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Drug-selected coexpression of human glucocerebrosidase and P-glycoprotein using a bicistronic vector

(multidrug resistance/retrovirus/gene therapy/lysosomes/Gaucher disease)

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ABSTRACT **Bicistronic cassettes under control of a single promoter have recently been suggested as useful tools for coordinate expression of two different foreign proteins in mammalian cells. Using the long 5′ untranslated region of encephalomyocarditis virus as translational enhancer of the second gene, a bicistronic unit composed of cDNA for human P-glycoprotein [the product of the multidrug resistance gene, *MDR1* (also called *PGY1*)] as selectable marker and cDNA for human glucocerebrosidase (GC; EC 3.2.1.45) (a membrane-associated lysosomal hydrolase) was constructed. NIH 3T3 cells transfected with a Harvey murine sarcoma virus retroviral vector carrying this bicistronic cassette (pHaMCG) express active P-glycoprotein and GC and expression of both proteins augments coordinately with selection for increased colchicine resistance. Percoll gradient analysis of homogenates showed that GC was targeted to the lysosomal fraction. The ability to select for expression of GC with natural product drugs after introduction of the pHaMCG retroviral vector may be useful in gene therapy strategies for Gaucher disease.**

Gaucher disease is caused by an inherited deficiency of the enzyme glucocerebrosidase (GC; EC 3.2.1.45), a membrane-associated acid hydrolase. In this lysosomal storage disorder glucosylceramide accumulates in macrophages of the reticuloendothelial system, which results in liver and spleen enlargement and lesions in the bones. Treatment of Gaucher patients by intravenous infusion of human carbohydrate-modified GC is extremely costly (1, 2). Allogeneic bone marrow transplantation is effective in treating Gaucher disease (2, 3) but has an associated high morbidity. The introduction of the human GC gene into Gaucher bone marrow by gene transfer should be a lower risk strategy.

For stable expression of genes in mammalian cells, most expression vectors include a dominant selectable marker that allows selection of successfully transformed cells. For gene therapy, the selectable gene should operate not only in tissue culture but also *in vivo* in animals or humans for the continuous selection of cells expressing the gene of interest. A gene with these properties is the human *MDR1* gene (multidrug resistance gene; also called *PGY1*), which encodes the 170-kDa P-glycoprotein, an efflux pump for a variety of naturally occurring cytotoxic agents (4). The *MDR1* gene can function as a selectable marker for gene transfer *in vitro* (5–7), and *in vivo* in transgenic mice expressing the *MDR1* gene in bone marrow (8). The bone marrow of these mice was shown to be resistant to doxorubicin, taxol, VP-16, and other cytotoxic substrates of P-glycoprotein (8, 9). Two recent reports demonstrate that the *MDR1* gene allows selection *in vivo* with taxol of mouse bone marrow cells transduced *in vitro* with an *MDR1* retrovirus (10, 11).

When selectable marker and the unselected gene are under control of separate promoters, expression of the unselected gene frequently does not occur (12). Concomitant expression of both the transgene of interest and the selectable marker in the form of a bifunctional fusion protein driven by a single retroviral promoter has provided one solution to this problem (7). It is also possible to coexpress two genes from a bicistronic transcript using cap-independent translation initiation of the second gene when it is preceded by the long 5′ untranslated region of a picornavirus, known as internal ribosome entry site (IRES); ribosome landing pad (RLP), or cap-independent translational enhancer (CITE) (13–15).

We have combined the utility of the *MDR1* gene as a dominant selectable marker and the efficiency of internal translation initiation to synthesize a bicistronic construct under control of the Harvey murine sarcoma virus promoter. We report the targeting and coordinate coexpression of active human GC and P-glycoprotein. Hence, the recombinant retroviral construct described in this study may prove useful for gene therapy of Gaucher disease.

MATERIALS AND METHODS

Expression Constructs. As shown in Fig. 1, retroviral vectors pHaMCG and pHaMCDG contain between the two long terminal repeats of Harvey murine sarcoma virus in a 5′ → 3′ orientation: (*i*) the human *MDR1* cDNA; (*ii*) a 510-bp fragment from the encephalomyocarditis virus 5′ untranslated region (IRES element); (*iii*) the human GC cDNA (pHaMCG) or a deleted version of GC cDNA in which 57 nt coding for the 19 amino acid leader sequence plus 365 nt encoding the amino-terminal portion of the protein were removed (pHaMCDG). Genetic engineering was carried out as follows: human GC cDNA was obtained from the American Type Culture Collection as a 2.2-kb *Eco*RI insert in the pBR322 plasmid (ATCC no. 65696). The GC cDNA was isolated as an *Eco*RI–*Sac* I fragment and subcloned into the pUC19 cloning vector creating pGC. An *Xho* I site was introduced at the 3′ untranslated region of the GC cDNA by digestion of pGC with *Tth*111 I–*Sac* I and religation in the presence of an adaptor formed by a 24-mer (5′-GAGTCTCGAGTGGTTCGTGCTGAGCT-3′) and a 19-mer (5′-CGCACGACCACTCGAGACT-3′) oligonucleotide. They provided *Tth*111 I and *Sac* I compatible ends containing a unique *Xho* I site. The resulting vector, pGCAX, was used as template for the introduction of an *Nco* I site in the ATG initiation codon of GC by PCR with oligonucleotide primers 5′ JA13 (5′-CCATGGCTTCCATGGCTGGCAGCCTCAGAGG-3′) and 3′ JA10 (5′-CAGCTGGCCATGGGTACCCG-

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Abbreviations: MDR, multidrug resistance; GC, glucocerebrosidase; IRES, internal ribosome entry site; CITE, cap-independent translational enhancer.

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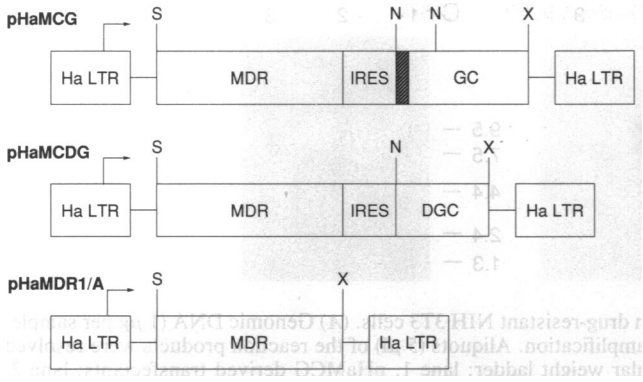

FIG. 1. Proviral structure of pHaMCG, pHaMCDG, and pHaMDR1/A retroviral vectors. The arrows indicate direction of transcription. The dashed box in pHaMCG shows the 57-nt GC leader sequence. Relevant restriction endonuclease sites are indicated: S, *Sst* II; N, *Nco* I; X, *Xho* I. Ha LTR, Harvey murine sarcoma virus long terminal repeats; *MDR*, multidrug resistance gene; GC, GC gene; DGC, deleted GC gene. Drawing is not to scale.

GATGATG-3′). The amplified 426-bp fragment was subcloned into the *Nco* I site of pGEM 5 (Promega) and its sequence was confirmed in both directions. pGPCAX was obtained by ligating the *Nco* I-digested PCR fragment to the *Nco* I-linearized dephosphorylated pGCAX. It contains the complete GC cDNA coding sequence and an *Nco* I site in its ATG starting codon. By partial *Nco* I digestion and complete *Sac* I digestion of pGPCAX, the 1.6-kb GC coding sequence was recovered and ligated into the *Nco* I–*Sac* I-digested pCITE plasmid (Novagen), which contains the encephalomyocarditis IRES sequence. The resulting plasmid, pCITE-GC, was digested with *Ase* I, yielding a 2.4-kb CITE-GC fragment, which was functional as demonstrated by *in vitro* transcription/translation.

The plasmid pMDR1/A (16), which includes the *MDR1* gene without 3′ poly(A) sequences, was linearized with *Xho* I. Both CITE-GC and pGEM 2-MDR fragments were blunt-ended using Klenow fragment and ligated. The resulting vector (pMCG) having the complete construct in the proper configuration was excised by *Sac* II–*Xho* I digestion and the bicistronic unit introduced in the *Sac* II–*Xho* I unique sites of pCO1 retroviral vector (16) creating pHaMCG. The pHaMCDG vector is identical to pHaMCG except that the GC fragment resulting from complete *Nco* I digestion of pGPCAX plasmid is missing a 422-bp *Nco* I fragment containing the leader sequence and 365 nt encoding the amino-terminal portion of GC. pHaMDR1/A (16) and pWE-GC (17) were described elsewhere.

Cell Transfections and Colchicine Selection. NIH 3T3 cells (6) were transfected by calcium phosphate coprecipitation with 10 μg of DNA per 3×10^5 cells (7). Forty-eight hour posttransfection cells were split 1:6 in selective medium containing 80 ng of colchicine per ml (pHaMCG, pHaMCDG, and pHaMDR1/A) or 1.5 mg of G-418 per ml (pWE-GC). After 10–12 days, drug-resistant colonies were pooled and expanded for characterization. Some cell populations initially selected with 80 ng of colchicine per ml were plated at low density (2×10^4 cells per ml) in 250, 500, or 1000 ng of colchicine per ml. After 10–12 days, resistant colonies in each selection were pooled, expanded, and analyzed.

Nucleic Acid Isolation and Analysis. Genomic DNA (7) was analyzed by PCR using 1 μg of genomic DNA template and 1 μM each of the primers JA11 (forward): (5′-CAACGCATTGCCATAGCTCGTG-3′) and JA4 (reverse): (5′-GATGGGCCCCATACTCAGC-3′). These amplify a 1.15-kb fragment comprising the whole encephalomyocarditis IRES, 300 bp of 3′ *MDR1* coding sequences and 225 bp of 5′ GC coding sequences. Amplification was for 40 cycles, with annealing at 55°C.

Total RNA from colchicine-resistant transfectants was isolated by acid guanidinium/phenol extraction. For Northern hybridization, RNA was resolved in a 0.8% formaldehyde gel. Equal loading and integrity of RNA samples were verified by visualization of the EtBr-stained ribosomal bands. After RNA transfer to a nitrocellulose filter (BA83; Schleicher & Schuell) the blot was probed with a random primed 1.1-kb GC cDNA fragment from the ATG initiation codon to the unique *Sal* I site. The blot was stripped and reprobed with a 0.8-kb *MDR1* probe obtained by *Eco*RI–*Hin*dIII digestion of the *MDR1* cDNA. Hybridization and washing conditions were as described (18).

Subcellular Fractionation of Colchicine-Resistant Transfectants on Percoll Gradient. Colchicine-resistant fibroblasts were homogenized and subfractionated in a discontinuous Percoll gradient according to the method of Neumann *et al.* (19) with some modifications. Cells grown to confluency in 500 ng of colchicine per ml in 5 × 150 mm dishes were scraped, washed three times in ice-cold PBS, and resuspended in 1.5 ml of isotonic homogenization buffer: 10 mM triethanolamine/1 mM EDTA/250 mM sucrose, pH 7.5. After 30 min on ice, cells were disrupted manually in a Tenbroeck tissue grinder (40 strokes on ice) with a tight-fitting pestle. Intact cells and nuclei were removed by centrifugation (10 min at 2000 rpm) and the post-nuclear supernatant was loaded on a preformed Percoll gradient consisting of 1 ml each of 7%, 12%, 17%, 22%, 25%, 28%, 31%, 40%, and 80% of a Percoll stock solution [90% Percoll (Pharmacia) in homogenization buffer] over a 1-ml cushion of 2.5 M sucrose. Gradient tubes were centrifuged at 24000 rpm for 1 hr (4°C) in a Beckman SW40 rotor. Fractions (750 μl) were collected from the top of the gradient and aliquots from each fraction were solubilized in 1% Triton X-100 before Percoll removal by centrifugation. The resulting supernatants were mixed with 2× Laemmli buffer and analyzed by Western blotting.

Western Analysis. Cell pellets from NIH 3T3 transfectants were extracted as described (21). Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Pierce). The supernatants were assayed by immunoblotting (20) with rabbit anti-human GC antiserum (21) at 1:10,000 dilution for human GC detection; monoclonal antibody C219 (22) at 1:1000 dilution for P-glycoprotein detection; rabbit anti-MEP antiserum (23) at 1:500 dilution for cathepsin L detection. Donkey anti-rabbit IgG horseradish peroxidase (HRP) and/or goat anti-mouse IgG-HRP (Amersham), both at 1:500 dilution, were used as secondary antibodies whose reactivity was detected by chemiluminescence (ECL kit; Amersham).

GC Activity Assay. Enzyme activity was assayed fluorimetrically from cell extracts using 4-methylumbelliferyl-β-D-glucoside (Sigma) as substrate (24). To distinguish the human GC activity from the mouse GC, duplicate samples were immunoabsorbed with 1 volume of anti-human GC antiserum or control rabbit serum (both diluted 1:50 in PBS) at 37°C for 1 hr before assay.

RESULTS

Integration and Expression of *MDR*-GC Bicistronic Constructs in NIH 3T3 Cells. For functional expression of GC from a bicistronic cassette containing the *MDR1* gene as a selectable marker, we constructed pHaMCG (Fig. 1). Two additional constructs, pHaMCDG (which has a 422-bp deletion at the 5′ end of the GC cDNA) and pHaMDR1/A (including the *MDR1* gene alone) (Fig. 1), were used as controls. Transfection into NIH 3T3 cells yielded colchicine-resistant colonies for all three retroviral vectors. The transfection efficiencies achieved

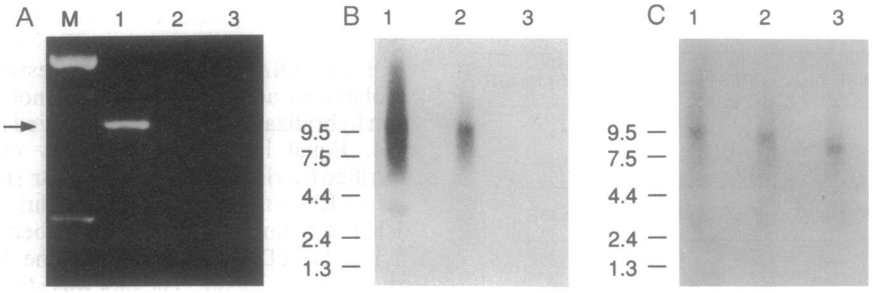

FIG. 2. Integration and expression of *MDR*-GC bicistronic constructs in drug-resistant NIH 3T3 cells. (*A*) Genomic DNA (1 μg per sample) isolated from pools of colchicine-resistant colonies was subjected to PCR amplification. Aliquots (5 μl) of the reaction products were resolved on a 1.5% agarose gel and stained with EtBr. Lane M, BRL low molecular weight ladder; lane 1, pHaMCG derived transfectants; lane 2, pHaMCDG derived transfectants; lane 3, pHaMDR1/A derived transfectants. The arrow at the left indicates expected band position. (*B*) Total RNA (20 μg per sample) isolated from colchicine-resistant cells was fractionated on a 0.8% formaldehyde/agarose gel and probed with a 1.1-kb specific "GC" probe. (*C*) The filter was stripped and reprobed with a 0.86-kb *Eco*RI–*Hin*dIII "*MDR*" fragment. Lanes 1, pHaMCG transfectants; lanes 2, pHaMCDG transfectants; lanes 3, pHaMDR1/A transfectants. Numbers on the left correspond to the size of RNA molecular weight markers.

with pHaMCG and pHaMCDG (7.9×10^{-4} and 7.5×10^{-4}, respectively) were similar to those obtained with pHaMDR1/A (8.7×10^{-4}), indicating that the IRES and GC sequences do not interfere with *MDR1* function.

PCR amplification of the *MDR*-GC transgene in drug-resistant transfectants demonstrated integration of pHaMCG into the mouse genome. The result in Fig. 2*A*, lane 1, indicate that the pHaMCG transfected population contains the specific 1.15-kb fragment.

To examine coexpression of GC and P-glycoprotein, RNA from drug-resistant transfectants was analyzed with *MDR1* and GC-specific cDNA fragments. When the GC probe was used, transcripts of about 10 kb and 9.5 kb were detected in pHaMCG and pHaMCDG transfected cells, respectively, but no signal was detected in pHaMDR1/A cells (Fig. 2*B*). The lower intensity of pHaMCDG transfectants (lane 2) compared to pHaMCG transfectants (lane 1) may be due to the absence of the amino end of GC in the GC-deleted bicistronic construct. As shown in Fig. 2*C*, the *MDR* probe also revealed diffuse mRNA bands with the strongest signal at 10 kb for pHaMCG, 9.5 kb for pHaMCDG, and 8 kb for pHaMDR1/A. Finding only a single mRNA transcript of the predicted size in the bicistronic transfectants indicates that no rearrangements or deletions were produced in the integrated retroviral transgene as a consequence of P-glycoprotein selection.

Expression of GC and P-glycoprotein Is Increased by Drug Selection in pHaMCG Transfectants. To determine if the MCG bicistronic transcripts produced GC and P-glycoprotein, we performed an immunoblot analysis on cell extracts from drug-resistant transfectants. Combined detection with rabbit anti-human GC antiserum and anti-P-glycoprotein C219 monoclonal antibody revealed coexpression of both GC (60-kDa band) and P-glycoprotein (170-kDa band) only in the pHaMCG derived transfectants (Fig. 3). As positive control for human GC expression we used NIH 3T3 cells transfected with the pWE-GC retroviral vector (17), which produces high levels of active human GC.

By single-step selection of the pHaMCG transfectant pool with different concentrations of colchicine we asked whether the stringency of drug selection influenced expression of both P-glycoprotein and GC. Fig. 4 shows that the expression levels of both proteins increased in parallel when the pHaMCG transfectants were selected in increasing colchicine concentrations.

To test whether the human GC protein expressed was functional, we measured its enzymatic activity in cell extracts. Fig. 5 shows that transfected cells exhibit human GC activity and that there is a gradual increase in human enzyme activity with more stringent colchicine selection, consistent with the expression data obtained from the Western blot analysis (Fig. 4). We conclude that the *MDR*-GC bicistronic construct efficiently expresses active GC and its level is increased by selection for MDR by colchicine.

Lysosomal Localization of Human GC in pHaMCG-Derived Transfectants. To determine the location of GC in pHaMCG transfected cells, homogenates were fractionated on a discontinuous Percoll gradient. The gradient conditions were chosen so that the plasma membrane, the endoplasmic reticulum, and the Golgi fractions were isopycnic (19). Fig. 6*A* shows the distribution profile of human GC (60-kDa band). The first two fractions are positive for human GC and represent the "solubilized" enzyme from broken organelles. The rest of human GC followed a bimodal distribution, with peaks of expression in the "microsomal," "endosomal," or "light lysosomal" fractions (lanes 4–7) ($\rho \approx 1.035$ g/ml) and in the "heavy lysosomal" fractions ($\rho \approx 1.085$ g/ml) (lanes 12 and 13). Similar heterogeneity in lysosomal enzyme distribution has been described (25, 26). By densitometry, about 30% of the enzyme was associated with the heavy lysosomal fractions. The broad fuzzy GC signal in the heavy lysosomal fractions is indicative of an extensively glycosylated protein (27, 28). Also, the slightly decreased molecular mass of the enzyme in the late gradient fractions (lanes 12 and 13) compared with the microsomal fractions (lanes 4–7) was previously observed for the lysosomal form of GC (17). The GC present in the microsomal fractions is probably immature enzyme being processed in the endoplasmic reticulum and Golgi before transfer to lysosomes.

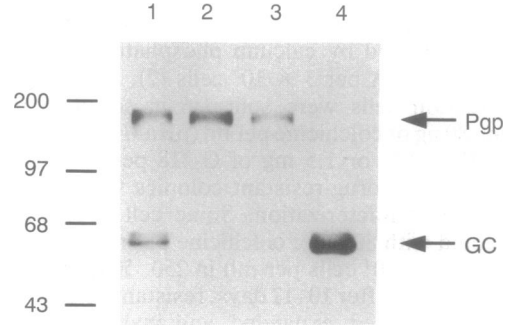

FIG. 3. Coexpression of GC and P-glycoprotein (Pgp) in pHaMCG transfected cells. Cells were harvested and extracted and 20 μg of protein per sample was fractionated by 8% SDS/PAGE and analyzed by immunoblotting with a mixture of anti-human GC antiserum and C219 monoclonal antibody. Lanes: 1, pHaMCG transfectants; 2, pHaMCDG transfectants; 3, pHaMDR1/A transfectants; 4, pWE-GC transfectants.

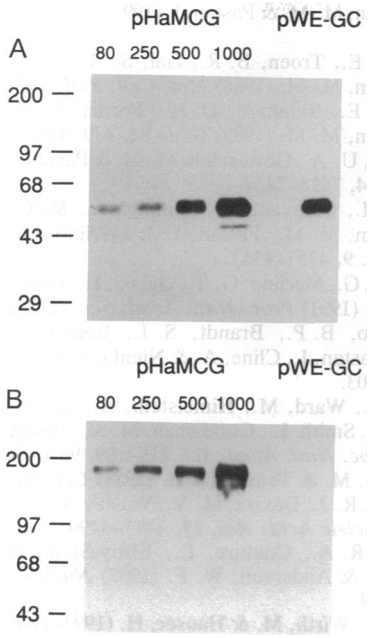

FIG. 4. Drug selection increases human GC and P-glycoprotein expression on pHaMCG derived transfectants. pHaMCG derived cells obtained by single-step selection with the indicated concentrations of colchicine (in ng/ml) were disrupted, electrophoresed in 8% SDS/polyacrylamide gels (10 μg of protein per lane), and analyzed by Western blotting. pWE-GC refers to transfectants control expressing human GC. (*A*) Human GC detected with anti-GC antiserum. (*B*) P-glycoprotein detected with C219 monoclonal antibody.

To confirm that human GC was targeted to lysosomes, we analyzed the same gradient fractions with a rabbit antiserum that reacts with endogenous mouse cathepsin L (Fig. 6*C*). This soluble lysosomal hydrolase initially synthesized as a 39-kDa precursor form is processed into 29- and 20-kDa polypeptides that are associated with the lysosomes (26) and colocalizes with recombinant GC in the high density fractions (lanes 12 and 13). Analysis of the gradient fractions with monoclonal antibody C219 showed that the 170-kDa band of recombinant P-glycoprotein is present in the light density fractions (Fig. 6*B*, lanes 4–6) as would be expected for a plasma membrane protein.

When homogenates from pWE-GC transfected mouse fibroblasts were analyzed, GC was found in the same fractions as in cells containing the bicistronic pHaMCG vector (data not shown). Taken together, these results indicate that P-glycoprotein is targeted to the plasma membrane and GC is targeted to lysosomes.

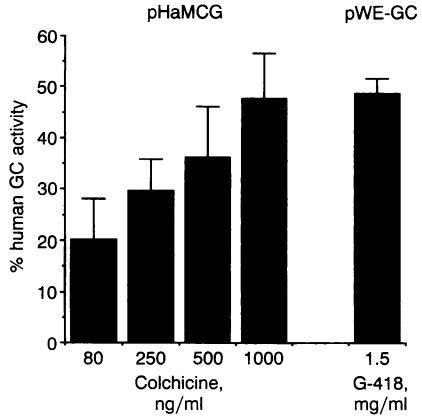

FIG. 5. Effect of the stringency of drug selection on the levels of human GC activity in pHaMCG derived cells. Single-step colchicine-selected pHaMCG transfectants and pWE-GC transfectants were extracted and GC activity was measured fluorimetrically. Values are means ± SD of duplicate samples from three separate experiments.

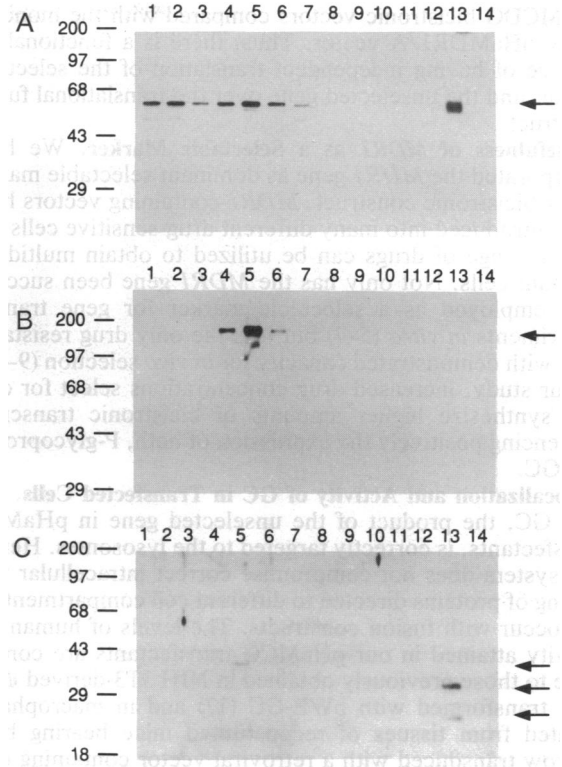

FIG. 6. Subcellular distribution of human GC, P-glycoprotein, and mouse cathepsin L in pHaMCG transfectants. Percoll fractionation of fibroblast homogenates was done as indicated in the text. From each gradient fraction, 1.6 μl was subjected to 10% SDS/PAGE and Western analysis. (*A*) Human GC detected with rabbit anti-GC antiserum. (*B*) P-glycoprotein detected with C219 monoclonal antibody. (*C*) Mouse cathepsin L detected with rabbit anti-MEP antiserum. Arrows on the right indicate the size of specific bands.

DISCUSSION

Our results demonstrate that a vector containing the human *MDR1* gene can be used to promote expression of an accompanying human GC gene by selection with colchicine. In the expression system used, the *MDR1* and the GC cDNAs are transcribed as a single mRNA driven by the strong Harvey sarcoma virus promoter but translated as independent entities combining cap-dependent (P-glycoprotein) and cap-independent (GC) translation mediated by the encephalomyocarditis IRES.

IRES Guarantees Expression of the Unselected Gene in Bicistronic Vectors. The capability of the 5′ untranslated regions of picornaviruses to direct internal translation initiation from bi- and polycistronic constructs has been reported recently (13–15). The coordinate coexpression of two otherwise unrelated genes under control of a single promoter overcomes negative interactions found when retroviral vectors carry the unselectable gene and the selectable marker in different transcriptional units (12, 29). The single, unspliced bicistronic *MDR*-CITE-GC transcript revealed by Northern analysis of the drug-selected population indicates equal amounts of mRNA for both genes. We previously have used the *MDR1* gene to express the adenosine deaminase gene by fusing both cDNAs to make a hybrid protein (7). However, the *MDR1*-ADA fusion gene gave lower transfection efficiencies than the *MDR1* gene alone (7). In the new vector system, similar transfection efficiencies were obtained from pHaMCG and

pHaMCDG bicistronic vectors compared with the monocistronic pHaMDR1/A vector. Thus, there is a functional advantage of having independent translation of the selectable marker and the unselected gene over the translational fusion construct.

Usefulness of *MDR1* as a Selectable Marker. We have incorporated the *MDR1* gene as dominant selectable marker in our bicistronic construct. *MDR1*-containing vectors have been introduced into many different drug-sensitive cells and a wide range of drugs can be utilized to obtain multidrug-resistant cells. Not only has the *MDR1* gene been successfully employed as a selectable marker for gene transfer experiments *in vitro* (5–7) but it is the only drug resistance gene with demonstrated capacity for *in vivo* selection (9–11). In our study, increased drug concentrations select for cells that synthesize higher amounts of bicistronic transcript, influencing positively the expression of both, P-glycoprotein and GC.

Localization and Activity of GC in Transfected Cells. Human GC, the product of the unselected gene in pHaMCG transfectants, is correctly targeted to the lysosomes. Hence, this system does not compromise correct intracellular trafficking of proteins directed to different cell compartments as can occur with fusion constructs. The levels of human GC activity attained in our pHaMCG transfectants are comparable to those previously obtained in NIH 3T3-derived ψ-am cells transformed with pWE-GC (17) and in macrophages isolated from tissues of reconstituted mice bearing bone marrow transduced with a retroviral vector containing only the human GC cDNA (30). Those same vectors have been used successfully to restore the human GC activity to normal levels in human Gaucher cells (17, 31).

Implications for Gene Therapy. Recent progress on sustained human GC expression *in vivo* has been obtained in murine models using retroviral vectors without capacity for positive selection (30, 32). Because transduction of pluripotent primate hematopoietic cells is significantly less efficient than its murine counterparts (33, 34), for any of the vectors to be used in gene therapy the inclusion of a versatile selectable marker such as the *MDR1* gene that possesses the capacity for positive selection *in vivo* seems necessary. Furthermore, in the pHaMCG vector the presence of the selectable marker not only guarantees expression of GC but also allows selection for its increased expression. Thus, the use of bicistronic vectors containing a therapeutically valuable gene, an intercistronic IRES element, and a selectable *MDR1* gene may have important applications for the gene therapy of many other disorders.

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