Use of Green Fluorescent Protein/Flp Recombinase Fusion Protein and Flow Cytometric Sorting to Enrich for Cells Undergoing Flp-Mediated Recombination

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ABSTRACT

Flp recombinase has been used extensively for in vivo manipulation of eukaryotic DNA at specific sequences designated as FRT sites. We developed a method to use Flp-mediated recombination without the need for drug resistance or metabolic selection of cells in which recombination has occurred. We generated expression plasmids directing expression of fusion proteins consisting of Flp recombinase and green fluorescent protein (GFP) coding sequences. When the plasmids were introduced into K562 cells containing Flp recombinase substrates and transfected cells were selected for by flow cytometric sorting, GFP-positive cells were enriched 5- to 30-fold for Flp-mediated recombination events compared with unsorted cells. These studies demonstrate the usefulness of GFP/Flp recombinase fusion proteins to manipulate chromosomal DNA in vivo without requiring drug resistance or metabolic marker genes.

INTRODUCTION

The bacteriophage P1 recombinase Cre and the yeast recombinase Flp have both been used to accomplish sequence-specific recombination of stably transfected DNA in vivo (11,12). These enzymes catalyze recombination between specific target sequences, *loxP* and *FRT* sites, respectively. The availability of two distinct recombinases makes it possible to design experiments in which two different recombination events can occur selectively in the same cell. For example, one could use Cre recombinase to target a plasmid construct to a specific chromosomal location containing a *loxP* site and then use Flp recombinase to remove a drug selection cassette flanked by *FRT* sites contained within the plasmid.

Most stable transfection strategies rely on drug resistance or metabolic selection to recover cells containing the correct DNA structure. However, in some circumstances, having drug resistance selection elements in the transfected DNA may be undesirable or impossible. As an alternative to drug selection for identifying cells in which site-specific recombination has occurred, a version of Cre recombinase fused to *Aequorea victoria* green fluorescent protein (GFP) was used in conjunction with flow cytometric sorting to enrich for cells in which recombination had occurred at *loxP* sites (3). Here, we describe the assembly of a GFP/Flp recombinase fusion protein that can be used in a similar fashion to enrich for cells with recombination occurring at *FRT* sites.

MATERIALS AND METHODS

Cell Lines

K562 cells were maintained in RPMI 1640 (Mediatech, Herndon, VA, USA) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, 50 µg/mL streptomycin, 5% characterized fetal calf serum and 5% defined fetal calf serum (the latter two both from HyClone Laboratories, Logan, UT, USA) in a 37°C humidified chamber containing 5% CO₂.

Plasmids

pNEOβGal, pFRTβGal and pOG44 (9) were obtained from Stratagene (La Jolla, CA, USA). The *EcoRI* restriction maps of pNEOβGal and pFRTβGal are shown in Figure 1. GFP-Flp fusion plasmids were constructed using PCR to amplify the 1280-bp Flp recombinase coding sequence from pFLP-Fix (a Flp expression plasmid derived from pFlp) (1) that contains a G to C substitution at nucleotide 557 and corrects a mutated residue present in pOG44 (GenBank® accession no. U46493), (1) (personal communication). The forward primer was 5'-GAAGATCTGCT-TCCACCATGCCACAATTTGATA-3' and the reverse primer was 5'-TCCC-CGCGGCAATATGCGTCTATTATGTAG-3' (Life Technologies, Rockville, MD, USA). The PCR product was inserted as a *Bgl/II-SacII* fragment into pBluescript® SK(-) (Stratagene) that had been modified to include a *Bgl/II* site; the insert was sequenced to

verify that no mutations had been introduced during PCR. The Flp sequences were then subcloned as *Bgl*II-*Sac*II fragments into pEGFP-N1 and pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA), which resulted in Flp sequences being placed 5' (pFlp-GFP) and 3' (pGFP-Flp) to GFP sequences, respectively, with the Flp coding sequences in frame with the GFP coding sequences. Expression of the fusion proteins is driven by the human cytomegalovirus (CMV) immediate early promoter (4).

Transfection

For stable transfection of K562 cells, 10^7 cells were suspended in 0.5 mL HEPES-buffered saline (HBS) (10), mixed with 10 μ g *Sca*I-digested pNEO β Gal, incubated on ice for 10 min and electroporated in 4-mm cuvettes using an ECM 600[®] electroporator (BTX, San Diego, CA, USA) set at 200 V, 600 μ F and 720 Ω . The cells were then incubated overnight in 5 mL RPMI complete medium. Twenty-four hours after transfection, the cells were plated in RPMI complete medium containing 0.25% (w/v) agar and 0.8 mg/mL G418

at a density of 10 000 viable cells/well in 6-well plates. After two to three weeks, macroscopic colonies were expanded for copy number determination.

For transient expression of GFP/Flp fusion proteins, K562 cells containing single integrated copies of pNEO β Gal were electroporated with 20 μ g supercoiled pGFP-Flp, pFlp-GFP or pOG44 using the previously described conditions (10).

Flow Cytometry

For flow sorting, cells were pelleted and resuspended in HBS to a density of $2-3 \times 10^6$ /mL. Both GFP-positive and GFP-negative cells were sorted with a Coulter Epics[®] Elite (Beckman Coulter, Fullerton, CA, USA) flow cytometer using 488-nm argon laser excitation, and 525-nm and 575-nm bandpass filters to detect fluorescein isothiocyanate (FITC) and phycoerythrin fluorescence emission, respectively. Sorted cells were expanded in culture in the absence of G418 for two to three weeks before assaying for Flp-mediated recombination.

Flow cytometric analysis of β -galactosidase (β -gal) activity (FACS-Gal) was performed as described (2,8). Ana-

lytical flow cytometry was performed with an Epics[®] XL-MCL (Coulter) flow cytometer using 488-nm argon laser excitation and a 525-nm bandpass filter to detect FITC fluorescence emission. For each analysis, 5000 events were collected.

Genomic DNA Blotting

Genomic DNA was isolated using the Easy DNA[®] nucleic acid isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's methods. DNA was quantitated by fluorometry (5). Ten micrograms of genomic DNA were digested by the appropriate restriction endonuclease and subsequent blotting, hybridization and washing were performed using Hybond-N[®] membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Radiolabeled pNEO β Gal plasmid was used as the probe. Cells containing single copies of pNEO β Gal were identified based on finding a single hybridizing *Sca*I fragment on the blot that was larger than pNEO β Gal (7 kb) and an absence of a 7-kb band indicating multiple tandem copies of the plasmid.

Figure 1. Experimental strategy for testing GFP-Flp fusion proteins. K562 erythroleukemia cells were stably transfected with pNEO β Gal and were selected in G418. Two clones containing single copies of pNEO β Gal were identified by genomic DNA blotting. The clones were transiently transfected with pGFP-Flp or pFlp-GFP, and GFP-positive and -negative cells were sorted by flow cytometry. After expanding the sorted or unsorted populations in culture without G418, recombination was measured by determining β -gal activity in individual cells by the FACS-Gal assay or in populations of cells by a chemiluminescent β -gal assay. In the plasmid maps, SV40 sequences are indicated by solid bars, *Neo* sequences by the bar with horizontal stripes, *LacZ* sequences by the stippled bars, plasmid sequences by thick lines and genomic sequences by thin lines. Sizes of *Eco*RI fragments detected in clone 2 when pNEO β Gal was used as a probe for DNA blots are indicated below each map (see Figure 3).

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β-Gal Enzyme Assay

Cells (2×10^6) were pelleted and washed twice with 1 mL PBS. The cell pellets were lysed in 500 μ L cell lysis buffer [100 mM potassium phosphate (pH 7.8), 6 mM MgSO_4 , 1 mM dithiothreitol, 0.1% (v/v) Triton® X-100]. The lysate was frozen at -70°C for at least 20 min. The β -gal assay was performed using the Galacto-Light Plus™ kit (Tropix, Bedford, MA, USA) according to the manufacturer's methods. A Monolight® 2010 luminometer (Analytical Luminescence Laboratory, Sparks, MD, USA) was used to inject 100 μ L accelerator solution. After a 2-s delay, light output was measured for 5 s. Protein assays were performed in triplicate as previously described (10). Absorbance at 570 nm was measured using a SPECTRAMax™ 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

RESULTS

Figure 1 shows the experimental strategy used to test the function of the GFP-Flp fusion proteins. For this study, the K562 erythroleukemia cell line (6) was used. K562 cells were first transfected with pNEOβGal (9), and two clones containing single integrated copies of the plasmid were identified by genomic DNA blotting. pNEOβGal contains a *Neo* cassette flanked by *FRT* sequences. Prior to Flp-mediated recombination, the simian virus 40 (SV-40) early promoter directs expression of the *Neo* gene, rendering the cells G418-resistant. Following Flp-mediated recombination, the *Neo* sequences are excised, bringing *LacZ* sequences under the control of the SV40 promoter. Thus, Flp recombinase activity can be measured by determining the number of cells that become β-gal-positive. The purpose of these experiments was to determine whether fusing GFP sequences to Flp recombinase would allow for flow cytometric enrichment of cells that have undergone Flp-mediated recombination.

The expression plasmids used were derivatives of pEGFP-N1 and pEGFP-C1, in which Flp coding sequences were cloned in frame 5' (pFlp-GFP) or

Table 1. Comparison of Recombinase Activities of Wild-Type Flp (pOG44) and GFP/Flp Fusion Protein

		FACS-Gal (% positive)	β-Gal specific activity ^a
Clone 1	Untransfected	0.3	12520
	pOG44	0.4	13345
	pGFP-Flp	0.5	12236
Clone 2	Untransfected	0.5	16175
	pOG44	0.7	18383
	pGFP-Flp	1.0	18804

^aRatio of light units to total protein in chemiluminescent assay

3' (pGFP-Flp) to GFP coding sequences, respectively. For reasons that were not clear, it was difficult to clone Flp sequences 5' to the GFP sequences, and the plasmids that were obtained gave poor plasmid DNA yields in *E. coli* strain DH5α. The CMV immediate early promoter (4) was used to drive expression of the fusion genes. These plasmids were transiently introduced into K562:pNEOβGal clones by electroporation, and fluorescence was determined 48 h after transfection.

Figure 2 shows an example of the

increase in green fluorescence obtained by transient transfection with pGFP-Flp. Cells electroporated in the absence of DNA are shown for comparison. Typically, 4%–10% of the transfected cells became GFP-positive following transfection with pGFP-Flp. pFlp-GFP was less efficient in generating fluorescent cells, with 1% of cells at most becoming GFP-positive. Both the GFP-Flp and Flp-GFP expression plasmids generated fewer fluorescent cells than the wild-type GFP expression plasmid (pEGFP-C1), which typically produced

Figure 2. Flow cytometric analysis of clone 1 cells 48 h after electroporation in the absence of DNA (top panels) or clone 1 cells 48 h after transfection with pGFP-Flp (bottom panels). Live cells were selected by forward (FS) vs. 90° (RA) light scatter (left, region labeled “viable cells”); fluorescent cells were identified by FITC vs. PE fluorescence (right, region labeled “GFP-positive”).

30% fluorescent cells.

To compare the relative recombinase activity of GFP-Flp to that of wild-type Flp, both clones of K562 cells containing single stably integrated copies of pNEO β Gal were transiently transfected with pGFP-Flp or pOG44 (9), a plasmid that directs expression of wild-type Flp recombinase. Recombinase activity was determined by measuring β -gal activity of the transfected cells, both at the single-cell and population levels. Single-cell β -gal activity was determined using the FACS-Gal assay (2,8), in which cells are permeabilized and loaded with fluorescein-di- β -D-galactopyranoside. Cells are

assayed for fluorescein fluorescence by flow cytometry, which allows determination of the distribution of β -gal activity across the cell population. β -gal activity for the entire cell population was determined using a chemiluminescent assay of cell lysates. As shown in Table 1, the proportion of transiently transfected cells becoming β -gal-positive following transfection with either

pOG44 or pGFP-Flp was 1% or less. The β -gal activity of the cell population barely increased with both plasmids (Table 1), which is consistent with the low transfection efficiency of electroporation when used with K562 cells.

To compensate for the low transfection efficiency, we used flow cytometry to enrich for transfected cells based on GFP fluorescence. The two K562

Figure 3. DNA blot analysis of transfected cells. Clone 2 cells were transiently transfected with pGFP-Flp, and GFP-positive and -negative cells were separated by flow cytometric sorting. Genomic DNA was isolated from untransfected cells (lane 1), unsorted cells (lanes 1 and 2), and each sorted population (lanes 3 and 4). The DNA was digested with *Eco*RI, transferred onto nylon membranes and hybridized with radiolabeled pNEO β Gal. A 6-kb band is obtained from cells that have undergone Flp-mediated recombination with concomitant loss of 4.0 and 3.2-kb bands.

clones containing single copies of pNEO β Gal were transiently transfected with pGFP-Flp and with pFlp-GFP in one experiment. Flow cytometric sorting was used to separate GFP-positive and GFP-negative cells. These two populations, as well as unfractionated cells, were expanded in culture for two to three weeks. Genomic DNA was isolated and subjected to DNA blotting using pNEO β Gal as a probe. Deletion of the *Neo* sequences removes approximately 1.3 kb sequence, including two *Eco*RI sites (Figure 1). Thus, the *Eco*RI fragments flanking the *Neo* sequences are lost, and a new larger fragment appears.

In the case of clone 2, cells in which the *Neo* sequences were deleted by Flp recombinase were detected by the disappearance of bands of 3.2 and 4.0 kb and the appearance of a 6-kb band on the DNA blot. As shown in Figure 3, unfractionated clone 2 cells that were transfected with GFP-Flp contain a small subpopulation that has undergone *Neo* deletion, which is demonstrated by the presence of a barely detectable 6-kb band in lane 2. This band is easily detected in DNA from the GFP-positive cells (lane 3), but is undetectable in DNA from the GFP-negative cells (lane 4). There is a corresponding decrease in intensity of the 4.0 and 3.2 kb bands derived from the unrearranged sequences in the GFP-positive cell population. Thus, enriching for GFP-positive cells by flow cytometry results in a population with increased Flp recombinase activity and an increase in the fraction of cells that have undergone deletion of sequences flanked by the *FRT* sites.

The efficiency of Flp-mediated recombination was determined by measuring β -gal activity, both at the single cell and population levels. Figure 4 compares the distribution of β -gal activity in clone 1 following transient transfection with pGFP-Flp before sorting and after separating GFP-positive and GFP-negative cells. The unsorted population contains 5.4% β -gal-positive cells. Cells sorted for high levels of GFP expression contained 67.4% β -gal-positive cells, a 12.5-fold enrichment. This increase was from a larger fraction of β -gal-positive cells rather than a global increase in fluorescence of the population, as evidenced by the two separate peaks of positive and negative fluorescence that are shown in Figure 4. Cells selected for lack of GFP expression had 1.8% β -gal-positive cells, which is less than that observed in the unsorted population.

The fluorescence observed in the FACS-Gal assay was not from residual GFP in the cells because no fluorescence was observed in the GFP-enriched cells from clone 1 that were transfected with pEGFP-C1, the GFP expression plasmid without added Flp sequences (Figure 5A). Three individual experiments were performed in which the two clones were transiently transfected with pGFP-Flp. In the second experiment, the clones were transfected with pFlp-GFP for comparison. The enrichment in β -gal-expressing cells averaged 9.3-fold (± 5.2) for GFP-Flp ($\bar{x} \pm \text{SD}$ based on data from three experiments with two clones, range 4.4- to 18.3-fold) and 27.3-fold (± 5.8) for

Figure 4. FACS-Gal assay showing enrichment of cells with Flp-mediated recombination. Clone 1 cells were transfected with pGFP-Flp, and cells were sorted as described in Figure 3. Unsorted cells (Panel A), GFP-positive cells (Panel B) and GFP-negative cells (Panel C) were loaded with a fluorogenic substrate for β -gal (FDG) and subsequently analyzed by flow cytometry after a brief incubation at 37°C.

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Flp-GFP (based on data from one experiment with two clones, range 23.2- to 31.4-fold).

Since it was not possible to determine the magnitude of increase in β -gal-specific activity using the FACS-Gal assay, the β -gal activity was

measured in lysates of the cell populations using a chemiluminescent enzyme assay; enzyme activity was normalized to total protein in the lysates. The data from three experiments are shown in Figure 5B. The increase in β -gal activity averaged 8.3-fold (± 7.1)

for sorted cells transfected with GFP-Flp ($\bar{x} \pm \text{SD}$ based on data from three experiments with two clones, range 2.4- to 22.2-fold) and 9.3-fold (± 3.4) for cells transfected with Flp-GFP (based on data from one experiment with two clones, range 6.9- to 11.7-fold).

Figure 5. (A) FACS-Gal analysis of cells transfected with GFP/Flp. Two independent clones of K562 cells containing single copies of pNEO β Gal were transiently transfected with the indicated expression plasmids, then sorted and analyzed as described in Figure 4. Controls include K562 cells lacking pNEO β Gal and clone 1 cells transfected with pEGFP-C1 (a plasmid directing expression of GFP alone). Data are expressed as the percentage of β -gal-positive cells. Three separate experiments are shown. (B) Chemiluminescent β -gal enzyme assay of the cell populations obtained from the experiments shown in (Panel A). Data are expressed as a ratio of light units to total protein.

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DISCUSSION

In this study, we have characterized Flp fusion proteins with GFP at both the carboxyl (Flp-GFP) and amino (GFP-Flp) termini. The presence of Flp sequences appeared to cause a small decrease in the fluorescence of the GFP moiety, but this did not affect the ability to enrich for cells expressing the fusion proteins. The presence of GFP sequences did not have a measurable effect on Flp recombinase activity. The C- and N-terminus fusion proteins were comparable in their recombinase activities, although Flp-GFP did not produce as high a proportion of GFP-positive cells as GFP-Flp following transient transfection. Because Flp-GFP is more difficult to propagate in *E. coli*, we have not investigated this phenomenon further.

Between 4% and 10% of electroporated K562 cells were found to be fluorescent 48 h after transfection. This figure is comparable to the 3.4%–9% of unsorted cells found to have undergone Flp-mediated recombination, based on flow cytometric detection of β -gal reporter gene expression. This proportion of β -gal-positive cells is somewhat lower than was found for CV-1 cells transfected with pOG44 (9). However, as in that study, the similar proportions of GFP-positive and β -gal-positive cells indicate that Flp-mediated recombination is highly efficient in those cells that receive the Flp expression plasmid.

Since Flp-mediated recombination is highly efficient, low transfection efficiency becomes the major barrier to obtaining cells with Flp-mediated recombination events. In the absence of selection, identifying a clone containing the desired recombination event would require extensive screening, generally by cloning transfected cells in a semi-solid medium or by limiting dilution, isolating DNA and performing PCR or DNA blot analysis to identify cells containing the desired rearranged DNA structure. Even if as many as 10% of cells contain the desired rearrangement, 29 clones would have to be screened to have a 95% probability of finding the desired clone (this is derived using the binomial distribution, in which the probability of finding the desired clone in N tries is equal to $1 - 0.9^N$).

In our experiments, a minimum of

25% of transfected cells became β -gal-positive after enriching for GFP-positive cells. Even with this minimum level of enrichment, only 11 clones would need to be screened to have a 95% chance of finding the desired recombination event. Since higher numbers of β -gal-positive cells were obtained in several transfections, it is likely that even fewer clones would need to be screened in most experiments. In addition, the efficiency of isolating cells with the desired recombination event could be improved further by more stringent selection for fluorescent cells at the time of flow sorting.

In the three experiments presented here, we observed considerable variability in the proportions of β -gal-positive cells obtained both before and after flow sorting. The variable β -gal activity in unsorted cells transfected with Flp recombinase is most likely due to differences in transfection efficiency between experiments. The variation observed in sorted populations is a function of both transfection efficiency and the actual gating strategy used for flow sorting. When selecting a cell population for sorting, one must weigh the stringency of selection against the time required to select for rare events by flow cytometry. In experiments where reasonable transfection efficiency is obtained, stringent selection for fluorescence intensity can yield acceptable numbers of cells and high-level enrichment for Flp-mediated recombination.

In summary, we have demonstrated that a GFP-Flp fusion protein retains both its fluorescence and recombinase activities when transiently introduced into a mammalian cell line. Since both Flp recombinase and GFP have been shown to be active in numerous cell types (7,11), it is reasonable to assume that the GFP-Flp fusion protein will function in a wide variety of cells, including embryonic stem cells. This reagent will provide additional options for designing experiments in which genomic DNA is manipulated in vivo.

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