Bifunctional Protein Conferring Enhanced Green Fluorescence and Puromycin Resistance


ABSTRACT

A new genetic marker was created in which sequences from enhanced green fluorescent protein were fused to those of puromycin N-acetyltransferase. The resulting fusion protein (EGFP-puro) conferred both green fluorescence and resistance to puromycin when expressed in mammalian cells. The utility of EGFP-puro as a selectable/screenable marker was demonstrated by the ease with which a recombinant guinea pig cytomegalovirus containing EGFP-puro was isolated by a combination of puromycin selection and screening for green fluorescence. We conclude that EGFP-puro is a compact and versatile marker that should prove useful for recombinant virus and transgenic cell line construction, particularly in applications in which coding capacity is limited.

INTRODUCTION

Genetic markers that provide either a visual or selectable phenotype are essential tools in molecular biology. In recent years, chimeric proteins have been created that combine screenable and selectable markers in a single marker protein (2,4,6,7,11,12,15,17). These chimeric markers are an advantage in retroviral or adenovirus-associated virus vectors where genetic coding capacity is limited (22) and where promoters for multiple genes can cause promoter suppression (9,10). Here, we describe EGFP-puro, a bifunctional marker consisting of enhanced green fluorescent protein (EGFP) (26) [an improved derivative of green fluorescent protein (23), reviewed in (24)] fused with puromycin N-acetyltransferase (Puro0) (8,25). We also show that EGFP-puro confers resistance to puromycin while providing rapid and convenient detection when viewed under UV light.

MATERIALS and METHODS

Cells and Cell Culture

Primary human foreskin fibroblast (HF) cells and guinea pig lung fibroblast (GLF) cells (ATCC no. CCL-158) were cultured as described elsewhere (13,18) using Eagle’s minimal essential media (EMEM) containing 10% fetal calf serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. Green fluorescence was visualized using a Nikon Diaphot 300 microscope (Nikon, Melville, NY, USA) equipped with a 470-525 nm UV filter.

Construction of pEGFP-puro

Plasmid pEGFP-puro was constructed by PCR amplification of Puro0 coding sequences from pPur (Clontech Laboratories, Palo Alto CA, USA) using upstream (5’-AAGTCCGGACTCAGATCTAGGAGACCGCATTCCA TGACCGAGT-3’) and downstream (5’-GGTATTCTAGGCTTGGATCCCGGGCACCAGGCTTGGGTCTATGCACCA-3’) primers, digestion of the PCR product with Bgl II and BanHI, and ligation into Bgl II/BanHI digested pEGFP-C1 (Clontech Laboratories).

Retrovirus Transduction

The retroviral plasmid LZRSpBMNZ was a gift from Gary Nolan (14,16). The lacZ sequences were removed from LZRSpBMNZ by EcoRI digestion and religation to produce pMA68. A BanHI fragment from pEGFP-puro was then inserted at a unique BanHI site in pMA68 to make the EGFP-puro retroviral vector pMA73.

Phoenix-AMPHO packaging cells were transfected using a calcium phosphate transfection system (16,21) according to the manufacturer’s instructions (Life Technologies, Rockville, MD, USA). Twenty-four hours before transfection, 2 x 106 cells were plated in 60-mm dishes. Three hours before transfection, the media was replaced with 3 mL/dish DMEM containing 25 µg/mL chloroquine. The cells were transfected with 15.5 µg carrier DNA and 5 µg pMA73 DNA. Five hours later, the cells were washed once and then incubated for 48 h with DMEM, at which time the culture media was removed, filtered through 0.45-µm filters, adjusted to 5 µg/mL polybrene (Sigma, St. Louis, MO, USA), and used to infect subconfluent HF cells.

Recombinant GPCMV Construction

Plasmid pGP47 contains the terminal HindIII O fragment of GPCMV cloned in pGEM3Zf+ (18). An EcoRI site was removed from pGP47 by digestion with EcoRI and XbaI, blunt-ending with T4 polymerase, and religation to make pGP104. A synthetic linker was inserted at the KpnI site of pGP104 (within GPCMV sequences) to introduce an EcoRI site adjacent to the KpnI site to make pGP104.1. An EcoRI/KpnI fragment from pEGFP-puro was then ligated into EcoRI/KpnI digested gpP104.1 to make pGP105. Twenty micrograms of pGP105 were linearized by MluI digestion and electroporated into GLF cells as previously described (18). Twenty-four hours later, the cells were infected with GPCMV strain 22122 (ATCC VR-682) at a multiplicity of infection of 3. Twenty-four hours post-infection (PI), the media was replaced with EMEM containing 1 µg/mL puromycin (Sigma). Five days PI, the culture supernatant was used to infect confluent GLF cells. Three hours PI, these cells were washed, incubated for 24 h in EMEM without puromycin, and then incubated for five days in EMEM containing 1 µg/mL puromycin. The culture supernatant was again used to infect a fresh flask of cells, and the selection procedure was repeated. Recombinant viruses were cloned from this culture supernatant by 96-well limiting dilution as previously described (19). The recombinant virus was considered pure when all virus-infected wells exhibited green fluorescence upon repeated 96-well limiting dilution.

RESULTS AND DISCUSSION

Construction and Characterization of a Bifunctional Fusion Protein

To construct a fusion protein con-
taining both EGFP and Puro\(^r\) sequences, Puro\(^r\) coding sequences were ligated into an existing expression cassette for EGFP. The resulting fusion protein was designated EGFP-puro and is contained in plasmid pEGFP-puro (Figure 1A). This plasmid contains the strong human cytomegalovirus immediate early promoter (\(P_{\text{hcmv}}\)), the EGFP-puro open reading frame, and the simian virus 40 (SV40) polyadenylation signal. The EGFP-puro protein contains the first 245 amino acids of EGFP. The C-terminal five amino acids of EGFP were deleted and replaced by four linker-encoded amino acids, followed by the complete 200-amino-acid Puro\(^r\) sequence. The C-terminus is formed by 11 amino acids encoded by vector sequences.

To determine if EGFP-puro retains both green fluorescence and the ability to confer puromycin resistance, the EGFP-puro expression cassette was cloned into a retroviral plasmid vector that was used to produce defective retroviral stocks. HF cells were then infected with this defective retrovirus. Green fluorescence was assessed by UV microscopy 48 h after infection, and puromycin resistance was determined by cell survival in the presence of 0.5 \(\mu\)g/mL puromycin. Consistent with previous findings using retroviral transduction of EGFP (20), green fluorescence produced by EGFP-puro was readily detected in retrovirally infected cells (Figure 1B) as compared to mock-infected cells (Figure 1C). Furthermore, infected cells exhibited normal morphology after culture for two weeks in the presence of puromycin (Figure 1D), whereas mock-infected cells exhibited severe cytotoxicity (Figure 1E). Thus, we conclude that EGFP-puro is able to produce visible green fluorescence while at the same time conferring resistance to puromycin.

**EGFP-puro as a Marker for Construction of Recombinant Herpesviruses**

Recombinant herpesviruses are conventionally constructed by homologous recombination and subsequent selection and/or screening to isolate the desired recombinants. To engineer a recombinant GPCMV, the EGFP-puro cassette from pEGFP-puro was inserted within plasmid-cloned GPCMV sequences from the right terminus of the viral genome. This plasmid was linearized and electroporated into GLF cells, which were subsequently infected with wild-type GPCMV. Viruses in which homologous recombination had transferred the EGFP-puro cassette into the viral genome were then selected by two rounds of viral propagation in the presence of 1 \(\mu\)g/mL puromycin. Within 24 h of the first round of selection, it

![Figure 1. EGFP-puro as a bifunctional marker.](image-url)
was possible to identify the presence of recombinant virus-infected cells by their green fluorescence. The effectiveness of puromycin selection could also be observed because green (recombinant virus-infected) cells exhibited normal infected-cell morphology, whereas uninfected or wild-type virus-infected cells, which failed to fluoresce, exhibited severe cytotoxicity (Figure 1F). Green fluorescence further served to monitor the extent of enrichment during selection and purification by rapidly allowing the determination of the ratio of recombinant to wild-type virus. Final purification was achieved by 96-well limiting-dilution, and green fluorescence again served to easily identify wells containing recombinant viral plaques (Figure 1G). Southern hybridization experiments confirmed that the viral DNA had the structure predicted for homologous recombination with the plasmid sequences (not shown).

These results demonstrate that the convenient visualization of EGFP and the potent selection of puromycin can be obtained from a single protein without loss of function. In the construction of recombinant herpesviruses, selection with puromycin was effective and economical, while green fluorescence allowed for the rapid determination of successful recombination, the identification of recombinant virus-infected cells or cultures, and the evaluation of recombinant virus purity.

Other chimeric screenable/selectable markers have been reported, including β-galactosidase, β-glucuronidase, alcohol dehydrogenase, and EGFP, fused to neomycin phosphotransferase II (Neo$^\text{r}$), phleomycin (zeocin)-resistance, guanine phosphoribosyltransferase, thymidine kinase, aminoglycoside 3′-adenyltransferase, hygromycin phosphotransferase (Hyg$^\text{r}$), and blasticidin-resistance (2–4,6,7) (Invitrogen and Clontech Laboratories). EGFP-puro compares favorably with these fusions in several respects. Its small size (1.4 kb) is comparable to several other chimeric markers (GFP::zeocin-resistance, GFP::blasticidin-resistance, and Neo$^\text{r}$::EGFP) but is half the size of Hyg$^\text{r}$::EGFP (2.7 kb) and substantially smaller than lacZ fusions (3–4 kb) (2,3) (Invitrogen and Clontech Laboratories). This may be particularly important for retroviral or adenov-associated virus vector applications when coding capacity is limited. As the process of selection often requires large culture volumes and frequent media changes, the costs involved in drug selection can also be an important consideration. Because of its low cost and high potency, puromycin selection is 10- to 100-fold less expensive than selection using drugs such as G418, zeocin, or hygromycin. The ease with which green fluorescence is detected is also an advantage over markers requiring fixation and enzymatic color development. Finally, Neo$^\text{r}$ has been reported to repress expression of neighboring promoters by 3- to 10-fold (1,5), whereas repression did not occur with Puro$^\text{r}$ (1). Thus, EGFP-puro is a compact, effective, and economical marker that should prove useful for a wide variety of molecular biology and genetic engineering applications.

REFERENCES


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**Generating Tandem Repeats by Cloning with Double Initiator Fragments**


**ABSTRACT**

The ability to generate tandem repeats of a DNA sequence has proven important for a large variety of studies of DNA structure and function. The most commonly used method to produce tandem repeats involves cloning of an oligomerized monomer sequence that contains asymmetric overlapping ends, but, in practice, this approach is inefficient because of the circularization of oligomers before they ligate into vector. Described here is a method that circumvents this problem by the use of two separate oligomerization reactions, each containing an initiator fragment onto which monomer polymerizes without circularization. Subsequent mixing of the two reactions permits circularization, generating a viable plasmid containing the sum of the added repeats from each reaction. A variation of this method is also demonstrated that permits the synthesis of constructs with a defined number of repeats.

**INTRODUCTION**

Synthesized tandem repeats of DNA sequences have been widely used for analyses of DNA structure and function as well as for affinity isolation of DNA binding proteins (4,6-9). While a number of strategies have been devised to produce such tandem repeats, one of the most effective is the cloning approach of Hartley and Gregori (3), which takes advantage of the fact that a restriction enzyme such as AvrI can recognize an asymmetric site. Ligation of such a cleaved asymmetric site can occur in one orientation only, with the result that oligomers cloned using such a site are necessarily in a head-to-tail direction. The simplest approach is to ligate monomers with the appropriate asymmetric ends into a compatible vector that has been treated with phos-