

Single Cell Analysis and Selection of Living Retrovirus Vector-corrected Mucopolysaccharidosis VII Cells Using a Fluorescence-activated Cell Sorting-based Assay for Mammalian β -Glucuronidase Enzymatic Activity*

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Mutations in the acid β -glucuronidase gene lead to systemic accumulation of undegraded glycosaminoglycans in lysosomes and ultimately to clinical manifestations of mucopolysaccharidosis VII (Sly disease). Gene transfer by retrovirus vectors into murine mucopolysaccharidosis VII hematopoietic stem cells or fibroblasts ameliorates glycosaminoglycan accumulation in some affected tissues. The efficacy of gene therapy for mucopolysaccharidosis VII depends on the levels of β -glucuronidase secreted by gene-corrected cells; therefore, enrichment of transduced cells expressing high levels of enzyme prior to transplantation is desirable. We describe the development of a fluorescence-activated cell sorter-based assay for the quantitative analysis of β -glucuronidase activity in viable cells. Murine mucopolysaccharidosis VII cells transduced with a β -glucuronidase retroviral vector can be isolated by cell sorting on the basis of β -glucuronidase activity and cultured for further use. *In vitro* analysis revealed that sorted cells have elevated levels of β -glucuronidase activity and secrete higher levels of cross-correcting enzyme than the population from which they were sorted. Transduced fibroblasts stably expressing β -glucuronidase after subcutaneous passage in the mucopolysaccharidosis VII mouse can be isolated by cell sorting and expanded *ex vivo*. A relatively high percentage of these cells maintain stable expression after secondary transplantation, yielding significantly higher levels of enzymatic activity than that generated in the primary transplant.

zymes responsible for the catabolism of glycosaminoglycans. A heritable deficiency of the acid hydrolase β -glucuronidase (β -D-glucuronide glucuronosohydrolase, EC 3.2.1.31) is responsible for the clinical manifestations of MPS type VII (Sly disease) (1), which include shortened life span, hepatosplenomegaly, skeletal deformities, and mental retardation (2). Animal models share many of the abnormalities found in human MPS VII (3, 4), including the distention of lysosomes by granular and fibrillar storage material. They have been used extensively as experimental systems to investigate pathogenesis and therapies.

The basis for therapy in MPS VII and most other lysosomal storage diseases is that secreted normal enzyme can be taken up into the lysosomes of diseased cells, where it can degrade substrate (5). This has been demonstrated in MPS VII mice by injection of recombinant enzyme (6) and by transplantation of bone marrow (7, 8) or neural progenitor cells (9). Somatic gene therapy using retrovirus vector-mediated transduction of hematopoietic stem cells followed by autologous transplantation of irradiated recipients showed that as little as 2% of normal enzyme levels could completely reverse the pathology in some major organs (10). Another strategy is autologous implantation in vector-corrected fibroblasts in neo-organs (11). This methodology has several technical advantages over stem cell transduction, including ease of harvest of autologous target cells by skin biopsy, ease of *ex vivo* expansion and retroviral infection, and ease of removal of the neo-organ. Some of the clinical manifestations of the disease, notably resolution of lysosomal storage lesions in the liver and spleen, were ameliorated by continuous intercellular exchange of the β -glucuronidase enzyme. The levels of normal β -glucuronidase seen were similar to those that were effective by hematopoietic stem cell transduction. However, there was no improvement in macroscopic characteristics, such as skeletal deformities. Similarly, enzyme secreted from fibroblast grafts in the brain can correct storage lesions in neural cells near the graft (12).

The efficacy of gene transfer has been limited, however, by the small number of fibroblasts transplanted and by down-regulation of vector expression (11, 12). One approach to overcoming these drawbacks may be to increase the level of enzyme secreted from each cell to supply more normal enzyme to the affected tissues (12, 13). Unfortunately, enhancing β -glucuronidase activity by generating vectors with strong or universal

The lysosomal storage disorders classified as mucopolysaccharidoses (MPS)¹ result from deficiencies in a number of en-

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¹ The abbreviations used are: MPS, mucopolysaccharidosis; MFI, me-

dian fluorescence value; FACS, fluorescence-activated cell sorting; MUGlcu, methylumbelliferyl-glucuronide; FDGlcu, fluorescein-di- β -D-glucuronide; PFB, pentafluorobenzoylamino.

genetic regulatory elements may not be a viable solution, as a number of enhancers gradually become inactivated in gene-corrected fibroblasts *in vivo* (12, 14, 15). Recently, several methodologies for the selection of retrovirus vector-transduced cells have been described, utilizing bicistronic vectors that encode transcriptional fusions between a therapeutic gene and a selectable marker (16). Two classes of selectable marker genes have been used, including those encoding proteins that confer resistance to otherwise cytotoxic drugs, or surface markers that allow for selection by fluorescence-activated cell sorting (FACS). However, a number of problems associated with these surrogate markers have arisen, including reduction in the translational efficiency of the downstream gene (17–19), reduction in retroviral titers as a result of the excessive size of the bicistronic DNA cassette (19), immunogenicity of the selectable marker (20), interference with normal cellular differentiation (in the case of expression of an endogenous surface protein) (21, 22), and repression of expression of the therapeutic gene due to the presence of transcriptional silencer elements in prokaryotic drug resistance encoding genes (23). The complications associated with the presence of surrogate markers might be avoided by selecting directly for expression of the therapeutic gene.

Previously, we described a novel reporter assay utilizing *E. coli* β -glucuronidase in mammalian cells (24). In this report, we describe the development of a FACS-based assay utilizing a fluorescein-based substrate for the detection of mammalian β -glucuronidase activity at the single cell level. This quantitative assay can be used to isolate viable cells with uniformly high β -glucuronidase activity from a population of cells showing heterogeneous levels of expression. Sorted cells can be expanded and maintain high levels of endogenous and secreted β -glucuronidase in culture. Utilizing the mouse model of MPS VII, this method was used to isolate a subpopulation of fibroblasts still expressing β -glucuronidase after *in vivo* subcutaneous passage in neo-organs. *Ex vivo* expansion and secondary transplantation of this subpopulation revealed that it was enriched for cells that stably express high levels of β -glucuronidase *in vivo*. We propose that this method may be a useful addition to retroviral-based gene therapy treatment of MPS VII.

EXPERIMENTAL PROCEDURES

Cells and Tissue Culture—Murine MPS VII skin fibroblasts (3521), fibroblasts isolated from a heterozygous mouse (3522) (13), and primary cultures of BL/6 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 50 nM 2-mercaptoethanol, 100 units/ml penicillin, 0.05 μ M streptomycin, and 2.0 mM glutamine. 3521 fibroblasts were transduced with the retroviral expression vector DCH β H (DCH β H-3521) as described previously (13). The DCH β H provirus encodes the human β -glucuronidase transcription unit 5' of the retroviral enhancer in each long terminal repeat and the neomycin resistance gene downstream of the 5' long terminal repeat. Where indicated, transduced cells were selected by culturing in the presence of 0.8 mg/ml G418 for 2 weeks. All cultures were maintained at 37 °C in 5% CO₂. Serum was heat-inactivated at 65 °C for 60 min to inactivate bovine β -glucuronidase. For cross-correction experiments, two-chamber, 24-mm plates with a 0.4 μ m filter (Transwell, Costar) separating the upper (donor cell) from the lower (target cell) chamber were used, as described (25). Briefly, 1×10^5 3521 target cells resuspended in 2.5 ml of complete media and an equivalent number of donor cells resuspended in 1.5 ml of complete medium were added to the bottom and top wells of the cluster plate, respectively. After 3.5 days in culture, supernatant and cells were harvested and assayed as described below.

Cell Lysate and Supernatant Analyses—Methylumbelliferyl-glucuronide (MUGlcu) (Molecular Probes, Eugene, OR) fluorometric lysate assays were carried out essentially as described (25). Briefly, adherent 3521 fibroblasts were washed in phosphate-buffered saline and harvested by incubation in the presence of 0.2–0.5 ml of 0.25% trypsin-EDTA for 5 min at 37 °C. After washing in phosphate-buffered saline, cell suspensions were centrifuged at $400 \times g$, and the cell pellets were

resuspended by vigorous vortexing in 200 μ l 0.2% (v/v) Triton X-100, 150 mM NaCl in H₂O and frozen at -70 °C. Immediately prior to analysis, the cell homogenate was thawed, vortexed, and centrifuged at $12,000 \times g$ for 3 min to remove cell debris. Fifty μ l of supernatant harvested from the lower target chamber was added to an equal volume of 10 mM MUGlcu in 0.1 M citrate buffer (pH 4.5). The reactions were carried out at 37 °C for various times and stopped by the addition of 150 μ l of 15 mM EDTA, 300 mM glycine (pH 11.2). For analysis of freshly sorted cells, 50 μ l of 6 mM MUGlcu (in 0.1 M citrate buffer, pH 4.8, plus 0.3% Triton X-100) was added to 50 μ l of sorted cells in phosphate-buffered saline. For analysis of secreted enzyme, cell supernatant was removed and centrifuged for 2 min at $12,000 \times g$ to remove cell debris. MUGlcu (10 mM) in 0.1 M citrate buffer (pH 4.8) was added to the supernatant, and the solution was incubated as described above. The fluorescent product, 4-methylumbelliferone, was measured on a 96-well plate with a Fluoroskan II fluorescence plate reader (Flow Laboratories, McLean, VA) (peak excitation at 355 nm, emission at 460 nm). A standard curve of 4-methylumbelliferone was assayed in parallel to calculate units (nmol of methylumbelliferone/h) per mg of protein for the lysate assay or per ml of supernatant. Cell protein content was measured in triplicate using the DC protein assay (Bio-Rad). Cell pellets had 0.1–0.16 mg of protein/24-mm well.

Cell Staining, FACS Analysis, and Sorting—Single cell suspensions were pelleted and resuspended at a concentration of 4×10^6 /ml in staining medium (biotin- and flavin-deficient RPMI supplemented with 4% v/v FCS). Fifty μ l of cells pre-incubated for 10 min at 37 °C were mixed 1:1 (v/v) with 2 mM fluorescein-di- β -D-glucuronide (FDGlcu) or pentafluorobenzoylaminofluorescein-di- β -D-glucuronide (PFBFDGlcu) dissolved in staining medium (also equilibrated to 37 °C). After incubating for 10–90 min at 37 °C, the loading was stopped by dilution with 10 volumes of ice-cold staining medium. For inhibition studies, 3521 fibroblasts were preincubated in 5 mM 1,4-saccharolactone (Sigma) or 0.3 mM chloroquine at 37 °C for 10–30 min prior to the addition of substrate. Cells were maintained in the presence of inhibitor throughout the experiment. Cells were pelleted to remove any fluorescent product in the medium and resuspended in 300 μ l of fresh staining medium supplemented with 1 μ g/ml propidium iodide to fluorescently stain dead cells. Cells were maintained on ice throughout the FACS analyses to prevent leakage of the fluorescein product. FACS set-up, analyses, and sorting were conducted as described (24, 26). Histochemical staining with naphthol AS-BI- β -D-glucuronide (Sigma) was conducted as described (25).

Generation and Harvesting of Neo-organs—The neo-organs were prepared as described (11). Briefly, Gore-Tex fibers (a gift from W. R. Gore, Inc., Taos, NM) were coated with collagen, sterilized by UV light, and coated with 10 μ g/ml recombinant fibroblast growth factor-2 (Promega). The cells were harvested by trypsinization and washed once with phosphate-buffered saline, and 5×10^6 cells/ml were resuspended in Dulbecco's modified Eagle's medium containing 10% FBS, 5 mM Hepes, 100 μ g penicillin/streptomycin/fungizone, 1 μ l (10 μ g/ml) fibroblast growth factor-2 (Promega), 0.25 μ l (0.8 mg/ml) epidermal growth factor (Boehringer Mannheim), and 0.5 ml (3 mg/ml) collagen in a dish with evenly separated fibers. After incubation at 37 °C for 30 min in the presence of 5% CO₂, 2 volumes of medium were added, and the incubation was continued for 2 days, by which time the neo-organ contracted into a semisolid gel. The neo-organs were transplanted subcutaneously through bilateral incisions on the back. After 4 weeks, individual neo-organ were removed, single cell suspensions were made by mincing and dispase digestion, and the explant cultures were evaluated for enzyme expression.

RESULTS

FACS Analysis of DCH β H-transduced 3521 Fibroblasts—Mammalian β -glucuronidase shows broad specificity for conjugated glucuronides, yielding D-glucuronic acid and alcohol hydrolysis products. Although the fluorogenic substrate FDGlcu (Fig. 1A) is efficiently hydrolyzed by *E. coli* β -glucuronidase (24), specific activity of the mammalian enzyme against this substrate has yet to be established. To determine whether FDGlcu is hydrolyzed by mammalian β -glucuronidase in viable cells, β -glucuronidase-negative 3521 fibroblasts or 3521 fibroblasts transduced with the β -glucuronidase double copy retrovirus vector DCH β H, which express high levels of β -glucuronidase (13), were incubated in the presence of FDGlcu and analyzed by FACS. Viable DCH β H-3521 cells showed high

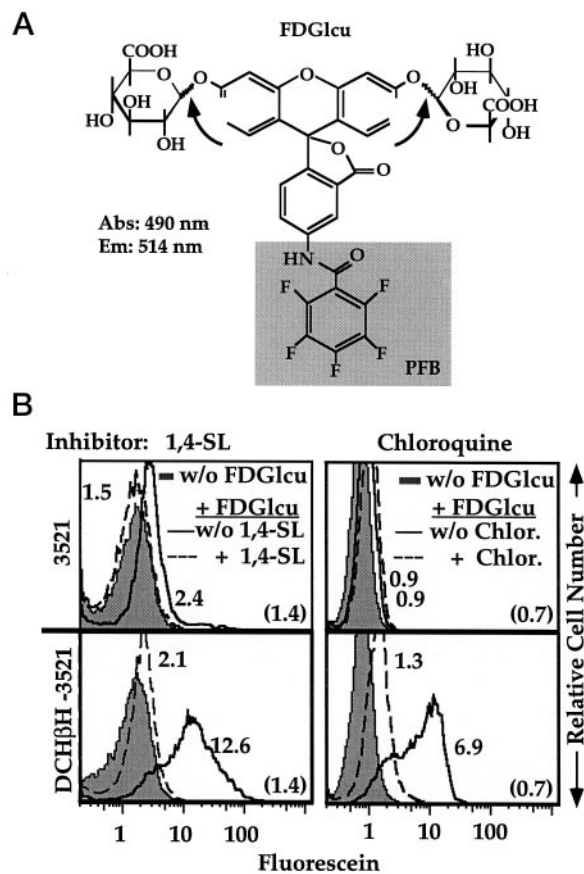
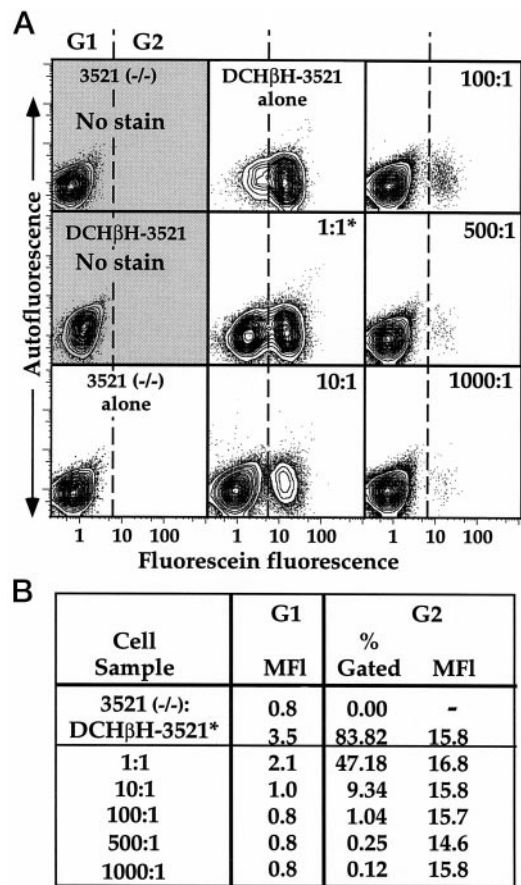


FIG. 1. Structure and β -glucuronidase-specific hydrolysis of FDGlcu. The fluorogenic diglycoside FDGlcu (A) includes two D-glucuronic acid residues covalently bound via β linkages at the first carbon to fluorescein. Addition of a pentafluorobenzoyl-amino group (*shaded*) yields the substrate PFBFDGlcu, which shows similar spectral properties but increased intracellular retention compared with FDGlcu (see under "Results"). For either substrate, hydrolysis of both acetal linkages (*arrows*) yields highly fluorescent fluorescein. B, 3521 (β -glucuronidase-negative) and G418-selected DCH β H-3521 (β -glucuronidase-positive) fibroblasts were preincubated in the presence of 5 mM 1,4-saccharolactone or 300 μ M chloroquine for 10 or 30 min, respectively, prior to the addition of 1 mM FDGlcu. Samples incubated in the absence of FDGlcu and inhibitor are also displayed. Viability was not influenced by the presence of substrate or inhibitor (data not shown). The MFI of each sample incubated in the absence of substrate is shown in parentheses. The MFI for each sample incubated in the presence of substrate is shown next to the relevant FACS histogram.

levels of fluorescein fluorescence (Fig. 1B). In contrast, non-transduced 3521 cells incubated in the presence of FDGlcu are not significantly more fluorescent than unstained controls. To establish that the fluorescence detected results from β -glucuronidase specific hydrolysis of the substrate, 3521 cells were incubated with the specific inhibitor 1,4-saccharolactone (27) prior to the addition of FDGlcu (Fig. 1B). In the presence of 1,4-saccharolactone, FDGlcu-loaded DCH β H-3521 cells showed fluorescence levels comparable to cells incubated in the absence of FDGlcu. These results show that β -glucuronidase is responsible for the hydrolysis of FDGlcu, and that the fluorescent product can be detected in viable cells by FACS.

β -Glucuronidase has optimal activity at pH 4.8, consistent with its localization and degradative function in the acidic lysosome (28). To determine whether the activity detected by FACS is primarily localized in the endosomal/lysosomal compartment, 3521 and DCH β H-3521 cells were incubated in the presence of the lysosomotropic weak base, chloroquine, prior to the addition of substrate. The accumulation of chloroquine leads to an increase in endosomal/lysosomal pH (29, 30), inhib-



* Mix = 3521: 3521 DCH β H

FIG. 2. Detection of rare β -glucuronidase-positive cells by FACS. 250 to 2.5×10^5 G418-selected DCH β H-3521 fibroblasts were mixed with 2.5×10^5 3521 fibroblasts and each population was incubated in the presence of 1 mM FDGlcu. A, 100,000 events were collected per sample and displayed as 5% probability contour plots with outliers. DCH β H-3521 and 3521 populations incubated in the absence of substrate are also shown (*gray plots*). B, an electronic gate in the fluorescein channel excluding all of the events collected in the 3521 sample was applied to determine the percentages and MFIs of fluorescein-positive (G1) and -negative (G2) populations in each sample. Autofluorescence was collected in the phycoerythrin channel.

iting the activity of acid hydrolases in these organelles (31, 32). In the presence of chloroquine, fluorescence development of FDGlcu-loaded DCH β H-3521 cells was significantly inhibited, to levels comparable to those found in nontransduced cells or in DCH β H-3521 cells incubated in the absence of FDGlcu (Fig. 1B). These results suggest that β -glucuronidase-mediated FDGlcu hydrolysis takes place primarily in the endosomal/lysosomal compartment.

Detection of Rare β -Glucuronidase-positive Cells—In contrast to bulk lysate protocols, FACS-based assays measure fluorescence on a single cell basis. As a result, very small populations of phenotypically distinct cells can be detected in a heterogeneous population. To determine the sensitivity of the FACS- β -glucuronidase assay, 3521 and DCH β H-3521 cells were mixed at ratios from 1:1 to 1000:1, loaded with FDGlcu, and analyzed by FACS (Fig. 2A). For each mixed population, the percentage of positive cells detected (G2) closely matched the predicted value (Fig. 2B). Comparison of the median fluorescence value (MFI) also allows for the quantitative determination of the level of product associated with uncorrected mutant cells. The MFI of 3521 cells (G1) decreased from 2.1 to 0.8 as the 3521:DCH β H-3521 ratio increased from 1:1 to 100:1, and remained at 0.8 as the ratio increased to 1000:1 (Fig. 2B).

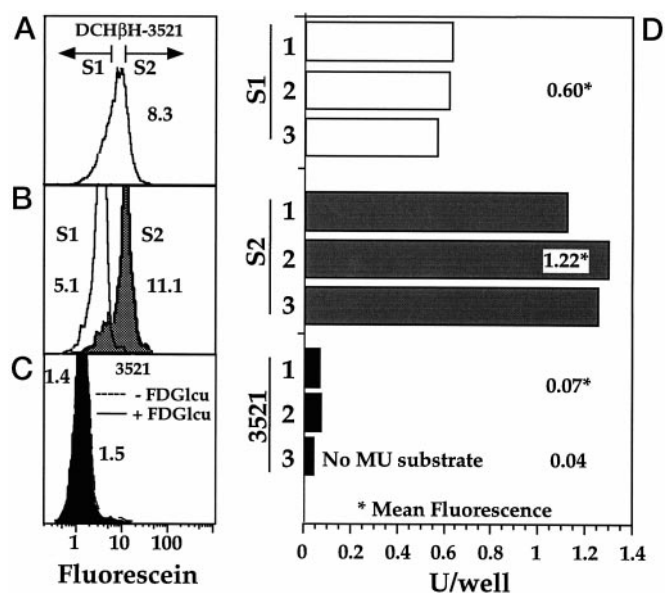


FIG. 3. DCH β H-3521 fibroblasts can be sorted on the basis of β -glucuronidase activity. DCH β H-3521 or 3521 fibroblasts were incubated in the presence of 1 mM FDGlcu as described in Fig. 1 and processed for cell sorting. A, two sets of electronic gates were applied to sort the duller (S1) and brightest (S2) viable cells. B, a total of 30,000 viable cells were sorted, per well, into staining medium and immediately reanalyzed by FACS. C, viable 3521 fibroblasts were sorted without gating in the fluorescein channel. D, alternatively, cells were sorted in triplicate using the same electronic gates directly into pH 4.5 lysate buffer and analyzed by lysate assay in the presence of 3 mM MUGlcu. Mean units/well/30,000 cell equivalents are shown for each sample. MFIs for each sample are displayed next to the relevant FACS histogram.

Although some component of this difference is contributed by a concomitant decrease in the number of DCH β H-3521 cells that fall in the G1 gate, the MFI of 2.2 primarily reflects the level of product internalized by the untreated mutant cells, and this figure is thus an overestimation of the true MFI of the 3521 population. Nevertheless, even at a ratio of 1:1, the majority of DCH β H-3521 cells show fluorescence values distinct from those of 3521 cells, with a MFI 7-fold greater than the latter. Most importantly, a population including 0.1% positive cells can be accurately enumerated with this method.

Cell Sorting Based on β -Glucuronidase Activity/Substrate Hydrolysis—The broad distribution of fluorescence in substrate-loaded DCH β H-3521 cells could result from heterogeneous substrate loading or from cell-to-cell differences in the levels of β -glucuronidase expression. Given the influence of the integration site (position effect variegation) on expression from the proviral long terminal repeat (33, 34), heterogeneity in expression levels would be expected from a population of cells comprised of a number of independent proviral integrants. To determine whether the fluorescence distribution reflects the intracellular β -glucuronidase expression level, DCH β H-3521 cells were loaded with FDGlcu, and viable cells were sorted on the basis of product fluorescence into lysis buffer for analysis using the independent MUGlcu assay (Fig. 3A). Reanalysis of sorted populations by FACS showed that the sorted fractions maintained their relative fluorescence profiles (Fig. 3B). Lysate analysis (Fig. 3D) of the bright sorted population (S2) showed levels of activity approximately 2-fold higher than an equivalent number of dull sorted cells (S1), a value consistent with that of the ratio of the corrected MFIs of these populations. Both S1 and S2 populations had significantly higher levels of activity than nontransduced 3521 cells when measured by FACS (Fig. 3C) and by lysate. Because dead cells were excluded during the sort, and the S1 and S2 populations show indistin-

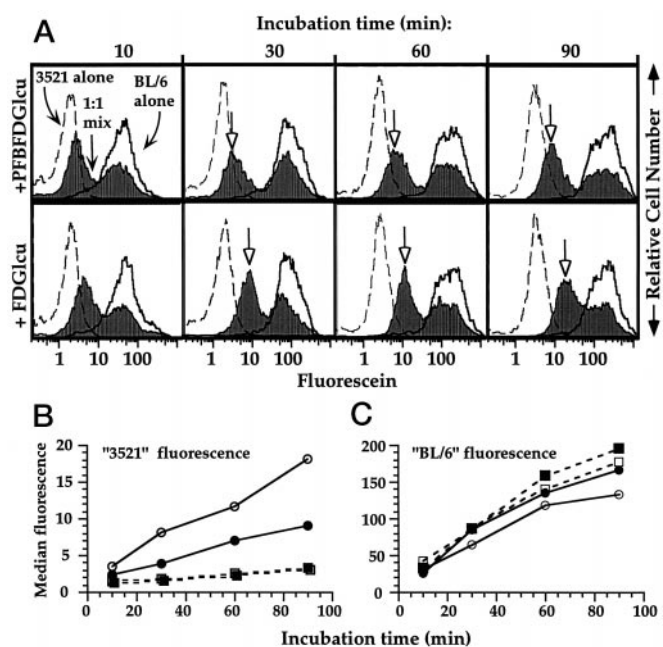


FIG. 4. *In vivo* kinetics of FDGlcu versus PFBFDGlcu hydrolysis and product retention. A, β -glucuronidase-negative 3521 cells (dashed line), β -glucuronidase⁺ BL/6 cells (solid line), or a 1:1 mix of the two (shaded histogram) were incubated in the presence of FDGlcu or PFBFDGlcu for 10–90 min and analyzed by FACS. For each time point, histograms of the homogeneous and mixed samples are superimposed. The MFIs of FDGlcu-loaded (open symbols) or PFBFDGlcu-loaded (filled symbols) 3521 (squares in B) or BL/6 (squares in C) samples were determined by calculating the 50th percentile of the fluorescence data. For the 1:1 mix samples (circles), the estimated MFIs of the 3521 (B) and BL/6 (C) subpopulations were determined by calculating the 25th and 75th percentiles of the fluorescence data values, respectively. These values approximate the MFIs of the 3521 and BL/6 subpopulations because the fluorescence distributions of the two are nearly completely resolved.

guishable forward and orthogonal scatter profiles (data not shown), the differences detected in β -glucuronidase activity were not the result of differences in cell viability within the DCH β H-3521 population. Thus, the broad distribution of fluorescence detected in the transduced population reflected the predicted heterogeneity in expression levels.

In Vivo Kinetics of Substrate Hydrolysis and Product Retention—We next wished to study in greater detail the kinetics of substrate hydrolysis, and the retention of the product fluorescein, in viable cells. During the course of this work we generated another fluorescein based glucuronide substrate, PFBFDGlcu, which includes a PFB moiety (Fig. 1A). We have established that PFB-based substrates react with reactive protein thiols *in vitro* and *in vivo*,² enhancing intracellular retention compared with conventional fluorescein-based substrates (35). Mutant 3521 fibroblasts, wild-type BL/6 fibroblasts, or a 1:1 mix of the two were loaded with FDGlcu or PFBFDGlcu for various times and analyzed by FACS (Fig. 4A). Analysis of the mixed samples reveals that both substrates yielded a bimodal fluorescence distribution at all time points. However, with increasing incubation time, resolution of the fluorescein dull (β -glucuronidase-negative) and bright (β -glucuronidase-positive) subpopulations is greater in the presence of PFBFDGlcu. The superior sensitivity of the PFB-based substrate is not due to an increase in substrate specificity, as the fluorescence distributions of the β -glucuronidase-negative samples stained with either substrate are indistinguishable. Thus, the disparity must be attributed to a difference in the intracellular retention

² Z. Diwa, unpublished observations.

of the fluorescent product in β -glucuronidase-positive cells, which becomes apparent only in the presence of β -glucuronidase-negative cells.

In order to evaluate the relative capabilities of the two substrates to resolve the negative and positive subpopulations, the 25th and 75th percentiles were calculated for each mixed sample. These values approximate the MFIs of the 3521 and BL/6 subpopulations, respectively, as there are equivalent numbers of each cell type present. By plotting these values, as well as the MFIs of 3521 and BL/6 samples stained alone with each substrate, the extent of leakage of the hydrolysis product from β -glucuronidase-positive cells and uptake by β -glucuronidase-negative cells can be determined. The MFI of the negative subpopulation is clearly lower (Fig. 4B) and that of the positive subpopulation higher (Fig. 4C) in the PFBFDGlc-loaded samples. However, a low level of product leakage does take place with PFBFDGlc staining, as revealed by the reduced MFIs of the BL/6 subpopulation in the mixed sample compared with the BL/6 sample stained alone.

Heritable High Levels of β -Glucuronidase Expression, Secretion, and Cross-correction by DCH β H-transduced Fibroblasts Sorted on the Basis of β -Glucuronidase Activity—We next wished to test whether cells sorted on the basis of high β -glucuronidase enzymatic activity show heritable levels of relatively high enzymatic activity, and if so, whether these sorted cells secrete higher levels of cross-correcting enzyme than the population from which they were sorted. To generate a population of cells with relatively high levels of β -glucuronidase activity, DCH β H-3521 fibroblasts were loaded with PFBFDGlc and gates were established such that the brightest 5% of fluorescein-positive cells were sorted (data not shown). After 30 days in log-phase culture, the selected cells and the DCH β H-3521 population from which they were sorted were reanalyzed by FACS. The sorted population maintained relatively high levels of β -glucuronidase activity, with 98% of the viable sorted cells showing fluorescence levels greater than or equal to the brightest 4% of viable DCH β H-3521 fibroblasts (Fig. 5A). The MFIs of 0.49, 0.81, 2.4, and 79.0 for 3521, 3522, DCH β H-3521, and sorted DCH β H-3521 cells, respectively, were comparable with those obtained on the day of sorting.

Murine 3521 Fibroblasts Efficiently Endocytose β -Glucuronidase—Using these β -glucuronidase deficient cells as targets for secreted enzyme, the cross-correcting enzyme in culture supernatants can be accurately measured. Cultures of 3521, 3522, DCH β H-3521, and sorted DCH β H-3521 donor fibroblasts, were established in Transwell plates that separated the donor from the target cells by a fluid but not cell permeable membrane, precluding enzyme transfer by cell-to-cell contact (25, 36). After 3.5 days in culture, the donor cells, supernatants, and target cells were harvested and analyzed with the MUGlc lysate assay and by FACS to compare the cross-correcting activity generated by sorted cells with that generated by the transduced, nonsorted population. Although enzymatic activity in the target cells was not detectable using the FACS assay (data not shown), lysate analysis revealed that the sorted population had a 2-fold greater level of intracellular β -glucuronidase activity than the cells from which they were sorted (Fig. 5B), demonstrating that the FACS assay can be used to enrich for cells expressing high levels of β -glucuronidase. Both the DCH β H-3521 and the sorted DCH β H-3521 populations showed significantly higher levels of expression than 3522 cells, which express normal levels of β -glucuronidase (13). The relative levels of β -glucuronidase secreted by each population was consistent with the donor cell lysate analysis (Fig. 5C). Enzymatic activity in the culture supernatant of the sorted DCH β H-3521 population was approximately 2-fold greater

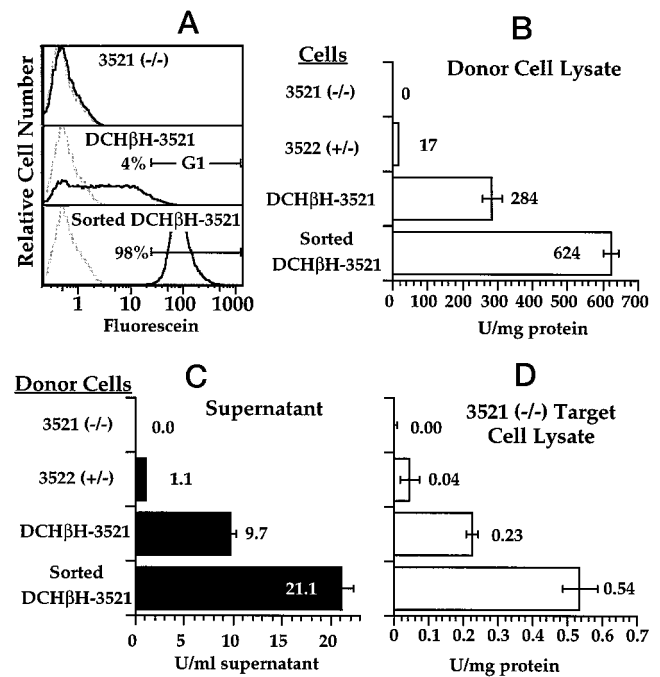


FIG. 5. Enhanced secretion of β -glucuronidase and cross-correction of β -glucuronidase deficient 3521 cells by DCH β H-transduced fibroblasts sorted on the basis of high β -glucuronidase activity. A, 3521, 3522, DCH β H-3521, and DCH β H-3521 fibroblasts sorted on the brightest 5% of cells (*sorted DCH β H-3521*) were reanalyzed by FACS 30 days post-sorting. Data for each population incubated in the presence (*light line*) or absence (*dark line*) of PFBFDGlc prior to FACS analysis are displayed. To measure the level of cross-correcting enzyme secreted by these cells, cultures of each were established in parallel on Transwell dishes with target 3521 cells plated in the lower chamber. Donor cells (B), supernatant (C), and target cells (D) were harvested after 3.5 days in culture, and β -glucuronidase activity for each sample was analyzed with the MUGlc assay. All analyses were conducted in triplicate, and the mean units (U) of activity per mg of protein or per ml of medium (harvested from the lower chamber) are shown for lysate and supernatant values, respectively. The S.E. is displayed for each measurement.

than that detected for the DCH β H-3521 population from which it was sorted and 20-fold greater than that detected for 3522 cells.

Target 3521 cells incubated in the presence of sorted DCH β H-3521 cells showed about 2-fold greater enzymatic activity than 3521 cells incubated in the presence of the unsorted transduced population (Fig. 5D), results that are consistent with enzyme levels detected in the supernatant. As expected, no activity was detected in target cells incubated in the presence of 3521 donor cells. The linear relationship between the level of β -glucuronidase in the supernatant and that endocytosed by β -glucuronidase deficient cells shows that saturation of the mannose-6-phosphate endocytic machinery (37) did not occur under the culture conditions used. Thus, the FACS can be used to select for cells with very high levels of β -glucuronidase expression and secretion, from a population already expressing a high level of β -glucuronidase.

Isolation and Secondary Transplantation of Fibroblasts Stably Expressing High Levels of β -Glucuronidase after *In Vivo* Passage in the MPS VII Mouse—Rapid silencing of retroviral expression after transplantation of genetically modified fibroblasts is well documented (12, 14, 15). However, a subpopulation of transplanted cells showing stable expression even after long term passage *in vivo* can be detected (11, 12, 15). The presence of rare expressing cells may reflect a stochastic process of silencing, whereby all proviruses have an equal probability of silencing over time. Alternatively, such cells may rep-

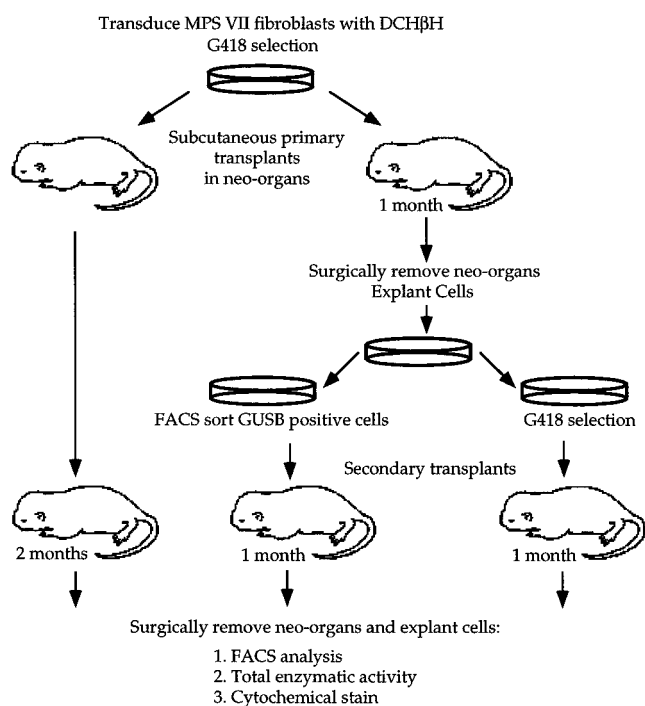


FIG. 6. Scheme for testing stability of vector expression by PFBFDGlcu FACS selection after *in vivo* passage. Neo-organs (see under "Experimental Procedures") of DCH β H-3521 cells were transplanted subcutaneously into adult MPS VII mice. One month later, the neo-organs from one mouse were surgically removed, and the cells were explanted and analyzed for β -glucuronidase expression. Cells from one neo-organ (6.4% β -glucuronidase-positive by FACS) were used for secondary transplants. Half of the cells were subjected to FACS (PFBFDGlcu substrate) to isolate β -glucuronidase-positive cells, and half were G418-selected to isolate only the cells expressing the vector. The cells were expanded, and new neo-organs were prepared from both populations for secondary transplantation into MPS VII mice. To analyze the cells for the same total length of time *in vivo*, the remaining primary grafts were removed 2 months after transplantation, and the secondary grafts were removed after 1 month. Explant cultures were established from each neo-organ and the cells were analyzed for total enzyme activity and proportion of positive cells.

resent clones in which the proviral integration site supports long term expression. If the latter explanation is correct, then the long term expressing population should show greater stability of expression upon secondary transplantation than the original transplanted population. To test this hypothesis, an *in vivo* experiment was conducted as described in Fig. 6. Neo-organs (11, 38) of G418 resistant DCH β H-3521 cells (80.0% FACS-positive, not shown) were transplanted into four adult MPS VII mice. The neo-organs from one mouse were removed 1 month after transplantation, and the cells were explanted. Cells from one of the neo-organs (5786R, 6.4% positive as measured by FACS, Table I) were used to generate new neo-organs for secondary transplantation. Half of the cells were loaded with the PFBFDGlcu substrate, and β -glucuronidase-positive cells were isolated by FACS. Because both vector-expressing and cross-corrected cells may be positive by this method, the other half of the explant culture was G418-selected to isolate only those cells expressing the vector. Neo-organoids were prepared from the sorted and from the G418-selected populations and transplanted as secondary grafts into MPS VII mice.

Although most silencing of vector expression occurs by 1–2 weeks after transplantation (12), the remaining three primary transplant recipients (six neo-organs) were kept for 2 months before analysis so the cells in the primary and secondary grafts were maintained *in vivo* for equivalent total lengths of time.

FACS analysis of cells derived from the six primary transplants revealed that a subpopulation of cells in each neo-organ stably expressed β -glucuronidase at percentages ranging from 0.7 to 20.2% with a mean of 5.2% (Table I). This degree of silencing *in vivo* was similar to our previous observations in the MPS VII mouse (10, 12).³ The secondary transplanted neo-organs were removed after 1 month and analyzed for β -glucuronidase expression (Table I). The secondary transplants derived from both the sorted and G418-selected populations had significantly increased percentages of β -glucuronidase-positive cells (17.2 and 44.8%, respectively) compared with the primary group (5.2%). The FACS analysis was verified by counting samples of the explanted cells stained by a histochemical reaction for β -glucuronidase enzymatic activity (39). Calculation of the mean percentage of positive cells for each transplant group revealed no significant differences between the two methods (Table I). The amount of β -glucuronidase enzymatic activity was generally proportional to the percentage of positive cells in individual neo-organs (Table I).

DISCUSSION

The development of several quantitative FACS-based mammalian reporter gene assays for bacterial hydrolytic enzymes (24, 31) prompted us to test a similar approach for the detection of known deficiencies in mammalian lysosomal hydrolases. The deficiency of β -glucuronidase causing MPS VII is an excellent test system because both mouse and large animal models are available, and they have been used extensively for enzyme replacement, transplantation, and gene therapy experiments. Furthermore, analysis of mammalian β -glucuronidase with a number of potential substrates has shown that this enzyme will hydrolyze almost any aglycone in a β -linkage to glucuronic acid (40). Here, we show that fluorescein- β -D-glucuronide substrates are internalized and hydrolyzed by human or murine β -glucuronidase in viable cells at levels sufficient for detection and isolation by FACS.

Acid hydrolases, including β -glucuronidase, are present in an acidic pre-lysosomal compartment of the endocytic pathway (41, 42), and the endosome is the primary site of initial exposure of endocytosed material to hydrolytic enzymes (43). Analysis of FDGlcu-loaded fibroblasts by confocal fluorescence microscopy revealed a punctate cytosolic staining pattern indicative of endosomal/lysosomal localization (data not shown). Thus, our results are most consistent with a model of substrate internalization involving fluid phase pinocytosis with subsequent hydrolysis of the endocytosed substrate in acidified endosomes and/or lysosomes. Consequently, the FACS assay described here may have greater physiological relevance than the lysate assay, which measures the entire cellular content of β -glucuronidase. However, as endocytic capacity and the size of the lysosomal compartment is dependent on the cell type (44) and state of activation (45), the fluorescence intensities detected under distinct culture conditions or in different cell types may not accurately reflect relative levels of intracellular β -glucuronidase. Furthermore, determination of the number of β -glucuronidase molecules within a cell using this methodology is confounded by several variables inherent to *ex vivo* analysis, including sequestration of the enzyme in compartments inaccessible to the endocytosed substrate, heterogeneity in autofluorescence levels, and heterogeneity in the pH of endocytic compartments, which strongly influences fluorescein fluorescence (29).

Analysis of heterogeneous samples of β -glucuronidase-positive and -negative fibroblasts allowed for direct comparison of the loading and retention properties of the substrates PF-

³ J. H. Wolfe, unpublished observations.

TABLE I

 β -Glucuronidase expression in DCH β H retrovirus vector-corrected fibroblasts after primary and secondary transplantation in neo-organs

Neo-organs of DCH β H-3521 cells were transplanted into adult MPS VII mice according the scheme shown in Fig. 6. The cells derived from the neo-organs after primary and secondary transplantation were analyzed for quantitative enzymatic activity and for proportion of positive cells, by two methods. The explanted primary cells were maintained in culture for a total of about 1 month before secondary transplantation (32 days for selected cells; 35 days for sorted cells), which included the times needed to establish the explant cultures, select or sort the cells, and expand the cultures to obtain enough cells to construct the secondary neo-organs. ND, not determined; 2°, secondary.

Transplant group	Total no. of days <i>in vivo</i>	Neo-organ no.	β -Glucuronidase activity	Cell counting	
				FACS analysis	Cytochemical stain
			<i>units/mg</i>	<i>% positive</i>	
Primary ^a	36	5786 L	0.4	0.7	0.1
Primary ^a	58	5786 R ^b	13.6	6.4	2.1
		5823 L	0.9	0.7	0.1
		5823 R	2.0	0.7	0.7
		5845 L	10.7	4.9	1.7
		5845 R	6.1	2.1	1.9
		5867 L	1.8	2.3	1.0
		5867 R	12.9	20.2	ND
		Mean \pm S.E.	5.8 \pm 2.1	5.2 \pm 3.1 ^c	1.1 \pm 0.3 ^c
Sorted secondary	65 ^d	6061 L	27.1	24.0	14.6
	(2° = 29 days)	6061 R	5.8	4.8	6.9
		6067 L	16.3	21.8	20.4
		6068 L	15.4	7.9	8.8
		6106 L	35.8	25.1	30.8
		6106 R	22.4	19.3	28.3
		Mean \pm S.E.	20.5 \pm 4.2 ^c	17.2 \pm 3.5	18.3 \pm 4.1
G418-selected secondary	69	6143 L	152.7	78.7	59.5
	(2° = 33 days)	6143 R	59.2	39.3	38.1
		6144 L	42.9	28.0	22.9
		6144 R	36.0	33.3	13.7
		Mean \pm S.E.	72.7 \pm 27.1 ^e	44.8 \pm 11.5	33.4 \pm 10.1

^a Cells used to construct the primary neo-organs were 80% positive by FACS and 67% positive by cytochemistry; uninfected 3521 cells were always negative by all assays.

^b Secondary organoids were derived from 5786R.

^c The differences between counting methods were not statistically significant within any transplant group.

^d The total number of days that secondary grafts were present *in vivo* equals the number of days (36 days) of the primary graft (5786R) plus the number of days of the secondary graft, shown in parentheses.

^e The differences between the primary and sorted secondary transplants, and between the primary and G418-selected secondary transplants, were statistically significant (all pairwise tests, $p < 0.03$) for enzyme activity, FACS counting, and cytochemical stain counting.

BFDGlcu and FDGlcu. The MFL ratio of β -glucuronidase-positive to -negative cells was significantly greater with the PF-BFDGlcu substrate than with FDGlcu, indicating that the signal to background ratio is superior for the former. The pentafluorobenzoyl moiety is covalently bound *in vitro* and *in vivo* to reactive thiols, such as glutathione.² Such a covalently modified substrate would be more hydrophilic and thus less likely to cross the plasma membrane, which could explain the superior retention properties of PF-BFDGlcu. Furthermore, the hydrolysis product pentafluorobenzoylamino fluorescein has a significantly lower pK_a than that of fluorescein ($pK_a = 6.0$ versus 6.7, respectively). As a result, a higher fraction of the former is fluorescent at the relatively low pH of the endosomal/lysosomal compartment, thus giving a stronger signal per molecule of substrate cleaved. In cells with low β -glucuronidase expression, the levels of intracellular fluorescein generated may be insufficient for detection by FACS. Although β -glucuronidase activity at 0 °C is very low, the enzyme shows a reduction in activity of only 9–10-fold at 15 °C versus 37 °C ($Q_{10} = 3.7$) (data not shown). Thus, the sensitivity of the assay can be increased by shifting the incubation to 15 °C, instead of 0 °C, allowing for further hydrolysis while preventing product efflux, which is inversely related to temperature (47).

An alternative method for detection of β -glucuronidase by flow cytometry, using monoclonal antibodies specific for the enzyme, was reported previously (48, 49). However, this method requires fixation and permeabilization of the cells, which precludes using it for enrichment of viable gene-corrected cells. Furthermore, the presence of cross-reactive material in MPS VII patients (50) clearly deficient in lysosomal enzymatic activity also limits the use of antibody staining,

which measures the level of β -glucuronidase protein (including mutant protein) present, rather than the physiologically relevant enzymatic activity level. Another approach is to co-express cell surface proteins, as surrogate markers, with the therapeutic gene from a vector (51, 52). However, several potential complications may preclude clinical use of such markers, including immunogenicity of the encoded protein (20) and interference with normal cellular differentiation (21, 22). Clearly, avoiding the requirement for surrogate markers by direct selection for expression of the functional therapeutic gene, is a desirable goal. Detection of enzymatic activity at the single cell level, and the potential for detecting very rare cells, makes this FACS assay superior to other methods for the detection of genetically corrected cells. Given the availability of a number of fluorescein-based hydrolase substrates, this assay may be applicable to the detection of other exoglycosidases responsible for lysosomal storage disorders, as we have shown with β -glucocerebrosidase-deficient Gaucher disease cells (35).

The efficacy of gene therapy for MPS VII depends on the level of enzyme secreted by the corrected cells (10–13), and the FACS method that we describe should be useful for isolating populations of cells producing high levels of β -glucuronidase prior to autologous transplantation. Because the amount of β -glucuronidase secreted by fibroblasts transduced with β -glucuronidase expression vectors is proportional to cellular expression levels (13, 25), we chose to test whether cells sorted on the basis of high β -glucuronidase expression produced higher levels of cross-correcting enzyme than the parental population from which they were sorted. Although most of the cells in the unsorted population were positive for DCH β H (data not shown), the broad heritable distribution of expression, attrib-

unable to position-effects (33, 34), allowed for the isolation by FACS of a subset of cells that stably expressed the highest levels of β -glucuronidase.

Although expression from Moloney-based proviral vectors is stable in the majority of transduced fibroblasts after long term passage in culture, even in the absence of selection, we and others have shown that silencing occurs relatively rapidly after transplantation in the mouse. However, in a small percentage of cells, expression can persist even after long term passage *in vivo* (10, 12, 14, 15). We applied the FACS assay to determine whether the population of transduced fibroblasts expressing β -glucuronidase after primary transplantation was resistant to silencing upon secondary transplantation. Neo-organs were used to simplify the retrieval of transplanted cells (11), which could then be sorted on the basis of β -glucuronidase expression and expanded *ex vivo* prior to secondary transplantation. Because the DCH β H vector also encodes the neomycin resistance gene, cells could also be selected on the basis of G418 resistance. Our results clearly demonstrate that *in vivo* passage followed by selection (either sorting or drug administration) yields a subpopulation of cells with a significantly higher probability of maintaining expression upon transplantation than the original population of transduced cells. We hypothesize that this population is composed of clones in which the proviral integration sites support long term expression of the therapeutic gene, and primary transplantation essentially serves as an *in vivo* selection step. Determining what factors are involved in retroviral silencing in the *in vivo* milieu would be of particular use in the development of *ex vivo* selection protocols.

The secondary transplants derived from the G418-selected population had higher percentages of long term stable expressing cells and proportionally higher levels of β -glucuronidase activity compared with the secondary transplants of sorted cells. We hypothesize that the sorted population included both transduced and cross-corrected cells, whereas the drug-selected population contained only vector-transduced cells. This was supported by the observation that the sorted primary cells used for the secondary neo-organ were 88.0% positive by cytochemical stain (94.4% by FACS) but decreased to 49.9% positive after several passages *in vitro* (data not shown). Because transduced cells show stable expression after *in vitro* passage (Fig. 5 and Refs. 13, 25, and 39), the cells that became negative were probably cross-corrected rather than vector-transduced. This is concordant with our previous experiments that showed that cross-corrected MPS VII cells rapidly lose β -glucuronidase activity when a high concentration source of donor enzyme is removed, as a result of normal proteolytic degradation of the endocytosed protein in the lysosome (25). The neomycin phosphotransferase protein is not transferred between cells and thus may provide a more stringent means of selection, but the disadvantages associated with the inclusion of a drug-selectable marker make the sorting approach more expedient for clinical applications. Furthermore, cells expressing the vector at low levels may be selected against by G418. This would be a disadvantage where it is desirable to transplant as many positive cells as possible, such as with hematopoietic stem cells (10, 46). The sorting method can be used to select the transduced cells from an infected population where no selectable marker is included in the vector design (35)³ and may thus result in better overall delivery of the therapeutic protein *in vivo*.

Our results demonstrate that fibroblasts showing stable proviral expression after passage *in vivo* can be isolated by cell sorting on the basis of PFBFDGlcu hydrolysis and have a higher probability of maintaining prolonged expression *in vivo* than the original drug-selected population. The implications of

these results for gene therapy-based treatments are significant. *In vivo* passage of transduced fibroblasts and perhaps other cell types, followed by an *ex vivo* selection step, such as cell sorting, may yield a subpopulation of cells harboring proviral integrants with a significantly higher probability of stably expressing the relevant therapeutic gene upon secondary transplantation. Incorporating such a selection step may improve the clinical outcome of somatic cell gene therapy-based approaches.

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