3326–3331 *Nucleic Acids Research, 1997, Vol. 25, No. 16*

Brief expression of a GFP*cre* fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions

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Received April 4, 1997; Revised and Acepted June 21, 1997

ABSTRACT

The Cre DNA recombinase of bacteriophage P1 has become a useful tool for precise genomic manipulation in embryonic stem (ES) cells that have been gene modified by homologous recombination. We have re-engineered the *cre* gene to allow ready identification of living Cre+ cells by constructing a functional fusion between Cre and an enhanced green fluorescent protein from *Aequorea victoria* (GFPS65T). The GFP*cre* fusion gene product rapidly targeted the nucleus in the absence of any exogenous nuclear localization signal. Moreover, GFPCre catalyzed efficient DNA recombination in both a mouse 3T3 derivative cell line and in murine ES cells. Fluorescence- activated cell sorting (FACS) of transiently GFP*cre*-transfected ES cells not only allowed rapid and efficient isolation of Cre+ cells after DNA transfection but also demonstrated that a burst of Cre expression is sufficient to commit cells to Cre-mediated 'pop-out' of *loxP*-tagged DNA from the genome. Thus, GFP*cre* allows rapid identification of living cells in which *loxP*- flanked DNA sequences are destined to be removed from the genome by Cre-mediated recombination without reliance on recombinational activation or inactivation of a marker gene at the target locus. In addition, the GFP*cre* fusion gene will prove useful in tracing tissue-specific Cre expression in transgenic animals, thereby facilitating the generation and analysis of conditional gene knockout mice.

INTRODUCTION

The generation of transgenic and gene modified mice by pronuclear injection of zygotes and homologous gene targeting in embryonic stem (ES) cells has rapidly advanced understanding of gene function in mammals and increasingly will permit the development of highly useful animal models of human disease and pathology. An important tool for genome modification *in vivo* is the Cre site-specific DNA recombinase of bacteriophage P1 (1–3). Cre catalyzes precise DNA recombination both intra- and intermolecularly between target 34 bp *loxP* sites and is proficient for recombination in a variety of eukaryotes. Efficient Cre-mediated excision of DNA between directly repeated *loxP* sites in developing transgenic animals has been harnessed both to activate expression of a dormant transgene (4) and to ablate a resident chromosomal gene (5). In both cases the timing and tissue localization of the recombination event can be precisely delimited by choosing a promoter to drive *cre* expression that has the spatial and temporal expression pattern desired. One other important use for Cre recombinase is the removal of unwanted DNA from the genome. For example, in some cases the selectable *neo* marker interferes with neighboring gene expression (6,7). Removal of the selectable marker eliminates potential complications in interpretation of an animal's phenotype after gene targeting and is easily achieved by using a selectable marker gene flanked by *loxP* sites so that it can be excised by transient expression of Cre recombinase either in ES cells (8–10) or in fertilized zygotes (11) or by simply mating the gene targeted animal with a transgenic mouse having either general (4,12) or zygote-specific expression of the *cre* gene (13).

Because of the binary nature of these site-specific recombination strategies, knowledge of the expression pattern of the *cre* transgene is critical for evaluation of the doubly transgenic animals that result from crossing a *cre* mouse with one having chromosomally positioned *loxP* sites. Cre protein can be detected in mammalian cells by *in situ* immunohistochemistry using polyclonal anti-Cre antibodies (S.Gagneten and B.Sauer, unpublished work) and with specific anti-Cre monoclonal antibodies (14). However, direct detection of Cre in living cells cannot be achieved by immunological methods. Yet in a variety of circumstances, for example excision or 'pop-out' of the selectable marker used for gene targeting in ES cells, it would be useful to identify and isolate live Cre-expressing cells for subsequent propagation or manipulation and analysis.

To provide a convenient and efficient way to identify Cre+ cells, we have fused Cre to the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (15). GFP has rapidly become an important new reporter of gene expression in a variety of organisms (16,17). The 238 amino acid protein requires no host cofactors and emits a green fluorescence (λ_{max} 508 nm) in living cells transfected with GFP cDNA after stimulation with UV light (λ_{max} 395 nm and a much weaker excitation λ_{max} 470 nm). Alteration of GFP Ser65 to Thr (18) results in a protein having substantially enhanced, red-shifted fluorescence (λ_{max} 511 nm)

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and is maximally excited with blue light (λ_{max} 490 nm). Because many flow cytometers employ an argon laser tuned to 488 nm for excitation, GFPS65T is more suitable for use in fluorescence-activated cell sorting (FACS) compared with wild-type GFP (19,20) and we have therefore used the GFPS65T mutant for construction of the GFP*cre* fusion.

We show here that the GFP*cre* fusion gene expresses Cre activity and allows the facile FACS separation of Cre^+ cells after transient transfection of both a cultured fibroblast cell line and of *loxP*-modified ES cells.

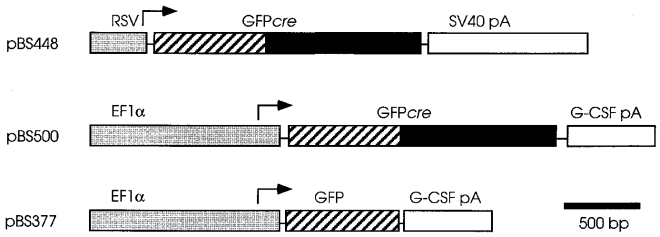

Figure 1. GFP and GFP*cre* expression vectors. The GFP coding sequences are indicated by the diagonal bars, the *cre* coding region by the black box, the RSV and EF1α promoter regions by the shaded boxes and polyadenylation regions (pA) by white boxes. G-CSF, granulocyte colony stimulating factor.

MATERIALS AND METHODS

DNA constructions

All DNA manipulations were performed by standard procedures (21). Plasmid pBS377 carries the wild-type GFP gene under the control of the strong EF1α promoter. It was constructed by cloning the *Asp*718–*Bsm*I GFP fragment of TU65 (16) into the cognate sites (underlined) of a polylinker 5′-AAT TGG ATC CAG ATA TCT GGT ACC ATG GCT AGC AGA ATG CCT CTA G-3′ inserted between the *Eco*RI and *Xba*I sites of a derivative of pEF-BOS (22) from which the *Hin*dIII fragment containing the SV40 origin had been removed.

A modified *cre* gene [carrying an optimized translational start (23), a neutral S2A mutation in the coding region and convenient flanking restriction sites] was amplified by Pfu DNA polymerase (Stratagene) from bacteriophage P1 DNA using the primers 5′-TTT TCA AGC TTG GAT GGT ACC ATG GCC AAT TTA CTG ACC G-3′ and 5′-TTC AGC TCT AGA GCA ATC ATT TAC GCG TTA ATG G-3′ and then cloned as a *Hin*dIII–*Xba*I fragment into pUC19 to obtain pBS353. The *Nco*I–*Sma*I *cre* fragment of pBS353 replaced the *neo* gene of RSV*neo* (24) using *Hin*dIII–*Nco*I bridging oligos (5′-AGC TTG GTC GAC GGA GAA AGC TCA GGC TCT GGC TCA GAG TCT GAC TCC ATG-3′, *Sal*I site underlined). The GFP gene with the S65T mutation (18) was fused in-frame with *cre* by cloning the *Xho*I fragment of pZA69 (a gift from Dr R.Dhar, NCI, Bethesda, MD) into the *Sal*I site to yield the RSV–GFP*cre* expression construct pBS448. In EF1α–GFP*cre* (pBS500) the GFP*cre* *Hin*dIII–*Xba*I fragment of pBS448 replaced the *Eco*RV–*Xba*I GFP fragment of pBS377. All cloned synthetic and amplified DNAs were verified by DNA sequencing.

Cell culture, DNA transfection and FACS analysis

Calf serum (CS), DMEM culture medium and leukemia inhibitory factor (ESGRO) were from Gibco BRL (Gaithersburg, MD). Fetal calf serum (FCS) was from Hyclone (Logan, UT). Cell line B-13, a derivative of NIH 3T3 cells (ATCC, Rockville, MD), carries a silent, but recombination activatable, *loxP*-modified *lacZ* cassette (B.Bethke and B.Sauer, manuscript in preparation) and was cultured in DMEM plus 10% CS. CHO-K1 cells were grown in DMEM + 10% FCS. The ES cell line H200 is a gene targeted *loxP*-flanked insertion at the HPRT locus (S.Gagneten, Y.Le and B.Sauer, unpublished work). Recombination between the two directly repeated *loxP* sites in H200 results in deactivation of the *neo* selectable marker. H200 cells were cultured in DMEM plus 16% FCS and 813 U/ml ESGRO and maintained on irradiated mouse embryonic fibroblasts (25). Cells were grown at 37°C in a humidified chamber with 5% CO_2.

DNA (18 μg/6 cm plate) was introduced into CHO and B-13 cells by co-precipitation with $CaPO_4$ (26). The procedure was slightly modified for ES cells: the DNA (9 μg)/$CaPO_4$ co-precipitate was mixed with 1.5×10^6 trypsinized cells in 5 ml ES medium and then plated on a 6 cm gelatinized plate. The DNA co-precipitate was removed after overnight incubation and replaced with fresh medium. A CMV*lacZ* plasmid, p324 (27), was used in non-GFP control transfections. Two days post-transfection cells were trypsinized and resuspended in culture medium for flow cytometric analysis and sorting with an Elite cytometer (Coulter, Miami, FL). Argon laser (488 nm) excitation and 525 nm bandpass emission filters were used to detect GFP expression. Analysis and sorting gates were chosen to identify and isolate cells which fluoresced at levels >3 SD above the mock-transfected controls. Sort logic and coincidence decisions were chosen for highest purity. Sorted and unsorted cells were replated to allow individual colony formation. B-13-derived colonies were analyzed by an *in situ* β-galactosidase assay (28) using Bluo-Gal (Gibco BRL).

Microscopy

Fluorescence was examined in both live and fixed (3% paraformaldehyde, 30 min) cells. Confocal microscopy was graciously performed by Dr A.Robbins (NIDDK) with a Zeiss Axiovert 100 microscope and a Zeiss LSM 410 imaging system, using a fluorescein filter set. A Nikon Optiphot-2 epifluorescence microscope with the Nikon B-2A filter block (excitation 450–490 nm, BA 520–560 nm) was used for routine monitoring of fluorescence in live cells.

DNA analysis

Individual ES cell colonies were expanded into 24-well dishes and DNA was prepared after cells had become confluent (29). Retention of *loxP*-flanked DNA after H200 transfection was detected with the PCR primers a (5′-ATA GCC GAA TAG CCT CTC CAG C-3′) and c (5′-TAA CAG CGT CAA CAG CGT GCC-3′). Primers b (5′-GTA GCC AAC GCT ATG TCC-3′) and d (5′-ACA GTA GCT CTT CAG TCT G-3′) were used to detect excision events. DNA was amplified with Taq DNA polymerase: 30 s, 61°C; 60 s, 72°C; 30 s, 94°C, for either 30 cycles (primers a + c) or 35 cycles (primers b + d).

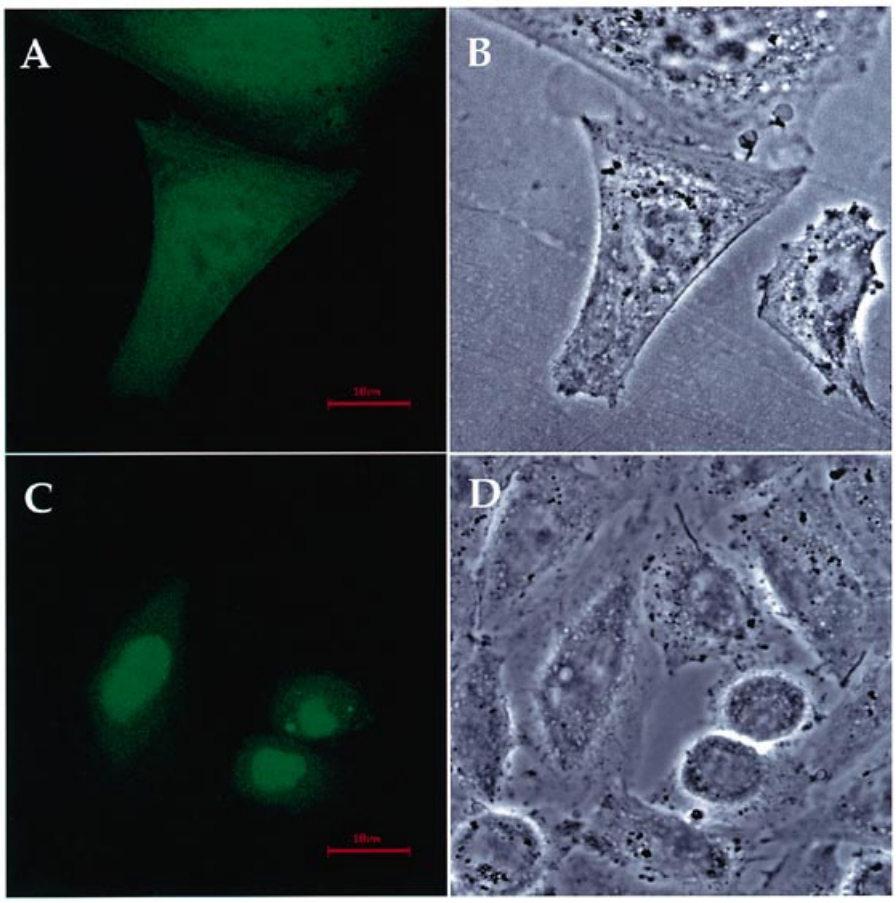

Figure 2. Detection of GFP by fluorescence microscopy. CHO-K1 cells were transiently transfected with either the wild-type GFP construct pBS377 (**A** and **B**) or the GFP*cre* construct pBS448 (**C** and **D**) and examined by both phase contrast (B and D) and fluorescence (A and C) confocal microscopy.

RESULTS

Expression of the GFP*cre* fusion gene

GFP carrying the S65T mutation was fused to the N-terminus of Cre. The GFPS65T mutant was chosen as a fusion partner for Cre both to give enhanced fluoresence and to permit excitation with blue light rather than UV in order to eliminate UV-induced damage to mammalian cells or mouse zygotes after visualizing expression of the GFP*cre* fusion gene (30). Initial work in *Escherichia coli* and in *Saccharomyces cerevisiae* showed that expression of GFP*cre* resulted in cellular fluorescence and Cre-mediated recombination (data not shown). The mammalian expression vectors pBS448 and pBS500 (Fig. 1) place the GFP*cre* fusion gene under the control of the strong RSV and EF1α promoters respectively. We first examined expression of the fusion gene in cultured mammalian cells by transient expression. Although of prokaryotic origin, Cre protein efficiently targets the nucleus of mammalian cells due to specific determinants in the wild-type Cre protein that direct nuclear localization/retention (S.Gagneten and B.Sauer, manuscript in preparation). We thus suspected that in cells transfected with the GFP*cre* fusion gene, only the nucleus would exhibit strong fluorescence. This is exactly the result observed. Transfection of CHO cells with pBS448 resulted in strong green fluorescence almost exclusively in the nucleus of productively transfected cells, whereas no subcellular localized fluorescence occurred after transfection with the wild-type GFP construct pBS377 (Fig. 2). These results indicated that fluorescence might be a convenient tag to identify Cre expression in living cells after transient expression.

FACS sorting of excision events in cultured fibroblasts

Transient expression of Cre recombinase efficiently evicts *loxP*-flanked DNA from the mammalian genome (31,32). The frequency of Cre-catalyzed excision is roughly equivalent to the DNA transfection efficiency, indicating that a brief burst of Cre expression commits a transfected cell to Cre-mediated excision of DNA. Because transient expression of the GFP*cre* fusion gene results in green fluorescence of cells that take up and express the GFP*cre* construct, we asked whether GFP*cre*-transfected cells could be sorted, based on their fluorescence, in order to enrich for cells that are destined to undergo Cre-mediated recombination.

To facilitate this analysis we exploited cell line B-13, an engineered derivative of NIH 3T3 cells containing a single copy of a recombination activatable *lacZ* gene (Fig. 3). B-13 cells are LacZ$^-$ (white) due to a *loxP*-flanked STOP cassette (4) inserted into the open reading frame at the N-terminus of the *lacZ* gene. Upon Cre-mediated excision the STOP cassette is removed and the *lacZ* reading frame is restored to allow production of β-galactosidase, thus producing blue colonies.

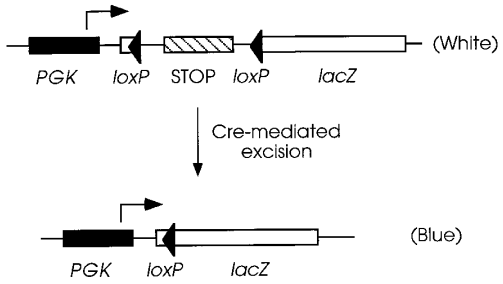

Figure 3. Recombination-activated gene expression of β-galactosidase. B-13 cells carry a single copy of a defective *lacZ* gene under control of the mouse *PGK* promoter. Deactivation of *lacZ* occurs from insertion of an out-of-frame *loxP*-flanked STOP cassette carrying the SV40 poly(A) signal into the 5′-end of the gene. Cre-mediated excision of STOP restores the *lacZ* reading frame.

To test the efficacy of FACS in selecting recombinants, B-13 cells were transfected with the EF1α–GFP*cre* construct pBS500. Two days post-transfection, FACS showed that 1–5% of the transfected cells exhibited a moderate to high level of fluorescence (data not shown) compared with non-transfected cells. This result was confirmed visually by fluorescence microscopy, which showed 5% or more of the cells to be fluorescent. The EF1α–GFP*cre*-transfected cells were sorted by FACS to eliminate ~99% of the least fluorescent cells. The resulting population of cells was thus expected to be highly enriched for cells that had been productively transfected by the GFP*cre* construct. Both the sorted and non-sorted transfected cells were replated and resulting colonies were stained *in situ* for β-galactosidase activity. Transfection of B-13 cells resulted in ~13% blue recombinant colonies derived from unsorted cells (Table 1), about twice the apparent (5%) transfection efficiency as determined by fluorescence. This suggests that even in those cells with expression too low to give bright intracellular fluorescence, there is still sufficient GFPCre protein to give chromosomal DNA excision. In contrast, 90% of the sorted cells gave rise to blue colonies that had undergone Cre-mediated recombination. Similar results (>90% recombinant colonies after FACS) were obtained with the RSV–GFP*cre* expression vector pBS448 (data not shown). Non-recombinant colonies may have come either from non-transfected cells contaminating the sorted population or from fluorescent cells that were not functionally Cre^+. FACS sorting thus permitted rapid enrichment of cells committed to Cre-mediated recombination from a population of GFP*cre*-transfected cells.

Selective enrichment of ES cell pop-outs by FACS

Although previous work had established that transient expression of Cre recombinase was sufficient for productive recombination in ES cells, the absolute frequency of obtaining such marker pop-outs was only a few percent, thus necessitating the use of a negative selectable marker, *tk*, interposed between the *loxP* sites in order to obtain an efficient yield of pop-outs (8). It is likely that this low absolute efficiency in detection of recombination derives not from an inherent inability of the recombinase to catalyze recombination, but rather from the low transfection efficiency of ES cells that results in only a small percentage of the transfected cell population actually expressing the enzyme.

Table 1. Cre-mediated *lacZ* activation by excisive recombination

Cells	Number of colonies			Excisive recombination (%)
	White	Blue	Total	
Not sorted	390	61	451	13.5
FACS sorted	51	460	511	90

To determine whether use of the GFP*cre* gene would facilitate retrieval of Cre-mediated DNA pop-outs, we examined GFP*cre*-catalyzed recombination in ES cell line H200 that carries a gene targeted *loxP*-flanked marker cassette at the HPRT locus (Fig. 5A). Transfection of H200 with the EF1α–GFP*cre* construct pBS500 resulted in ~1% of the cells exhibiting green fluorescence after 2 days, as determined by fluorescence microscopy. Similarly, FACS analysis indicated fluorescence in ~1.4% of the transfected cells (Fig. 4B) compared with the control (Fig. 4A). After sorting, FACS analysis revealed a dramatic enrichment to ~95% fluorescent cells (Fig. 4C).

Both sorted and non-sorted EF1α–GFP*cre*-transfected ES cells were replated and resulting colonies were analyzed for Cre-mediated recombination by PCR. Of 15 randomly picked colonies from the sorted population of GFP^+ cells, 12 (80%) gave the PCR product diagnostic of excision (Fig. 5B). The three remaining colonies exhibited the PCR product diagnostic of no recombination event having occurred. In contrast, analysis of randomly picked non-sorted colonies showed that only one of 15 (6.7%) had undergone Cre-mediated recombination. No mosaic colonies, exhibiting both excision and non-excision chromosomes, were observed, confirming that transient expression of the GFP*cre* gene efficiently commits cells to Cre-mediated recombination.

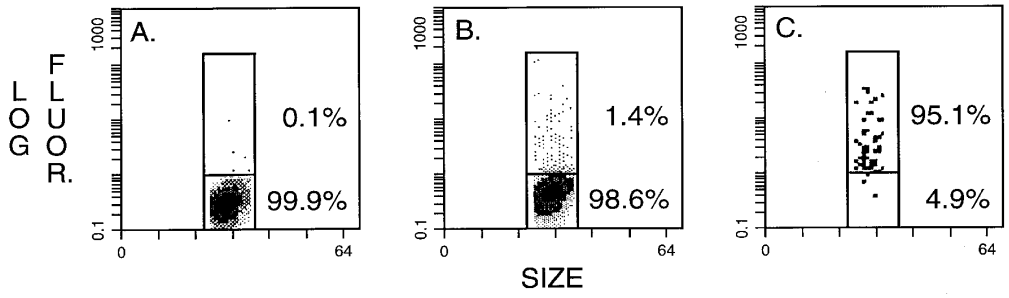

Figure 4. Flow cytometric analyses of GFP*cre* transfected cells. Non-GFP control plasmid-transfected (**A**) and pre-sort (**B**) versus post-sort (**C**) pBS500-transfected ES cell analyses are shown. Rectangles and percentages define the negative (autofluorescent) and positive (GFP*cre* expressing) cells.

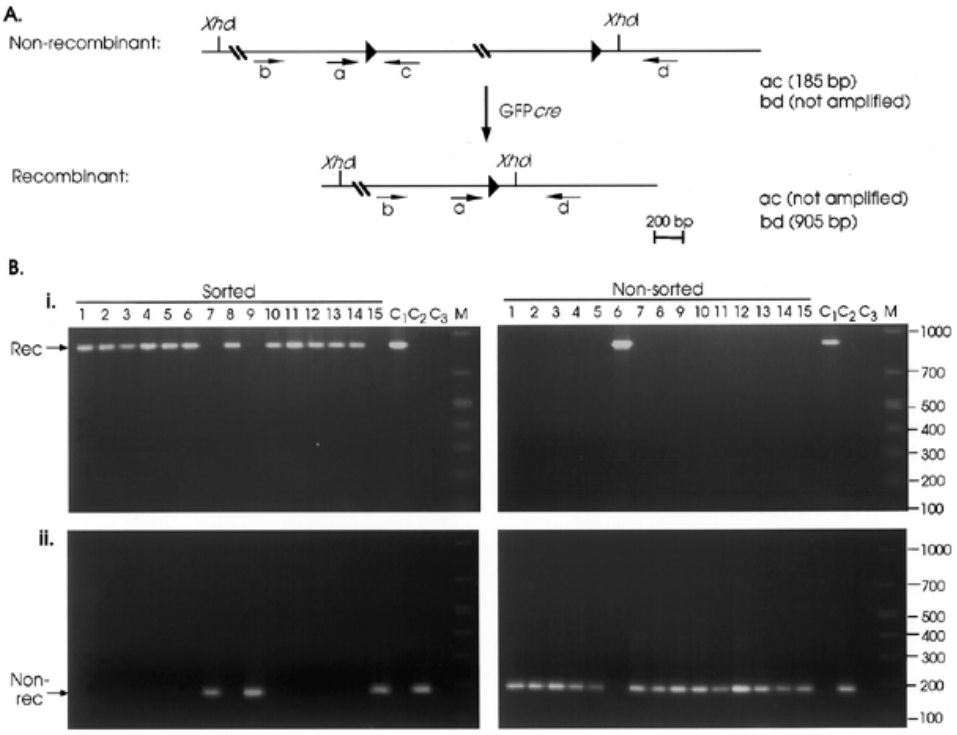

Figure 5. Detection of excision in the ES cell line H200. (**A**) PCR strategy. Black triangles represent the *loxP* sites. Primers b and d amplify a 905 bp fragment from the Cre-mediated recombinant genome, whereas primers a and c amplify a 185 bp fragment specific for the non-recombinant genome. The 4.4 kb PCR product from the non-recombinant genome with primers b and d is too large to be amplified under the PCR conditions used. (**B**) PCR analysis of GFP*cre* transiently transfected ES cells. DNA from 15 colonies derived from either sorted or unsorted GFP*cre*-transfected H200 ES cells was amplified with primers diagnostic for excision (i) and for no recombination (ii). Controls: C_1, a pop-out derivative of H200; C_2, parental H200; C_3, no DNA. DNA size markers in bp (Gibco BRL) are shown to the right.

DISCUSSION

We have constructed a functional GFP*cre* fusion gene and shown that transient expression after DNA transfection of mammalian cells results in cells that concomitantly exhibit bright nuclear fluorescence and are phenotypically Cre^+. Moreover, enrichment of GFP*cre* fluorescent cells by FACS resulted in a population of cells in which the vast majority are committed to Cre-mediated excisive recombination. We have applied this procedure to ES cells that carry a chromosomal *loxP*-flanked DNA cassette and shown that Cre^+ cells destined to pop-out the DNA between the two directly repeated *loxP* sites could be easily and efficiently obtained by transient expression of the GFP*cre* construct and subsequent FACS. This is particularly valuable for situations in which only a low percentage of the cells productively take up DNA, as is often the case for ES cells.

The task of ridding the ES cell genome of a no longer needed selectable marker after homologous targeting is greatly facilitated by the use of GFP*cre*. Rapid identification and enrichment of phenotypically Cre^+ cells by FACS obviates the need for a negative selectable marker like *tk* to be present on the DNA segment targeted for removal by Cre. This is advantageous not only because it avoids a negative drug selection step, but also because it frees the *tk* marker to be used for positive/negative selection, if desired, in the initial gene targeting step in order to enhance recovery of homologous recombinants (33). As an alternative to the use of FACS, purification of $GFPCre^+$ ES cells in good yield may also be achieved using direct visualization by fluorescence microscopy and micromanipulation (T.Larson, Y.Le and B.Sauer, unpublished work).

Use of the GFP fusion approach is advantageous for the detection and isolation of rare transfection events by flow cytometry. High specificity can be attained by elimination of background fluorescence from non-specific binding with antibodies or the fluorescent substrates used to detect reporter gene expression. Moreover, it should be possible to precisely select cell populations based on the level of GFP*cre* transgene expression by simple differential gating of the sort histogram. This has certain practical applications. For example, in certain cases ES cells may be modified to carry a selectable marker flanked by directly repeated *loxP* sites and also a third *loxP* site *in cis* (5) designed to delete the targeted gene by Cre-mediated recombination (a conditional knockout). To remove the selectable marker in ES cells without deleting the targeted gene itself thus requires partial Cre-mediated excision. Because the recombinational potential of a transfected cell correlates with the level of Cre expression (34), FACS-mediated retrieval of cells expressing only a moderate level of Cre should enhance the recovery of cells that have excised only the selectable marker, leaving the targeted gene intact but flanked by two directly repeated *loxP* sites.

In addition to simple marker removal, Cre can also be used to engineer more extensive, precisely determined chromosomal deletions by targeting (by homologous recombination) correctly oriented *loxP* sites to span the exact chromosomal interval to be deleted. Although the efficiency of large deletion generation (>200 kb) in the mammalian genome by Cre may vary between

different loci, the use of GFP*cre* to identify functionally Cre^+ cells will clearly allow rapid enrichment of the desired deletions by eliminating non-expressing or weakly expressing cells. Finally, because expression of GFP does not appear to be toxic in transgenic animals (35), the GFP*cre* gene should also prove useful in tracing tissue-specific expression of Cre recombinase in transgenic animals.

ACKNOWLEDGEMENTS

We are grateful to Dr A.R.Robbins for confocal microscopy, to M.Klessman for assistance with cell culture and to J.Njoroge for FACS expertise. We thank Dr E.Ginns for thoughtful comments on the manuscript.

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