DNA AND CELL BIOLOGY
Volume 28, Number 3, 2009

Pp. 103–108
DOI: 10.1089/dna.2008.0792

Original Research Articles

Generation of Recombinant Vaccinia Viruses via Green Fluorescent Protein Selection

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We developed a rapid method to generate recombinant vaccinia viruses (rVVs) based upon a bicistronic cassette encoding the gene for green fluorescent protein (GFP) and a foreign gene of interest separated by an internal ribosome entry site (IRES). As proof-of-concept, we inserted a mutant *env* gene of human immunodeficiency virus (HIV) into the cassette, which was cloned into the vaccinia virus (VV) insertion vector pSC59 under the control of the early-late VV synthetic promoter and flanked by disrupted *tk* gene sequences. To generate rVVs, 293T cells were inoculated with wild-type (wt) VV, followed by transfection of the modified pSC59 vector containing the bicistronic cassette, which allows expression of GFP and the protein of interest. Next, GFP-positive cells were isolated by flow cytometry or by picking under a fluorescent microscope. Thymidine kinase–deficient (Tk^-) 143B cells were then exposed to lysates of GFP-positive 293T cells and cultured in the presence of bromodeoxyuridine. This selection allows only Tk^- rVV to remain viable. We demonstrated the success of this GFP selection strategy by expressing high levels of mutant HIV Env. Our approach shortens the time needed to generate rVVs and represents a practical approach to generate recombinant proteins.

Introduction

Protein expression via recombinant vaccinia viruses (rVVs) is widely used to express foreign genes in mammalian cells, where posttranslational modifications, such as glycosylation, can occur and lead to correct folding of foreign proteins (Moss and Earl, 2001; Bleckwenn *et al.*, 2003). The rVV technology has been in use since 1984 (Mackett *et al.*, 1984) and has proven to be a reliable approach to express foreign proteins (Moss, 1996; Terajima *et al.*, 2002). Various rVV strains have also been employed as live vaccines in animal model studies (Hu *et al.*, 1992; Doria-Rose *et al.*, 2003; Edghill-Smith *et al.*, 2005; Zhan *et al.*, 2005; Li *et al.*, 2008).

Important aspects involving the usage of rVVs include generating the viral clones and maintaining the purity of viral stocks derived from them (Mackett *et al.*, 1984). The standard experimental protocols to generate and purify rVVs are labor intensive and time consuming (Lorenzo *et al.*, 2004). Further, the yield of recombinant gene products expressed under the control of conventional vaccinia virus (VV) promoters is relatively low (Mackett *et al.*, 1984; Davison and Moss, 1989a, 1989b). To shorten the time required to generate and clone rVVs and to increase the level of expression of the gene of interest, we employed a bicistronic cassette under the control of a single promoter. This strategy is based on the usage of the internal ribosomal entry site (IRES) sequence that permits both the gene of interest and the selection marker to be translated from a single bicistronic mRNA (Borman *et al.*, 1997; Gaines and Wojchowski, 1999).

In our study, we took advantage of green fluorescence protein (GFP) as a selection marker to isolate and purify rVVs. As a test gene of interest, we chose a mutant version of *env* derived originally from a primary strain of human immunodeficiency virus (HIV) clade C, termed *envC* (Song *et al.*, 2006). The *envC* and GFP genes were separated by IRES and positioned under the control of the VV early-late synthetic promoter in the VV insertion vector pSC59 (Chakrabarti *et al.*, 1997). This new approach allowed us to generate the desired rVVs in less than 10 days. The newly created rVV was able to express the foreign gene of interest at high levels.

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Materials and Methods

Plasmids and bacterial strains

The insertion vector pCS59 (Chakrabarti *et al.*, 1997) was kindly provided by Dr. B. Moss (NIAID, NIH, Bethesda, MD). An expression vector containing IRES and the GFP gene, pIRES2-EGFP, was purchased from Clontech Laboratories (Mountain View, CA). A recombinant plasmid encoding a mutant HIV *env*C was created by subcloning SHIV-1157ip *env* (Song *et al.*, 2006) into vector pJW4303 and subsequent site-directed mutagenesis to remove the variable loops V1–V3; the plasmid was propagated in *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA).

Cell lines

The thymidine kinase–deficient (Tk^-) human osteosarcoma cell line 143B Tk^- (ATCC, Manassas, VA) and the human embryonic kidney cell line 293T (ATCC CRL-11268) were propagated in DMEM supplemented with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO), 2 mM of glutamine (Invitrogen), and 100 units/mL penicillin and 100 μg/mL streptomycin mix (Invitrogen) at 37°C in the presence of 5% CO_2.

Vaccinia viruses

Wild-type (wt) VV (strain WR) was obtained from Dr. B. Moss (NIAID, NIH). The control rVVs expressing GFP (rVV–GFP) was generated by us (Sergei Popov, unpublished results). Crude stocks of both strains were prepared on 143B Tk^- cells.

Primers, polymerase chain reaction, and molecular cloning

To obtain the sequence of the mutated *envC*, we designed primers flanking the *env* gene of HIV-1. To clone *env* into the IRES-containing vector, we added tails containing an *Xho*I site to the sense primer, while the antisense primer incorporated a *Bam*HI site (sense-primer: 5′ GGGGCTCGAGATGCG CGTGAAGGAGAAGTA 3′; antisense-primer: 5′ GCGCGG ATCCTCACAGCAGGATGCGCTC 3′). Thermostable high fidelity DNA polymerase and dNTPs were obtained from Roche Diagnostics (Mannheim, Germany). Polymerase chain reaction (PCR) was carried out in 50 μL thin-wall polypropylene tubes (Axygen Scientific, Union City, CA) using a thermocycler (PerkinElmer, Shelton, CT) under the following conditions: denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s, elongation at 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products were visualized in 1% agarose gel with ethidium bromide staining, cut out, and purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA). Next, the fragment was digested with *Xho*I and *Bam*HI, purified in a 1% agarose gel, and cloned into vector pIRES2-EGFP, which had been linearized with *Xho*I and *Bam*HI (New England Biolabs, Ipswich, MA). The resulting construct was used as a template to obtain the expression cassette GFP–IRES–envC by PCR. To amplify this cassette, the primer containing the *Xho*I site described above, and the primer with the *Eco*RI site (5′ CCCCGAATTCTTACTTGTACAGCTCG TC 3′) were used under the same PCR conditions. The cassette was cloned into the insertion vector pSC59 (Fig. 1A) giving rise to the final construct, termed pSC–GFP–envC, and used to generate rVVs.

Generation of recombinant VVs

Transfection experiments were set up in six-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) using subconfluent monolayers of 293T cells cultured for 12 h. First, cells were infected with 3 μL of a crude stock of wt VV (strain WR) (10^7 plaque-forming units [pfu]/mL). Infected cells were incubated for 2 h at 37°C in a 5% CO_2 incubator. Then, cells were transfected by standard calcium phosphate precipitation with the pSC–GFP–envC construct (5 μg of DNA/250 μL of transfection mix), incubated for 6 h, and washed with DMEM with 5% of FCS, and incubated for another 12 h at 37°C in the presence of 5% CO_2. As negative control, 293T cells infected with wt VV were transfected with plasmid pSC59. When GFP-positive cells appeared, cells were sorted by flow cytometry as described (Popov *et al.*, 2005).

Preparative scale culture of rVVs expressing EnvC

To generate large numbers of cells (10^9 cells) expressing the foreign gene of interest, HIV *envC*, we inoculated 143B Tk^- cells in 800 mL tissue culture flasks (Becton Dickinson Labware) with crude stock of rVV–envC at a high multiplicity of infection (MOI; 10 pfu/cell; 50 μL/flask); 10 such flasks were used. The cells were maintained in DMEM supplemented with 10% FCS and bromodeoxyuridine (BrdU; 50 μg/mL) to suppress replication of wt VV and monitored for infection under a fluorescent microscope. When 75% of the infected cells expressed GFP, cells were harvested and the pellet was stored at −80°C.

Western blot analysis

To examine the expression of HIV clade C Env (HIV EnvC) in infected cells, 10^5 cells were resuspended in 100 μL of phosphate-buffered saline (PBS) mixed with 2× Laemmli buffer (BioRad Laboratories, Hercules, CA), boiled for 4 min, and mildly sonicated to degrade chromosomal DNA. A 50 μL aliquot was subjected to gradient PAGE (4–12%) (PerkinElmer, Woodbridge, Ontario, Canada) and transferred to a PVDF membrane (BioRad Laboratories). To detect EnvC, polyclonal IgG from serum of an HIV-positive individual was used (1:2000) followed by staining with anti-human IgG labeled with horseradish peroxidase (1:2500). Specific bands were detected by chemoluminescence (PerkinElmer Life Sciences, Boston, MA).

Purification of HIV clade C envelope glycoproteins

To isolate HIV EnvC, frozen pellets of 10^9 infected 143B Tk^- cells were resuspended in hypotonic buffer (10 mM Tris pH 8.0; 15 mM NaCl; 0.5 mg/L Leupeptin/0.7 mg/L Pepstatin/0.01% [w/v] aprotinin; 0.02% sodium azide) and homogenized with a Dounce homogenizer. Cell membranes pelleted by centrifugation were resuspended in lysis buffer (20 mM Tris pH 7.4; 500 mM NaCl; 0.5 mg/L Leupeptin/0.7 mg/L Pepstatin/0.01% [w/v] aprotinin; 0.02% sodium azide; 1.0% [v/v] Empigen) and sonicated on ice. Clarified cell lysate was adjusted to 0.25% Empigen and fractionated by lentil lectin affinity and size exclusion chromatography as described (Earl *et al.*, 1994). Postchromatography fractions were subjected to Western blot analysis;

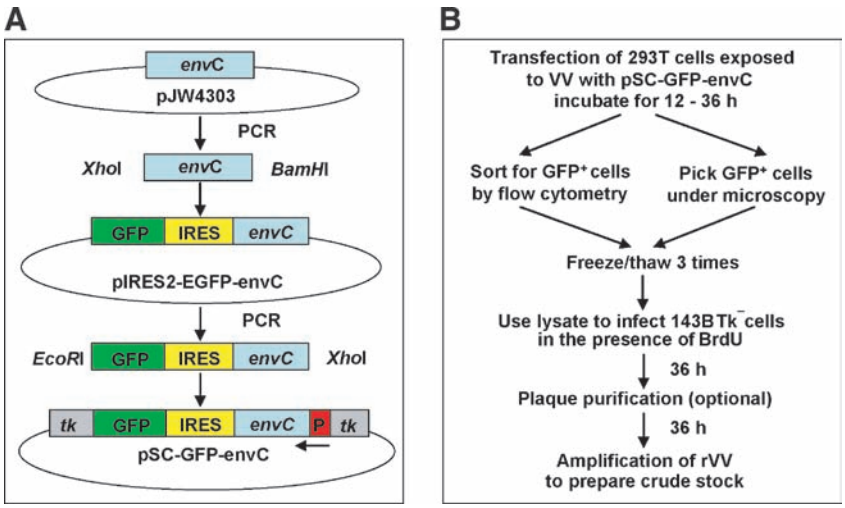

FIG. 1. Strategy to generate insertion vector for rapid selection of rVVs. (**A**) The sequence of the mutated HIV clade C *env* gene, termed *envC*, was obtained by PCR (blue box) and cloned into the *Xho*I and *Bam*HI sites of the vector pIRES2-GFP. In the second step, the sequence of the expression cassette *envC*–IRES–GFP was PCR amplified and cloned into vector pCS59 (sites *Xho*I–*Eco*RI) under the control of strong synthetic early/late VV promoter (red box). Gray boxes, flanking sequences of the *tk* gene used for targeting the insertion vector and thereby disrupt the normal VV *tk* gene. As a consequence, rVVs containing the desired insert are no longer able to express thymidine kinase, and thus cells infected with rVVs become resistant to the toxic effects of BrdU. (**B**) Experimental protocol to accelerate the generation and purification of rVVs. First, 293T cells are exposed to wt VV, followed by transfection of the insertion vector encoding the gene of interest (*envC* in our example). After 12 h, the GFP-positive cells are selected using flow cytometry. As an alternative to FACS sorting, rVVs can also be isolated by picking of green 293T cell plaques under a fluorescent microscope. After freezing and thawing, the GFP-positive 293T cell lysates are used to infect 143B Tk$^-$ cells in the presence of BrdU (the latter prevents replication of wt VV). To obtain pure clonal rVV stocks, several rounds of plaque purification can be performed. Finally, the amplified rVVs are collected and frozen in aliquots.

positive fractions were combined and dialyzed against PBS. The EnvC concentration was determined using a Bradford assay kit (BioRad Laboratories).

Results

Rapid generation of rVVs expressing HIV-1 gp160

Conventional methods to generate rVVs generally require at least 3 weeks for obtaining a pure stock of rVVs, as several plaque purification steps are involved (Lorenzo *et al.*, 2004). We devised a novel experimental strategy that significantly streamlines the procedure and contains practical short cuts (Fig. 1B). Our key reagent is a new insertion vector, a derivate of plasmid pSC59, which was modified to include a bicistronic cassette encoding eGFP and a foreign gene of interest separated by an IRES. This cassette was placed under the control of the early-late VV synthetic promoter (Fig. 1A). The final construct was designated as pSC–GFP–envC.

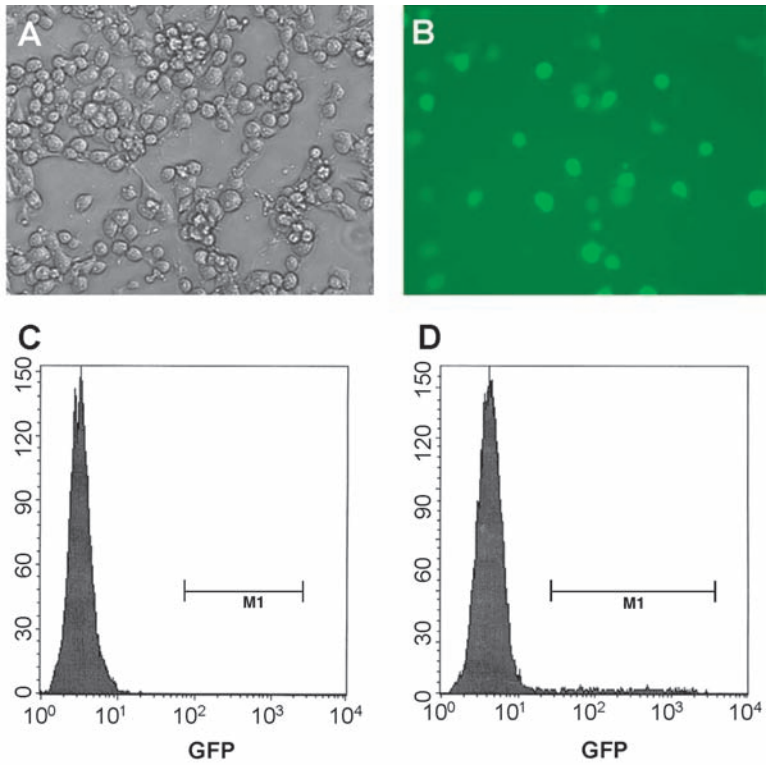

FIG. 2. Selection of rVVs using flow cytometry. (**A**) Phase-contrast microscopy and (**B**) fluorescent microscopy of 293T cells infected with rVVs expressing HIV-1 clade C envelope glycoproteins (EnvC) and GFP. Flow cytometry histograms of 293T cells infected with (**C**) wt VV (negative control) and (**D**) rVV–GFP–envC. The brightest cells were sorted and cloned by limiting dilution; viral particles were released by repeated cycles of freezing–thawing and used for infection of 143B Tk$^-$ in the presence of BrdU.

The experimental steps in our new strategy include (1) generating the initial rVV stock after transfection of the insertion vector into cells previously inoculated with wt VV; (2) visual monitoring of infected cultures by fluorescent microscopy to assess the level of rVV infection and to determine the optimal time to harvest cells; and (3) isolation of the foreign protein. Our experimental system allowed efficient expression of correctly processed recombinant polypeptides at a relatively large scale.

Recombinant VV expresses two genes from a single promoter

When pSC–GFP–envC was transfected into 293T cells that had been previously exposed to wt VV, spots of brightly green cells appeared within 12 h (Fig. 2A, B). This result implied that VV RNA polymerase driven by the synthetic promoter was able to read the GFP sequence, and that the resulting mRNA was successfully translated.

Next, we proceeded to the rapid purification and cloning of rVVs expressing GFP by taking advantage of flow cytometry (Fig. 2C, D). After sorting, GFP-positive 293T cells were placed into 24-well plates at 1, 10, and 100 cells/well and expanded (for 24–48 h). After three cycles of freezing–thawing of GFP-positive 293T cells, their lysates were used to infect 143B Tk^- cells (5×10^4 cells) in the presence of BrdU, which blocks the replication of wt VV, thus allowing the propagation of rVVs in a short period of time. The following day, abundant GFP expression was seen in the 143B Tk^- cell monolayers. Lysate obtained from one sorted GFP-positive 293T cell was sufficient to yield GFP-positive 143B Tk^-cells, and the yield of GFP-positive 143B Tk^-cells increased proportionally to the numbers of sorted GFP-positive cells used to prepare the lysates. The reason for using three different numbers of GFP-positive starting cells is to find a culture in which sparse, individual green plaques can be identified. As an alternative to FACS sorting, rVVs can also be isolated by picking of green 293 T cell plaques under fluorescent microscopy (Fig. 1B); either way represents a rapid method to generate rVVs for preparative protein expression that substantially shortens conventional protocols.

Next, we demonstrated EnvC expression in 143B Tk^- cell lysates by Western blot analysis using a polyclonal HIV-positive human serum. A specific band with a molecular weight of 140 kDa (gp140), corresponding to the expected size of the truncated HIV clade C envelope glycoproteins, was detected, while no signal was seen in 143B Tk^- cells infected with control virus expressing GFP only (Fig. 3A). Further, no bands were detected on the membrane containing lysates of 143B Tk^- cells infected with rVV–GFP–envC or rVV–GFP, when serum from a healthy donor was used to probe the membrane (Fig. 3B). These data demonstrate that rVV–GFP–envC was able to express two foreign genes under the control of a single promoter. To demonstrate that this strategy is reproducible, we constructed another insertion vector by introducing a codon-optimized *env* gene derived from a pediatric HIV clade C isolate 1084i (Grisson *et al.*, 2004; Rasmussen *et al.*, 2006) into the pSC–GFP plasmid. Similarly, after transfection of 293T cells that had been previously exposed to wt VV, spots of brightly green cells appeared within 12 h (Supplemental Fig. 1, available online at www.liebertonline.com). Western blot analysis of cell extracts showed that the resulting rVV expressed HIV1084i envelope glycoproteins (Supplemental Fig. 1).

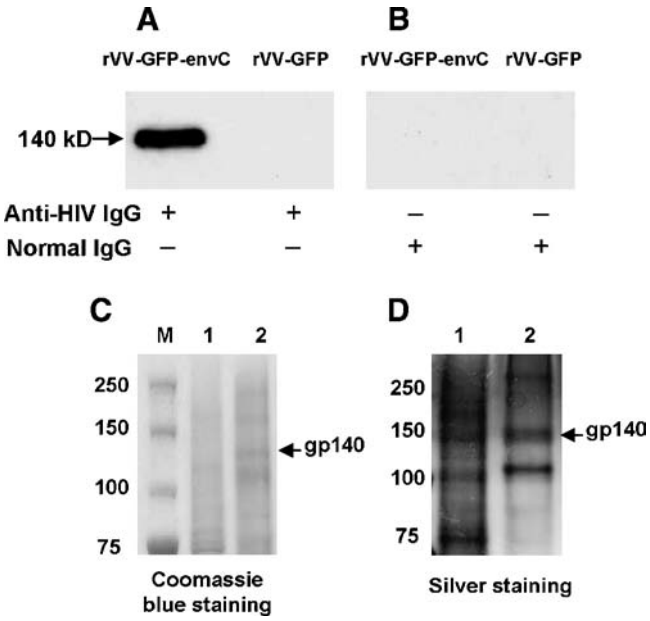

FIG. 3. Expression and purification of EnvC from 143B cells infected with rVV–GFP–envC. (**A–B**) Infected cells were solubilized in Laemmli buffer and fractionated in gradient PAGE gel (4–12%); proteins were transferred to the PVDF membrane and probed with (**A**) anti-HIV-1 human IgG isolated from serum of an HIV-1–infected patient and (**B**) normal human IgG. (**C–D**) Analysis of purified mutant HIV envelope glycoproteins produced in 143B cells infected with rVV–GFP–envC. The mutant HIV EnvC yielded the expected band of approximately 140 kDa under denaturing conditions. (**C**) SDS-PAGE Coomassie blue staining. Lane 1, 10 μg of total protein from mock-infected cells; lane 2, 10 μg of protein purified by lentil lectin affinity column and Superdex-S200 size exclusion column chromatography using extracts of cells infected with rVV–GFP–envC. (**D**) Silver staining. Lane 1, total protein (2 μg) after the lentil lectin affinity column purification; lane 2, purified protein (2 μg) after size exclusion column chromatography purification.

Expression and purification of recombinant EnvC on a preparative scale

To generate recombinant envelope glycoprotein on a preparative scale, we exposed 10^9 of 143B Tk^- cells to rVV–GFP–envC at a high MOI (10 pfu/cell) and monitored the infection by fluorescent microscope. When 70% of the cells were GFP positive, they were harvested, and lysates were subjected to a two-step purification procedure using lectin and size exclusion chromatography methods described previously (Earl *et al.*, 1994). Polypeptides of the expected size (140 kDa) were observed after purification (Fig. 3C, D). After the appropriate fractions were pooled, the yield of EnvC was 1 mg. We further characterized the purified protein using Western blot analysis with anti-HIV human serum (Supplemental Fig. 2A, available online at www.liebertonline.com) and anti-HIV gp120 C-terminal monoclonal antibody (Supplemental Fig. 2B, available online at www.liebertonline.com). Both antibodies could detect the purified EnvC (gp140)

(lane 3 in Supplemental Fig. 2A, B) and a control, full-length HIV gp160 (lane 2) at the expected size, suggesting that the rVV–GFP–envC-infected cells produced correctly processed EnvC. Further, the purified EnvC was able to induce specific antibody responses in animals (data not shown).

Discussion

We have established an expeditious strategy to generate rVV strains. The novelty includes the following: (1) use of a bicistronic cassette in the insertion vector with an IRES positioned between the reporter gene GFP and gene of interest and (2) use of GFP as a selection marker to isolate cells harboring recombinant virus by flow cytometry. These modifications substantially reduced the time to obtain cloned rVVs that express the gene of interest. Moreover, GFP expression in infected cells provides a reliable, straightforward methodology to maintain viral stocks free from contamination with spontaneous VV revertants by rapid selection of GFP-positive cells.

We believe that our results are of interest not only because of the technical advance. Since VV RNA polymerase was able to generate functional, bicistronic mRNA in the cytoplasm and this mRNA yielded the expected functional protein products, a single rVV may potentially be engineered to express several genes of interest. Given that the VV genome can accept inserts of >20 kB of foreign genetic material (Moss and Earl, 2001), designing rVVs that express up to four foreign genes using our IRES methodology may be feasible. If so, the possibility to design polyvalent vaccines based upon this vector may be considered. The rVV system has advantages over DNA plasmid vaccines and other viral vectors because the use of rVVs eliminates the risk of integrating foreign genetic material into the host genome, although potential side effects of rVVs in immunocompromised individuals may raise safety concerns.

Finally, we believe that our novel GFP-based vector construct may represent a practical tool to study VV infection and pathogenesis in animal models. Previous studies have used rVV-mediated GFP expression to monitor infection *in vitro* (Dominguez *et al.*, 1998) or *in vivo* (Norbury *et al.*, 2002); these studies did not use bicistronic cassettes. Our bicistronic cassette gives the added advantage of precise identification of cells expressing a gene of interest; GFP localization will thus give the opportunity to study host–virus interactions at the single-cell level *in vivo.* Such studies may have added significance given the renewed interest in vaccines against small pox (Qutaishat and Olson, 2003; Stanford and McFadden, 2005).

To conclude, we designed a new, expeditious approach to generate and purify rVVs based upon the use of a bicistronic insertion vector. Our method substantially reduces the time required to obtain constructs expressing the gene of interest.

Acknowledgments

We thank Dr. Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH) for the gift of VV (strain WR) and pSC59 vector, and Susan Sharp for assistance in the preparation of the manuscript. This work was supported by NIH Grant P01 AI048240 to R.M.R.

Disclosure Statement

No competing financial interests exist.

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Received for publication July 21, 2008; received in revised form November 7, 2008; accepted November 10, 2008.