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RAPID COMMUNICATION

An Integral Membrane Green Fluorescent Protein Marker, Us9-GFP, Is Quantitatively Retained in Cells during Propidium Iodide-Based Cell Cycle Analysis by Flow Cytometry

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Previously, we described GFP-spectrin, a membrane-localized derivative of the green fluorescent protein that can be employed as a marker during the simultaneous identification of transfected cells and cell cycle analysis by flow cytometry (Kalejta *et al.*, *Cytometry* 29: 286–291, 1997). A membrane-anchored GFP fusion protein is necessary because the ethanol permeabilization step required to achieve efficient propidium iodide staining allows cytoplasmic GFP to leach out of the cell. However, viable cells expressing GFP-spectrin are not as bright as cells expressing cytoplasmic GFP and their fluorescence intensity is further diminished after ethanol treatment. Here, we demonstrate that the fluorescence intensity of cells expressing an integral membrane GFP fusion protein (Us9-GFP) is similar to that of cells expressing cytoplasmic GFP and is quantitatively maintained in cells after ethanol treatment. By allowing an accurate assessment of the expression level of GFP, Us9-GFP allows a more precise analysis of the effects of a cotransfected plasmid on the cell cycle and thus represents an improvement upon the original membrane-associated GFP fusion proteins employed in this assay.

Key Words: **green fluorescent protein; cell cycle; flow cytometry; transient transfection; pseudorabies virus; Us9.**

INTRODUCTION

Transient transfection of cells to overexpress genes of interest is commonly employed to study the effect of the protein on cell cycle progression or apoptosis by flow cytometry. Since only a minority of cells express the genes offered in transfections, a method to identify the subset of transfected cells is required. If an antibody to the protein is not available, cells are often cotransfected with both the gene of interest and a plasmid expressing a marker gene. Commonly utilized markers for flow cytometric applications are the B cell-specific receptor molecules CD19 and CD20, which can be identified by direct or indirect immunofluorescence using commercially available antibodies to these cell surface antigens [1]. However, this protocol is somewhat laborious and expensive and suffers from a marked loss of cell surface fluorescence following ethanol treatment in preparation for propidium iodide (PI) staining and cell cycle analysis.

We developed the use of the green fluorescent protein (GFP, reviewed in Ref. 2) as a marker for transfected cells during PI-based cell cycle analysis by flow cytometry [3]. Propidium iodide cannot enter intact cells unless they are first permeabilized and ethanol is often employed to fix and permeabilize cells in preparation for PI staining. Due to its small size, cytosolic GFP is lost from cells upon fixation and permeabilization with ethanol. However, GFP fusion proteins that localize to the inner leaflet of the cell membrane are retained in cells after ethanol treatment and thus can serve as markers for transfected cells during PI-based cell cycle analysis by flow cytometry. Two EGFP [4] fusion proteins have been employed in this type of assay, one fused to the pleckstrin homology domain of spectrin [3, 5] and one containing the palmitoylation and farnesylation signal of the RAS protein [6, 7].

Here we report the use of a plasmid expressing an integral membrane EGFP fusion protein as a marker for transfected cells in cell cycle analysis by flow cytometry. In this construct, the EGFP gene is fused to the pseudorabies virus Us9 gene and thus is localized to the Golgi apparatus, cytoplasmic vesicles, and the plasma membrane [8]. This fusion protein has unique properties in ethanol-treated cells that make it a markedly more sensitive marker protein in comparison to the GFP fusion proteins that are currently employed for the simulta-

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neous identification of transfected cells and PI-based cell cycle analysis by flow cytometry [3, 6].

MATERIALS AND METHODS

Cells and transfections. The human osteosarcoma cell line U-2 OS (ATCC) was cultured in DMEM (Sigma) 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 5×10^6 cells/ml. In single plasmid transfections, a 700-μl aliquot of the cells was mixed with 2 μg of plasmid DNA and electroporated (960 μF, 220V) using a Bio-Rad gene pulser (Bio-Rad, Hercules, CA). In cotransfection assays, 1 μg of the GFP expression plasmid and 20 μg of the test plasmid were electroporated employing the same conditions. Cells were plated immediately after electroporation. Nocodazole treatment was as previously described [3].

Plasmids. pSP72 is from Promega (Madison, WI). All green fluorescent proteins used in this work were FACS-enhanced mutants [4] and were expressed from the cytomegalovirus promoter. pEGFPN-1 is from CLONTECH Laboratories Inc. (Palo Alto, CA), pEGFPF [6] was kindly provided by W. Jiang and T. Hunter, and pCMVEGFPspectrin has been previously described [3]. Plasmid pBB14 expresses a pseudorabies virus wild-type Us9-EGFP fusion protein (Us9-GFP) which localizes to the Golgi, cytoplasmic vesicles, and plasma membrane as previously described [8]. Plasmids pAB35 and pAB37 encode fusions of engineered mutants of Us9 fused to EGFP [9]. In pAB35, amino acids 46–55 of Us9 are deleted and in pAB37, leucines at positions 30 and 31 are replaced with alanines. The fusion protein encoded by pAB37 localizes to both the Golgi and the plasma membrane, whereas the one encoded by pAB35 is located almost exclusively in the plasma membrane [9]. The human cytomegalovirus UL69 open reading frame was amplified by PCR from viral strain AD169 genomic DNA and cloned as an approximately 2.2-kb *Xba*I/*Bam*HI fragment into pCGN [10], which adds an amino-terminal hemagglutinin (HA) tag, creating pCGNUL69. Expression of UL69 from this vector in transiently transfected cells was confirmed by Western immunoblot with anti-HA (Babco) and anti-UL69 antibodies (data not shown).

Sample preparation and analysis. Sample preparation and analysis were performed exactly as described [3]. Briefly, cells were collected by trypsinization, pelleted, and resuspended in phosphate-buffered saline (PBS) to a final concentration of about 1×10^6 cells/ml. An aliquot (viable cells) was analyzed immediately. To the remaining sample, two volumes of cold, absolute ethanol were added and the samples were stored at −20°C until the day of analysis. At that time, cells were pelleted and resuspended in PBS. For cell cycle analysis, ethanol-fixed cells were resuspended in staining solution (50 μg/ml PI, 100 μg/ml RNase A in PBS). Samples were stored at 4°C for at least 1 h in the dark prior to analysis. Flow cytometric analysis for GFP and PI fluorescence was performed using a four-color FACScan [custom product; Becton–Dickinson Immunocytometry Systems (BDIS), San Jose, CA] and CellQuest software with doublet discrimination (BDIS). For each analysis 50,000 to 200,000 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 shortpass dichroic mirror and collected with a 530/30 bandpass (FL1, GFP) and 572/26 bandpass (FL2, PI). Spectral overlap of GFP into the PI signal was corrected by compensation where appropriate. Data analysis was performed using CellQuest (BDIS) and Modfit LT (Verity Software House, Topsham, ME). GFP fluorescence was collected on a logarithmic scale and PI fluorescence was collected on a linear scale both with 1024 channel resolution. All mean fluorescence intensity values were determined as linear values from the CellQuest software.

RESULTS

Figure 1 shows the FL1 channel fluorescence of viable (dashed lines) U-2 OS cells transfected with a control plasmid, pSP72 (Fig. 1A), or each of four different green fluorescent protein (GFP) expression plasmids: pEGFPN-1 (EGFP, Fig. 1B), pCMVEGFP-spectrin (GFP-spectrin, Fig. 1C), pEGFPF (EGFPF, Fig. 1D), and pBB14 (Us9-GFP, Fig. 1E). The EGFP expressed from pEGFPN-1 is distributed throughout the cell. GFP-spectrin, a fusion between EGFP and the pleckstrin homology domain of spectrin [5], localizes to the plasma membrane and is the marker we have previously utilized for PI-based cell cycle analysis of transfected cells by flow cytometry [3]. pEGFPF expresses EGFP fused to the palmitoylation and farnesylation signals of RAS and is thus localized to the plasma membrane [6]. The Us9-GFP protein expressed from pBB14 is a fusion of EGFP to the Us9 gene of pseudorabies virus and is found in both the Golgi apparatus and the plasma membrane. Us9-GFP is a type II transmembrane protein where the GFP portion is found in the lumen of the Golgi and on the outside of the cell. The fusion protein is anchored in the membrane by the single hydrophobic membrane-spanning domain of Us9 [8].

Cells transfected with one of the four GFP expression plasmids were harvested by trypsinization, resuspended in PBS, and analyzed immediately by flow cytometry. The GFP-expressing cells can be clearly identified as a broad peak of highly fluorescent cells (Figs. 1B–1E, dashed lines) which are well resolved from the GFP-negative cells (compare to cells transfected with pSP72, Fig. 1A, dashed line). However, the fluorescence intensity of the GFP-spectrin-expressing cells is attenuated compared to cells expressing EGFP. EGFPF-expressing cells also display a slightly attenuated fluorescence. Only Us9-GFP shows an almost identical fluorescence pattern to EGFP (compare dashed lines in Figs. 1E and 1B). Quantitation of the histograms in Fig. 1 is presented in Table 1.

When an aliquot of these same cell populations is fixed and permeabilized overnight with ethanol (Figs. 1A–1E, bold lines), only Us9-GFP retains its original fluorescence intensity. As we have shown previously [3], EGFP is completely lost from the cells (Fig. 1B, bold line). Cells expressing GFP-spectrin retain fluorescence, however the fluorescence intensity is reduced compared to nonfixed cells. In the pEGFPF transfected cells, ethanol treatment dramatically reduces the fluorescence intensity of the GFP-positive cells (Fig. 1D). Also, there is a corresponding increase in the fluorescence intensity of the negative cell population. These changes were also seen after incubation in ethanol for as short as 1 h (data not shown). Since after ethanol treatment a small population of highly fluorescent EGFPF-expressing cells still exists, this plasmid can

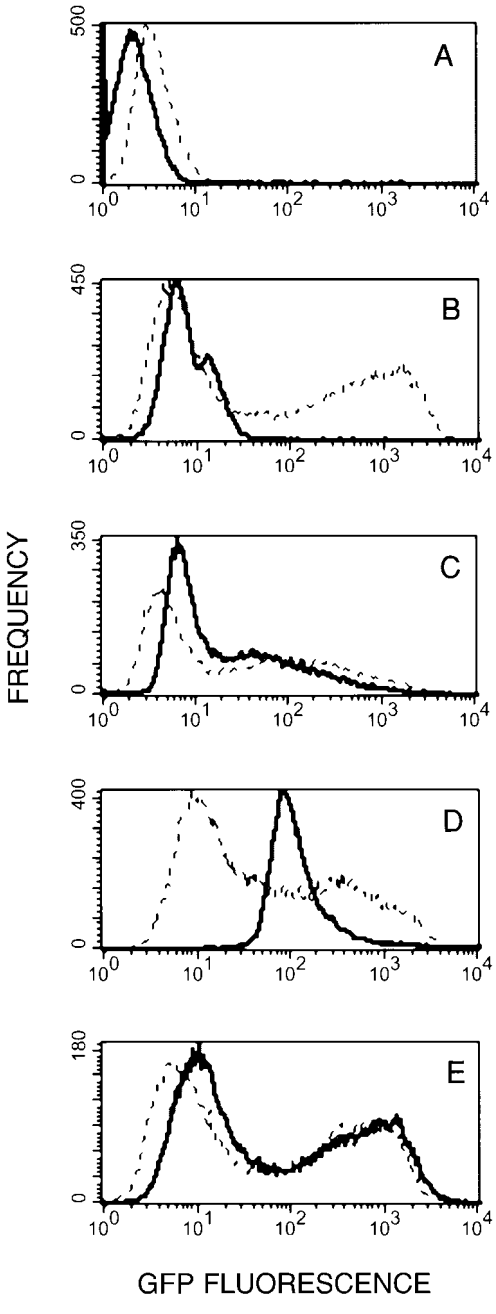

FIG. 1. The fluorescence intensity of Us9-GFP-expressing cells is similar to that of cytoplasmic GFP-expressing cells and is quantitatively retained after ethanol fixation/permeabilization. Single-parameter FL1 histogram overlays of viable (dashed lines) and ethanol-treated (bold lines) U-2 OS cells transfected with either the control plasmid pSP72 (A) or each of the GFP-expression plasmids pEGFPN-1 (B), pCMVEGFPspectrin (C), pEGFPF (D), and pBB14 (E). Samples were prepared as described under Materials and Methods. Only the US9-GFP protein encoded by pBB14 displays a fluorescence intensity similar to EGFP in viable cells and is quantitatively retained in cells after ethanol treatment.

be employed as a marker of transfected cells [6]. However, the abilities to identify the GFP-positive cells and to distinguish positive from negative cells are severely compromised when EGFPF-expressing cells are treated with ethanol. Us9-GFP-expressing cells show essentially identical fluorescence patterns before and after ethanol treatment and thus, because of the ability to discriminate between dim and bright GFP-positive cells, allow for a correlation between fluorescence intensity and the expression level of a cotransfected test plasmid. Since ethanol-treated cells expressing Us9-GFP most accurately depict the GFP expression levels of the cells prior to ethanol treatment, pBB14 represents the preferred marker plasmid in this type of experiment.

Overexpression of Us9-GFP has no effect on cell cycle progression (see Fig. 2 and Discussion). To demonstrate that Us9-GFP can serve as a marker in the identification of proteins which affect the cell cycle, we utilized the same assay system that we employed to show the utility of GFP-spectrin [3]. U-2 OS cells were cotransfected with pBB14 and an expression plasmid (pCGNUL69) for an HA-tagged derivative of the human cytomegalovirus UL69 gene. We have previously shown by a variety of methods that untagged UL69 induces cells to arrest in the G1 phase of the cell cycle [11]. Twenty-four hours after transfection, the cultures were treated with nocodazole to arrest cycling cells in mitosis. Twenty-four hours later, attached and floating cells were harvested and analyzed by flow cytometry to compare the DNA histograms of the GFP-positive and GFP-negative cells in the population. Figure 2 shows a typical analysis.

Single cells were identified from a plot of FL2-width versus FL2-area (R1, not shown) and gated onto a dual-color dot plot of PI fluorescence (FL2-H) versus GFP fluorescence (FL1-H). This plot (Fig. 2, top panels) is used to gate GFP-negative cells (R1 and R2) and GFP-positive cells (R1 and R3). These subpopulations are displayed as DNA histograms (FL2-H), which are then analyzed for the percentage of cells in each stage of the cell cycle (Fig. 2, bottom panels). Cells transfected with a vector control (pCGN) show essentially no cells in G1 (3.6%) after 24 h of nocodazole treatment (Fig. 2A), indicating that Us9-GFP alone does not arrest cells in G1. However, the GFP-positive subpopulation of cells transfected with pCGNUL69 (Fig. 2B) displays a substantial number of cells trapped in G1 (16.8%). The significant number of cells observed in the S period (19.7%) presumably arises from cells that formerly were arrested in G1, but have eventually escaped the UL69-enforced block and now continue to advance toward the nocodazole block. The GFP-negative population of cells does not show this effect, indicating the specificity of the method. With this assay, we have also identified a viral protein that stimulates cell cycle progression (Kalejta and Shenk, manuscript in preparation), indicating that Us9-GFP (pBB14) can be

TABLE 1

Effect of Ethanol Fixation and Permeabilization on the Retention of GFP and GFP-Fusion Proteins by U-2 OS Cells

	Viable			Ethanol-treated		
Plasmid	MFI GFP(−)	% GFP(+)	MFI GFP(+)	MFI GFP(−)	% GFP(+)	MFI GFP(+)
SP72	3.7 ± 0.9	0	ND	2.1 ± 0.4	0	ND
EGFP	5.5 ± 0.5	47.7 + 0.1	414.0 ± 3.4	6.0 ± 0.5	1.2 ± 0.8	ND
Spectrin	4.4 ± 0.2	46.9 ± 1.2	117.4 ± 3.9	5.7 ± 0.5	38.8 ± 1.0	109.0 ± 0.1
EGFP-F	8.8 ± 0.3	37.5 ± 0.8	192.1 ± 0.4	63.0 ± 7.4	7.2 ± 0.9	397.6 ± 1.2
BB14	5.7 ± 0.1	49.4 ± 0.1	300.4 ± 1.7	7.1 ± 0.3	50.0 ± 0.3	305.5 ± 3.5

Note. Data are expressed as means ± standard error for duplicate samples and are representative of three experiments. MFI, mean fluorescence intensity; GFP(+), GFP-positive cells; GFP(−), GFP-negative cells; ND, not determined.

used to identify different classes of cell cycle regulators in transient transfections analyzed by flow cytometry.

Since Us9-GFP is the only fusion protein tested here that is quantitatively maintained in ethanol-treated cells and is also the only integral membrane protein, it is likely that the insertion of this fusion protein into the membrane efficiently prevents it from leaching out of the cell as a result of ethanol treatment. However, it is unclear why only this protein shows identical fluorescence intensity to the wild-type protein in viable cells (Fig. 1). Other GFP fusion proteins that localize only to the Golgi (GalT-GFP, Ref. 12) or to the cell membrane (GFP-spectrin, EGFPF) display attenuated fluorescence in viable cells (Fig. 1 and Ref. 3). We have begun a mutational analysis of Us9-GFP to determine whether the dual localization of this protein to both the Golgi and plasma membrane is responsible for its unique fluorescence properties in both viable and ethanol-treated cells.

We analyzed two mutant Us9-GFP fusion proteins. One (AB37-GFP encoded by pAB37) maintains a dual localization, but the equilibrium is shifted toward the plasma membrane. The other mutant protein (AB35-GFP encoded by pAB35) resides almost exclusively in the plasma membrane. Each of these mutants displays fluorescence properties very similar to Us9-GFP in both viable and ethanol-treated cells (Fig. 3, quantitation shown in Table 2). AB35 (Fig. 3C) shows both a slight reduction in the fluorescence intensity of the GFP-positive cells and a small increase in the background fluorescence upon treatment with ethanol (Table 2). However, the small changes observed lead us to conclude that localization may not be as important as other features of this molecule for retaining fluorescence intensity. For instance, since Us9 is a component of the pseudorabies virus virion [8], both the viral protein and the Us9-GFP fusion protein are most likely very stable, as is GFP itself [2]. Perhaps the other fusions to GFP increase its instability, resulting in decreased fluorescence even in viable cells. An analysis of additional mutant Us9-GFP proteins, especially those that may be less stable or localize only to the Golgi, is needed to confirm the molecular mechanism behind the remarkable fluorescent properties in both viable and ethanol-treated cells of this useful marker.

DISCUSSION

The ease with which positive cells can be identified makes EGFP a powerful tool for flow cytometric studies. However, when combined with cell cycle analysis using impermeant dyes such as PI, care must be taken to ensure that the small GFP molecule is not lost from the cell during the required permeabilization step. Although fixation to retain cytosolic GFP inside the cell is one alternative, we and others have had difficulty achieving high-resolution DNA histograms after paraformaldehyde fixation [3, 13–16]. To avoid this additional fixation step and the problems associated with it, membrane-localized GFP fusion proteins, which are retained in the cell after ethanol permeabilization, are now commonly employed in this type of assay.

Three types of membrane-localized GFP fusion proteins have been used to identify transiently transfected cells during PI-based cell cycle analysis by flow cytometry. We reported the first such molecule, GFP-spectrin [3], in which the pleckstrin homology (PH) domain from human spectrin [5] was fused to EGFP. PH domains are protein motifs found in many cytoskeletal and signaling molecules that bind to both membrane proteins and lipids [17], mediating the membrane association of these proteins.

In another marker plasmid (pEGFPF), the EGFP gene is fused to the palmitoylation and farnesylation signals from the RAS protein [6]. These signals direct the posttranscriptional attachment of lipids to many signal transduction proteins, helping to anchor the protein to the membrane, and in some cases promote protein–protein interactions [18, 19].

EGFPF appears to be the least stable in ethanol-treated cells (Fig. 1). This may be because the only contacts that the fusion protein makes with the mem-

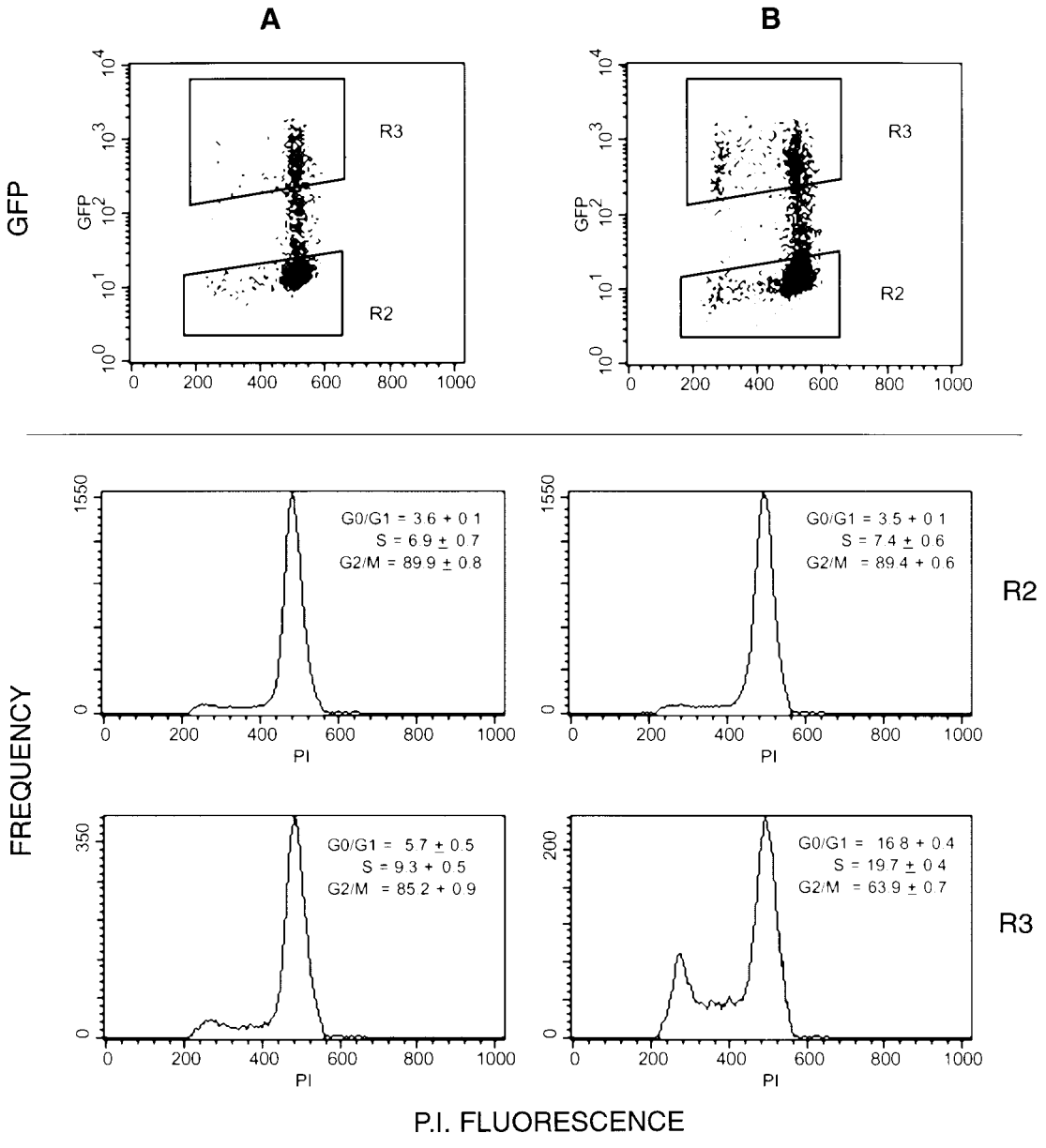

FIG. 2. pBB14 (Us9-GFP) can serve as a marker of transfected cells during PI-based cell cycle analysis by flow cytometry. Asynchronous U-2 OS cells were cotransfected with pBB14 and either pCGN (A) or pCGNUL69 (B). Twenty-four hours later, the media were replaced with media containing 40 ng/ml nocodazole. Floating and attached cells were harvested for analysis 24 h later. Single cells identified from a dot plot of FL2-width vs FL2-area (R1, not shown) are gated onto a dot plot of PI vs GFP. The GFP-negative (R1 and R2) and GFP-positive (R1 and R3) cells can be clearly resolved. DNA histograms (FL2-H) of these subpopulations show that a significant proportion of the cells that received DNA (GFP-positive, R3) in the pCGNUL69 transfection are arrested in G1.

brane are mediated through the attached lipid molecule. Since many lipids are soluble in ethanol, fixation and permeabilization with ethanol may destabilize the interaction of EGFPF with the plasma membrane, leading to loss of fluorescence intensity in the positive cell population. Interestingly, RAS proteins are found in the soluble phase of an ethanol extraction step employed during their purification [20], indicating that ethanol fixation of EGFPF-expressing cells may lead to the release of the fusion protein from the plasma membrane. If ethanol permeabilization allows EGFPF to escape from the cell in which it was synthesized and associate with other, nonexpressing cells in the population, this would result in a decrease in the fluorescence intensity of the positive cells and an increase in the background fluorescence, as we have observed (Fig. 1D). Although we have not investigated the mechanism responsible for the instability of EGFPF in cells

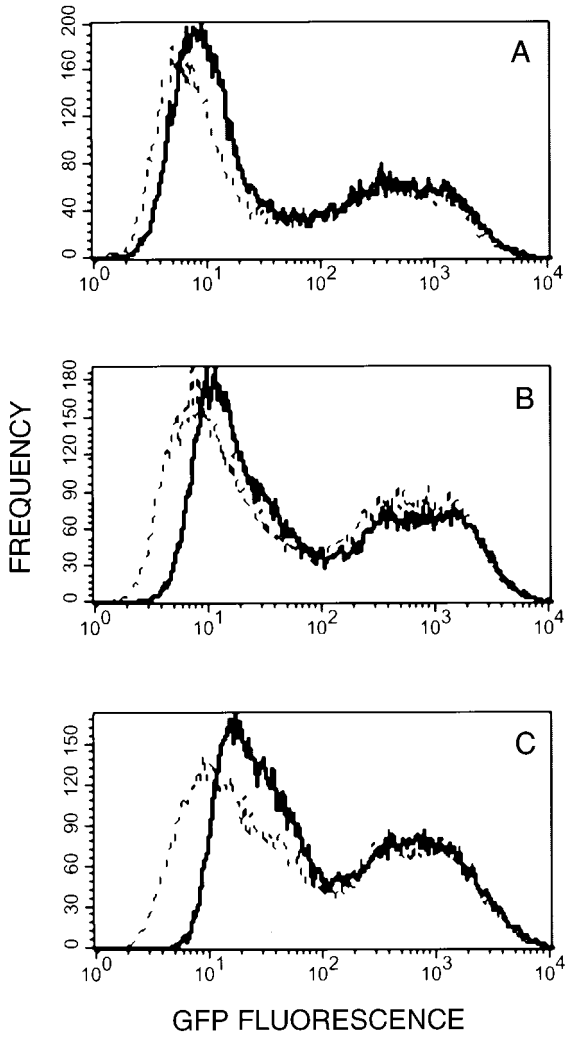

FIG. 3. Localization is not the only factor affecting the fluorescent properties of Us9-GFP. Single-parameter FL1 histogram overlays of viable (dashed lines) and ethanol-treated (bold lines) U-2 OS cells transfected with each of the GFP expression plasmids pBB14 (A), pAB37 (B), and pAB35 (C). Samples were prepared as described under Materials and Methods and the different cellular localizations of the US9-GFP derivatives encoded by each of the plasmids are described in the text.

treated with ethanol, small patches of bright fluorescence have been observed by microscopy on all cells in a population that was transfected with pEFGPF and subsequently fixed in ethanol (M. Kasten, personal communication). This phenomenon was not observed with unfixed cells or with the cytoplasmic GFP protein. Although the increased background can be diminished by washing the cells prior to staining and analysis by flow cytometry (M. Kasten, personal communication), the dramatic loss of fluorescence from the positive cells still occurs.

In this report, we describe a novel marker for use in the cell cycle analysis of transiently transfected cells, the Us9-GFP fusion protein encoded by plasmid pBB14. In this fusion protein, the GFP moiety is located in the lumen of the Golgi and on the exterior of the cell. The fluorescence intensity of viable cells expressing Us9-GFP most closely resembles that of cells expressing cytoplasmic GFP. Moreover, this protein is quantitatively retained in cells upon ethanol permeabilization. Neither an increase in the background fluorescence of GFP-negative cells nor a loss of fluorescence intensity of the GFP-positive cells is observed. As such, Us9-GFP represents the most attractive marker molecule for use in the identification of transfected cells during PI-based cell cycle analysis by flow cytometry. However, the availability of three expression plasmids (pCMVEGFP-spectrin, pEGFPF, and pBB14) increases the flexibility one has in choosing an appropriate marker molecule for a given experiment. This may become important if, for instance, these markers have unique stabilities in different cell types.

While any effect of these markers on cell cycle progression has never been observed [3, 6, 11], it remains a formal possibility that differing levels of expression or cell type-specific interactions may reveal some cryptic effect of these proteins on the cell cycle. For example, many molecules involved in signal transduction at the cell membrane are palmitoylated, farnesylated, or contain PH domains [17–19]. Thus, overexpressing palmitoylated or farnesylated proteins (EGFPF) or those with PH domains (GFP-spectrin), in certain cell types or under certain culture conditions, may impact upon intracellular signaling and hence the cell cycle.

From the sequence and properties of Us9, it is not obvious how any cryptic effect on the cell cycle may be mediated by this protein. Although the function of Us9 is unknown, its primary localization to the Golgi sequesters it from most of the cell cycle machinery. We have not detected any effect of Us9-GFP on the cell cycle in transient assays [11], nor in cells that constitutively express this fusion protein (data not shown). In addition, the activities of both of the novel cell cycle regulatory proteins identified in our laboratory by this method have been confirmed by independent assays (Kalejta and Shenk, manuscript in preparation, and Ref. 11). While truncated fusion proteins which may not retain any Us9-specific biological activity may avoid any potential cell cycle-related problems, they may also be less stable in ethanol-treated cells and thus not as useful in this assay. Once again, because these three markers all target to membranes by different mechanisms, any hidden cell cycle effects will probably be unique to each molecule, emphasizing the importance of having three to choose from. Finally, it is important to stress that the proper positive and negative controls be employed in this cotransfection assay to ensure the validity of the results and that the effects

TABLE 2

Effect of Ethanol Fixation and Permeabilization on the Retention of Wild-Type and Mutant Us9-GFP Fusion Proteins

	Viable			Ethanol-treated		
Plasmid	MFI GFP(−)	% GFP(+)	MFI GFP(+)	MFI GFP(−)	% GFP(+)	MFI GFP(+)
BB14	7.3 ± 0.4	38.7 ± 0.7	359.1 ± 17.0	9.7 ± 0.4	40.1 ± 0.1	378.8 ± 2.5
p37.1	9.0 ± 0.5	45.0 + 1.0	415.0 ± 12.0	12.2 ± 0.9	45.7 ± 1.1	420.1 ± 5.6
p35.1	10.7 ± 0.5	50.0 ± 0.2	401.0 ± 6.0	18.0 ± 0.2	35.6 ± 1.4	353.0 ± 6.6

Note. Data are expressed as means ± standard error of duplicate samples and are representative of four experiments. MFI, mean fluorescence intensity; GFP(+), GFP-positive cells; GFP(−), GFP-negative cells.

of any cell cycle modulators discovered by this technique be confirmed by additional, independent assays.

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