Reports

Use of a negative selectable marker for rapid selection of recombinant vaccinia virus

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Vaccinia virus has been a powerful tool in molecular biology and vaccine development. The relative ease of inserting and expressing foreign genes combined with its broad host range has made it an attractive antigen delivery system against many heterologous diseases. Many different approaches have been developed to isolate recombinant vaccinia virus generated from homologous recombination; however, most are time-consuming, often requiring a series of passages or specific cell lines. Herein we introduce a rapid method for isolating recombinants using the antibiotic coumermycin and the interferon-associated PKR pathway to select for vaccinia virus recombinants. This method uses a negative selection marker in the form of a fusion protein, GyrB-PKR, consisting of the coumermycin dimerization domain of *Escherichia coli* gyrase subunit B fused to the catalytic domain of human PKR. Coumermycin-dependent dimerization of this protein results in activation of PKR and the phosphorylation of translation initiation factor, eIF2 α . Phosphorylation of this factor leads to an inhibition of protein synthesis, and an inhibition of virus replication. In the presence of coumermycin, recombinants are isolated due to the loss of this coumermycin-sensitive gene by homologous recombination. We demonstrate that this method of selection is highly efficient and requires limited rounds of enrichment to isolate recombinant virus.

Vaccinia virus (VACV), the poxvirus used as the vaccine against smallpox, has gained widespread use as a general vector for expressing foreign proteins in mammalian cells. The ability to take up large inserts of DNA and express high levels of foreign protein in a wide variety of cell lines has made VACV an attractive delivery vehicle for expressing antigens and analyzing protein function (1). The standard method of generating VACV recombinants is through homologous recombination between replicating viral DNA and a transfected plasmid or PCR product containing the protein coding sequence of interest flanked by viral sequences (1). The frequency of recombination accounts for ~0.1% of total virus, and isolation of the recombinants typically requires a series of selections using a genetic marker (1,2).

Many different selection schemes have been developed for detection of VACV recombinants. Such examples include the use of thymidine kinase-negative and

-positive selection (3,4), reversal of host range mutations (5–7), neomycin resistance (8), and transient dominant selections using mycophenolic acid (MPA) (1). These methods have proven to be quite efficient, but they often require special cell lines or serial passages in selection media and therefore can be time-consuming.

Herein we demonstrate a novel, alternative method for rapid isolation of recombinant VACV using the antibiotic coumermycin and a coumermycin-sensitive VACV, VACV Δ E3L::GyrB-PKR. The VACV Δ E3L::GyrB-PKR expresses the coumermycin dimerization domain of *Escherichia coli* gyrase B (GyrB, residues 1–220) fused to the catalytic domain of mammalian dsRNA-dependent protein kinase, PKR (residues 258–551), in the viral locus of interest for gene insertion. The GyrB-PKR system has been characterized previously (9). In this heterologous system, the gyrase domain binds to coumer-

mycin in a 2:1 stoichiometric ratio, thereby causing the dimerization of the PKR kinase domain (Figure 1A) (10). This dimerization mimics the endogenous activation of PKR and results in the phosphorylation of translation initiation factor eIF2 α , which leads to an inhibition of protein synthesis (11,12). For this study, we used the E3L locus of VACV to express GyrB-PKR. In this system, recombinants are generated by standard homologous recombination between a plasmid containing the insert of interest surrounded by sequences that flank the E3L gene and the parental VACV Δ E3L::GyrB-PKR. Recombinants are resistant to the effects of coumermycin, whereas the replication of VACV Δ E3L::GyrB-PKR is inhibited following treatment with coumermycin. Therefore, this system exclusively allows the viability of viruses that undergo a double recombination event that result in loss of the GyrB-PKR gene and retention of the desired insert.

Materials and methods

Cells, virus and reagents

Baby hamster kidney (BHK-21-CCL10) and rabbit kidney cells (RK13-CCL37) (ATCC, Manassas, VA, USA) used in the experiments were maintained in MEM (Mediatech, Manassas, VA, USA) supplemented with 5% FBS (HyClone, Logan, UT, USA) and 10 µg/mL gentamicin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO₂. Coumermycin A1 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO (Invitrogen) at a stock concentration of 5 mg/mL and diluted with PBS. Vectors pC939 and pC940 containing GyrB-PKR and GyrB-PKRK296H, respectively, were provided by Tom Dever (National Institutes of Health, Bethesda, MD, USA) and have been described (9). These vectors contain the residues 1–220 of *E. coli* gyrase B fused to the kinase domain (residues 258–551) of human PKR. The catalytic inactive mutant contains a lysine to histidine mutation at residue 296 of the PKR domain. Viruses used in this study expressed the GyrB-PKR or its mutant protein in either the E3L or the J2R locus. Isolation of VACV expressing GyrB-PKR was by transient dominant selection using mycophenolic acid as describe previously (13,14). Individual mycophenolic acid-resistant plaques were tested for sensitivity to coumermycin, and the most coumermycin-sensitive viruses were further characterized. VACV strains used were Western Reserve (WR) and NYVAC. All infections, plaque purifications, virus amplifications and viral genomic extraction for sequencing were carried out as previously described (14).

Coumermycin sensitivity of VACVΔE3L, VACVΔE3L::GyrB-PKR, VACVΔE3L::GyrB-PKRK296H, and VACVΔJ2R::GyrB-PKR

RK13 cells grown in six-well dishes were treated with coumermycin A1 at doses ranging 0–100 ng/mL in media for 16 h. The cells were infected with 100 pfu VACVΔE3L, VACVΔE3L::GyrB-PKR, or VACVΔE3L::GyrB-PKRK296H, and the infections were carried out in media supplemented with the corresponding concentration of coumermycin for 48 h. To test the sensitivity of the VACVΔJ2R::GyrB-PKR to coumermycin, RK13 cells were mock-treated or treated with coumermycin A1 at a concentration of 100 ng/mL for 24 h and then infected with 10-fold serial dilutions of the virus stock. Infections were carried out for 48 h in media with or without 100 ng/mL of coumermycin A1. Cells were stained with crystal violet and plaques were analyzed by a standard plaque assay.

Figure 1. Coumermycin/GyrB-PKR system as a negative selection marker for isolation of VACV recombinants. (A) Schematic representation of the coumermycin/PKR system. Coumermycin-induced dimerization of GyrB-PKR fusion protein results in activation of the fused PKR catalytic domain. The GyrB-PKR fusion protein consists of the first 220 amino acids of the *E. coli* gyrase B coumermycin-dependent dimerization domain (GyrB-DD) fused to residues 258–551 of the catalytic domain of human PKR (PKR-KD). The GyrB domain binds to coumermycin in a 2:1 ratio, resulting in the dimerization and activation of the kinase domain of PKR. This leads to the phosphorylation of translation initiation factor eIF2α, causing an inhibition of protein synthesis. (B) Expression of GyrB-PKR and GyrB-PKRK296H in recombinant VACV. RK13 cells were infected at an MOI of 5 with the indicated VACV mutants. At 6 h post-infection, cell extracts were harvested and subjected to Western blot analysis using antibodies that recognize human PKR.

Western blot analysis for GyrB-PKR and eIF2α phosphorylation

For GyrB-PKR expression analysis, subconfluent RK13 cells were mock-treated or treated with 100 ng/mL coumermycin A1. After 16 h, the cells were infected with VACVΔE3L, VACVΔE3L::GyrB-PKR or VACVΔE3L::GyrB-PKRK296H at a multiplicity of infection (MOI) of 5 pfu/cell. At 6 h post-infection, the cells were scraped into media and pelleted by centrifugation at 500× *g* for 10 min at 4°C. The cells were lysed by addition of RIPA lysis buffer (1× PBS, 0.1% SDS, 1% nonyl phenoxypolyethoxyethanol (NP40), 0.5% sodium deoxycholate, and 100 mM sodium fluoride) followed by incubation on ice for 10 min. The lysates were centrifuged at 10,000× *g* for 10 min at 4°C and the supernatant transferred to new tube. Equal amounts of 2× SDS loading buffer was added to the lysates and then separated on a 12% SDS PAGE gel under denaturing conditions and then transferred onto PVDF membrane. Following transfer, the membranes incubated in blocking buffer (3% nonfat dry milk, 140 mM NaCl, 3 mM KCl, 20 mM Tris pH 7.8, 0.05% Tween 20) for 1 h at room temperature. GyrB-PKR expression was determined by using rabbit antibodies directed against total PKR and the phosphorylation of eIF2α by phospho-specific eIF2α antibodies (Cell Signaling, Danvers, MA, USA) at a concentration of 1:1000 diluted in blocking buffer. Secondary goat anti-rabbit antibodies conjugated to HRP (Sigma-Aldrich) were added at 1:15,000 in blocking buffer

followed by chemiluminescent development (Supersignal West Dura, Thermo Scientific, Rockford, IL, USA).

Generation of VACVΔE3L::LacZ by coumermycin system

In vivo recombination (IVR) of the following viruses occurred accordingly: subconfluent BHK cells (2 × 10⁶ cells total) were transfected with 2 µg pMPLacZ using Lipofectamine and Plus Reagent (Invitrogen) per the manufacturer's directions and coinfecting with parental VACVΔE3L::GyrB-PKR at an MOI of 0.05 pfu/cell. At 30 h post-infection, cells were scraped and virus was released by three rounds of freeze-thaw treatment followed by sonication. RK13 cells pretreated with or without coumermycin A1 at 100 ng/mL for 16 h were infected with dilutions of the IVR extract, and overlaid with media containing coumermycin if required. After 48 h post-infection, the cells were covered with an agarose overlay consisting of 1× MEM, 1.5% agarose, X-gal substrate (10 mg/mL), and neutral red. At 24 h post-infection, recombinant plaques (blue) were quantified and compared with the number of parental plaques (clear).

Results and discussion

Parental virus (VACVΔE3L::GyrB-PKR) was constructed to express the first 220 amino acids of *E. coli* GyrB fused to residues 258–551 of the kinase domain of human PKR in the E3L locus of VACV. As described in the “Materials and methods” section,

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Figure 2. VACV expressing GyrB-PKR is sensitive to the effects of coumermycin. (A) Coumermycin sensitivity of VACVΔE3L::GyrB-PKR. RK13 cells were pretreated with increasing doses of coumermycin A1 for 16 h and then infected with 50–100 pfu indicated viruses. At 48 h post-infection, cells were stained with crystal violet and the plaques quantitated. (B) Coumermycin sensitivity of VACVΔJ2R::GyrB-PKR. RK13 cells were mock-treated or treated with 100 ng/mL coumermycin A1 for 24 h. The cells were infected with 10-fold serial dilutions of VACVΔJ2R::GyrB-PKR. At 48 h post-infection, cells were stained with crystal violet and the number of plaques quantitated.

GyrB-PKR was inserted into VACVΔE3L by transient dominant selection for mycophe-
nolic acid resistance. Since the kinase activity
of this PKR fusion protein is dependent
on interaction with coumermycin (9), this
virus would be expected to be coumer-
mycin-sensitive (*cmr*^s). In addition to the
coumermycin-sensitive virus, a PKR cataly-
tically inactive mutant, VACVΔE3L::GyrB-
PKRK296H, was constructed, which
was unable to phosphorylate eIF2α. To
confirm that the viruses were able to express
the chimeric proteins, RK13 cells were
infected with VACVΔE3L::GyrB-PKR,
VACVΔE3L::GyrB-PKR K296H, and
VACVΔE3L and extracts prepared at
6 h post-infection. Immunoblot analysis
using antibodies against PKR illustrate
that only VACVΔE3L::GyrB-PKR and
VACVΔE3L::GyrB-PKRK296H viruses
express the GyrB-PKR protein (Figure 1B).
To determine whether the GyrB-PKR system
was able to function in the context of a
vaccinia virus infection in cells in culture,
the sensitivity of VACVΔE3L::GyrB-PKR,
VACVΔE3L, and VACVΔE3L::GyrB-
PKRK296H to coumermycin was compared.
RK13 cells were pretreated with increasing
concentrations of coumermycin and infected
with 50–100 pfu of each virus (Figure 2A).
For VACVΔE3L::GyrB-PKR, sensitivity
to coumermycin was observed at concen-
trations >5 ng/mL. As expected, both

VACVΔE3L and the catalytically inactive
mutant, VACVΔE3L::GyrB-PKRK296H,
were resistant to all doses of coumermycin,
thereby supporting that the observed sensi-
tivity of VACVΔE3L::GyrB-PKR to coumer-
mycin was dependent on the function of the
chimeric protein, GyrB-PKR. To determine
if the coumermycin-sensitive phenotype was
not limited to the expression of the protein
in the E3L locus, we also tested the sensitivity
to coumermycin with VACV expressing the
GyrB-PKR protein in the J2R (thymidine
kinase) locus. Clearly this recombinant virus
was sensitive to coumermycin by ≥100,000
fold when compared with untreated cells
(Figure 2B), demonstrating the versatility
of this system for selection of recombinant
viruses.

It has been established that the treatment
of coumermycin results in the dimerization
and activation of GyrB-PKR and leads to
the subsequent inhibition of translation
(9,15). This inhibition occurs by phospho-
rylation of a key mediator of translation,
eIF2α. To determine that the sensitivity of
VACVΔE3L::GyrB-PKR to coumermycin
was the result of eIF2α phosphorylation, the
level of eIF2α phosphorylation between the
virus infections in the presence or absence of
coumermycin was determined. RK13 cells
treated with or without coumermycin at
100 ng/mL for 16 h were infected at an MOI
of 5 to ensure all cells were infected. At 6 h

Figure 3. Western blot analysis of eIF2 α phosphorylation in RK13 cells infected with VACV Δ E3L::GyrB-PKR in the presence of coumermycin. RK13 cells were pretreated with 100 ng/mL coumermycin A1 for 16 h and then infected at an MOI of 5 with the indicated viruses. At 6 h post-infection, cell extracts were harvested and subjected to Western blot analysis using antibodies against ser51-phospho-eIF2 α .

post-infection, extracts were prepared and assayed for eIF2 α phosphorylation. Figure 3 shows that without coumermycin, infection with either VACV Δ E3L::GyrB-PKR or VACV Δ E3L::GyrB-PKR Δ 296H led to low levels of eIF2 α phosphorylation compared with mock-infected cells. The low levels of phosphorylation could be attributed to the loss of the E3L gene, which acts to sequester viral dsRNA preventing endogenous PKR activation during a VACV infection (13,16). Comparatively, in the presence of coumermycin, infection with VACV Δ E3L::GyrB-PKR infection led to an increase in eIF2 α phosphorylation. As expected, infection with the catalytically inactive mutant, VACV Δ E3L::GyrB-PKR Δ 296H, did not lead to an increase in eIF2 α phosphorylation following coumermycin treatment. These results suggest that coumermycin mediated dimerization and activation of GyrB-PKR effectively increased eIF2 α phosphorylation and therefore resulted in the sensitivity of VACV Δ E3L::GyrB-PKR to the antibiotic.

Next, the VACV Δ E3L::GyrB-PKR system was tested for the ability to isolate recombinant viruses. Ideally, the antibiotic should effectively inhibit the replication of viruses that do not contain the desired insert and allow only recombinant viruses to replicate (Figure 4A). pMP Δ E3L-lacZ was used as the donor plasmid, which upon recombination would place the β -galactosidase gene in the E3L locus, in place of GyrB-PKR. This would allow for screening and quantitation of the recombinants in the presence of X-gal substrate, resulting in blue plaques. In the absence of coumermycin, the ratio of parental virus to recombinants was $\sim$ 4000:1 (4×10^6 parental to 1×10^3 recombinants),

yielding a recombination efficiency of 0.025% (Figure 4B). Upon treatment with coumermycin, only 10 parental viruses were detected. However, the number of recombinants detected with or without coumermycin treatment was comparable, giving 1000 recombinant plaques without coumermycin to 800 plaques with coumermycin treatment. In the presence of coumermycin, the ratio of parental virus to recombinant virus was $\sim$ 1:80 (10 parental to 800 recombinants). It should be noted that the plaque morphology of the parental virus differed from the recombinant VACV Δ E3L::LacZ in the presence of coumermycin, where VACV Δ E3L::GyrB-PKR plaques appeared as distinct foci in the monolayer, rather than forming clear plaques free of cells. This phenotype only occurred in the presence of the antibiotic. VACV Δ E3L::LacZ, a well-characterized virus with a very limited host range, formed plaques easily distinguishable from VACV Δ E3L::GyrB-PKR in the presence of coumermycin (data not shown). Individual plaques picked after coumermycin selection routinely yielded only blue recombinant viruses upon replating (zero nonblue plaques out of 213 second round plaques assayed). This demonstrates that this selection method can be used to obtain pure virus cultures after minimal rounds of plaque purification. Overall, these results demonstrate that this system is highly efficient in generating recombinant viruses containing genes of interest in a rapid manner. In this example a plasmid containing the gene of interest flanked by homologous arms was used as the donor for homologous recombination. However, since the donor DNA does not need to contain any extraneous sequences, a PCR product containing the gene of interest

flanked by homologous arms can be used as the DNA donor using this method.

This study demonstrated that the coumermycin/GyrB-PKR negative selection method is an effective system for the isolation of recombinant VACV viruses. Once a coumermycin-sensitive parental virus was obtained, pure recombinant viruses were rapidly isolated following minimal rounds of plaque purification. The sensitivity to coumermycin was not limited to the GyrB-PKR gene being expressed from the E3L locus, as this phenotype was maintained when GyrB-PKR was expressed from the TK locus (Figure 4), demonstrating the versatility of the system. The GyrB-PKR selection scheme has many advantages over selection methods that result in the rapid isolation of recombinants. Markers such as neomycin (8), hygromycin (17), and puromycin (18), which confer antibiotic resistance, function to enrich the population of recombinants that have taken up the desired DNA through homologous recombination. Enrichment of these recombinants requires multiple rounds of passage and these methods often require the retention of the markers. The use of transient dominant selection markers, such as MPA (1) and GFP (19), requires multiple passages for selection of marker gene uptake followed by resolution for the desired recombinant. In addition, the GyrB-PKR system does not require special cells for the isolation of recombinants unlike thymidine kinase selection (3,4). Furthermore, coumermycin is relatively innocuous to eukaryotic cells, unlike other systems such as MPA and herpes simplex thymidine kinase, which use nucleoside analogs that are potentially mutagenic.

Figure 4. Coumermycin/GyrB-PKR selection of VACV recombinants. (A) Parental cmr^R VACV expressing GyrB-PKR in the locus of interest undergoes homologous recombination with foreign genetic material flanked by homologous sequences to the locus of interest. Only recombinants that replaced the negative selection marker are resistant (cmr^R) to the effects of coumermycin, allowing for their enrichment in the presence of the antibiotic. (B) VACV Δ E3L::LacZ was generated by in vivo recombination between pMPLacZ and parental virus VACV Δ E3L::GyrB-PKR. Progeny virus was then plaque out in RK13 cells in the presence or absence of coumermycin. A comparison of the number of recombinants (blue plaques) to parental virus (clear plaques) in the presence or absence of coumermycin was determined. VACV Δ E3L::GyrB-PKR and recombinant VACV Δ E3L::LacZ were screened using X-gal and neutral red staining.

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Competing interests

The authors declare no competing interests.

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