

Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β -D-galactosidase activity after transduction of *Escherichia coli lacZ*

(reporter molecule/fluorescein digalactoside/retrovirus/promoter assay)

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ABSTRACT We demonstrate that individual cells infected with and expressing a recombinant retrovirus carrying the *Escherichia coli* β -galactosidase gene (*lacZ*) can be viably stained, analyzed, sorted, and cloned by fluorescence-activated cell sorting based on the levels of *lacZ* expressed. To accomplish this we have devised a method to enzymatically generate and maintain fluorescence in live mammalian cells. Accumulation of fluorescent products in cells is linear with time, with a direct correlation of fluorescence to enzymatic activity. This technology for β -galactosidase detection is more sensitive than other available cytochemical or biochemical methods. We have used this procedure to show that the expression of ψ -2-MMuLVSVnslacZ in the T-cell lymphoma BW5147 and the B-cell hybridoma SP2/0 is not completely stable and that subclones selected by the fluorescence-activated cell sorter for low *lacZ* activity demonstrate distinctly lower average expression of LacZ. These findings indicate the utility of β -galactosidase as a reporter molecule at the single-cell level for studies of gene regulation, including studies of promoter efficacy, enhancer activity, trans-acting factors, and other regulatory elements.

The *Escherichia coli lacZ* gene has previously been adapted for use in mammalian cells and has shown utility as a marker for the expression of chimeric genes (1, 2). In these previous studies, the chromogenic β -galactoside analog *o*-nitrophenyl β -D-galactopyranoside (ONPG) was used as an alternative to the chloramphenicol acetyltransferase assay (3) to quantitate gene expression from heterologous promoters fused 5' proximal to the *lacZ* structural gene. However, in both the chromogenic LacZ and radioactive chloramphenicol acetyltransferase assays, extracts of bulk populations of cells are used to quantitate an average promoter strength/enzyme activity and, thus, do not give any indication of the heterogeneous expression patterns that could exist within complex cell populations. These other assays also do not provide one with a means to select viable cells expressing known quantities of gene product.

lacZ has also proven effective as a marker in the genetic tagging of cell lineage founders and subsequent tracing of progeny in tissue sections using a cytochemical stain for expression of LacZ (4, 5). Using recombinant, replication-defective Moloney murine leukemia viruses (Mo-MuLV) in which the expression of *lacZ* is permitted from an internal simian virus 40 (SV40) promoter, Sanes *et al.* (4) were successful in the infection and determination of cell lineages in the postimplantation mouse embryo. Owing to the mobile nature of immune system cells, cytochemical *in situ* stains for

expression of LacZ have limited use in cell lineage determinations with these cells.

The fluorescence-activated cell sorter (FACS) has proven invaluable in the analysis and viable sorting of complex cell populations (6). We have now developed a fluorogenic assay for detection of LacZ as a quantitative genetic reporter molecule in single cells using FACS. Several problems had to be solved before LacZ could be used as a fluorogenic marker in live mammalian cells. First, although mammalian cells do not express cytosolic β -galactosidase activity, many do express lysosomal β -galactosidases (7). Therefore, one must be able to distinguish between cytosolic LacZ and any endogenous lysosomal β -galactosidases. Second, one must be able to load cells to a sufficient concentration of fluorogenic substrate to saturate the enzyme. Third, the enzymatically liberated fluorochrome must be retained within the cells. Fourth there must be no transfer of fluorochrome from LacZ⁺ to LacZ⁻ cells. Finally the staining procedure must maintain cell function and/or viability.

Previous attempts to quantitate *lacZ* expression in single, viable, eukaryotic cells either failed because of inadequate quantitation (8) or, in the case of yeast, provided useful expression information but resulted in dead cells (9). Jonkind *et al.* (10) were able to provide a rough correlation by FACS for endogenous lysosomal β -galactosidases but could not compare the activities of different cell types.

Here, we use the β -galactoside analog fluorescein di- β -D-galactopyranoside (FDG) (11) in a protocol, termed "FACS-FDG," that sensitively distinguishes LacZ⁺ cells from LacZ⁻ cells and allows time-dependent fluorescence-activated cell analysis and sorting. FDG is cleaved by β -galactosidase in LacZ⁺ cells to yield fluorescein, which can be detected by FACS. We find that the kinetics of fluorescence accumulation within LacZ⁺ cells, after FDG substrate loading, shows a linear dependence over time until maxima are approached because of exhaustion of substrate. The rate of fluorescence accumulation is directly proportional to β -galactosidase activity in cell extracts as quantitated by standard enzyme assays. We show that cells above a fluorescence intensity threshold determined in the FACS-FDG assay will be scored as LacZ⁺ (blue) with the 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) cytochemical stain. We have cloned cells by FACS based on the absolute level of their expression of β -galactosidase and verified that these clones express the expected levels of β -galactosidase. We also show that *lacZ* expression from our retroviral

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Abbreviations: FU, relative fluorescence unit(s); MuLV, murine leukemia virus; Mo-MuLV, Moloney MuLV; FACS, fluorescence-activated cell sorter; FDG, fluorescein di- β -D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside; ONPG, *o*-nitrophenyl β -D-galactopyranoside.

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construct, MMuLVSVnlsLacZ, displays a lack of tight transcriptional control, presumably due to superimposed epigenetic factors.

MATERIALS AND METHODS

Cells and Tissue Culture. NIH 3T3 cells were obtained from M. Dieckmann and P. Berg (Stanford University). The cell lines ψ -2-MMuLVSVnlsLacZ (ψ -2-21C) and ψ E-34(4) were derived by recombinant retroviral infection of ψ -2 (12) and maintained as described (13). The T-cell line BW5147 and the SP2/0 B hybridoma were maintained in complete RPMI 1640 medium (10% added fetal calf serum) at 5% CO₂/95% air.

Retroviral Infections. Retrovirus producer cells ψ -2-21C were plated to a density of 10⁶ cells per 100-mm plate in 7 ml of fresh Dulbecco's modified Eagle's medium. After 24 hr, 6 ml of virus-containing medium was filter-sterilized, and Polybrene was added to 10 μ g/ml. Virus (3 ml) was added to 10⁶ cells in 3 ml of RPMI 1640 medium. After 4 hr, cells were washed and replated.

Chemicals. FDG was generously supplied by R. Haugland (Molecular Probes, Eugene, OR; catalog no. 1179). Contaminating fluorescent fluorescein mono- β -D-galactopyranoside, which develops during dry storage (11), was bleached with 488-nm light from an argon laser. X-Gal and ONPG were obtained from Boehringer Mannheim.

Fluorescence Activated Cell Analysis and Sorting. FACS was set up as described (6). We compensated for the autofluorescence of cultured cell lines as described (14). Multiparameter data were collected and analyzed by using FACS-DESK run on a Digital VAX-780 configured as described (15). Fluorescence intensity of individual cells was measured as relative fluorescence unit(s) (FU).

X-Gal Staining. After 8 hr of X-Gal staining as described (4), blue cells were scored visually.

FDG Staining for β -Galactosidase. Exponentially growing fibroblast cells were treated with trypsin (GIBCO no. 610-5400; diluted to 1 \times solution) in phosphate-buffered saline until they could be removed from the plate with mild agitation. BW5147 and SP2/0 suspension cells in exponential phase were pelleted and resuspended in phosphate-buffered saline. Cells for staining were counted and brought to 10⁷ per ml in RPMI 1640-deficient medium (no. 9826, Applied Scientific, San Francisco) containing 2% (vol/vol) fetal calf serum, 10 mM Hepes (pH 7.3). The protocol for staining cells is as follows: (i) add 100 μ l of cells at 10⁷ per ml to a 5-ml polystyrene tube; (ii) bring the cell suspension to 37°C in a water bath for 5 min; (iii) add 100 μ l of 2 mM FDG in H₂O, prewarmed to 37°C; (iv) mix gently but thoroughly and rapidly place back into the 37°C water bath for 1 min; and (v) place the tube on ice and add 1800 μ l of ice-chilled isotonic incubation medium and 1 μ M propidium iodide.

RESULTS

Specific Staining of NIH 3T3 LacZ⁺ Cells with FDG.

Initially, we incubated LacZ⁺ ψ E34 and LacZ⁻ control cells in the presence of 20 μ M FDG and used FACS to measure cellular fluorescence. Under these conditions, LacZ⁺ ψ E-34 cells develop strong fluorescence (Fig. 1A). LacZ⁻ cells remain at background autofluorescence levels. However, a 1:1 mixture of LacZ⁻ and LacZ⁺ cells incubated under the same conditions for 1 hr resulted in a single peak because of leakage of fluorescein generated in LacZ⁺ cells and its passive uptake by LacZ⁻ cells (Fig. 1B). Therefore, we altered the procedures as follows.

FDG Substrate Loading via Hypotonic Shock at 37°C and Incubation at 4°C. Fluorescein passes the cell membrane >200 times faster at 37°C than at 5°C (16), whereas the V_{max} of β -galactosidase is lowered by only a factor of 10 over this

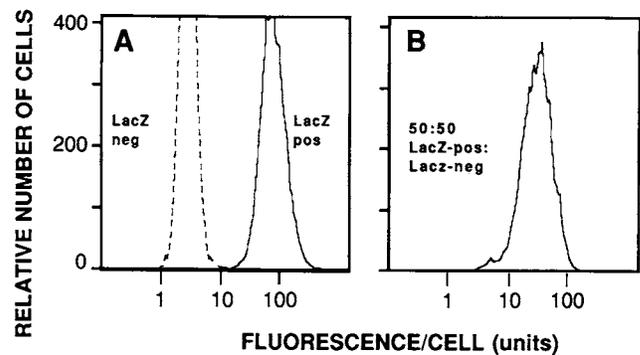


FIG. 1. During incubation at 37°C, fluorescein produced in LacZ⁺ cells leaks into LacZ⁻ cells. (A) Fluorescence distribution of LacZ⁺ (—) and LacZ⁻ (---) cells after separate exposure to 20 μ M FDG at 37°C for 1 hr. (B) Fluorescence distribution of a 1:1 mixture of LacZ⁺ and LacZ⁻ cells exposed to 20 μ M FDG at 37°C for 1 hr. LacZ⁻ cells are NIH 3T3 fibroblasts; LacZ⁺ cells are ψ E34.

temperature differential (17). We reasoned that loading the substrate at high concentrations under hypotonic conditions at 37°C, with a subsequent rapid switch to cold temperature (thus allowing enzyme activity to proceed while preventing fluorescein or FDG leakage), could effect resolution of a mixture of LacZ⁺ and LacZ⁻ cells. Therefore, we loaded cells in the presence of 1 mM FDG at 50% of the isotonic concentration at 37°C for 1 min. Cells were then rapidly cooled by 1:9 dilution with ice-cold isotonic medium and placed at 4°C to allow fluorochrome generation. Fig. 2 shows that this procedure gives excellent generation and resolution of peaks. LacZ⁻ cells do not develop any fluorescence above the autofluorescent background (Fig. 2A). After 60 min,

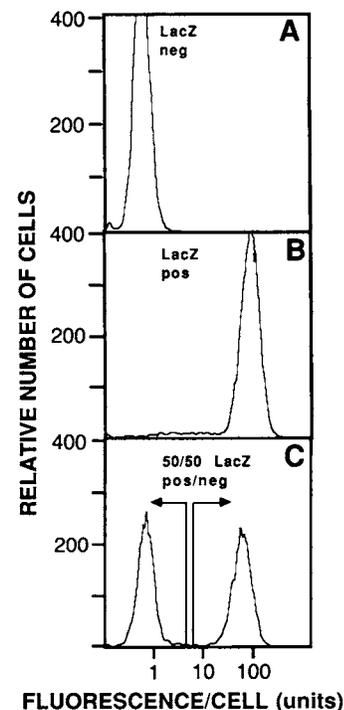


FIG. 2. All cells are uniformly loaded with substrate; LacZ⁺ cells can be clearly resolved from LacZ⁻ cells in a mixed population. In the FACS-FDG assay, cells were loaded with FDG at 37°C and allowed to generate fluorescein at 4°C for 60 min. (A) LacZ⁻ cells stained alone do not develop appreciable fluorescence. (B) LacZ⁺ cells develop considerable uniform fluorescence. (C) A 1:1 mixture of LacZ⁺ and LacZ⁻ cells is clearly resolved. Arrows indicate positive and negative sorts. LacZ⁺ cells are ψ -2-21C; LacZ⁻ cells are NIH 3T3 fibroblasts.

LacZ⁺ cells generated a narrow peak of fluorescence with a minor "trailing" population of cells (Fig. 2B), whereas a mixture of equal numbers of LacZ⁺ and LacZ⁻ cells stained together generated two discrete peaks (Fig. 2C). When incubation at 4°C was extended beyond 60 min, the major peak in Fig. 2B did not move, but the trailing population gradually merged with the major peak, indicating that the major peak represents cells that contain sufficient enzyme to have hydrolyzed all intracellular FDG and the trailing population represents cells with much lower levels of β -galactosidase activity (data not shown).

The two major populations in Fig. 2C were sorted by setting the gates shown at a sort rate of about 3000 cells per sec. Subsequent staining of these sorted populations with X-Gal for postmortem analysis of LacZ content showed sorting efficiencies that were theoretically expected (6): percent positives from the positive sort = 98.5%; percent negatives from the negative sort = >99%.

We also obtained excellent discrimination of LacZ⁺ and LacZ⁻ cells of other developmental lineages after infection with the recombinant LacZ retrovirus ψ -2-21C. We infected 1×10^6 SP2/0 and BW5147 cells with this retrovirus and, after waiting 3 days for integration of the retrovirus and expression of LacZ, performed the FACS-FDG assay. Mock-infected control cells had low autofluorescence, between 0.1 and 1.0 FU. However, 4% of the infected BW5147 cells and 2% of the infected SP2/0 cells had fluorescence of 1.0–100 FU. To separate LacZ⁻ from infected LacZ⁺ cells, we sorted cells with <1.0 FU (LacZ⁻) and >2.0 FU (LacZ⁺) from both BW5147 and SP2/0 populations. The sorted cells were immediately fixed and stained with X-Gal. The LacZ⁻-sorted populations did not contain any blue cells, whereas the LacZ⁺-sorted populations included cells that stained white, light blue, or dark blue. Thus, none of the cells defined as LacZ⁻ by the FACS-FDG assay stain with X-Gal. However, only some cells defined as LacZ⁺ by the FACS-FDG assay stained blue with X-Gal.

Clones derived from these infected BW5147 populations had similar X-Gal staining phenotypes. In another experiment, ψ -2-21C-infected BW5147 and SP2/0 cells were again assayed by FACS-FDG, and those cells fluorescing >2.0 FU (after 1 hr of incubation) were cloned by using the single-cell deposition capability of the FACS (6). After growing clones for 1 week, cytochemical staining with X-Gal revealed that 70 of 79 BW5147-21C clones selected by FACS for β -galactosidase activity display a wide range of percentages of blue cells: from <1% blue to >95% blue. Pictures of representative clones BW5147-21C.28 and BW5147-21C.42 stained with X-Gal are shown in Fig. 3. All 20 SP2/0-21C clones examined had only a small population of cells, <1%, that stained blue. Therefore, cells expressing LacZ can be viably cloned by FACS, but cells within the clones exhibit apparent variable expression. We decided to focus on an understanding of the relationship between the intensity of blue staining with X-Gal and β -galactosidase activity as determined by FACS-FDG in the BW5147-21C clones.

Fluorescence Accumulation Within Cells Is Directly Proportional to β -Galactosidase Enzymatic Activity and Is a More Sensitive Indicator of LacZ Expression Than X-Gal. Although many cells within some LacZ⁺ BW5147-21C clones could not be shown to have detectable β -galactosidase activity when assayed with the X-Gal histological stain (Fig. 3), almost all of the cells within these clones were seen to express some β -galactosidase by using the FACS-FDG assay. Histograms of fluorescence distribution per cell of representative clone BW5147-21C.28 at three time points after FDG loading are shown in Fig. 4 A–C. By 11 min, >90% of the cells in clone 28 were fluorescing more than the negative control population. X-Gal staining of clone 28 revealed only 23% of the cells that could be visually deter-

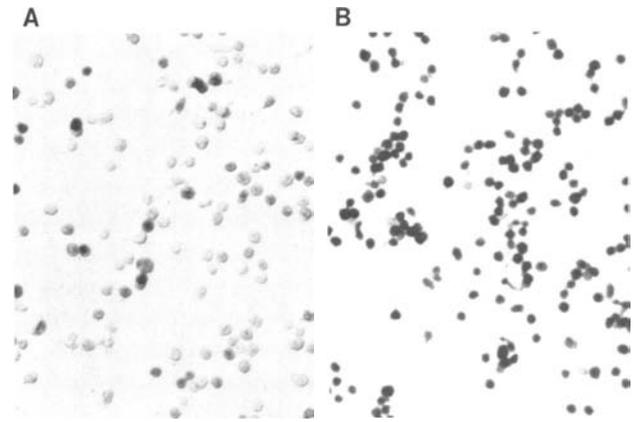


FIG. 3. BW5147 clones expressing ψ -2-21C retrovirus. Cells (1×10^6) of each clone were fixed and stained with X-Gal. (A) BW5147-21C.28 displays a heterogeneous staining pattern: cells stain white, light blue, and dark blue. (B) BW5147-21C.42 stains homogeneously blue, with few or no white cells.

mined as blue (Fig. 3A), indicating FACS-FDG detects β -galactosidase activity in cells that X-Gal does not.

From these histograms we calculated the arithmetic mean fluorescence per cell. Plots of the mean fluorescences versus time for BW5147-21C.28 and several other BW5147-21C clones revealed that the fluorescence increase is clone-dependent, initially linear, and eventually plateaus (Fig. 4D). This plateau is due to substrate exhaustion and is a measure of the total amount of FDG actually taken up during loading (data not shown).

The broad fluorescence profiles (a representative histogram is shown in Fig. 4A) displayed within these clonal populations after 6 months of growth could reflect epigenetic variations in the level of expression of LacZ. To determine if this variable β -galactosidase activity is heritable and whether the amount of fluorescence within each cell is proportional to the enzyme activity, we subcloned BW5147-21C.28 using FACS. Gates were set to sort FDG-loaded cells having 0.1–1 FU per cell after 10 min of incubation at 4°C, with the expectation that these cells and their progeny would express low levels of LacZ activity. The β -galactosidase activity in the FACS-FDG assay of three representative subclones, the parental line, clone 28, and clone 42, is shown in Fig. 5A. As expected, each subclone generated fluorescence at a much lower rate than did the parental clone 28, while clone 42 maintained its high-activity phenotype. Cells (5×10^6) of each clone and subclone were lysed, and the total β -galactosidase activity was determined by the chromometric ONPG assay. The rate of ONPG cleavage in the extract versus the mean rate of fluorescence increase in individual cells showed excellent correlation over a 200-fold range of β -galactosidase activities (Fig. 5B), indicating that FACS-quantification of FDG cleavage is an accurate measurement of β -galactosidase content determined conventionally. Comparison of the respective time scales and numbers of cells analyzed indicated that the FACS-FDG assay is at least 8 orders of magnitude more sensitive than the ONPG assay.

We assessed whether the property of individual cells to stain blue with X-Gal was related to the FU intensity in the FACS-FDG assay. The percentage of blue X-Gal-stained cells within each clone was determined and used to calculate the threshold FU above which there was a corresponding percentage of cells in the respective histograms of FU per cell at 2 min. Table 1 shows that an average of these threshold values, 7.3 FU per cell at 2 min, provides a value that predicts the percentage of blue X-Gal-stained cells determined visually within each clone.

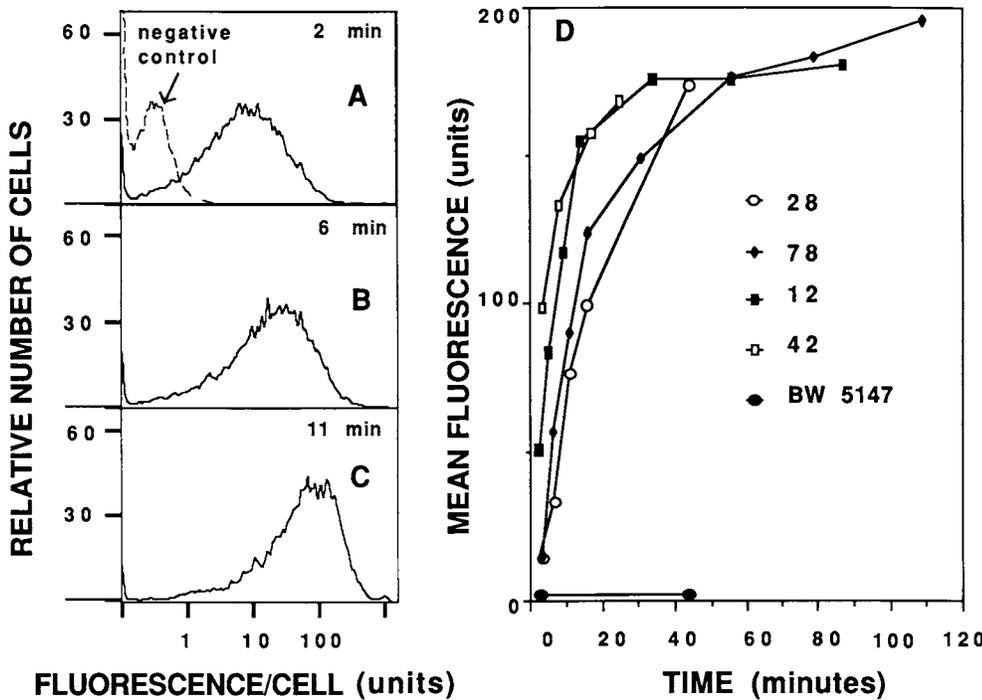


FIG. 4. Kinetics of fluorescence accumulation in BW5147-21C LacZ⁺ clones. (A-C) Fluorescence distribution of BW-5147-21C.28 at three time points (dashed negative control in A is BW5147). (D) Arithmetic mean fluorescence per cell for each LacZ⁺ clone and LacZ⁻ BW-5147 is plotted versus the time after FDG loading. Clones used are indicated in the figure.

DISCUSSION

With FACS and the fluorogenic β -galactosidase substrate FDG, expression of *E. coli lacZ* in individual mammalian cells can be rapidly analyzed, quantitated, and used as a basis to select viable cells expressing specified levels of β -galactosidase activity. FACS-FDG detection of LacZ reporter molecule is faster, is much more sensitive than, and can substitute for many applications of the X-Gal and other β -galactosidase detection methods (8-10). Although data relating average gene expression in bulk cell populations as quantified by previous methods (chloramphenicol acetyltransferase, ONPG/ β -galactosidase) have provided significant insights into gene regulation, a finer analysis of gene expression on a cell-by-cell basis and the ability to select viable cells expressing known quantities of a reporter molecule will generate a more precise understanding of gene regulation processes.

We noted several important parameters regarding the enzymatic generation of fluorescein in live mammalian cells. To avoid the problem of fluorescein leakage from LacZ⁺ cells and its passive uptake by LacZ⁻ cells at temperatures above 15°C, β -galactosidase activities were measured at temperatures below the membrane freezing point (18) (Fig.

2). The rate of fluorescence increase at 4°C is linear and directly proportional to extracted enzymatic activity (Fig. 5). Fluorescence eventually reaches a plateau, after complete hydrolysis of the substrate, in LacZ⁺ cells at a level indicating the amount of substrate loaded (Fig. 4). With the FDG loading protocol presented in this paper, cells are loaded to $\approx 3 \times 10^{-4}$ M FDG (data not shown). This is adequate to provide a concentration of substrate in excess of the K_m , given a K_m of 17×10^{-6} M of β -galactosidase LacZ for FDG (19), so that the initial rate of hydrolysis is not dependent upon substrate concentration.

We have not measured the lowest number of β -galactosidase molecules detectable; however, based on published turnover rates (19, 20) estimated at 4°C to be 0.75 or 7 substrate molecules per sec, and if the enzyme has free access to the substrate, one enzyme molecule per cell can theoretically be detected by FACS at 4°C in some 30-180 min. Because of the high sensitivity of this enzymatically amplified assay, we expect that it will be useful to quantify expression from weak promoters or to rapidly assess gene activation kinetics.

We used the FACS-FDG method to directly select BW5147 infected with the recombinant *lacZ*-containing retrovirus ψ -2-21C. BW5147-21C clones exhibited broad varia-

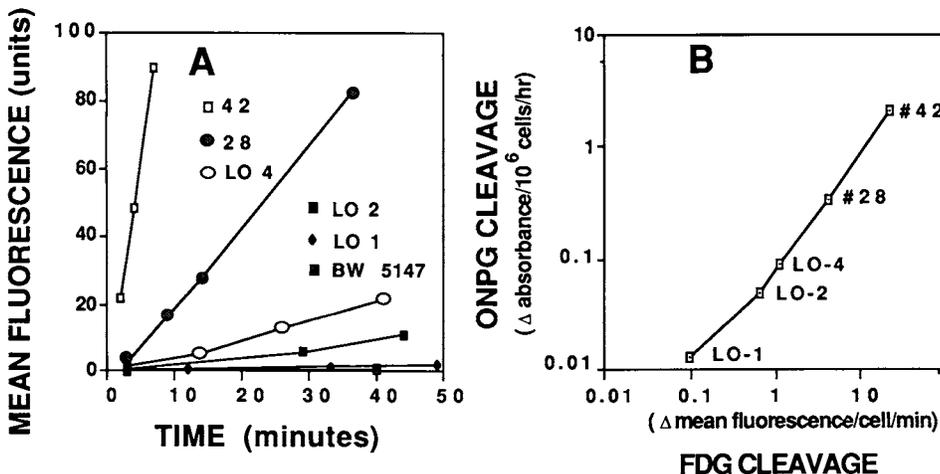


FIG. 5. Rate of fluorescence increase is linear and directly proportional to β -galactosidase activity. (A) Plot of mean fluorescence per cell versus time for LacZ⁻ BW5147, LacZ⁺ BW5147-21C clones 28 and 42, and subclones of clone 28 selected for low β -galactosidase activity, 28lo1, 28lo2, and 28lo4. (B) Rate of mean fluorescence increase for each clone assayed in A versus the ONPG cleavage rate in total cellular extracts of each clone. Note the difference in scales.

Table 1. Prediction of the percentage of blue cells using a calculated fluorescence threshold

Clone	% blue cells observed	Threshold, FU	% blue cells predicted
42	93	4	87
28	23	8	25
28lo4	4	10	9
28lo2	<1	—	0.87
28lo1	<1	—	0.28

Columns from left to right: BW5147.21C clones and subclones used in this experiment; percentage of blue cells determined visually after staining with X-Gal; calculated fluorescence threshold in FU at 2 min, derived from respective fluorescence histograms for each clone, above which there is a percentage of cells corresponding to the percentage of blue cells in the preceding column; and the average of the three fluorescence thresholds applied in a regression analysis to predict the percentage of blue cells expected.

tion of *lacZ* expression between and within clones. We readily subcloned lines by FACS-FDG that displayed fluorescence histogram and X-Gal-staining characteristics distinct from their parental clone, indicating that epigenetic factors can play a significant role in expression. Subcloned lines developed large coefficients of variation which, though lower than the parental clone, suggest that consistent transcriptional rates in cells with these constructs are not strictly regulated.

All SP2/0 clones selected for *lacZ* expression portray the same phenotype: the majority of cells show no staining with X-Gal, whereas a few cells stain dark blue. Whether this can be explained by frequent promoter shutdown or rare activation of gene expression remains to be determined. Promoter shutdown in retrovirally transduced, cultured cell lines and in *in vivo* differentiating immune lineage cells has proven to be an imposing barrier to studying gene expression (21–23). It has been suggested that transcriptional variation of retroviral genomes is a feature of developmental availability of trans-acting factors, cis-acting retroviral sequences, and changes in chromatin structure or is influenced by the integration site (24). The results from our work indicate that variable expression can be cell-type-dependent and that differentiation is not a prerequisite for frequent loss of or variation in expression.

The FACS-FDG assay is more sensitive than staining with X-Gal. We can reliably define a fluorescence threshold that corresponds to the minimum detectable blue staining with X-Gal (Table 1). Given the difficulty of visually distinguishing intensity variations of blue coloration in fixed cells and the relative insensitivity of X-Gal to detect β -galactosidase activity, FACS-FDG will be useful to simultaneously quantify both *lacZ* expression and transfection efficiency.

We see application of this basic method to a variety of problems. For instance, the marriage of a retrovirus marker to multiparameter FACS analysis of surface phenotype provides useful lineage and functional markers in isogenic systems for immunology and developmental biology. It should now be possible to adapt the system to quantitate cell type-specific promoter strength in mixed populations of cells by multiparameter FACS. Along these lines, we have proven that the system will be amenable to multiparameter FACS analysis using FDG in combination with membrane-bound fluorochromes (unpublished data). Other enzyme/fluorogen combinations are possible, making available the opportunity to analyze several promoters simultaneously with multiparameter FACS.

Having shown the utility of the FACS-FDG assay for LacZ enzyme expression studies in several mammalian cell types (we also have used this system to successfully analyze expression of LacZ in embryo carcinoma cells, *in vivo* infected peritoneal lymphocytes, and several other cell types; unpublished data), we expect that adaptations of the methodology provided here can be applied to other cell systems to quantitate β -galactosidase reporter molecule expression on a cell-by-cell basis for studies of gene regulation.

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