Journal of Virological Methods 156 (2009) 37–43

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Marker gene swapping facilitates recombinant Modified Vaccinia Virus Ankara production by host-range selection

Giulia Di Lullo[a,1], Elisa Soprana[a], Maddalena Panigada[a,b], Alessio Palini[a], Volker Erfle[c], Caroline Staib[c,2], Gerd Sutter[c,3], Antonio G. Siccardi[a,b,*]

[a] *Dipartimento di Biologia e Genetica per le Scienze Mediche, Università di Milano, Italy*
[b] *DIBIT-HSR, San Raffaele Scientific Institute, Milano, Italy*
[c] *GSF - Institute of Virology, Technical University, Munich, Germany*

Article history:
Received 19 June 2008
Received in revised form 14 October 2008
Accepted 16 October 2008
Available online 10 December 2008

Keywords:
Recombinant MVA
Host-range selection
Marker gene swapping
K1L*gfp*
Fluorescent proteins

A B S T R A C T

Modified Vaccinia Virus Ankara (MVA) is employed widely as an experimental and human vaccine vector for its lack of replication in mammalian cells and high expression of heterologous genes. Recombinant MVA technology can be improved greatly by combining transient host-range selection (based on the restoration in MVA of the deleted vaccinia gene K1L) with the differential expression of fluorescent proteins. Recombinant virus results from swapping a red protein gene (in the acceptor virus) with a cassette of the transfer plasmid comprising the transgene and the green marker K1L*gfp* (a chimeric gene comprising K1L and EGFP). Recombinant selection is performed in the selective host RK13. Finally, in the non-selective host BHK-21, a single crossover between identical flanking regions excises the marker gene. The three types of viruses involved (red parental, green intermediate and colourless final recombinant) are visualized differentially by fluorescence microscopy or fluoro-imaging of terminal dilution microcultures, leading to a straightforward and efficient purification protocol. This method (Red-to-Green gene swapping) reduces greatly the time needed to obtain marker-free recombinant MVA and increases the reliability of the construction process.

1. Introduction

Modified Vaccinia Virus Ankara (MVA), obtained after over 570 serial passages in chicken embryo fibroblasts (CEF) (Mayr et al., 1975), is characterized by an attenuated phenotype, a severe host restriction and six major genomic deletions (Meyer et al., 1991; Antoine et al., 1998). Although unable to multiply in mammalian cell lines (with the only exception of BHK-21 cells) (Carroll and Moss, 1997; Drexler et al., 1998), MVA retains unimpaired expression of viral and heterologous genes (Sutter and Moss, 1992). This feature, together with the lack of pathogenicity to humans (including immunocompromised individuals) (Mayr and Danner, 1979) and the adjuvant effect for immune responses (Ramirez et al., 2000), makes recombinant MVA (rMVA) an ideal vector for both prophylactic and therapeutic vaccines; indeed, it has proved to be protective in animal models of vaccination against several infectious diseases (Barouch et al., 2001; Hirsch et al., 1996; Schneider et al., 1998; Sutter et al., 1994a; Weidinger et al., 2001; Wyatt et al., 1996) and tumors (Carroll et al., 1997; Drexler et al., 1999) and is used widely in prime-boost strategies (Amara et al., 2002) and clinical trials (McConkey et al., 2003; Cosma et al., 2003; Bejon et al., 2007; Jaoko et al., 2008).

Originally, in order to obtain rMVA, the vectors included β-galactosidase or β-glucuronidase genes and required the visual selection (and manual picking) of cell foci infected by recombinants (Sutter and Moss, 1992; Schneider et al., 1998; Drexler et al., 1998). Subsequently, co-expression of the *E. coli* xanthine-transferase gene allowed the selection for mycophenolic acid resistance. More recently, "growth rescue" methods have been described, such as E3L-based selection of rMVA in CEF cells (Hornemann et al., 2003) and K1L-driven "Transient Host-Range Selection", in RK13 cells (Staib et al., 2000, 2003). In the latter system, the K1L gene, deleted in the MVA genome, is reinserted in the genome of recombinants, conferring the potential to replicate in the permissive rabbit cell line RK13 (Sutter et al., 1994b). Two identical sequences flanking the K1L gene marker drive its excision by homologous recombination under non-selective growth conditions (*e.g.*, on BHK-21 cells), leaving the

* Corresponding author at: DIBIT-HSR, Via Olgettina 58, I-20132 Milano, Italy. Tel.: +39 02 2643 4787; fax: +39 02 2643 4723.
E-mail address: siccardi.antonio@hsr.it (A.G. Siccardi).
[1] Present address: Institute for Research in Biomedicine, Bellinzona, Switzerland.
[2] Present address: Genelux GmbH, Product Development, D-82347 Bernried, Germany.
[3] Present address: Division of Virology, Paul-Ehrlich-Institute, Langen, Germany.

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.10.026

transgene as the only heterologous sequence in the rMVA genome. Albeit much more efficient than other methods, host-range selection is, anyway, a rather laborious method, since it cannot bypass the carry-over of non-recombinant ($K1L^-$) virus, rescued by ($K1L^+$) recombinants.

Aim of the present study was the introduction of two different fluorochromes in the host-range selection system, in order to conveniently distinguish and monitor the three virus types involved in the procedure, i.e. the parental virus (red), the $K1L^+$ intermediate recombinant (green) and the marker-free final recombinant (colourless).

2. Materials and methods

2.1. Cells and viruses

Baby hamster kidney BHK-21 (ATCC CCL-10) and rabbit kidney RK13 cells (ATCC CCL-37) were grown in RPMI-1640 supplemented with 10% foetal calf serum (FCS). Cells were cultured at 37 °C with 5% CO_2. For viral infections, FCS was lowered to 2%.

MVA(II_{new}) (derived from MVA, isolate F6) lacks any residual K1L sequence (Staib et al., 2003). MVA-Tat_{HIV} (unpublished) and MVA-HuTYR (Staib et al., 2003) (both derived from isolate F6) harbour the transgenes in deletion II. MVA-Env_{HIV} (unpublished, derived from isolate F6) harbours the HIV-1 LAI *env* gene in deletion VI. MVA-KG (derived from MVA, isolate F6) harbours the K1L and EGFP genes, respectively, in deletions III and VI (unpublished).

Viral lysates were prepared either from single plaque isolates, after 1-day amplification on a cell monolayer in a 96-well plate, or from terminal dilution cultures in 96-well plates. Lysates were obtained by 3 rounds of freezing–thawing–vortexing, followed by two rounds of micro-centrifugation, i.e. clearing at 2000 rpm/10 min and pelleting at 13,000 rpm/30 min. Virus pellets from 96-well microcultures were resuspended in 100 μL of PBS and contained approximately 10^6 pfu. Larger stocks were obtained by conventional methods on fresh CEF cultures.

Terminal dilution titration and cloning of rMVA was carried out in 96-well plates, infecting 8, 12, or 16 replicas with serial (10-fold or 3.2-fold) dilutions of viral lysates. In order to diffuse viruses and infected cells throughout the monolayers, after 24 h, microwell cultures were "scrambled" (with the plastic tips of multichannel pipettes, by scraping the monolayers and pipetting up and down). Finally, after 48 h, the wells were monitored by fluorescence microscopy or by whole-plate fluoro-imaging (Typhoon, GE Healthcare) to distinguish uninfected from infected cultures. A convenient macroscopic record of viral titrations is obtained by acetone–methanol fixation, extensive washing with water and Crystal Violet (or Giemsa) staining of the microcultures (only uninfected monolayers persist and are stained). For the rows containing a fraction (f) of uninfected replica cultures, the mean number of infecting viruses per well (m) can be calculated by Poisson's distribution ($m = -\ln f$).

2.2. Plasmids

Host-range transfer plasmids pΔIII-HR-P7.5 and pΔIII-HR-sP (differing for the promoter in the multiple cloning site) have been described (Staib et al., 2000, 2003). Plasmid pSW13as (unpublished) carries a chimeric gene coding for the fusion protein K1L*gfp*, under control of the authentic K1L promoter. The K1L*gfp* ORF encodes 527 amino acids: residues 1–284 are from K1L (Sutter et al., 1994b); VTRAGA are linker residues 285–290; residues 291–527 correspond to residues 3–239 of EGFP (Clontech Laboratories). Plasmid pHcRed1-1 (Clontech Laboratories) carries the gene for the red fluorescent protein HcRed 1-1. Plasmid pAS-E (unpublished) carries the chimeric gene tMoIgE, a truncated form of the mouse membrane IgE, whose ORF contains 312 amino acids: residues 1–21 are a mouse Ig intron-less signal peptide (Li et al., 1997); residues 22–312 correspond to residues 288–485 of the constant region of murine membrane IgE (domains CHε3-CHε4-M1-M2) (Anand et al., 1997).

2.3. PCR analysis of viral DNA

Viral DNA was isolated from infected cells as described (Staib et al., 2000). Oligonucleotide primers, annealing within the flanking sequences, were used to specifically amplify DNA fragments within deletions II, III, or VI. ΔIII-K1L PCR was designed to specifically amplify a fragment between flank 2 of deletion III and a K1L sequence. tMoIgE PCR was designed to amplify an internal sequence of the construct. The PCR protocols (95 °C/2 min; 30 cycles: 95 °C/30 s, T_{ann}/30 s, 72 °C/1 min; 72 °C/7 min) differed only for the annealing temperatures (T_{ann}). Primers and T_{ann} are listed in Table S1 of Supplementary Materials.

2.4. Construction of MVA-Red

The HcRed1-1 gene was excised from plasmid pHcRed1-1 using restriction endonuclease NotI, followed by Klenow filling and by a second restriction with BamHI, yielding a 711-bp fragment. The transfer plasmid pΔIII-HR-sP was restricted by ClaI, (followed by Klenow filling), then by BamHI to remove the K1L marker, then treated with alkaline phosphatase. Insert and vector DNA were ligated by T4 ligase, generating the transfer plasmid pIII-sP-Red. Correct expression of the HcRed1-1 gene under the vaccinia virus (VV) synthetic promoter sP was verified by DNA lipofectamine-driven transfection (Lipofectamine 2000, Life Technologies, GIBCO BRL, UK) into MVA-infected (m.o.i. = 10) BHK-21 cells. To obtain MVA-Red, a lysate was prepared after pIII-sP-Red DNA transfection into MVA(II_{new})-infected BHK-21 cells (m.o.i. = 0.05). Subsequently, isolated red foci were selected from BHK-21 6-well cultures infected with serial 10-fold dilutions of the lysate. Such cultures were put "under-agarose", i.e. overlaid with medium containing 1% agarose 2 h after the infection. Virus from red foci was amplified in BHK-21 cells, and purified after a second round of focus-selection (under-agarose) and 1 round of terminal dilution in 96-well microtiter cultures. Finally, the absence of parental virus was verified by ΔIII PCR.

The HcRed1-1 gene of pIII-sP-Red was also introduced into deletion III of 3 rMVA strains already carrying genes in deletion II (MVA-Tat_{HIV} and MVA-HuTYR) or in deletion VI (MVA-Env_{HIV}). In all cases, 2 rounds of red focus-selection (under-agarose) and 1 round of terminal dilution cloning (in 96-wells) were sufficient to obtain double recombinants (MVA-Red-Tat_{HIV}, MVA-Red-Env_{HIV} and MVA-Red-HuTYR). All recombinants were analysed by PCR.

2.5. Construction of Transfer Plasmid-Green (pΔIII-K1Lgfp-P7.5)

Transfer plasmids carrying K1L*gfp* were obtained by replacing the K1L gene of transfer plasmid pΔIII-HR-P7.5 with the K1L*gfp* gene of plasmid pSW13as. The latter plasmid was digested with BamHI, blunted with T4 polymerase and digested again with PsiI to obtain a 1631-bp fragment. pΔIII-HR-P7.5 was digested with HpaI and PsiI, followed by alkaline phosphatase treatment. Insert and vector were ligated by T4 ligase, resulting in Transfer Plasmid-Green (pΔIII-K1L*gfp*-P7.5). Correct expression of the green fusion protein gene was verified by lipofectamine-driven DNA transfection into MVA-infected BHK-21 cells (m.o.i. = 10).

The gene tMoIgE from plasmid pAS-E was inserted into pΔIII-K1L*gfp*-P7.5 by digesting both insert and vector with BamHI and PmeI. T4 DNA-ligase treatment resulted in transfer plasmid