

# Enzyme-Generated Intracellular Fluorescence For Single-Cell Reporter Gene Analysis Utilizing *Escherichia coli* $\beta$ -Glucuronidase

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We report the development of a new fluorescence-activated cell sorter (FACS)-based reporter gene system utilizing the enzymatic activity of the *E. coli*  $\beta$ -glucuronidase (*gus*) gene. When loaded with the Gus substrate fluorescein-di- $\beta$ -D-glucuronide (FDGlcu), individual mammalian cells expressing and translating *gus* mRNA liberate sufficient levels of intracellular fluorescein for quantitative analysis by flow cytometry. This assay can be used to FACS sort viable cells based on Gus enzymatic activity, and the efficacy of the assay can be

measured independently by using a fluorometric lysate assay. Furthermore, both the  $\beta$ -glucuronidase and the previously described *E. coli*  $\beta$ -galactosidase enzymes have high specificities for their cognate substrates, allowing each reporter gene to be measured by FACS independently.

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**Key terms:**  $\beta$ -Glucuronidase,  $\beta$ -galactosidase, reporter gene, fluorescence-activated cell sorting, selectable marker

The measurement of *lacZ* reporter gene activity on the fluorescence-activated cell sorter (FACS-Gal) has proven to be a useful tool in studies of gene regulation at the single-cell level (5,15,17). The FACS-Gal system permits the quantitative, sensitive analysis of gene expression on a single-cell basis and allows for simultaneous measurement of other cellular parameters, such as cell size, surface immunophenotype, DNA content, etc. (6). In addition, the sorting capability of the FACS can be used to isolate viable single cells based on *lacZ* expression, making the FACS-Gal assay a nonlethal selectable marker system as well as a quantitative reporter gene system.

Recent technological advances in the methodologies of introducing genes into whole organisms has made the study of gene expression in vivo a reality. FACS-Gal can be used to isolate cells expressing *lacZ* from transgenic or chimeric animals, as long as those cells can be processed into single-cell suspensions (15). Soriano et al. have generated several murine transgenic strains with the *lacZ* gene under the control of endogenous promoters with ubiquitous expression patterns (7,26).  $\beta$ -gal Activity was detected in the lymphocytes of several of these transgenic lines, using the FACS-Gal system (26; W.T. Kerr, personal communication).

Although *lacZ* has proven to be of general utility in the study of developmentally regulated gene expression in mice (1),  $\beta$ -gal activity has not been detected in lymphocytes isolated from a large number of independently gen-

erated transgenic or chimeric mice, in which the *lacZ* gene was inserted into the genome under the control of promoters/enhancers known to be expressed in lymphocytes. This absence of activity has been described for *lacZ* under the control of tissue-specific (25) or ubiquitous (for review, see 5,22) regulatory elements. In several mice that were examined, *lacZ* mRNA was not detected, suggesting that the block is due to transcriptional silencing or to mRNA instability (5). The clear lack of  $\beta$ -gal expression in developing lymphocytes in many systems is an impediment to analysis of immune function. Establishment of a reporter system not subject to this limitation would be of general use to those interested in studying lymphocyte gene expression in vivo.

There are several requirements for an enzymatic reporter system to be useful for flow cytometry studies. These include 1) an enzyme with stable in vivo activity; 2) a fluorogenic substrate that can be loaded into viable

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cells which, upon hydrolysis, yields a highly fluorescent product that is retained in cells; 3) a low level of endogenous enzymatic activity for this fluorogenic substrate; and 4) low levels of cellular fluorescence at the wavelengths at which the product fluorescence is detected. We limited our search for new reporter genes to hydrolases due to the availability of greater than 300 cloned and characterized members of this class of enzyme (10). Furthermore, interest in hydrolases has led to the development of a number of commercially available fluorogenic hydrolase substrates.

Of ten cloned hydrolases, including cellulases,  $\beta$ -glucosidases, and *E. coli*  $\beta$ -glucuronidase (*gus*), only the *Gus* enzyme and a fluorescein-based substrate satisfied the above criteria. The *gus* gene has been used extensively as a reporter gene in plants, because there is no endogenous cellular glucuronidase activity in the majority of plants tested (11,12). Although high levels of lysosomal  $\beta$ -glucuronidase activity have been reported for a number of mammalian tissue types (19), we find very low *Gus* activity in lysates of several mammalian cell lines. In this study, we show that, in combination with FACS, a *gus* reporter gene system can be used both in quantitative expression studies and as a selectable marker. Furthermore, we show that the  $\beta$ -galactosidase and  $\beta$ -glucuronidase enzymes will hydrolyze only their cognate substrates, allowing for the development of a dual reporter system based on these hydrolases.

## MATERIALS AND METHODS

### Cells and Tissue Culture

NIH 3T3 cells (ATCC CCL 163) and BOSC 23 retroviral producer cells (21) were maintained in Dulbecco's modified Eagle's medium (DMEM). 70Z/3 cells (ATCC TIB 155) were maintained in RPMI 1640 medium. All media were supplemented with 10% (v/v) 1:1 fetal calf serum, 50 nM 2-mercaptoethanol, 100 units/ml penicillin, 0.05 mM streptomycin, and 2 mM glutamine. All cultures were maintained at 5% CO<sub>2</sub>.

### Transient Transfection and Retroviral Infections

BOSC 23 producer cells were plated at a density of  $2 \times 10^6$  cells per 60 mm plate in 3 ml of fresh supplemented DMEM. After 24 h, these cells were calcium-phosphate transfected, as previously described (21), with 5  $\mu$ g of plasmid DNA. Forty-eight hours later, retroviral supernatant was harvested and pelleted (5 min at  $\times 1,200$  g) to remove cell debris. One milliliter of retroviral supernatant was added to  $10^6$  NIH 3T3 or 70Z/3 cells preincubated for 5 min in the presence of 10  $\mu$ g/ml polybrene (21). After 8 h, the cells were washed, resuspended in polybrene-free growth medium, and cultured for at least 48 h prior to reporter gene expression analyses.

### Chemicals

5-Bromo-4-chloro-3-indoylgalactoside (X-gal) was obtained from Sigma. 5-Bromo-4-chloro-3-indoylglucuronide (X-GlcU), fluorescein-di- $\beta$ -D-galactoside (FDG), fluorescein-di- $\beta$ -D-glucuronide (FDGlcU), methylumbel-

liferyl-galactoside (MUG), methylumbelliferyl-glucoside (MUGlcU), methylumbelliferyl-glucuronide (MUGlcU), and the  $\beta$ -gal inhibitor phenylethylthio-galactoside (PETG) were generously supplied by R. Haugland and Ian Johnson (Molecular Probes, Eugene, OR). The fluorescein and methylumbelliferyl (MU) substrates were stored as previously described (22).

### MUG, MUGlcU, and MUGlcU Cell Lysate Analyses

MU assays were carried out as described for MUG (22). Briefly,  $2 \times 10^4$  cells were lysed in a 0.125% (v/v) Triton X-100-based lysis buffer, and the MU substrates were added to a final concentration of 0.6 mM. The reactions were carried out at 25°C before the addition of stop buffer (15 mM EDTA, 300 mM glycine, pH 11.2). The fluorescent MU product was measured on a 96-well plate with a Fluoroskan II fluorescence plate reader (Flow Labs, McLean, VA; excitation at 355 nm, emission at 460 nm). Substrates were titrated into lysates of *gus*<sup>+</sup> or *lacZ*<sup>+</sup> 3T3 cells for the purpose of determining *K<sub>m</sub>* values for  $\beta$ -glucuronidase and  $\beta$ -gal, respectively. Fluorescence intensities of at least eight samples per titration were plotted by using the Michaelis-Menten equation and were fitted by nonlinear least-squares regression.

### FDG and FDGlcU Staining and FACS Analysis and Sorting

70Z/3 cells or trypsinized 3T3 fibroblasts were pelleted and resuspended at a concentration of  $4 \times 10^6$ /ml in 50–100  $\mu$ l of staining medium (biotin- and flavin-deficient RPMI supplemented with 4% v/v FCS) at 37°C. Cells were mixed 1:1 (vol:vol) with 2 mM substrate (FDG or FDGlcU) dissolved in water (also equilibrated to 37°C), exposing the cells to 50% tonicity. After incubating for 1 min at 37°C, the loaded cells were restored to isotonicity with the addition of 10 volumes of ice-cold staining medium [which prevents passage of the substrate and product(s) across the cell membrane]. The cells were pelleted and resuspended twice in 2 ml of 0°C staining medium to remove any fluorescent product that might have leaked out of cells during the loading procedure. The final resuspension medium was supplemented with 1  $\mu$ g/ml propidium iodide for fluorescent staining of dead cells. The 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 (17).

### Fluorescence Microscopy

NIH 3T3 fibroblasts were plated on chambered borosilicate coverglass plates (Nunc Inc., Naperville, IL) and loaded by using the conditions described for FACS analysis. Loaded cells were fixed with 0.05% glutaraldehyde in phosphate-buffered saline (PBS), washed with 2 ml PBS, and observed on a fluorescence microscope (Zeiss) through a  $\times 63$  immersion objective. A mercury vapor lamp in combination with a 465–495 nm band-pass filter was used for fluorescein excitation. Emitted light was collected with a 522–547 nm band-pass filter. For the

brightfield images, the emission band-pass filter was removed, leaving a 480 nm high-pass filter. Photographs were taken with a Minolta X-370 camera.

#### Specific Inhibition of $\beta$ -Glucuronidase With 1,4 Saccharolactone (1,4-SL), and $\beta$ -Gal With PETG

Prior to FDGlcu substrate loading, 100  $\mu$ l of NIH 3T3 fibroblasts were resuspended in staining medium plus 1,4-SL and were incubated for 10 min at 37°C. The cells were maintained in 1,4-SL at a final concentration of 5 mM (unless otherwise indicated) throughout the course of the experiment. Cells were loaded as described above by mixing with 100  $\mu$ l of a 2 mM FDGlcu + 1,4-SL solution. The cells were washed in the presence of the inhibitor and were resuspended in staining medium, including 1  $\mu$ g/ml propidium iodide and 1,4-SL. Diminution of  $\beta$ -gal activity with PETG was carried out as described (12) by using a final concentration of 100  $\mu$ M PETG.

## RESULTS

### Analysis of Candidate Reporter Genes

Lysate assays of *E. coli* harboring expression vector plasmids of ten candidate reporter enzymes revealed that only *E. coli*  $\beta$ -glucuronidase (*Gus*), the thermophilic bacterium *Clostridium thermocellum* cellulase (*CelE*; 9), and the *Butyrivibrio fibrosolvens*  $\beta$ -glucosidase (*bglA*; 28), isolated from high-arctic Svalbard reindeer, showed high specificity for their cognate substrates (data not shown). The *Gus*, *CelE*, and *bglA* genes were subsequently cloned into the Moloney-based retroviral expression vector MFG (4) and were tested for mammalian expression. Only the *Gus* gene showed detectable activity when transfected into the human embryonic kidney 293 cell line (data not shown), and this gene was chosen for further analysis.

### Expression of the $\beta$ -Glucuronidase and $\beta$ -Galactosidase Enzymes

To maximize expression of the *E. coli Gus* gene in mammalian cells, several modifications were made at the 5' end of the gene by using polymerase chain reaction (PCR) mutagenesis. The 5' primer was designed to alter 1) the nucleotides surrounding the wild type met, codon to conform to the core Kozak consensus sequence for translational initiation (CCATGG) in mammalian cells (14) and 2) the second codon to encode valine, a stability conferring residue, as established by Varshavsky's N-end rule (2; Fig. 1A). The amplified 1,809-base-pair, full-length gene was cloned into the MFG vector (Fig. 1B), permitting expression from the long terminal repeat (LTR) enhancer. Several clones tested positive for the *Gus* insert by using PCR and restriction analysis, and they were isolated for further study. One of these plasmid clones (MFG-Gus6), when transiently transfected into mammalian 293 cells, showed  $\beta$ -glucuronidase activity, as determined by the MUGlcu assay. This plasmid was used in all further studies.

To study  $\beta$ -glucuronidase expression in mammalian

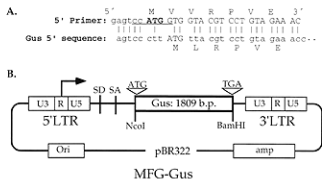


Fig. 1. Construction of pMFG-Gus. The 1,809-base-pair *E. coli*  $\beta$ -glucuronidase gene was amplified by using polymerase chain reaction (PCR) and was cloned into the NcoI-BamHI sites of MFG (see Materials and Methods). A: The 5' primer was designed to include 1) the consensus nucleotides for mammalian translational initiation (underlined) and 2) the amino acid valine at the second codon, conferring protein stability in mammalian cells. The template *E. coli*  $\beta$ -glucuronidase (*Gus*) gene sequence is shown on the lower strand, including the wild type Met, codon. Vertical lines indicate complementary base pairs. Amino acids are indicated by the single letter code. B: The orientation of the  $\beta$ -glucuronidase gene with respect to the MMULV-derived retroviral vector MFG is shown, including the translational start and stop codons. The splice donor (SD) and splice acceptor (SA) sequences are located upstream of the inserted *Gus* gene, and the 5' and 3' long terminal repeats (LTRs) are also shown.

cells, several cell lines with stable expression of the *Gus* gene were generated by retroviral transfer. The MFG-Gus6 clone was transiently transfected into BOSC producer cells, and the viral supernatant was isolated 48 h postinfection. Subsequently, cells of the adherent murine fibroblast line NIH 3T3 were infected by using the viral supernatant. One week later, infected cells were tested for the efficiency of infection with the X-Glcu histological stain (11,13; data not shown). Greater than 90% of the cells stained blue, indicating that most, if not all, of the target cells were stably infected with MFG-Gus6 and were expressing the reporter enzyme from an integrated provirus. In contrast, mock-infected cells showed no detectable X-Glcu staining.

Because the FDGlcu substrate is chemically similar to FDG, we reasoned that FDGlcu could be used with a protocol analogous to FACS-Gal to distinguish *Gus*<sup>+</sup> from *Gus*<sup>-</sup> cells based on the levels of fluorescein fluorescence. FACS analysis of FDGlcu-loaded, MFG-Gus6-infected cells showed two distinct populations at a ratio roughly equivalent to that revealed by the X-Glcu stain (data not shown). By using the single-cell deposition capability of the FACS (20), clones were generated by sorting MFG-Gus6-infected cells that fluoresced when loaded with FDGlcu. After expanding the cloned cells for 2 weeks, 30 clones were tested for  $\beta$ -glucuronidase activity by using the substrate MUGlcu in the quantitative cell lysate assay (22). All of the clones showed *Gus* activity above background levels (data not shown). The 3T3 MFG-Gus6-6 clone was chosen on the basis of a high level of expression for further study. A similar approach was used to isolate *Gus*<sup>+</sup> clones of the nonadherent murine

Table 1  
Determination of  $\beta$ -Gal, Gus, and Endogenous Enzymatic Activity in 70Z/3 Pre-B Cells and NIH 3T3 Fibroblasts

Substrate	$K_m^a$	Uninfected		MFG-Gus6		MFG-lacZ	
		70Z/3	3T3	70Z/3	3T3	70Z/3	3T3
MUG <sup>b</sup>	107	41	53	58	73	2,050	4,050
MUGlcu	119	93	10	6,520	13,300	91	17
FDG <sup>c</sup>	17	2.9	1.3	2.3	1.6	59.0	342.0
FDGlcu	133	7.2	1.4	26.0	33.0	nd	1.5

<sup>a</sup> $K_m$  values are expressed in  $\mu$ M substrate. The nonlinear least-squares regression coefficient was greater than 0.99 for each determination.  $\beta$ -gal,  $\beta$ -galactosidase; gus,  $\beta$ -glucuronidase.

<sup>b</sup> $1 \times 10^6$  70Z/3 cells or  $2 \times 10^5$  3T3 cells were harvested and lysed in Z-buffer. Lysates were incubated in the presence of methylumbelliferyl (MU) substrate for 25 min before the addition of stop buffer. The values indicated are relative fluorescence units, as measured by fluorometry, per lysed sample. Typically, errors in measurement are less than 10% using these assays. For substrate abbreviations, see Materials and Methods.

<sup>c</sup>Fluorescence-activated cell sorter (FACS) analysis of fluorescent substrate hydrolysis was conducted as described in Materials and Methods. Tabulated values show the median FACS fluorescence for each stained cell population. nd, not done.

pre-B 70Z/3 cell line as well as several  $\beta$ -gal<sup>+</sup> 3T3 and 70Z/3 clones.

### Specificity of $\beta$ -Glucuronidase and $\beta$ -Galactosidase for Their Cognate Substrates

To establish whether Gus enzymatic activity can be detected independently of  $\beta$ -Gal enzymatic activity, and vice versa, we characterized the enzymatic specificities of both enzymes vs. several glucuronide- and galactoside-based substrates. To ensure that the initial rates of reaction were not dependent on substrate concentration, the  $K_m$  values of  $\beta$ -gal with MUG and of  $\beta$ -glucuronidase with MUGlcu in the context of cell lysates were determined. A concentration of the substrate that was well above the  $K_m$  value of either reporter enzyme, 0.6 mM (Table 1), was chosen as a suitable concentration for the lysate experiments.

$\beta$ -Glucuronidase and  $\beta$ -galactosidase specificities were analyzed with the cell lysate assay using MU-based substrates and with the FACS-Gal assay using fluorescein-based substrates. Under both experimental conditions, 70Z/3 and 3T3 parental (untransfected) cells showed low or undetectable levels of enzymatic activity vs. these substrates (Table 1), indicating that the endogenous activity of either enzyme is very low. Furthermore, cells expressing  $\beta$ -gal or  $\beta$ -glucuronidase hydrolyzed only the galactoside-based substrates (MUG and FDG) or the glucuronide-based substrates (MUGlcu or FDGlcu), respectively (Table 1). Similar results were found by using the chromogenic X-gal (23) and X-Glu assays (data not shown). Thus, each of these enzymes can be quantitated independently of the expression of the other.

### FACS Detection of NIH 3T3 *gus*<sup>+</sup> Cells Loaded With FDGlcu

Our initial results showing that Gus activity could be detected in individual cells prompted us to explore the

broader utility of this enzyme-substrate system. We used the FACS-Gal procedure to load NIH 3T3 parental cells, NIH 3T3 MFG-Gus6-6 cells, or a mixture of the two cells with FDGlcu. We chose to use 2 mM FDGlcu in the hypotonic loading step, because the  $K_m$  of Gus for FDGlcu is comparable to that of  $\beta$ -gal for FDG (Table 1), and 2 mM FDG is sufficient to detect  $\beta$ -gal in the FACS-Gal assay (6).

Consistent with the results of the lysate experiments, NIH 3T3 parental cells loaded with FDGlcu are no more fluorescent than unstained cells (Fig. 2A). However, *gus*<sup>+</sup> cells loaded with FDGlcu developed fluorescence levels 40-fold greater than *gus*<sup>-</sup> parental cells (compare Fig. 2A and Fig. 2B). When a 1:1 mix of *gus*<sup>+</sup> and *gus*<sup>-</sup> cells is loaded with FDGlcu, the two populations can be resolved clearly (Fig. 2C). Furthermore, there is no leakage of fluorescein from the *gus*<sup>+</sup> into the *gus*<sup>-</sup> cells, because the *gus*<sup>-</sup> cells loaded in the presence of *gus*<sup>+</sup> cells are no more fluorescent than *gus*<sup>-</sup> cells loaded alone (compare Fig. 2A and Fig. 2C). Thus, *gus*<sup>+</sup> cells generate significant fluorescein based on intracellular recombinant reporter protein expression. Comparable results were obtained by using  $\beta$ -gal in the FACS-Gal procedure (17).

### Subcellular Localization of Fluorescein After Gus-Mediated FDGlcu Hydrolysis

To determine the subcellular localization of the fluorescein product of Gus-mediated FDGlcu hydrolysis, parental (Fig. 3A) or MFG-Gus6-6 (Fig. 3B) NIH 3T3 cells were loaded with FDGlcu and analyzed by fluorescence microscopy. Parental cells show low or undetectable levels of green fluorescence (Fig. 3C). Any signal is likely to be due to autofluorescence, because cells imaged in the absence of substrate show a similar low-level fluorescence pattern (data not shown). In contrast, *Gus*<sup>+</sup> cells show bright, exclusively cytoplasmic fluorescence (Fig. 3D) when they are imaged under identical conditions.

### Temperature Dependence of $\beta$ -Glucuronidase Activity

An inherent difficulty in the use of fluorescein-based substrates is the fact that the cell membrane is permeable to fluorescein under physiological conditions. However, fluorescein leaks out of cells >200 times slower at 0°C than at 37°C (24). To avoid fluorescein staining of *lacZ*<sup>-</sup> cells as a result of leakage from *lacZ*<sup>+</sup> cells, the FACS-Gal protocol calls for incubation of FDG-loaded cells at 0°C rather than at 37°C after loading. Because  $\beta$ -gal still has appreciable enzymatic activity at 0°C (17), hydrolysis of the FDG substrate is not prevented under these conditions.

To establish conditions suitable both for  $\beta$ -glucuronidase catalyzed FDGlcu hydrolysis and for intracellular fluorescein retention, the temperature dependence of the Gus enzyme was determined in an MU cell lysate assay (Fig. 4). Although Gus is not active at 0°C, the enzyme shows a reduction in activity of only twofold at 10°C vs. 37°C (Fig. 4). Thus, if hydrolysis beyond the first min of loading is required, then incubation can be carried

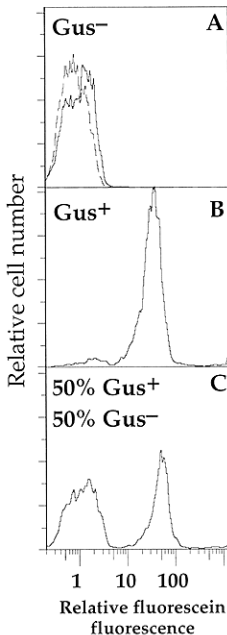


FIG. 2. 3T3 MFG-Gus6-6 (*gus*<sup>+</sup>) cells can be distinguished from 3T3 parental (*gus*<sup>-</sup>) cells by using the fluorescence-activated cell sorter (FACS). Cells were loaded with 2 mM fluorescein-di- $\beta$ -D-glucuronide (FDGlcu) for 1 min at 37°C, as described in Materials and Methods. A: Unstained 3T3 parental cells (dashed line) vs. 3T3 parental cells loaded with FDGlcu (solid line). B: NIH 3T3 MFG-Gus-infected cells loaded with FDGlcu. Note that approximately 10% of these cells are *gus*<sup>-</sup>, as measured by 5-bromo-4-chloro-3-indoilyglucuronide (X-Glcu), accounting for the low percentage of cells that appear to be *gus*<sup>-</sup> by using FACS. C: 3T3 MFG-Gus and 3T3 parental cells were mixed 1:1 (prior to loading with FDGlcu) and were analyzed by using FACS.

out at or above 10°C. To test whether fluorescein passes the cell membrane at 10°C, 3T3 cells were loaded with fluorescein, as described above for FDG, washed, and incubated for up to 4 h at 0°C or at 10°C before FACS

analysis. Fluorescence levels of cells maintained at 10°C were indistinguishable from those of cells maintained at 0°C after loading (data not shown), indicating that the fluorescein product is retained within the cell at 10°C.

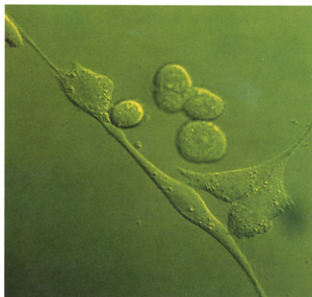
#### The Competitive $\beta$ -Glucuronidase Inhibitor 1,4-SL Inhibits Hydrolysis of the FDGlcu Substrate *In Vivo*

To maintain a correlation between enzyme activity and fluorescence, the enzymatic reaction must not lead to the hydrolysis of all of the available substrate within a cell. In our 3T3 *gus*<sup>+</sup> clones, no time-dependent increase in the fluorescence levels of stained cells was observed at 10°C (data not shown), suggesting that these cells express levels of Gus sufficient to hydrolyze all of the available FDGlcu during the loading step. Thus, the distribution of fluorescence levels seen in Figure 2B is not a reflection of the enzymatic activity within the *gus*<sup>+</sup> 3T3 clone but, rather, it is an indication of the uniformity of loading of the FDGlcu substrate.

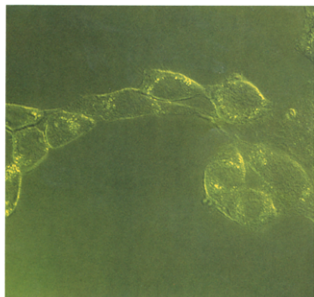
Distinguishing between 3T3 cells expressing relatively low vs. high levels of Gus requires slowing the reaction to avoid the hydrolysis of all available substrate into fluorescent product. A previously identified  $\beta$ -glucuronidase inhibitor, 1,4-SL (16), was tested for its loading properties and for its effect on the hydrolysis of FDGlcu by  $\beta$ -glucuronidase in viable cells. Pools of MFG-Gus6-infected cells were hypotonically loaded with mixtures of 2 mM FDGlcu and several different concentrations of 1,4-SL and were analyzed by FACS. In the absence of inhibitor, the median fluorescence of the *gus*<sup>+</sup> cells was >30-fold above background (Fig. 5B). In the presence of concentrations of 1,4-SL from 0.2 to 5.0 mM, there was a clear concentration-dependent decrease in the fluorescence distribution of the *gus*<sup>+</sup> cells (Fig. 5C-E). These results indicate that loading cells with 5 mM 1,4-SL sufficiently inhibits  $\beta$ -glucuronidase activity to allow quantitative analysis of expression by FACS.

#### Relative Gus Activity as Determined by FACS is Correlated With Relative Activity as Determined by the MUGlcu Lysis Assay

When loaded with FDGlcu in the presence of 1 mM 1,4-SL, the clonally mixed pool of 3T3 MFG-Gus6-infected cells shows a broad distribution of FACS-measured fluorescence (Fig. 5D). To test whether this distribution is indicative of the relative Gus activity of cells within the *gus*<sup>+</sup> population, 13 fractions were sorted on the basis of fluorescein fluorescence (Fig. 6A), such that each fraction included cells of a unique, narrow range of fluorescence. The sorted populations were then lysed and assayed by the MUGlcu assay to determine the relative enzymatic activity of each fraction. Plotting the FACS-determined mean fluorescence of each sorted sample against the fluorescence as determined in the lysate assay reveals a strong linear correlation between these independent assays (Fig. 6B). However, the positive X-intercept suggests that a fraction of the reporter enzyme is accessible to substrate only upon lysis of the cell. Never-



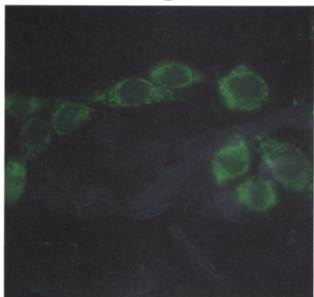
A



B



C



D

Fig. 3. Subcellular localization of the hydrolysis product, fluorescein. Cells were loaded with 2 mM FDGlu for 2 min at 37°C and were processed as described in Materials and Methods. Brightfield images of loaded 3T3 parental (A) or MFG-Gus6-6 (B) cells were taken by using differential interference contrast optics with exposures of approximately

theless, this experiment indicates that fluorescence measured by FACS reflects Gus activity *in vivo*.

#### DISCUSSION

Several properties of the Gus reporter system are useful in studies of mammalian gene expression. The *Gus* gene is only 1,800 base pairs in size (vs. the 3,400-base-pair *lacZ* gene), allowing for its use in DNA constructs with functional size constraints, such as retroviral vectors. The Gus enzyme, like  $\beta$ -gal, is extremely stable under a variety of conditions (e.g., in the presence of 0.1% v/v Triton

3 s. Fluorescence images of 3T3 parental (C) or MFG-Gus6-6 (D) cells were taken of the same fields as in A and C, respectively. Fluorescence images were exposed for 2 min. Cells imaged in the absence of the glutaraldehyde fix showed similar fluorescence distributions.

X-100) and over a broad pH range (13,15). The Gus and  $\beta$ -gal hydrolases are evolutionarily related (10), with a 30% amino acid identity (determined by using the FASTDB-Intelligenetics suite) over a region previously shown to be conserved in such hydrolases (8). Because they have evolved in the same organism, these enzymes have presumably been selected to operate optimally under comparable biochemical conditions. Although the Gus enzyme is tetrameric in its native state, it retains enzymatic activity when fused to the C-terminus of heterologous polypeptides (13), much like  $\beta$ -gal (18),

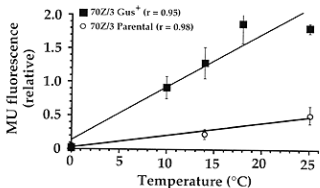


Fig. 4. Temperature dependence of  $\beta$ -glucuronidase. 70Z/3 MFG-Gus6 cells (squares) were lysed in Z-buffer, and titrations of cell extract were equilibrated to 0, 10, 14, 18, and 25°C. MU/Glcu was added to a final concentration of 0.6 mM. At 30, 90, and 240 min, aliquots were removed from each titration point (equivalent to  $2.5 \times 10^5$ ,  $1.25 \times 10^5$ , and  $6.25 \times 10^4$  cell equivalents), and the reactions were stopped by the addition of ice-cold stop buffer. Each point on the graph represents the reaction rate as measured at each time point on a Fluoroskan fluorometer, normalized to  $2.5 \times 10^5$  cell equivalents for all samples. 70Z/3 parental (*gus*<sup>-</sup>) cells (circles) were also measured to determine endogenous  $\beta$ -glucuronidase activity at 0, 14, and 25°C. Vertical bars denote the standard deviation of the fluorescence levels when normalized to  $2.5 \times 10^5$  cell equivalents.

allowing for translational fusions to genes of interest in heterologous promoter expression studies.

When cells are loaded with 2 mM FDGlcu in the presence of 1 mM 1,4-SL, a strong linear correlation is found between the relative fluorescence of the *gus*<sup>+</sup> population as measured by FACS, with the fluorescence as measured in the lysis assay (Fig. 6). This is in contrast to the *lacZ* system, which, for unknown reasons, shows exponential enzyme kinetics in vivo (22). Nevertheless, the high specificities of the Gus and  $\beta$ -gal enzymes for FDGlcu and FDG, respectively, suggest that the "FACS-Gus" system could be used in combination with FACS-gal in sequential analyses.

The hypotonic-loading experiments indicate that FDG is loaded into cells at a significantly higher concentration than the FDGlcu substrate (data not shown). Because the fluorescein moiety is identical in these substrates, the distinct loading properties of the different substrates must be due to the different chemical structures of the glucuronide and galactoside sugars (a carbonyl group vs. a hydroxyl group, respectively). 70Z/3 MFG-Gus6 cells loaded with FDGlcu show a lower signal-to-background ratio after complete substrate hydrolysis than do the NIH 3T3 cell lines (similar to results obtained with FACS-Gal). Thus, although sufficient levels of FDGlcu are loaded into fibroblast cells, allowing for resolution of *gus*<sup>+</sup> from *gus*<sup>-</sup> cells (Fig. 2), smaller cells, such as lymphocytes, might require longer loading times to reach FDGlcu substrate levels sufficient for the complete separation of *gus*<sup>+</sup> from *gus*<sup>-</sup> cells.

We found that millimolar concentrations of the glucuronidase inhibitor 1,4-SL were required to inhibit the hydrolysis of FDGlcu to levels sufficient for quantitative

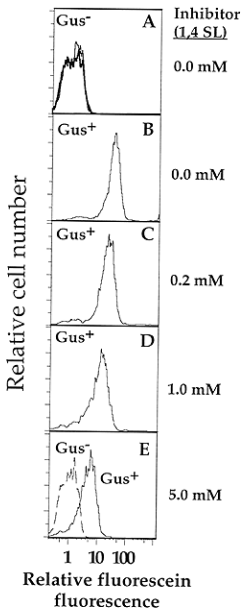


Fig. 5. The inhibitor 1,4 saccharolactone effectively inhibits  $\beta$ -glucuronidase activity in viable MFG-Gus6-infected cells. NIH 3T3 MFG-Gus6-infected (*gus*<sup>+</sup>) and NIH 3T3 parental (*gus*<sup>-</sup>) cells were centrifuged and resuspended in staining medium alone or in the presence of 1,4 saccharolactone (1,4-SL). Each sample was transferred to 37°C and incubated for 10 min prior to loading. A: *gus*<sup>-</sup> Cells were loaded in the presence (thick line) or absence of FDGlcu. *gus*<sup>+</sup> Cells were preincubated with and loaded in the presence of no inhibitor (B), 0.2 mM inhibitor (C), 1 mM inhibitor (D), or 5 mM inhibitor (E). Included in E is the fluorescence profile of parental NIH 3T3 cells loaded in the presence of FDGlcu and 5 mM 1,4-SL (dashed line). The median fluorescence measurements for each sample were as follows: A, +FDGlcu 1.1, -FDGlcu 1.0; B, 33.0; C, 20.2; D, 11.3; E, *gus*<sup>+</sup>, 5.2, *gus*<sup>-</sup>, 1.0.

analysis by FACS (Fig. 5). By titrating 1,4-SL in the presence of a constant concentration of MU/Glcu and *Gus*<sup>+</sup> cell extract, we determined the  $K_i$  to be approximately 100  $\mu$ M (data not shown). These results suggest that cells are loaded with a concentration of inhibitor significantly

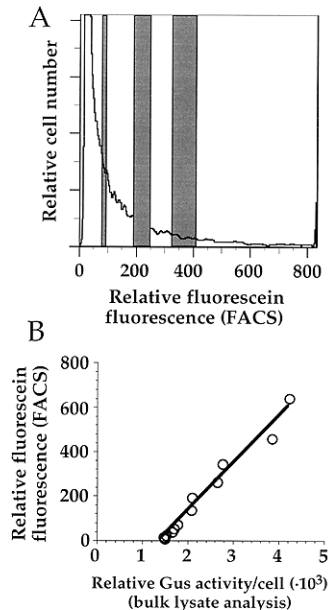


Fig. 6.  $\beta$ -Glucuronidase activity determined on a cell-by-cell basis is an accurate reflection of enzymatic activity in lysates. MFG-Gus-6-infected NIH 3T3 cells were loaded with 2 mM FDGlu in the presence of 1 mM 1,4-SL, as described in Materials and Methods. Linear gating mode ( $\times 2$ ) was set to sort 13 pools of  $5 \times 10^4$  to  $2 \times 10^4$  cells spanning the distribution on the basis of fluorescein fluorescence levels into a 96-well dish. A: Three examples (shaded bars) of the 13 sets of gates used in the experiment are shown. B: Z-buffer was added to the sorted pools to lyse the cells followed by the addition of MUGlu to a final concentration of 0.6 mM. Each data point represents the mean fluorescence, as determined by FACS for each sorted population plotted, vs. the total  $\beta$ -gal activity of the sorted pools, as revealed by the methylumbelliferone (MU) fluorescence of the corresponding cell lysate sample (normalized to 1,000 cell equivalents). The diagonal line indicates the linear least-squared regression.

lower than is present in the surrounding medium, even when the inhibitor is present during the hypotonic treatment of cells.

Previous application of the FACS-Gal system demonstrated the utility of an assay that provides information on gene expression on a single-cell basis. Because the *gus* gene shares only limited homology with *lacZ*, and the *Gus* enzyme has a different glycolytic substrate specificity than  $\beta$ -gal, it is possible that the *Gus* reporter system will circumvent the limitations ascribed to *lacZ* in hematopoietically derived cells. The FACS-Gus system described in this study should be useful as an alternative to the FACS-Gal system in studies where  $\beta$ -gal is inadequate and in combination with the FACS-Gal system under conditions where both genes are expressed.

Recently, we have explored the utility of the FACS-Gus assay in measuring levels of endogenous  $\beta$ -glucuronidase activity in whole animals. Absence of  $\beta$ -glucuronidase activity in mouse and man results in the lysosomal storage disorder Mucopolysaccharidosis type VII (MPSVII; 3). By using the murine model of MPSVII (27), we have established conditions that allow for the quantitation of endogenous lysosomal activity as well as for sorting of cells based on this activity (manuscript in preparation). This approach may be used in gene transfer therapy to enrich cells that have been transduced with  $\beta$ -glucuronidase prior to reinfusion of patients with the disease.

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