gpt-gus Fusion Gene for Selection and Marker in Recombinant Poxviruses

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Poxviruses have become a useful eukaryotic expression vector system and have been used to express a variety of viral (especially poxviral), eukaryotic and bacterial proteins and as recombinant vaccines (11). During construction, the identification of recombinant poxviruses is greatly facilitated by co-expression of either a dominant selection gene or a color screening marker. Examples of the former are *E. coli* guanine phosphoribosyl transferase gene (*gpt*) (4) and the neomycin-resistance gene derived from Tn5 (5), and the *E. coli* genes *lacZ* (encoding β-galactosidase) (2,10) and *gusA* (encoding β-glucuronidase) (1) have been used for color screening. Although the dominant selection offers the advantage of selective amplification of the recombinant virus, the phenotype of recombinant virus plaques is usually similar to the parental wild-type virus. Consequently, a second screening is required. Color screening markers make recombinant viruses produce easily identifiable plaques. However, they do not offer a selective advantage, and the subsequent isolation and purification can be troublesome. In this communication, we report construction of a fusion gene comprised of *E. coli gpt* and *gusA* genes, which can offer both dominant selection and color screening. Under the control of a single promoter, the complete cassette can be easily transferred into other vectors by blunt-end ligation.

E. coli GPT, which can initiate a salvage pathway for purine synthesis inhibited by mycophenolic acid (MPA), has been widely used for dominant selection of recombinant poxviruses (4,9). The *E. coli gusA* gene, which had been used effectively as a color reporter in plants (7), invertebrates (8) and mammalian cells (6), has recently been introduced as a color screening marker for isolation of the recombinant vaccinia virus (1). The *gusA* gene is significantly smaller than *lacZ* and lacks several of the restriction sites present in the latter, which have often complicated cloning strategies. A further advantage of *gusA* is that it may be used as a color marker in viruses that already express β-galactosidase (1). The strategy used to construct the *gpt-gusA* fusion gene is illustrated in Figure 1 with primer sequences shown in Figure 2. The fusion gene (Figure 2C) encodes an intact GPT, an extra amino acid (alanine) as a linker and GUS with the alteration of only the second amino acid (leucine → valine). Initially, the construct was tested by transient assays, which demonstrated GUS activity in the transfected poxvirus-infected cells. Since *gusA* is the C-terminal half of the fusion gene, this suggested that the whole fusion protein was produced.

Therefore, this cassette, containing the *gpt-gusA* fusion gene regulated by a synthetic early/late promoter (E/L) (1), was isolated by digestion with *Sca*I and cloned into the ectromelia virus (EV) homolog of the vaccinia virus F3L gene. A recombinant EV was produced

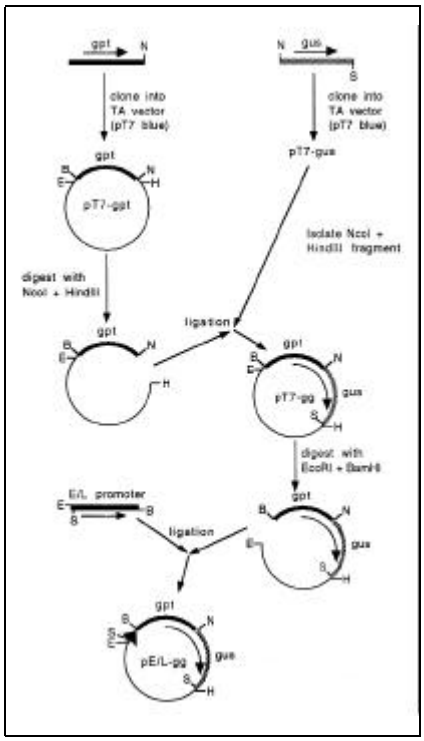

Figure 1. Synthesis of *gpt-gusA* fusion gene. The *gpt* gene was synthesized using PCR from plasmid pSBgpt/R (kindly provided by K. Mossman, Department of Biochemistry, University of Alberta) with an *Nco*I site at the C-terminus. The *gus* gene was synthesized using PCR from plasmid pBI101 (CLONTECH Laboratories, Palo Alto, CA, USA) with an *Nco*I site at the N-terminus and an *Sca*I site at the C-terminus. PCR products were cloned into pT7/Blue (Novagen, Madison, WI, USA). The *gus* gene was isolated from the pT7/gus by *Nco*I and *Hin*dIII digest and cloned into *Nco*I- and *Hin*dIII-digested pT7/gpt. A strong synthetic vaccinia early and late (E/L) promoter (1) with a 5′ *Eco*RI cohesive end and a 3′ *Bam*HI cohesive end, constructed by annealing two synthetic oligonucleotides, (sequence shown in Figure 2) was cloned into *Eco*RI- and *Bam*HI-digested pT7/gg. B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; N: *Nco*I; and S: *Sca*I.

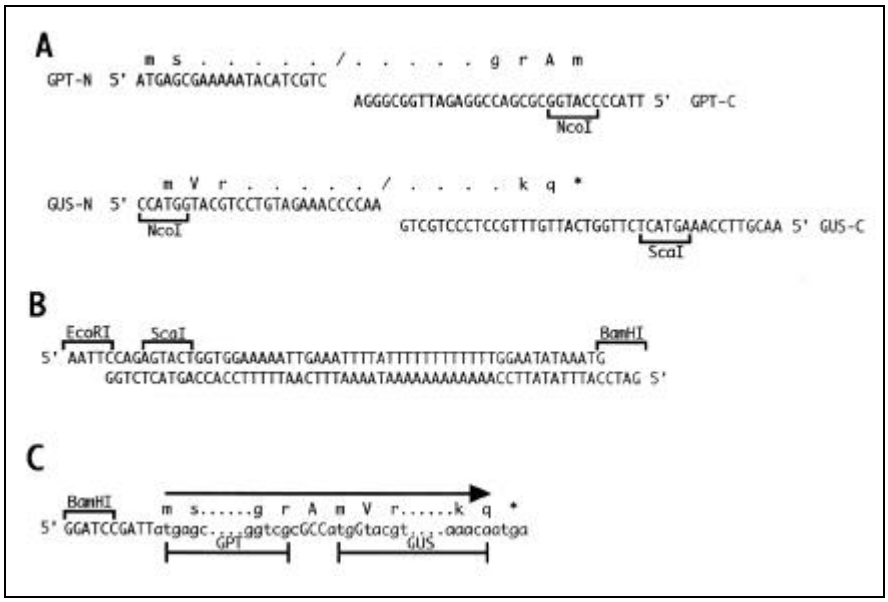

Figure 2. Organization of primers and genes. Amino acids at the start and end of ORFs are shown above DNA sequences (upper case denotes a change from original protein). (A) Oligonucleotides used for PCR of *gpt* and *gus* genes. (B) Sequence of E/L promoter cassette. (C) Overall gene arrangement.

by standard procedures for poxvirus homologous recombination (3). The recombinant EV was amplified 3 times in the MPA selection medium (DMEM containing 10% newborn calf serum, MPA at 25 µg/mL, xanthine at 250 µg/mL and hypoxanthine at 15 µg/mL; tissue culture reagents from Life Technologies, Burlington, ON, Canada) and plaque-purified 3 times using MPA-selection medium containing 1% LMP agarose and X-glucuronide X-glu (Gold BioTechnology, St. Louis, MO, USA) at 200 µg/mL (prepared as 20 mg/mL stock in DMF) (1).

The pure recombinant virus as characterized by polymerase chain reaction (PCR) was isolated after 3 rounds of plaque purification (data not shown). The GPT activity of the fusion protein was demonstrated by the inhibited growth of the wild-type parental virus in comparison with the recombinant virus in the MPA selection medium (data not shown). The recombinant virus formed easily visible foci after 3 days in selection medium, although the virus yield was reduced to approximately 30% of that obtained in the absence of the drug. For virus purification, the foci were stained by including X-glu (200 µg/mL) in the overlay (not shown). As observed previously (1), we noticed that the intensity of staining for foci in overlays was variable; however, identical virus preparations produced evenly stained foci after fixation in the absence of overlay (Figure 3). This suggests that the staining variability seen with the overlays is probably an artifact due to virus growth, cell lysis or GUS activity in the agarose overlay rather than being a virus phenotype. Staining after fixation is a useful way to assess the purity of recombinant virus preparations, and with only a slight loss in final color intensity, the concentration of X-glu could be reduced to 200 µg/mL although the development times were increased to several hours. The optimal concentration of X-glu is likely to be dependent on the virus and promoter used and should be tested empirically to avoid wasting the reagent.

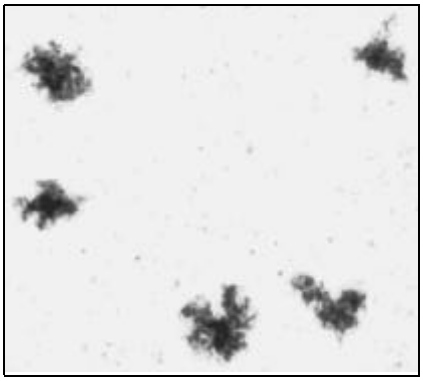

Figure 3. BGMK cells with recombinant virus. After the recombinant virus foci became visible on the BGMK cell monolayer, cell monolayers were washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde at room temperature for 5 min. The cells were then incubated at 37°C with the substrate buffer (5 mM ferricyanide, 5 mM ferrocyanide, 2 mM magnesium chloride, 1 mg/mL of X-glu in PBS).

In conclusion, the *gpt-gusA* fusion gene reported in this communication provides both the advantage of dominant selection and the ability to rapidly identify recombinant poxviruses by color screening. The promoter-gene cassette is portable and can easily be cloned into other plasmids. This construct demonstrates the ability to make functional fusion proteins with both *gpt* and *gus*, which may have potential uses in other systems.

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Simple Method for High Sensitivity Chemiluminescence ELISA Using Conventional Laboratory Equipment

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Enzyme-linked immunosorbent assays (ELISAs) have a unique importance in numerous different analytical procedures due to their specificity and sensitivity. Moreover, the use of the 96-well format allows the quantification of high numbers of samples in parallel. Most commonly, the binding of antibodies to antigens in an ELISA is detected by the cleavage of a chromogenic substrate by an enzyme conjugate, for instance alkaline phosphatase or horseradish peroxidase (HRP) coupled to either an antibody or to streptavidin. Thus it is usually a colored reaction product that is quantified by measuring the absorbance. Assays with higher sensitivities can be set up based on the principle of chemiluminescence (2–5), but equipment for chemiluminescence measurements in a 96-well format are more expensive than the usual ELISA plate readers and therefore not available in many laboratories.

We developed a chemiluminescence ELISA system with a very simple detection of the immobilized HRP activity that does not require any sophisticated equipment and which is still much more sensitive than a conventional photometric assay with *o*-phenylenediamine as the substrate. Since the chemiluminescence detection that we used is based on the HRP immobilized to the bottom of the plate only, the volume of the coating antibody (or protein) and all subsequent antibody solutions can be as low as 50 µL (or even 30 µL, which is about the minimal volume to cover the bottom surface).

For a model system, we used an ELISA for the detection of cyclophilins (1) with a cyclosporine/bovine serum albumin (BSA)-conjugate coated to flat-bottom plates (Immuno MaxiSorp™; Nunc, Roskilde, Denmark). In brief, the plates were coated overnight at 4°C with the cyclosporine/BSA conjugate (1 µg/mL; 50 µL/well) in coating buffer (50 mmol/L $NaHCO_3$, pH 9.6). Unspecific binding was blocked by incubation with 2% BSA in phosphate-buffered saline (PBS)/0.5% Tween® 20 (PBS/T) for 1 h at room temperature. Then the plates were washed three times with PBS/T (all washing steps were carried out with 200 µL per well). This was followed by incubation with different concentrations of cyclophilin A (50 µL per well) for 1 h at 37°C, then by 7 washing steps as above, incubation with rabbit anti-cyclophilin A (polyclonal antibody against human cyclophilin A at 50 µL per well) at 1:100 overnight at 4°C, washing as above and finally incubation with peroxidase-conjugated anti-rabbit IgG [50 µL per well, $F(ab')_2$ fragments; Amersham International, Little Chalfont, Bucks, England, UK] at 1:2000 for 1 h at 37°C. After thorough washing, the plates were then either processed conventionally by incubation with *o*-phenylenediamine substrate solution, or the chemiluminescence-based detection was carried out. The conventional assay was done by adding 50 µL per well substrate buffer (1 mg/mL *o*-phenylenediamine in buffer containing 7.3 g/L Na_2HPO_4, 5.64 g/L citric acid monohydrate, pH 5.0, and 0.4 µL/mL 30% H_2O_2). After stopping the reaction with 10 µL per well with 2 mol/L H_2SO_4, the color was measured at 492 nm with reference reading at 620 nm with an ELISA reader (SLT Labinstruments, Salzburg, Austria).

For the chemiluminescence detection, 50 µL of substrate solution were added per well (enhanced chemiluminescence solution 1 and 2 from Amersham in the ratio 1:1), and the plate was exposed as soon as possible to a suitable film (Hyperfilm™-ECL; Amersham) in the darkroom. The film was cut somewhat smaller than the size of the plate and put on the plastic cover of a 96-pipet tip box. Thus, the plate could be put onto the film in a way that the flat bottom of the wells was in direct contact with the film. After various exposure times, the film was developed and fixed according to the manufacturer's protocol. A blackening of the film was observed exactly in the size of the

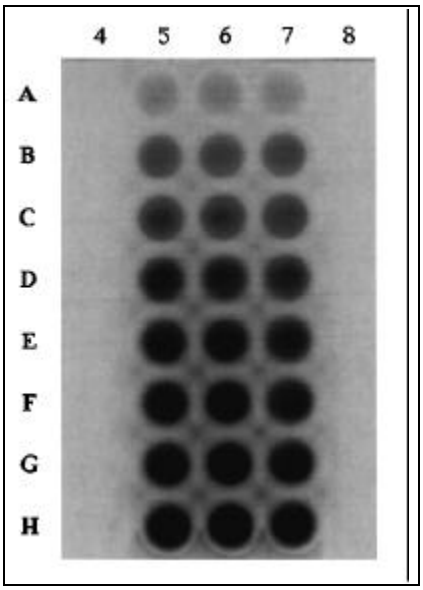

Figure 1. Film after exposure to chemiluminescence ELISA plate for 5 s (triplicates of cyclophilin standard dilutions: 10, 25, 33, 50, 62.5, 83, 125 and 250 ng per well). Note that no signal is observed at adjacent well positions without samples, indicating that there is no cross-talk of signal.