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## Original Articles

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# Use of a Membrane-Localized Green Fluorescent Protein Allows Simultaneous Identification of Transfected Cells and Cell Cycle Analysis by Flow Cytometry

Robert F. Kalejta,<sup>1</sup> Thomas Shenk,<sup>1,2</sup> and Andrew J. Beavis<sup>3\*</sup>

<sup>1</sup>Department of Molecular Biology, Princeton University, Princeton, New Jersey

<sup>2</sup>Howard Hughes Medical Institute, Princeton University, Princeton, New Jersey

<sup>3</sup>Flow Cytometry Core Facility, Princeton University, Princeton, New Jersey

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The simultaneous detection of the green fluorescent protein (GFP) and DNA content using propidium iodide (PI) by flow cytometry is made difficult because of the unique nature of these 2 fluorogenic reagents. For PI to enter cells efficiently and to stain DNA quantitatively, the cells must first be permeabilized; ethanol treatment is a routine method to achieve this. However, this permeabilization step causes GFP, which is normally found in the cytoplasm, to leach out of the cells. Although the use of paraformaldehyde-based fixatives allows GFP to be maintained in cells and retain its fluorescence even after ethanol permeabilization, the protocol we commonly employ results in inefficient PI staining and poor quality DNA histograms. To circumvent these

difficulties, we have employed a GFP-fusion protein which localizes to the cellular membrane and as such is retained in cells upon ethanol permeabilization without prior fixation. This allows the GFP signal to be detected in cells treated with ethanol in preparation for PI staining and cell cycle analysis. This property facilitates the use of GFP as a marker for transfected cells in experiments designed to characterize the effects of ectopic expression of cellular or viral genes on cell cycle progression. *Cytometry* 29:286–291, 1997. © 1997 Wiley-Liss, Inc.

**Key terms:** green fluorescent protein; fusion protein; propidium iodide staining; cell cycle; flow cytometry; transient transfection

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The investigation of the biological roles of cell cycle regulatory proteins is often hindered by the activities of the proteins themselves. For example, since proteins such as the cyclin-dependent kinase (cdk) inhibitor p21 (4) inhibit cell cycle progression and thus cell division, it is impossible to make cell lines that constitutively express these proteins. To combat this problem, promoter systems have been developed that can express such proteins in a regulated fashion (e.g., the tetracycline system; ref. 7). While these promoters have been used successfully to study a few cell cycle proteins (13,14), the isolation and characterization of appropriate cell lines is a long and tedious process. Moreover, because cell lines need to be established, immortalized cells as opposed to primary cultures must be employed.

Transient transfection assays allow one to avoid these problems and to employ primary cultures to identify and study cell cycle regulatory proteins. Since only a minority of cells actually take up and express the genes offered in

transient transfections, a method to identify the subset of transfected cells is required. This is usually accomplished by including a plasmid that expresses a marker gene in the transfection cocktail. When transfection cocktails contain more test plasmid than marker plasmid, the majority of cells that express the marker have also taken up the test plasmid, but few if any randomly selected clones have incorporated the test DNA alone (18). Since transfection markers usually encode very stable proteins and the cotransfected test plasmid may encode a labile one, it is a common practice to include a molar excess of test plasmid over marker plasmid, to ensure for both cotransfection and that the protein encoded by the test plasmid accumulates to a high level in the transfected cell population (8,11,16,20).

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\*Correspondence to: Andrew J. Beavis, Lewis Thomas Laboratory, Washington Road, Princeton University, Princeton, NJ 08544-1014.  
E-mail: abeavis@molecular.princeton.edu

The green fluorescent protein (GFP) is an excellent choice for a marker gene in such experiments since it can be expressed and maintained in the cytoplasm of heterologous cell types (1,12). There is a mutant GFP that absorbs strongly at 488 nm (3) facilitating its detection by flow cytometry on the single-laser benchtop analyzers that are typically equipped with an argon laser. Furthermore, the green emission of this GFP allows its use in combination with other fluorochromes, such as propidium iodide (PI), which emit toward the red region of the spectrum. Such a combination of GFP and PI would prove useful in allowing one to perform cell cycle analysis on transiently transfected cells by flow cytometry.

In this report, we describe the development of a method for quantification of GFP as a marker of transfected cells and cell cycle analysis by flow cytometry. In this assay, cell cycle position is identified by the DNA content of cells as determined by PI staining. Although PI does not enter untreated cells with great efficiency, permeabilization and fixation with ethanol allow the dye access to the nucleus resulting in quantitative DNA staining. However, we show here that cells treated with ethanol lose the GFP fluorescence signal, which precludes the simultaneous detection of transfected cells and PI-based cell cycle analysis. The loss of GFP fluorescence presumably occurs because the protein leaches from the cell upon ethanol permeabilization. The use of a fixative which allows the retention of cytosolic GFP results in inadequate staining of the DNA even after ethanol treatment and thus provides data of insufficient resolution for quantitative cell cycle analysis. To avoid this difficulty, we developed a simple assay employing a membrane-localized GFP-fusion protein that facilitates the detection of GFP fluorescence in ethanol-treated cells allowing the simultaneous detection of GFP as a marker of transfected cells and PI for cell cycle analysis. By gating histograms based on GFP fluorescence, the DNA content of cells that either do or do not coexpress the genes delivered by transfection can be analyzed independently of one another. We show that this analysis is effective in demonstrating the cell cycle arrest caused by a known cell cycle inhibitory protein and suggest that this approach should prove useful in identifying novel genes that are critical for proper cell cycle regulation.

## MATERIALS AND METHODS

### Cells and Transfections

Primary human foreskin fibroblasts (PHFFs) between passages 6 and 14 and the human osteosarcoma cell line U-2 OS (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). For electroporations, cells were collected by trypsinization and resuspended in fresh media to a final concentration of  $5 \times 10^6$  cells/ml. In single plasmid transfections, a 700  $\mu$ l aliquot of the cells was mixed with 5  $\mu$ g of plasmid DNA and electroporated (960  $\mu$ F, 250 V) using a BioRad gene pulser (BioRad, Hercules, CA). In cotransfection assays, 2  $\mu$ g of the GFP expression plasmid and 20  $\mu$ g of the test plasmid

were electroporated at 960  $\mu$ F and 220 V. Cells were plated immediately after electroporation.

### Plasmids

The pSP72 is from Promega (Madison, WI) and the pCEP4 is from Invitrogen (Carlsbad, CA). All GFPs used in this work were the FACS-enhanced mutants (3) and were expressed from the cytomegalovirus promoter. pEGFPN-1 is from Clontech (Palo Alto, CA). pCMVhgalEGFP (galT-GFP) expresses a fusion protein of the first 60 amino acids of human  $\beta$ -1,4-galactosyltransferase to GFP (ref. 2; provided by N. Cole and J. Lippincott-Schwartz). We cloned the pleckstrin homology domain from human  $\beta$ I $\Sigma$ II spectrin (ref. 17; provided by D.-S. Wang and G. Shaw) as a fusion protein to the carboxy terminus of GFP (GFP-spectrin). Briefly, the multiple cloning site in pEGFPN-1 was deleted and a linker containing an Eco RI site in frame with GFP was inserted into the Bsr GI/Xba I sites. The Eco RI/Sal I spectrin fragment was inserted into the linker to generate plasmid pCMVVEGFPspectrin. The wild-type p53 expression plasmid pRc/CMVhp53 and a plasmid expressing a mutant p53 (pRc/CMVh273hp53) with a single amino acid change from arginine to histidine at position 273 (10) were provided by J. Lin and A. Levine.

### Sample Preparation

Cells were collected by trypsinization, pelleted, and resuspended in phosphate-buffered saline (PBS) to a final concentration of about  $1 \times 10^6$  cells/ml. Two volumes of cold, absolute ethanol were added and the samples were stored at  $-20^\circ\text{C}$  until the day of analysis. At that time, cells were pelleted and resuspended in staining solution (50  $\mu$ g/ml PI, 100  $\mu$ g/ml RNase A in PBS) to about  $1 \times 10^6$  cells/ml. Samples were stored at  $4^\circ\text{C}$  for at least 1 h in the dark prior to analysis. When fixation was employed, trypsinized cells were washed in PBS and resuspended in PermeaFix (Ortho Diagnostics, Raritan, NJ) to a final concentration of  $3 \times 10^6$  cells/ml. These cells were incubated at room temperature for 30 min, after which they were pelleted and washed twice with the indicated solutions (either PBS, PBS plus 0.05% sodium dodecyl sulfate, or PBS plus 0.05% Tween) prior to ethanol fixation.

### Flow Cytometry

Flow cytometric analysis for GFP and PI fluorescence was performed using a 4-color FACScan [custom product; Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA] and CellQuest software with doublet discrimination (BDIS). For each analysis, 30,000–100,000 gated events were collected to permit cell cycle analysis of both the GFP(+) and GFP(–) cell subpopulations. The GFP and PI fluorescence signals were separated with a 560 short-pass dichroic mirror and collected with a 530/30 bandpass (FL1, GFP) and 572/26 bandpass (FL2, PI). Data analysis was performed using CellQuest (BDIS) and Modfit LT (Verity Software House, Topsham, ME). The GFP fluorescence was collected on a logarithmic scale and the PI fluorescence was collected on a linear scale, both with

1,024 channel resolution. All mean fluorescence intensity values were determined as linear values from the CellQuest software.

## RESULTS

Figure 1A–D shows the FL1 channel fluorescence of PHFFs transfected either with a control plasmid (pSP72, Fig. 1A) or a GFP expression plasmid (pEGFPN-1, Fig. 1B–D). The GFP-transfected sample shows a broad peak of highly fluorescent cells (approximately 15% of the population; data not shown), which represents those cells that have taken up and are expressing GFP (Fig. 1B). These GFP(+) cells are clearly resolved from their GFP(–) counterparts in the same sample. However, when GFP-transfected cells were permeabilized with ethanol in preparation for PI staining, the GFP fluorescence signal was lost (Fig. 1C). The loss of GFP fluorescence most likely occurs because the cytoplasmically localized GFP can escape from cells upon permeabilization, since fixing cells prior to ethanol treatment allows detection of the GFP signal (Fig. 1D). However, such a fixation step results in poorly resolved histograms of PI-stained DNA, even if the cells are extensively washed after fixation with either PBS (Fig. 1E) or PBS plus detergents (data not shown) prior to ethanol permeabilization (compare to a DNA histogram of cells treated only with ethanol, Fig. 1F).

It has been shown by fluorescence microscopy that a membrane-associated GFP-fusion protein was retained in cells even after methanol treatment (17). In these experiments, GFP was fused to the pleckstrin homology domain of the membrane-associated protein spectrin. Based on this finding, we investigated whether a membrane-associated GFP-fusion protein would remain in cells after ethanol permeabilization, allowing its visualization by flow cytometry in synchrony with PI-based cell cycle analysis. We initially analyzed 2 GFP-fusion proteins (see Materials and Methods for details), one that is localized to the Golgi apparatus (galT-GFP) and one to the plasma membrane (GFP-spectrin). GFP fluorescence was detected when any one of the 3 GFP-expressing plasmids were transfected into PHFFs and the cells were collected by trypsin and resuspended in PBS (Fig. 2A). The mean fluorescence intensity (MFI) of the GFP(+) cells following transfection with pEGFPN-1 was 388.9 (Fig. 2A, curve 1). Constraining GFP to the Golgi (Fig. 2A, curve 3) resulted in a decreased magnitude of the fluorescence pattern (MFI = 86.3), reducing the resolution between the GFP(+) and GFP(–) cells. Although localization to the plasma membrane (Fig. 2A, curve 2) resulted in a slightly lower fluorescence intensity (MFI = 103.5) compared to cytoplasmic GFP, the GFP(+) cells are still well resolved from the GFP(–) cells in the population. None of the GFP expression plasmids had any effect on cell cycle progression (data not shown).

As stated previously, the cytoplasmic GFP leaks from the cells after ethanol treatment which prevents the simultaneous detection of GFP fluorescence and PI staining (Fig. 2B). However, the membrane-localized GFP, remains in cells even after ethanol permeabilization, allowing simultaneous detection of GFP(+) cells and PI staining (Fig. 2C). Furthermore, the percentage of cells expressing GFP

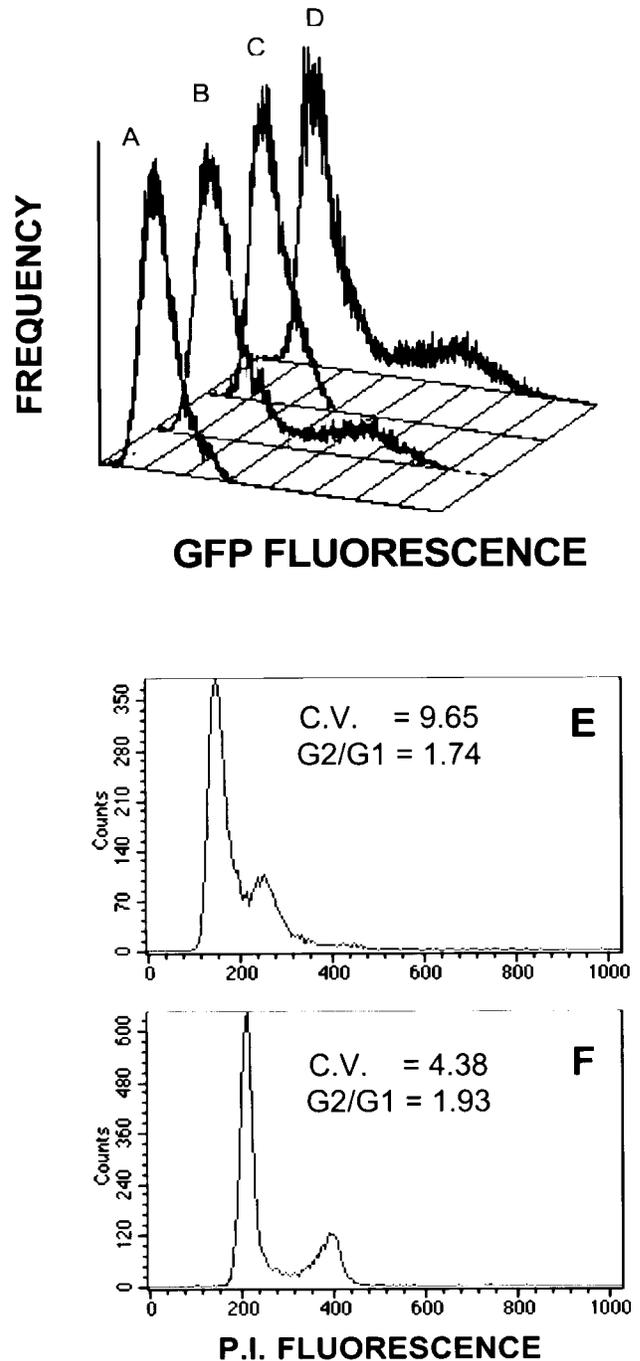


FIG. 1. The fixation step required to trap cytoplasmic GFP in cells impairs PI staining and DNA content analysis. Single parameter histograms of GFP fluorescence for PHFFs transfected either with pSP72 (A) or GFP expression plasmid pEGFPN-1 (B) collected by trypsinization and resuspended in PBS. Ethanol permeabilization of GFP-transfected cells prior to analysis washes the GFP out of the cells (C), unless they are first treated with a fixative, as described in Materials and Methods (D). Histogram analysis of DNA content of PI-stained, GFP-transfected cells fixed with Permeafix and washed with PBS prior to ethanol permeabilization (E), or of unfixed cells permeabilized with ethanol (F).

following transfection with GFP-spectrin was comparable for unfixed cells (GFP(+) = 22.6; Fig. 2A, curve 2) and in ethanol-treated cells stained with PI (GFP(+) = 21.9, Fig.

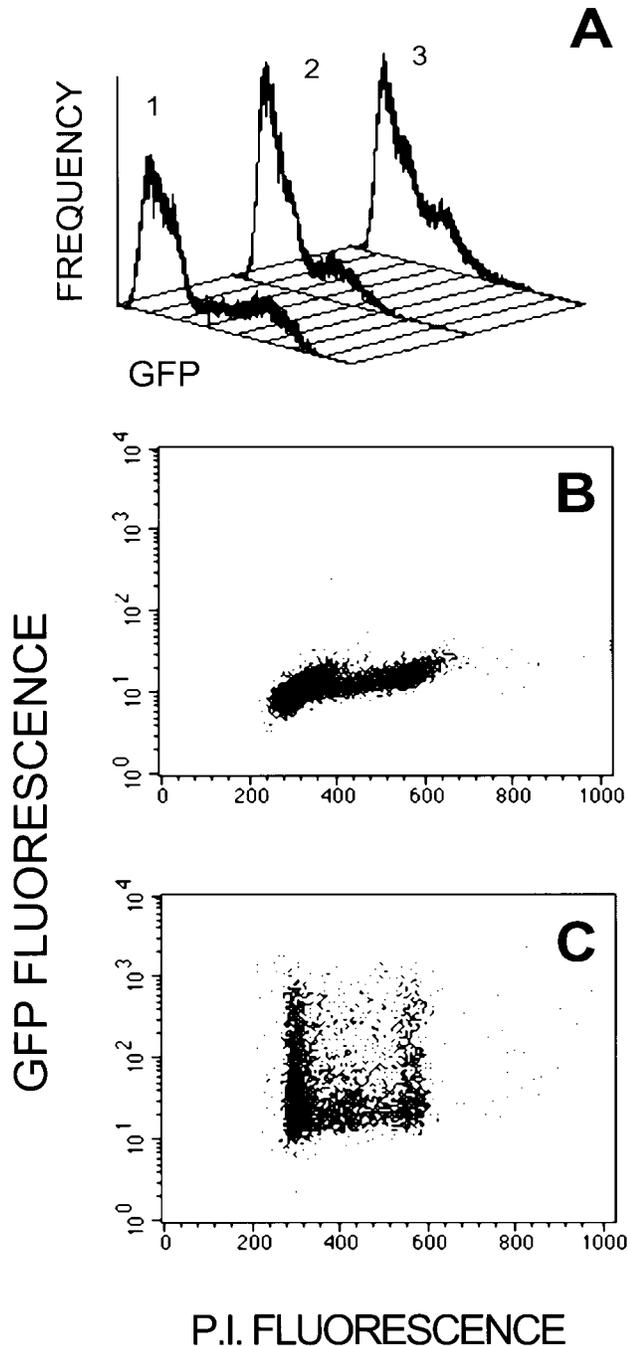


FIG. 2. A plasma membrane-localized GFP-fusion protein allows simultaneous detection of GFP and PI fluorescence by flow cytometry. **A:** Single parameter histogram overlays of GFP fluorescence for PHFF cells. PHFFs were transfected with either pEGFPN-1 (curve 1), GFP-spectrin (curve 2), or galT-GFP (curve 3) expression plasmids and 48 h later were prepared for flow cytometric analysis by trypsinization and resuspension in PBS. Dual parameter dot plots of PI versus GFP fluorescence for PHFFs transfected with pEGFPN-1 (**B**) or the GFP-spectrin expression plasmid (**C**). Cells were collected 48 h after transfection, stained with PI for cell cycle analysis as described in Materials and Methods, and analyzed by flow cytometry.

2C). This property facilitates the use of GFP-spectrin as a marker of transfected cells in flow cytometry cell cycle analysis.

Since the effects of cell cycle inhibitory proteins on PHFFs are not well documented, we employed the human osteosarcoma cell line U-2 OS to illustrate the utility of our GFP-based assay. These cells have been shown to arrest in G1 when transfected with a p53 expression plasmid (19). Additionally, high transfection efficiencies are routinely achieved with this cell line. Cytosolic, Golgi-, and membrane-localized GFP proteins showed similar properties in U-2 OS cells as in PHFFs (data not shown). Asynchronous U-2 OS cells were transfected with GFP-spectrin and expression plasmids for either wild-type p53, a mutant p53 that does not bind DNA (10), or a control plasmid pCEP4. When overexpressed in cells, p53 binds to the promoter of the cdk inhibitor p21 and stimulates its transcription resulting in a G1 cell cycle arrest (reviewed in 9). Since the mutant p53 employed in this experiment does not bind DNA, it should not inhibit cell cycle progression and serves as a negative control.

Due to the fact that asynchronous populations already have a large percentage of cells in G1, it is sometimes difficult to observe and quantitate a G1 arrest. Additionally, proteins which may slow progression through, but not arrest cells in G1, may be mistakenly concluded to arrest the cell cycle if asynchronous cells are analyzed. To avoid these problems, we chose to employ a nocodazole (G2/M) arrest in our experiments. Twenty-four hours after transfection (to allow time for expression of the transfected plasmids), the medium on the cells was changed to medium containing 40 ng/ml nocodazole. Twenty-four hours later (to allow time for even slow-moving cells to arrest at G2/M), cells were harvested and prepared for flow cytometry.

Figure 3A shows the results from transfection of U-2 OS cells with GFP-spectrin and p53, and the subsequent nocodazole block. The top dot plot shows PI versus GFP and was used to gate cells into 3 different populations: GFP(-) (R2), GFP(dim+) (less than 10-fold brighter than the negative cells; R3), and GFP(bright+) (more than 10-fold brighter than the negatives; R4). The DNA histograms for each population are shown underneath. For cells transfected with the p53 plasmid, the R2 and R3 populations show no G1 arrest, whereas the R4 population contains a significant number of cells arrested in G1. Neither the p53 mutant (Fig. 3B), nor the control plasmid pCEP4 (Fig. 3C), produced a G1 arrest in any population. Thus, this GFP-based technique can accurately identify plasmids delivered by transient transfection that do, or do not, inhibit cell cycle progression.

## DISCUSSION

This report describes the use of GFP as a marker of the subpopulation of transfected cells in the PI-based cell cycle analysis of transiently transfected cell cultures. We have demonstrated certain technical limitations to the use of GFP and PI in combination. The non-permeant PI requires that cells be permeabilized (e.g., in ethanol) to allow high resolution, quantitative DNA staining. During this permeabilization step, cytosolic GFP leaches from the cells. While fixation allows cytosolic GFP to be retained in cells, the procedure we commonly employ impairs the

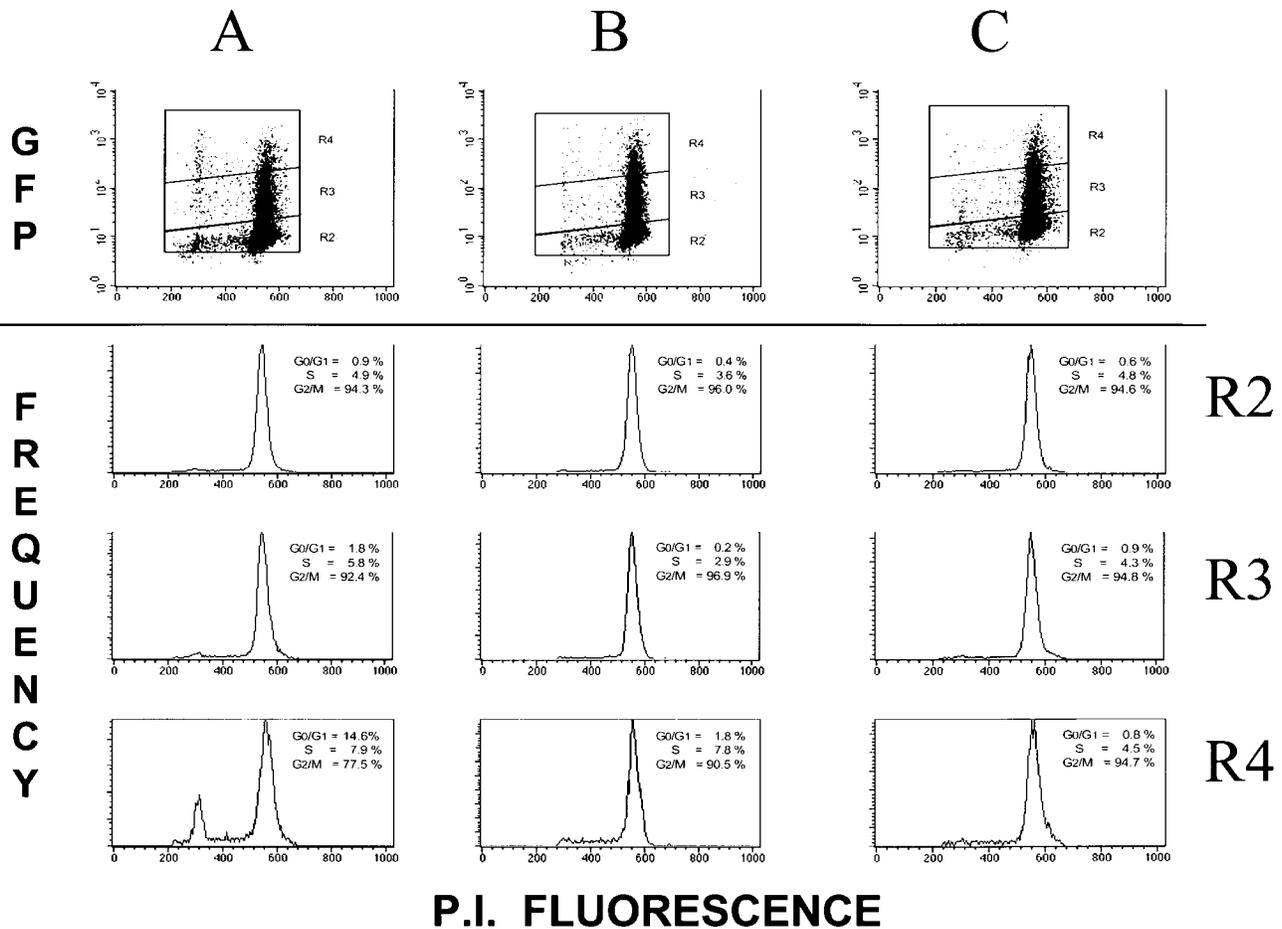


FIG. 3. The p53-mediated G1 arrest is observed when GFP-spectrin is used as a marker of transfected cells. **A**: Twenty-four hours after transfection with GFP-spectrin and the p53 expression plasmid, nocadazole was added, and the cells were harvested 24 h later. Single cells are displayed on a dot plot of PI versus GFP, which was then used to gate

GFP(-) (R2), GFP(dim+) (R3), and GFP(bright+) (R4) cells. DNA histograms for each of those populations are shown below. Control experiments in which a mutant p53 plasmid (**B**), or a vector control (**C**), was cotransfected with GFP-spectrin are displayed as in **A**. Data are representative of 4 experiments.

ability of PI to stain DNA quantitatively. Although it is possible that altered fixation protocols may allow for improved PI staining, we have avoided this additional step by employing a membrane-localized GFP in our assay. This GFP-spectrin fusion protein is retained in ethanol-treated cells without prior fixation, permitting PI-based cell cycle analysis of GFP(+) cells. The utility of this assay was demonstrated by observing the p53-induced G1 arrest in U-2 OS cells. Interestingly, we only detected an efficient arrest in the cells that expressed high levels of GFP (and, presumably, high levels of the gene on the cotransfected plasmid). The significance of this phenomenon, and whether or not it is observed with other transfection markers or cell cycle inhibitors, is unclear.

The ability to detect GFP and PI simultaneously by flow cytometry allows these 2 fluorochromes to be used in combination for cell cycle analysis, bringing together the most commonly used stain for cell cycle analysis (15), and a marker molecule (GFP) with intrinsic fluorescence permitting its detection without the addition of fluorogenic compounds. The other markers commonly employed in such experiments, lac Z (5) and CD20 (20),

require the addition of substrate or antibody for detection, respectively, adding another step and expense to the experiment. Furthermore, employing paraformaldehyde-based fixatives can preclude the visualization of apoptotic cells (6) using PI staining. Since our method avoids this step, it may be employed to quantitate apoptosis by the appearance of a sub-G0/G1 peak by flow cytometry and to investigate the role of specific proteins on the apoptotic process. Finally, preliminary experiments indicate that a GFP-fusion protein that is actually inserted into the cell membrane by the classic secretory pathway may be more stable in ethanol-treated cells than the membrane-associated GFP-spectrin (Kalejta and Banfield, unpublished observations). This may allow brighter fluorescence to persist for longer times after transfection.

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