposome:DNA complex.

Previous studies in our laboratory have shown that a liposomal delivery system can mediate successful *MDR*1 transfection of mouse bone marrow cells and in vivo expression of functional P-gp in hematopoietic cells (97). The introduction via liposomes into hematopoietic cells of an *MDR*1 gene driven by Harvey murine sarcoma virus long-terminal repeat sequences (Ha-MSV-LTR) was achieved either ''directly'' by intravenous administration into mice, or ''indirectly'' by adoptive transplantation of previously in vitro-transfected bone marrow cells. In these studies, using a cationic liposome complex consisting of dioctadecylamidoglycyl spermidine (DOGS) and dioleoylphosphosphatidyl ethanolamine (DOPE), *MDR*1 transfection was detected in up to 30% of unselected and 66% of vincristine preselected murine bone marrow cells as demonstrated by drug resistance in an in vitro, colony-forming unit assay. Although transfection into human bone marrow cells is likely to be much less efficient, the potential of obtaining drug-selectable mouse bone marrow progenitor cells after gene transfer using such a liposome delivery system may eventually make it possible to protect cancer patients undergoing chemotherapy from bone marrow toxicity of anticancer drugs.

Liposome-mediated gene transfer can also be used for in vivo delivery of Adeno-Associated-Vectors (AAV)-*MDR*1-based vectors. Recently, drug-selected coexpression of both P-gp and glucocerebrosidase (GC) was achieved with an AAV vector containing the *MDR*1-IRES-GC fusion delivered to NIH 3T3 cells by lipofection (96). Moreover, a single intravenous injection of this bicistronic vector complexed with cationic liposomes into recipient mice allowed detection of GC and *MDR*1 sequences by PCR in all organs tested 7 weeks later.

For nonintegrating DNA vectors such as EBV-based systems (see Section IV) and the AAV system (96), liposome-based gene delivery usually results in transient transgene expression due to the episomal nature of the transfected plasmid and loss of the plasmid when the cells proliferate (215,216). Use of a selectable marker such as *MDR*1 may make it possible to maintain nonintegrated episomal forms in proliferating cells (see Section IV). Since only cells carrying such episomal *MDR*1-based vectors would survive the selection, this advantage should be useful for gene therapy with episomal *MDR*1 vectors in vivo. Combining liposomes with AAV- or EBV-based vectors and *MDR*1 as a selectable marker may make it possible to expand the population of expressing cells by *MDR*1-drug selection.

We are developing a gene therapy model to treat Fabry disease (123) using intravenous injections of a pHa-aGal-IRES-MDR bicistronic vector complexed to cationic liposomes into α-galactosidase A deficient mice (T. Shoshani and M. M. Gottesman, unpublished results). Both human α-Gal and *MDR*1 were detectable in the lungs of the recipient Fabry mice by Southern blot analysis 7 days after injection. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of total RNA extracted from the kidneys of recipient Fabry mice showed the presence of both human α-Gal and *MDR*1 mRNA. The expression in the kidneys was specific to the α−galactosidase-A-deficient mice, where renal tubule cells may be damaged by an accumulation of glycosphingolipids. In situ hybridization analysis localized the mRNA expression to the renal distal tubule epithelial cells. Higher RNA expression was obtained in Fabry mice that were injected 3 times every third day. The repeated administration is tolerated by the recipient mice and no toxic effects were obtained. It remains to be determined whether selection in vivo will allow expansion of cell populations expressing human α-Gal by repeated administration of cytotoxic *MDR*1 substrates.

VI. ENGINEERING MDR VECTORS TO IMPROVE EFFICIENCY OF DRUG SELECTION

One of the goals of gene therapy is to modify cells genetically such that they can supply a useful or necessary function to the cell (3). One of the most promising applications of the *MDR*1 gene in therapeutic vectors as a selectable marker in vivo is the protection of bone marrow cells during intensive chemotherapy. During chemotherapy, the *MDR*1 gene is transduced or transfected into drug-sensitive bone marrow cells and selected for by exposure to MDR agents. The untransfected/untransduced cells will necessarily be killed and those containing the *MDR*1 gene will expand. The efficacy of this therapy depends on the interaction between P-gp and the selecting agent employed. Thus, it is important to be able to distinguish between the endogenous P-gp and the exogenously introduced molecule. Furthermore, it obviously would be beneficial to create a P-gp molecule that would confer very high levels of resistance to certain drugs, giving an advantage to transduced cells/tissues compared to wild-type P-gp. Studies of a number of mutations made in P-glycoprotein have suggested that it should be possible to construct mutant ''designer'' transporters useful for *MDR*1-based gene therapy.

One of the hallmark characteristics of the multidrug transporter is its extremely broad substrate specificity. Over the past several years, the identification of specific domains and amino acid residues involved in substrate recognition has contributed to our present understanding of the mechanism of action of P-gp. The major sites of interaction have been shown to reside in transmembrane domains (TM) 5 and 6 in the N-terminal half of the protein and in TMs 11 and 12 in the C-terminal half and the loops that conjoin them (217–221). For the purposes of chemoprotection, the design of a P-gp that has increased resistance to chemotherapeutic agents compared to the endogenous P-gp would be most useful because increased doses of the agent could be administered without harming the bone marrow cells expressing the exogenous P-gp molecule. To date, a number of these types of mutations have been described.

Mutations in TM domains of P-gps from both rodent and human have demonstrated significant alterations in substrate specificity (3,222). An F338A mutation in hamster P-gp enhances resistance to vincristine, colchicine, and daunorubicin but has little impact on resistance to actinomycin D (223,224). An F339P mutation in the same molecule only increases actinomycin D resistance. However, the double F338A/F339P mutant demonstrates an increased level of resistance to actinomycin D and vincristine but a lowered level of resistance to colchicine and daunorubicin (223,224). Of these mutants, the F338A may prove most useful because it confers increased resistance to a wider range of chemotherapeutic agents. In human P-gp, however, a homologous mutation at F335 confers greater resistance to colchicine and doxorubicin but causes a severe reduction in resistance to vinblastine and actinomycin D (225,226) . Additionally, cells expressing a Val>Ala mutation at position 338 also exhibit preferential resistance to colchicine and doxorubicin but are severely impaired for vinblastine (226). Resistance to actinomycin D, however, is unaffected. Alanine scanning of TM 11 in mouse P-gp encoded by *mdr*1a revealed that two mutants, M944A and F940A, show an increase in resistance to doxorubicin and colchicine while maintaining wild-type levels of resistance to vinblastine and actinomycin D (227). For certain treatment protocols, it is conceivable that increased resistance to certain agents would be desirable, and the reduction in levels of resistance to other compounds would not be problematic, especially if a well-defined chemotherapy regimen was being employed.

Although the majority of residues that increase resistance to various chemotherapeutic agents reside in the TM domains, a number of residues in the putative cytoplasmic loops also have been implicated in defining drug resistance profiles for cytotoxic drugs. The best characterized of these mutations is the G185V mutant that confers an increased resistance to colchicine and etoposide but decreased resistance to actinomycin D, vinblastine, doxorubicin, vincristine, and taxol

(228–231). Interestingly, and perhaps relevant clinically, when this mutation is made in conjunction with an Asn->Ser mutation at residue 183, increased resistance to actinomycin D, vinblastine, and doxorubicin is achieved without loss of the increase in colchicine resistance (229). Mutations of Gly-141, 187, 288, 812, or 830 to Val in human P-gp increase the relative resistance of NIH3T3 cells to colchicine and doxorubicin but do not alter resistance to vinblastine (232). Only the mutations at positions 187, 288, and 830 confer decreased resistance to actinomycin D to cells in culture.

Due to its broad substrate specificity, P-gp not only interacts with chemotherapeutic compounds but also with reversing agents and inhibitors. In combination chemotherapies, reversing agents increase the efficacy of cytotoxic agents in *MDR*1-expressing cancers. Two of the most potent reversing agents currently in use or in clinical trials are cyclosporin A and its nonimmunosuppressive analog PSC833. Recently, a number of mutants have been described that affect sensitivity to these agents. Cells expressing a human P-gp containing a deletion at Phe335 or Phe334 are substantially resistant to cyclosporin A and PSC-833 [(233), Hrycyna, C.A., Pastan, I., and Gottesman, M.M., unpublished data]. A similar phenotype has been observed for a transporter containing 5 mutations in the region including TM 5 and TM6, namely Ile299Met, Thr319Ser, Leu322Ile, Gly324Lys, and Ser351Asn (234). Additionally, in hamster P-gp, the substitution of an alanine at position 339 with proline results in a transporter that confers lowered sensitivity to cyclosporin A (224). From these studies, it appears that TM6 plays an important role in the recognition of cyclosporin A and its analogs. The decreased sensitivity to these reversing agents observed in cells expressing the TM6 mutations could help protect bone marrow stem cells transduced with the mutant *MDR*1 gene from the toxic effects of chemotherapy given with reversing agents to sensitize *MDR*1-expressing tumors.

The *cis* and *trans* isomers of flupentixol, a dopamine receptor antagonist, have also been shown to inhibit drug transport and reverse drug resistance mediated by P-gp (235,236). The substitution of a single phenylalanine residue at position 983 with alanine (F983A) in TM 12 affects inhibition of P-gp-mediated drug transport by both isomers of flupentixol (59,60,237). Both isomers were found to be less effective at reversing P-gp mediated drug transport of daunorubicin and bisantrene. However, the inhibitory effects of other reversing agents such as cyclosporin A were not affected. The reduced sensitivity of the F983A mutant to this compound coupled to the apparent lack of clinical toxicity of (*trans*)-flupentixol (235), suggests that this mutant may be useful in combining *MDR*1 gene therapy with chemotherapy including *trans*-flupentixol as a chemosensitizer. This approach, in theory, should allow for effective treatment at lower doses of chemotherapeutic agents while maintaining bone marrow protection.

The use of *MDR*1 gene therapy in bone marrow chemoprotection protocols has undergone preliminary analysis in clinical trials (89,90,238). Results indicate a low efficiency of marking bone marrow cells using retroviral vectors, but some selective advantage manifested as an increased percentage of positive cells after chemotherapy (89). In the future, with the generation of higher resolution structures of human P-gp, it should be feasible to model and synthesize new, more effective cytotoxic drugs or modulators capable of blocking P-gp function clinically. However, until that time, the analysis of spontaneously occurring or engineered mutants, coupled to our knowledge of the current battery of anticancer and reversing agents, offers an opportunity to begin designing second-generation vectors for use in these trials.

VII. CONCLUSIONS AND FUTURE PROSPECTS

We have argued in this review that drug-selectable marker genes may be helpful for gene therapy in two ways: first, to protect bone marrow progenitor cells (and other sensitive cells) from the cytotoxicity of anticancer drugs, thereby allowing safe chemotherapeutic treatment at reduced risk of severe side effects, and second, to enrich the expression of otherwise nonselectable genes in drug-sensitive cells to overcome low or unstable gene expression in vivo. Given the current instability of expression of genes from existing vectors, especially episomal vectors, such selectable markers may be an essential component of gene therapy protocols.

We are still in the early stages of vector development, and until transduction efficiencies into human tissues such as bone marrow are improved and shown to be safe, long-term human gene therapy will not be feasible. The combination of more efficient gene transfer targeted vector systems, and effective, relatively nontoxic selection systems to maintain gene expression may make long-term correction of human genetic defects feasible and safe.

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