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Strategies to obtain multiple recombinant modified vaccinia Ankara vectors. Applications to influenza vaccines

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ABSTRACT

As a vaccination vector, MVA has been widely investigated both in animal models and humans. The construction of recombinant MVA (rMVA) relies on homologous recombination between an acceptor virus and a donor plasmid in infected/transfected permissive cells. Our construction strategy "Red-to-Green gene swapping" – based on the exchange of two fluorescent markers within the flanking regions of MVA deletion ΔIII, coupled to fluorescence activated cell sorting – is here extended to a second insertion site, within the flanking regions of MVA deletion ΔVI. Exploiting this strategy, both double and triple rMVA were constructed, expressing as transgenes the influenza A proteins HA, NP, M1, and PB1. Upon validation of the harbored transgenes co-expression, double and triple recombinants rMVA(ΔIII)-NP-P2A-M1 and rMVA(ΔIII)-NP-P2A-M1-(ΔVI)-PB1 were assayed for *in vivo* immunogenicity and protection against lethal challenge. *In vivo* responses were identical to those obtained with the reported combinations of single recombinants, supporting the feasibility and reliability of the present improvement and the extension of Red-to-Green gene swapping to insertion sites other than ΔIII.

1. Introduction

Due to its established safety and high-level transgene (TG) expression in infected cells, MVA is a promising vaccination vector, widely investigated both in animal models (Calvo-Pinilla et al., 2014; Hessel et al., 2011; Kamlangdee et al., 2014; Sutter et al., 1994; van den Doel et al., 2014) and humans (Chen et al., 2005; Corona Gutierrez et al., 2002; Di Bisceglie et al., 2014; Kimani et al., 2014; Sutter and Staib, 2003). Increasing the number of TGs carried by a single recombinant virus might improve vaccination effectiveness, making recombinant vaccines more similar to traditional vaccines, which usually contain a collection of antigens. Multiple TG expression by a single vector might therefore provide higher protection.

The major flaw of rMVA construction is that recombination is a rare event, making the recovery of recombinant clones from a vast excess of parental virus the most critical and time consuming issue of the procedure. To increase selection efficiency, several methods have been designed so far (Falkner and Moss, 1988; Holzer et al., 2005; Hornemann et al., 2003; Mackett et al., 1984; Staib et al., 2000; Staib et al., 2004; Staib et al., 2003; Sutter et al., 1994). Red-to-Green gene swapping, the method developed by our group (Di Lullo et al., 2009; Di Lullo et al., 2010; Soprana et al., 2011), makes use of fluorescent markers inserted into the flanking regions of deletion III (ΔIII). The markers are red in the acceptor virus and green in the donor plasmid: fluorescence activated cell sorting (FACS) of infected permissive cells (Chicken Embryo Fibroblasts, CEF) allows separation of cells infected by recombinant viruses (fluorescing green) from cells infected by parental viruses (fluorescing red). The process is then completed by terminal dilution cloning and fluorographic screening of microcultures infected by recombinant virus clones. The method is demonstrably

Abbreviations: MVA, Modified vaccinia Ankara; rMVA, Recombinant MVA; CEF, Chicken embryo fibroblast; TG, Transgene; FACS, Fluorescence activated cell sorting; TPG, Transfer plasmid green; MCS, Multiple cloning site; P2A, Peptide 2A

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reliable and cuts down production times considerably.

The aim of this article is to further develop Red-to-Green gene swapping toward the construction of multivalent rMVAs containing TGs in another insertion site (deletion VI; ΔVI) besides ΔIII (Fig. S1). Additional transgenes can be added to one or both insertion sites as polyproteins by exploiting the technology of self-cleaving peptides. The advantage of expressing several antigens in a single vector, rather than using mixtures of individual recombinants, is to obtain multivalent vaccination protocols with a single viral vector and the consequent reduction of the amount of virus to administer.

The immediate application has been in the field of experimental vaccines based on influenza internal proteins, which show a much lower degree of variation as compared to surface antigens (principally HA and NA) and are therefore likely candidates to elicit (cellular) immune responses of wider specificity.

Single recombinants, rMVA(ΔIII)-NP, rMVA(ΔIII)-M1 and rMVA(ΔIII)-PB1 were described in a previous report where they were used separately, and in combination, as vaccines in AAD mice (Di Mario et al., 2017). rMVA(ΔIII)-NP and rMVA(ΔIII)-M1, but not rMVA(ΔIII)-PB1 provided significant levels of protection against lethal challenge. In the present study, in order to reduce the number of individual rMVAs necessary to study vaccine combinations, double and triple rMVA were constructed.

2. Materials and methods

2.1. Cell cultures

Fresh CEF (Chick Embryo Fibroblast) were prepared from 11-day-old embryonated SPF (Specific Pathogen Free) eggs (Charles River, Paris, France) and maintained in a serum-free medium containing 10 ng/mL EGF (VP-SFM, GIBCO), supplemented with 2% glutamine and 1% Pen/Strep, as previously described (Di Lullo et al., 2010). BHK-21 (ATCC CCL-10) and HEK-293T (ATCC CRL-11268) cells were maintained in RPMI-1490 (GIBCO) and DMEM (GIBCO) respectively, both supplemented with 10% FBS (Fetal Bovine Serum, Euroclone) and 1% Pen/Strep (Euroclone). Cells were cultured at 37 °C, 5% CO2.

3. Plasmid construction

All of the influenza genes used in this paper were derived from the A/California/04/2009(H1N1) influenza strain (accession numbers: HA – FJ966082, NP – FJ969512, M1 – FJ969513, PB1 – FJ966080).

3.1. ΔIII-flanking-region targeting plasmids

TPG (Transfer Plasmid Green; carrying ΔIII flanking regions and EGFP under the vaccinia promoter sP) (Di Lullo et al., 2010), was improved by inserting Bsu36I, a directional restriction site, in the MCS. This new plasmid, more suitable for bulk-cloning, was called TPG(ΔIII).

TPG(ΔIII)-HA, TPG(ΔIII)-NP and TPG(ΔIII)-M1, the donor plasmids used in the construction of rMVAs carrying HA, NP or M1 influenza genes in ΔIII, were assembled through Bsu36I-cloning of the V5-tagged influenza genes from plasmids pMK-RQ(HA), pMK-RQ(NP) and pMK-RQ(M1) (obtained from Mathias Knauf and Stephen Norley, Robert Koch Institute, Berlin) into TPG(ΔIII). PB1 DNA was produced by GenScript USA Inc. (NJ, USA).

To assemble TPG(ΔIII)-NP-P2A-M1, the donor plasmid used in the construction of the double rMVA virus carrying both NP and M1 influenza genes in ΔIII, the Porcine Tesho Virus-1 (PTV1) 2A peptide (P2A) sequence (Kim et al., 2011) flanked by AscI and ApaI sites at the 5′-end and BamHI site at the 3′-end (ggcgcgccgggcccGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCggatcc) was synthetized by GenScript USA Inc. (NJ, USA) and inserted into AscI- and BamHI-cut TPG(ΔIII), yielding TPG(ΔIII)-P2A. The NP gene, deprived of its stop codon and of the V5-tag, was amplified by PCR from TPG(ΔIII)-NP and BamHI-cloned at the N-end of the polyprotein. The orientation of the insert was checked by PCR. Finally, the M1 gene, containing a V5-tag sequence before the stop codon, was cloned at the C-end of the polyprotein by insertion of a ApaI- and AscI-flanked PCR amplicon (from TPG(ΔIII)-M1). The final construct, TPG(ΔIII)-NP-P2A-M1 was tested by restriction site analysis and sequencing.

3.2. ΔVI-flanking-region targeting plasmids

To address homologous recombination in the ΔVI flanking region by Red-to-Green gene swapping, ΔVI-flanking-region-carrying plasmids, homologous to p(ΔIII)-RED (previously reported as pIII-sP-Red in Di Lullo et al., 2009) and TPG(ΔIII), were constructed.

p(ΔVI)-RED, carrying HcRED1.1 under the vaccinia promoter sP within the ΔVI flanking regions, was constructed ligating the PCR fragments obtained from plasmids p(ΔIII)-RED and pVIdHR-sP/P7.5 (see Table S1 for the primers, sP-red for & rev and pVI for & rev), exploiting the Bsu36I restriction site (underlined in primer sequences). Sequencing was carried out to exclude PCR-induced point mutations (PRIMM S.r.l., Milan, Italy). Plasmid pVIdHR-sP/P7.5 was originally obtained from Professor Gerd Sutter, TU, Munich.

TPG(VI), carrying ΔVI flanking regions, sP-EGFP and the Bsu36I-carrying MCS, was constructed cloning the whole TPG(ΔIII) recombination cassette (Z,sP-EGFP,Z,MCS; Fig. S1) within the ΔVI flanking regions of pVIdHR-sP/P7.5. The recombination cassette was excised from TPG(ΔIII) through a SnaBI-BstBI partial digestion and subsequently Klenow-blunted, while the ΔVI-flanking-region-containing plasmid backbone was isolated through a complete PmeI-SnaBI digestion of pVIdHR-sP/P7.5.

As for the construction of the donor plasmid targeting the ΔVI flanking regions, the V5-tagged NP influenza gene contained in TPG(ΔIII)-NP was inserted into TPG(ΔVI) using BamHI and AscI sites, leading to TPG(ΔVI)-NP, while the V5-tagged PB1 gene was Bsu36I-excised from TPG(ΔIII)-PB1 and cloned into TPG(ΔVI), obtaining TPG(ΔVI)-PB1. Dephosphorylation of Bsu36I-cut TPG(ΔVI) was carried out using Antarctic Phosphatase (NEB #M0289L).

3.3. PCDNA-based plasmids construction

In order to easily transfer genes between TPG- and pCDNA-based plasmids, pCDNA3.1(+) plasmid (Invitrogen) was engineered through insertion of the Bsu36I restriction site in the MCS. Briefly, the 235 bp BamHI-EcoRI fragment contained in TP(ΔIII) was excised and directly cloned into BamHI-EcoRI-cut pCDNA3.1(+), leading to pCDNA3.1(+)-Bsu36I.

Consequently, NP-P2A-M1 and PB1 fragments were Bsu36I-excised from TPG(ΔIII)-NP-P2A-M1 and TPG(ΔIII)-PB1, respectively, and cloned into pCDNA3.1(+)-Bsu36I, leading to pCDNA-NP-P2A-M1 and pCDNA-PB1.

4. Virus construction and characterization

All rMVA viruses described in this work were derived from a 1977 MVA vaccine sample (MVA-Mu77; obtained from the Department of Health of the Bavarian Government) through homologous recombination by Red-to-Green gene swapping (Di Lullo et al., 2010), or its adaptation to ΔVI (Fig. S1).

4.1. Construction of MVA(ΔIII)-RED and MVA(ΔVI)-RED

Plasmids p(ΔIII)-RED and p(ΔVI)-RED were infection/transtection crossed with MVA-Mu77; acceptor viruses MVA(ΔIII)-RED and MVA(ΔVI)-RED were obtained by two rounds of sorting of “red” CEF, followed by terminal dilution cloning monitored by fluorography of microcultures (Fig. S2 reports the characterization of p(ΔVI)-RED and MVA(ΔVI)-RED). The acceptor vector MVA(ΔVI)-RED, constructed as a