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Modified H5 promoter improves stability of insert genes while maintaining immunogenicity during extended passage of genetically engineered MVA vaccines

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ABSTRACT

We have engineered recombinant (r) Modified Vaccinia Ankara (MVA) to express multiple antigens under the control of either of two related vaccinia synthetic promoters (pSyn) with early and late transcriptional activity or the modified H5 (mH5) promoter which has predominant early activity. We sequentially passaged these constructs and analyzed their genetic stability by qPCR, and concluded that rMVA expressing multiple antigens using the mH5 promoter exhibit remarkable genetic stability and maintain potent immunogenicity after serial passage. In contrast, rMVA expressing antigens using engineered vaccinia synthetic E/L (pSyn I or II) promoters are genetically unstable. Progressive accumulation of antigen loss variants resulted in a viral preparation with lower immunogenicity after serial passage. Metabolic labeling, followed by cold chase revealed little difference in stability of proteins expressed from mH5 or pSyn promoter constructs. We conclude that maintenance of genetic stability which is achieved using mH5, though not with pSyn promoters, is linked to timing, not the magnitude of expression levels of foreign antigen, which is more closely associated with immunogenicity of the vaccine.

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1. Introduction

Modified Vaccinia Ankara, a highly attenuated poxvirus does not propagate in most mammalian cells [1]. This property minimally impacts viral or foreign gene expression, because MVA continues to replicate its DNA with concomitant robust transcriptional activity until its life cycle is interrupted by a late block in viral assembly. In addition, MVA has a large foreign gene capacity as a result of multiple deletions that were created in its original development during passage in chicken embryo fibroblasts (CEF) [2]. MVA has a well-established safety record and history of use as a vaccine [3–7]. The virus has superior properties of inducing potent humoral and cellular immunity which has led to MVA based vaccines for treatment of infectious disease and cancer, with some having successfully entered Phase I/II clinical trials [8–13].

MVA only replicates its DNA in the cytoplasm of cells while exclusively using its own vaccinia transcriptional system, including its own promoters used to direct foreign antigen gene expression [14]. Two examples of vaccinia promoters widely used to direct for-

eign gene expression in rMVA are the synthetic promoter (pSyn), which contains both vaccinia early and late promoter sequences optimized for high level protein expression [15] and the modified H5 promoter (mH5) which contains both native early and late vaccinia promoter regions [16]. pSyn has stronger overall promoter activity than mH5, but the early activity of the mH5 promoter is three–five fold stronger than the pSyn series [16]. While MVA as a viral vector has virtues including its large foreign gene capacity and multiple integration sites [17], only a few investigations of genetic stability of rMVA have been reported [16,18–23].

Our laboratory has developed MVA as a viral vector for delivering antigens into mouse and rhesus macaque models for infectious disease and cancer [24–28]. We have recently generated rMVA expressing CMV antigens {pp65, IE1/exon4 (e4) and IE2/exon5 (e5)} under control of either the pSyn or mH5 promoters. These viral vectors promote substantial immunogenicity either when evaluated in vitro to propagate existing T cell memory populations, or in vivo in mouse models as primary immunizations [26]. In this report, we demonstrate that rMVA expressing CMV antigens under control of pSyn promoter are genetically unstable after serial passage; however, rMVA expressing the same antigens under control of mH5 promoters exhibits marked genetic stability that translates into comparable levels of immunogenicity after extended virus passage.

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2. Materials and methods

2.1. Cells, virus, peptides, and mice

Primary CEF cells prepared from specific pathogen-free chicken eggs were purchased from Charles River SPAFAS (North Franklin, CT, USA). BHK-21 cells (ATCC CCL-10) were purchased from American Type Cell Collection (Manassas, VA, USA) and maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum in a 37 °C incubator containing 5% CO₂.

Wild type (wt) MVA virus stock, pLW51 and pSC11 transfer plasmids were kindly provided by Dr. Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH). rMVA expressing CMV pp65 alone (pSyn-pp65-MVA) or together with IE1/e4 under control of pSyn promoter (pSyn-pp65-IE1/e4-MVA) were generated by our laboratory and described previously [27]. rMVA expressing CMV pp65, IEFusion protein (IE1/e4 and IE2/e5) under control of pSyn promoter (pSyn-pp65-IEfusion-MVA) were also developed via a homologous recombination method [29].

2.2. Construction of MVA transfer plasmids and viruses containing mH5 promoters

pZWIIA transfer vector containing two pSyn promoters was constructed as described previously [27]. Additional MVA transfer plasmids were constructed after replacement of pSyn with the mH5 promoter. We first replaced the two pSyn promoters in pZWIIA with one mH5 promoter. Briefly, a 228 bp DNA fragment including the 70 bp mH5 promoter sequences and multiple cloning sites was synthesized (Genebank accession # FJ386852) and cloned into pZERO-2 (Integrated DNA Technologies, Coralville, IA). This 228 bp DNA fragment was excised with Xho I and Not I, gel purified and cloned into pZWIIA to yield mH5-pZWIIA. We then modified mH5-pZWIIA and replaced the bacterial *gus* (β -glucuronidase) marker gene with the VenusTM fluorescent marker gene (Clontech, Mountain View, CA, USA) to improve the speed of rMVA screening. The CMV pp65 gene was cloned into mH5-pZWIIA to yield mH5-pp65-pZWIIA. The IEFusion gene was cloned into mH5-pZWIIA to yield mH5-IEfusion-pZWIIA, an MVA transfer plasmid used to generate mH5-IEfusion-MVA. To make rMVA expressing both pp65 and IEFusion protein simultaneously, a new MVA transfer vector that contained mH5 promoter and targets MVA deletion III region was constructed based on the pLW51 plasmid. We modified pLW51 by replacing the original expression cassette by excision at XhoI and AscI sites and inserted the mH5 promoter followed by the CMV pp65 gene to yield mH5-pp65-pLW51. The structure of MVA transfer vectors (mH5-pp65-pZWIIA, mH5-IEfusion-pZWIIA and mH5-pp65-pLW51) were verified by restriction enzyme digestion and DNA sequence analysis.

mH5-pp65-MVA was generated by transfecting mH5-pp65-pZWIIA into wtMVA infected BHK-21 cells and screened based on VenusTM fluorescent marker to eliminate wtMVA according to our published procedures [25]. mH5-pp65-IEfusion-MVA was generated in two steps. Briefly, we first generated mH5-IEfusion-MVA by transfecting mH5-IEfusion-pZWIIA into BHK-21 cells infected with wtMVA in six-well plates. mH5-IEfusion-MVA was screened to eliminate wtMVA based on VenusTM fluorescent marker expression. mH5-IEfusion-MVA was expanded on BHK-21 cells after 8–10 rounds of screening to create a stock for the 2nd round of gene insertion after verification that wtMVA was eliminated. The 2nd step was the transfection of mH5-pp65-pLW51 into BHK-21 cells simultaneously infected with mH5-IEfusion-MVA. mH5-pp65-IEfusion-MVA was screened based on the bacterial *Gus* gene marker for 8–10 rounds until parental virus (mH5-IEfusion-MVA) was removed completely. mH5-pp65-MVA and mH5-pp65-IEfusion-MVA were

expanded on BHK-21 cells to create virus stocks that were stored long term at –80 °C.

2.3. Stability analysis of individual rMVA isolates from passage 10

rMVA with expression cassettes based on mH5 (mH5-pp65-MVA, mH5-pp65-IEfusion-MVA) or pSyn promoters (pSyn-pp65-IE1/e4-MVA, pSyn-pp65-IEfusion-MVA) were consecutively passaged 10 times on either CEF or BHK-21 cells. Briefly, a 150 mm tissue culture dish of either CEF or BHK-21 cells was infected with rMVA at MOI = 0.1 (multiplicity of infection). rMVA was harvested 48 h after infection, resuspended in 1.0 ml of MEM containing 2% fetal calf serum (MEM-2) and subjected to 3× freeze/thaw cycles followed by sonication to release the virus. The virus from each passage was subsequently titrated on either CEF or BHK-21 cells and after adjustment to an MOI of 0.10, it was used for the next passage. DNA samples of each passage were obtained for qPCR analysis using the QiagenTM column purification kit according to manufacturer's instructions (Valencia, CA, USA). Cell lysates of each passage used for western blot (WB) analysis were prepared from 100 mm dishes of either CEF or BHK-21 cells infected with the same number of pfu of rMVA of each serial passage.

In order to further characterize virus plaques from passage 10 (P10) of pSyn-pp65-IE1/e4-MVA, individual plaques were isolated from P10 virus stock by plaque purification. Briefly, P10 virus stock of pp65-IE1/e4-MVA (pSyn) was titrated by immunostaining using anti-vaccinia polyclonal sera (AbD serotech, Raleigh, NC, USA), diluted and distributed at 0.5 pfu per well into 96-well plates. At 4 days post-infection, 18 wells that appeared to be infected by no more than one virus isolate were collected, expanded and analyzed by WB for CMV-pp65 expression levels. We also randomly isolated 6 individual plaques from P1 using the same method.

2.4. WB detection of rMVA protein expression

Protein expression levels of the CMV-pp65, IE1/e4 and IEFusion genes from pSyn-pp65-IE1/e4-MVA, pSyn-pp65-IEfusion-MVA and mH5-pp65-IEfusion-MVA infected cells were measured by WB using the Amersham ECL PlusTM detection kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Cell lysates were separated by SDS-PAGE on 10% gels. After electro-transfer of proteins from the gel onto PVDF membranes (Bio-Rad, Hercules, CA, USA), the blots were incubated with purified mAb 28-103 [30] against CMV-pp65, mAb p63-27 [31] against IE1, or mAb 19C2 against MVA-BR5 [32] then washed and further incubated with HRP-labeled goat anti-mouse polyclonal Ab according to the manufacturer's instructions (Amersham Pharmacia Biotech).

2.5. Southern blot detection of CMV-pp65 and IE1/e4 insertion gene in rMVAs

To determine the presence of the CMV-pp65 and IE1/e4 gene in individual pp65-IE1/e4-MVA isolates after P10, Southern blot (SB) was performed. Briefly, a 150 mm culture dish of BHK-21 cells was infected with individual pp65-IE1/e4-MVA isolates at MOI = 1 and incubated at 37 °C for 24 h. The MVA viral genomic DNA was isolated according to a described method [33]. Cells were homogenized in 1.25 ml hypotonic buffer (10 mM Tris-HCl, pH 7.8 and 12 mM KCl followed by incubation with 450 units of micrococcal nuclease (Sigma-Aldrich St. Louis, MO, USA) for 1 h at 25 °C to digest cellular DNA. The reaction was stopped by adding EGTA (glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid). Cell lysates were treated with proteinase K for 1 h at 25 °C to release MVA viral DNA and then extracted using the phenol/chloroform method. For SB, MVA viral DNA was digested with Pme I and Nhe I restriction enzymes to excise the 3.9 kb fragment

Table 1

List of qPCR primers. The qPCR primers to measure pp65, IEfusion and MVA Thymidine Kinase (TK) genes were designed based on standard qPCR conditions using Primer Express Software Version 3.0 (ABI, Foster City, CA, USA).

Target gene	Primer sequences
CMV pp65	Forward: 5' A TCAAACCGGGCAAGATCTCGC 3' Reverse: 5' ATCGTACTGACG CAGTTCACG 3'
CMV IE1	Forward: 5' CCATCGCCGAGGAGTCAGAT 3' Reverse: 5' AGTGTCTCTCCGCTCCTCT 3'
CMV IEfusion	Forward: 5' AAGTTGCCCC AGAGGAAGAG 3' Reverse: 5' CTGCTAACGCTGCAAGAGTG 3'
MVA TK	Forward: 5' TGTGAGCGTATGGCAAACGAA 3' Reverse: 5' TCGATGTAACACTTCTACACCGATT 3'

containing the foreign gene cassette, separated on a 1% agarose gel and transferred to nylon membrane. This filter was hybridized with a ³²P-labeled DNA probe specific for both CMV-pp65 and IE1 e4 gene and exposed to HyPerfilm (Amersham Bioscience, Piscataway, NJ, USA).

2.6. qPCR to measure DNA copy number

MVA viral DNA was extracted using a Qiagen QIAmp miniprep kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). The plasmid DNA used to generate the standard curve was made by inserting both the CMV-pp65 and IEfusion gene into the pSC11 vector containing the TK gene [34]. The absolute concentration of the plasmid was measured by two independent means: OD₂₆₀ by UV spectrophotometry and a fluorophore-based method using Quant-iT™ Picogreen® dsDNA kit (Invitrogen, Carlsbad, CA, USA). The concentration was converted to plasmid copy # using the molecular weight of the plasmid DNA. The primers to measure CMV-pp65, IEfusion gene and MVA backbone copies were designed based on standard qPCR conditions using Primer Express Software Version 3.0 (Applied Biosystems Inc., Foster City, CA, USA) and listed in Table 1. qPCR was performed using an ABI 7300 real-time PCR system and Power SYBR green master mix kit (ABI). Briefly, 5 μl of MVA genomic DNA was amplified in a mixture of 25 μl containing 1 μM forward, 1 μM reverse primers and SYBR green containing solution. The thermal cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and ending with one cycle at 60 °C for 30 s. Gene copy numbers were calculated using ABI sequence detection system software (SDS). The ratio of insert CMV genes and MVA backbone (ratio = CMV gene copy number/MVA backbone computed copy number) was calculated for each passage.

2.7. Immunogenicity of mH5-pp65-IEfusion-MVA using human PBMC

Human peripheral blood mononuclear cells (PBMC) were collected by the City of Hope Donor/Apheresis Center, ficoll-purified and cryopreserved at –80 °C. All human blood samples were considered discard and anonymous, except for HLA A and B typing information provided to investigators without other identifiers. IVS of PBMC using rMVA was performed according to previously described methods [27]. Briefly, cryopreserved PBMC were rapidly thawed and cultured with both CpG-A ODN 2216 and CpG-B ODN 2006 (TriLink BioTechnologies, San Diego, CA, USA). After 3d, PBMCs were infected with rMVA for 6h, γ-irradiated (2500 rad) and used as APC incubated with autologous PBMC for 7d.

PBMC harvested at 8d post-IVS were incubated with either CMV-pp65 or IEfusion peptide library for 12h in the presence of brefeldin A, then washed and labeled with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4 antibodies, fixed, and permeabilized (Cytotfix-Cytoperm; BD Biosciences) before they were labeled with

APC-conjugated antibody to IFN-γ. The stained cells were analyzed on a FACSCanto flow cytometer (BD Biosciences). Comparison of paired data between P1 and P7 of mH5-pp65-IEfusion-MVA was performed using the Student *t*-test based on two-tailed procedure. *P* values were considered significant if <0.05.

2.8. Immunogenicity of rMVA in HHD II Tg mice

HHD II mice (Tg HLA A2.1) were used at 6–12 weeks for immunization and were bred and maintained under SPF conditions in a centralized animal care facility. HHD II mice were immunized with 5×10^7 pfu of purified rMVA by the intraperitoneal (i.p.) route. Spleens were removed three weeks post-immunization and were stimulated *in vitro* for one week using a simplified protocol with HLA-matched human EBV-LCL [35] as antigen presenting cells (APC), loaded either with the relevant CMV-CTL epitope HLA-A*0201 IE-1_{316–324} (IE1-A2), pp65_{495–503} (pp65-A2) [36–38] or, IE2 CMV-peptide library (4 μg/ml) made in our laboratory [29].

ICC was used to measure CMV-pp65, IE1 and IE2 IFN-γ⁺/CD4⁺ or IFN-γ⁺/CD8⁺ T cells from the stimulated splenocytes according to previously described methods [39,40]. $0.5\text{--}1 \times 10^6$ events were acquired for each sample on a FACSCanto™ (BD Biosciences, San Jose, CA, USA). Analysis was performed using FCS Express version 2 software (De Novo, Ontario, Canada). The number of double-positive cells is expressed as a percentage of the CD8⁺ T-cell population.

2.9. Comparison of mH5 and pSyn promoter activity in rMVA infected cells

To determine either early or total transcriptional activity of mH5 and pSyn promoters in rMVA infected cells, pSyn-pp65-MVA and mH5-pp65-MVA were used to infect BHK-21 cells for 24h in the absence or presence of cytosine arabinoside (ara-C). Briefly, BHK-21 cells were seeded at 0.6×10^6 per well onto a 6-well plate. The cells were infected with either pSyn-pp65-MVA or mH5-pp65-MVA at moi 5 in the absence or presence of 40 μg/ml of Ara-C and incubated for 24h at 37 °C in a 5% CO₂ incubator. The infected cells were harvested and lysed in 0.2 ml SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8, 2.8 mM β-mercaptoethanol, 2% SDS, 10% glycerol, 0.4% Bromophenol Blue). Cell lysates were separated by SDS-PAGE on 10% gels. After electro-transfer of proteins from the gel onto PVDF membranes (Bio-Rad, Hercules, CA, USA), the blots were incubated with purified mAb 28–103 [30] against CMV-pp65 and mAb against β-tubulin (Sigma–Aldrich), then washed and further incubated with HRP-labeled goat anti-mouse Ab according to the manufacturer's instructions. To measure CMV-pp65 protein expression levels, the blots were incubated with chemifluorescence substrate solution in ECL Plus detection kit (Amersham, CA) for 30' and were scanned using Typhoon™ 9410 workstation and analyzed by ImageQuant TL (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA).

2.10. Pulse-chase (PC) metabolic labeling and immunoprecipitation (IP)

PC and IP were performed based on modification of described methods [41,42]. Briefly, subconfluent cultures of CEF or BHK-21 cells grown in 6-well plates were infected at an MOI of 10 with mH5-pp65-MVA or pSyn-pp65-MVA. At 1h postinfection (hpi) cells were washed and incubated with Cys-free and Met-free DMEM (Invitrogen, Carlsbad, CA, USA) medium containing 5% dialyzed fetal calf serum (FCS; Invitrogen) for 1h. Afterwards, cells were metabolically labeled (100 μCi/mL/well) for 30 min with a mixture of [³⁵S]Cys + [³⁵S]Met [Express Protein Labeling Mix™ (1000 Ci/mmol) PerkinElmer, Boston, MA, USA]. After labeling, the

cells were washed twice with PBS and either harvested immediately or chased in RPMI medium with 10% FCS (ISC-BioExpress, Kaysville, UT, USA) supplemented with excess unlabeled methionine (1 mM) and cysteine (5 mM) up to 10 h. After each time point, cells were immediately pelleted, then lysed in 1.0 mL PBS containing 1.0% Triton X-100, 1.0% sodium deoxycholate (Sigma, St. Louis, MO, USA) and 0.1% SDS in the presence of Protease Inhibitor Cocktail (Roche, Nutley, NJ, USA). Supernatants (0.5 mL) were pre-cleared once with 50 μ L of protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h. Sequential incubation with 2.4 μ g purified mAb against CMV-pp65 (mAb 28–103 [43]) was followed by an isotope-specific mAb (19C2 [32]) for 2 h. Immune complexes were captured by incubation for 1 h with 50 μ L of protein A/G beads. The immune complex bound Protein A/G beads were washed 4 times with 0.1% Triton X-100 in PBS and bound proteins were eluted by boiling in 0.2% SDS, 5 mM DTT, 40 mM sodium phosphate buffer (pH 6.5) into SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Proteins were separated by 10% SDS-PAGE and detected by autoradiography using X-OMAT film (Kodak, Rochester, NY, USA).

3. Results

3.1. Serial passage of pSyn-pp65-IE1/e4-MVA

pp65-IE1/e4-MVA (pSyn) was generated using pZWIIA transfer plasmid as previously described [27]. The CMV-pp65 and IE1 gene expression cassettes were integrated into the del (deletion) integration site II of the MVA genome via homologous recombination as shown in Fig. 1A. The virus titer was measured and growth rate of each passage was measured after pSyn-pp65-IE1/e4-MVA was sequentially passaged for 10 rounds on CEF. No significant change was found in virus titer and growth rate during serial passage (data not shown). Cell lysates of each passage were prepared in parallel from 100 mm culture dishes of cells infected with the same amount of virus established by titration on CEF. CMV-pp65 and IE1/e4 protein levels progressively decreased during passage, and were significantly reduced after ten serial passages (Fig. 1B). The constitutively expressed MVA protein BR5 was also probed at each passage from the same lysates using the 19C2 mAb [32], and as shown in Fig. 1B, its steady state expression level was unchanged

during the 10 passage evaluation. Serial passage of pSyn-IE1/e4-MVA was also carried out on CEF with similar results (data not shown).

3.2. Preparation and expression analysis of 18 individual P10 isolates

It was hypothesized that gradual decrease of the CMV-pp65 and IE1 expression levels during serial passage could be caused by genetic changes that result in non-expressing viral isolates [22]. To test this hypothesis, individual isolates were obtained from passage 10 (P10) by plaque purification. 18 wells that appeared to have cyto-pathologic effects (CPE) were collected at 4d post-infection. Each virus sample was considered to be a single isolate, because the equivalent of 0.5 pfu of virus was distributed in each well. Viral infection from these collected samples was confirmed by continuous virus growth and virus titration. CMV-pp65 expression levels were measured, and it was discovered that 40% (8 of the 18) individual isolates had lost CMV-pp65 expression from P10 (Fig. 1C). In contrast, 100% (6 of 6 isolates) individual isolates from P1 all had similar strong levels of CMV-pp65 expression (data not shown).

3.3. Deletion of the pp65 and IE1/e4 gene was the cause of loss of pp65 and IE1/e4 protein expression from individual virus isolates of pSyn-pp65-IE1/e4-MVA

The cause of the loss of CMV-pp65 and IE1 protein expression was investigated. Mutations or total deletion of the CMV-pp65 and IE1 genes during serial passage was hypothesized to be responsible for the loss of protein expression. Two isolates with full expression levels, two isolates that lost CMV-pp65 protein expression (#4, #6 in Fig. 1C) and two isolates that retained CMV-pp65 expression from P10 (#7, #13 in Fig. 1C) were further investigated. WB blot was performed to detect both CMV-pp65 and IE1 protein expression levels, and Southern blot (SB) to detect CMV-pp65 and IE1 expression cassettes from viral DNA. Similarly, a mAb specific for the MVA BR5 viral protein was used to probe lysates in a separate WB to detect endogenous viral gene expression to control for virus input in all six samples (Fig. 2A, iii). The two individual isolates

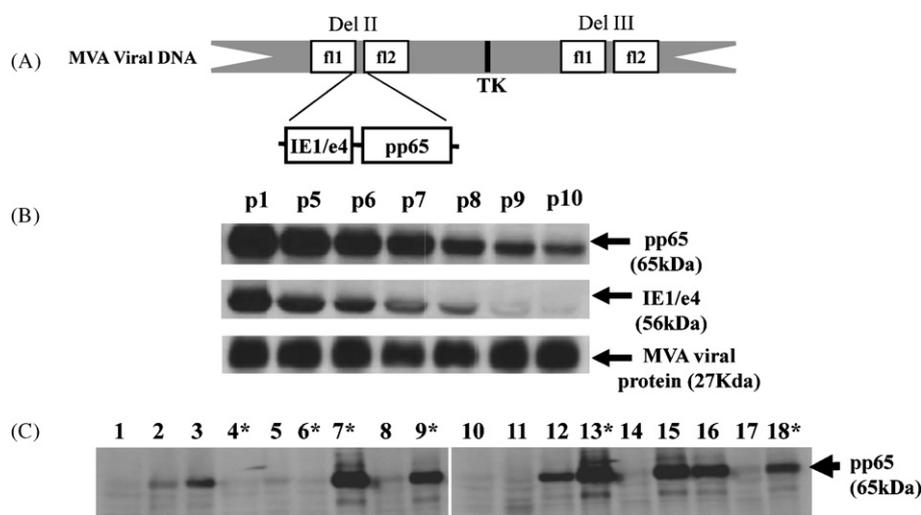


Fig. 1. Schematic map of pp65 and IE1/e4 gene expression cassette of pSyn-pp65-IE1/e4-MVA and WB detection of pp65 and IE1 e4 expression levels of pSyn-pp65-IE1/e4-MVA. (A) Schematic map of viral DNA genome of pSyn-pp65-IE1/e4-MVA generated via homologous recombination as described previously [27]. (B) Western blot detection of pp65 and IE1/e4 expression level of pp65-IE1/e4-MVA infected CEF cells of serial passages 1–10. Top panel shows a membrane blotted with mAb 28–103 specific for pp65; middle panel shows a membrane blotted with p63–27 specific for IE1/e4; and the bottom panel shows a membrane blotted with mAb 19C2 that detects VV-BR5. (C) WB detection of pp65 expression of 18 pSyn-pp65-IE1/e4-MVA individual isolates. 18 individual pSyn-pp65-IE1/e4-MVA viruses were isolated from passage 10 by virus plaque purification and expanded on CEF cells to prepare cell lysates for WB. Each lane represented single individual isolate from passage 10. Samples #4, #6, #7 and #13 marked with a star were selected for viral genomic DNA extraction and Southern blot analysis as described in Section 2.

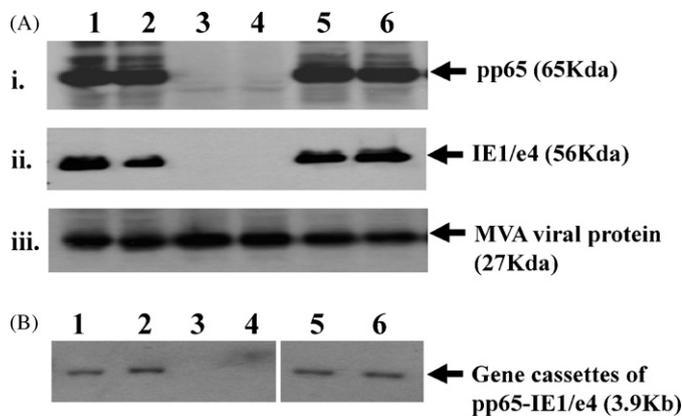


Fig. 2. Western blot detection of pp65 and IE1 e4 protein expression and Southern blot detection of pp65 and IE1 e4 gene insertion in pSyn-pp65-IE1/e4-MVA individual isolates. (A) Western blot detection of pp65 and IE1 e4 protein expression of selected individual isolates of pSyn-pp65-IE1 e4-MVA. The membrane shown in Panel A (i) was blotted with mAb 28–103 specific for pp65, the membrane shown in Panel A (ii) was blotted with p63–27 specific for IE1/e4 and the membrane shown in Panel A (iii) was blotted with mAb 19C2 specific for the VV-BR5 viral protein. (B) Southern blot detection of pp65 and IE1 e4 gene insertion of selected individual isolates of pSyn-pp65-IE1/e4-MVA. MVA viral genomic DNA was digested with restriction enzymes to excise 3.9 kb fragments of pp65-IE1 gene expression cassettes, separated by 1% agarose gel and transferred to nylon membrane filter. This filter was hybridized with the 32 P-radiolabeled DNA probe specific for both pp65 and IE1 e4 gene and exposed to x-ray film. Notes: Lanes 1 and 2 (Panels A and B) are two individual isolates selected randomly from passage 1 of pSyn-pp65-IE1/e4-MVA. Lanes 3 and 4 (Panel A and B) are the two individually isolates of #4 and #6 marked with asterisk from Fig. 2 with no expression of pp65 and IE1 e4. Lanes 5 and 6 (Panels A and B) are the two individual isolates #7 and #13 marked with asterisk in Fig. 2 with elevated pp65 and IE1/e4 expression levels.

from P10 that maintained CMV-pp65 expression also expressed IE1 at similar level as P1 (Fig. 2A, i). In contrast, the two isolates from P10 that lost CMV-pp65 expression also coordinately lost IE1 protein expression (Fig. 2A, i and ii). Interestingly, in all 4 cases, there was either coordinate expression of both antigens or their absence, suggesting that the whole cassette was either maintained or inactivated by deletion or mutation when protein expression was not detected. In contrast, the expression of the MVA BR5 protein remained uniformly unchanged (Fig. 2A, iii).

An SB was performed to detect the CMV-pp65 and IE1 genes to establish the relationship of protein expression levels and the presence of the genes. Equal amounts of DNA from each viral isolate was digested with Pme I and Not I DNA restriction enzymes to excise CMV-pp65 and IE1/e4 gene expression cassettes (3.9 kb), which were detected by a 32 P-radiolabeled DNA probe. The gene expression cassette was detected in two individual virus isolates from P1 and P10 (lanes 1, 2, 5, 6 in Fig. 2B), but not detected in two viral isolates from P10 that also lost protein expression (lanes 3 and 4 in Fig. 2B). The del II site of MVA was further analyzed by DNA restriction endonuclease analysis of MVA genomic DNA and by PCR using a series of primers that target the surrounding del II region. It was found that CMV-pp65 and IE1 gene expression cassettes together with the surrounding MVA del II region were absent (data not shown). The possibility was excluded that the two non-expressing mutants were contaminant wt MVA virus that was introduced and amplified during the serial passage using additional qPCR primers (data not shown). The isolates that maintained CMV-pp65 and IE1/e4 expression shown in Fig. 2A (lanes 1 and 2) were tested to see if they represented stabilized forms of each gene in the virus, pSyn-pp65-IE1/e4-MVA, during serial passage. The two p10 isolates were sequentially passaged for an additional 10 rounds on CEF. We discovered that both CMV-pp65 and IE1 protein expression still decreased at the conclusion of additional serial passage (data not shown). These results demonstrate that high expressing iso-

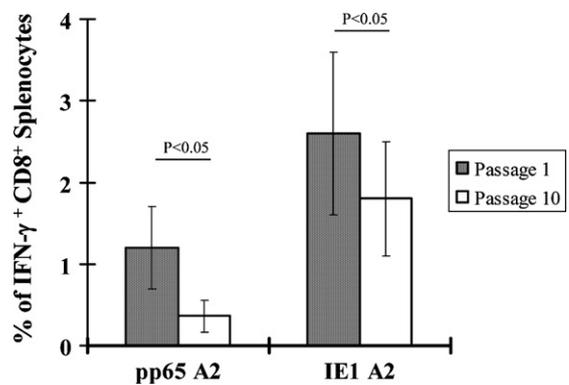


Fig. 3. Immunogenicity of pp65-IE1/e4-MVA passage 1 and passage 10 immunized HHD II mice (HLA A2.1). Splenocytes from HHD II mice immunized with pSyn-pp65-IE1/e4-MVA from passage 1 (P1) or passage 10 (p10) were subjected to IVS separately with either pp65 A2 or IE1 A2 peptides loaded blast cells for one week. After IVS, the splenocytes were incubated with mock A2, pp65A2 or IEA2 peptides overnight and harvested for ICC described in Section 2. Average levels of IFN-γ producing specific for the CMV pp65- or IE1-A2 epitope (x-axis) for all immunized mice is shown in Y-axis. IFN-γ producing CD8+ T-cells to mock during the ICS procedure were subtracted. Error bars represent the SEM for all immunized mice.

lates from P10 are not stabilized forms of pSyn-pp65-IE1 e4-MVA, and are subject to deletion during passage.

3.4. Immunogenicity of pSyn-pp65-IE1/e4-MVA is reduced after serial passage

It was investigated if reduction of CMV-pp65 and IE1 protein expression impacted immunogenicity by expanding P1 and P10 virus stocks for mouse immunizations. HHD II mice were separately immunized with both P1 and P10 passage strains for 3 weeks, and splenocyte immune response was assessed by ICC to detect IFN-γ expression. Immunodominant HLA A2-restricted CMV-pp65 and IE1 CTL epitopes were used to evaluate the HLA A2-restricted CD8+ T cell response. We found a statistically significant diminution of CMV-pp65 and IE1 specific-IFN-γ producing CD8± splenocytes in P10 compared to P1 immunized groups (Fig. 3).

3.5. Genetic stability of pSyn-pp65-IE1/e4-MVA measured by qPCR

Since progressive loss of CMV-pp65 and IE1/e4 protein expression is correlated with the deletion of gene expression cassettes, the kinetics of the loss of both genes by qPCR was measured to help explain the instability of the gene cassettes. The genetic stability of rMVA can be assessed by computing the ratio of the foreign gene insert and the MVA backbone copy number. The ratio of gene insert to MVA backbone at initial passage was normalized to unity, and a gradual reduction of the ratio was found during serial passage. Only 20% of the rMVA genomes retained CMV-pp65 and IE1/e4 gene inserts after round P10 (Fig. 4A). This measurement establishes a correlation between the loss of both antigen genes that is confirmed by qPCR, lower protein expression levels and reduced immunogenicity of the passaged viral population.

3.6. Genetic stability of pSyn-pp65-IEfusion-MVA measured by qPCR

We constructed rMVA expressing three CMV antigens (CMV-pp65, IE1/e4 and IE2/e5) under control of two separate pSyn promoters (pSyn-pp65-IEfusion-MVA) to expand the representation of early genes and epitope diversity [29]. pSyn-pp65-IEfusion-MVA includes the IE2/e5 gene which is fused to IE1/e4. pSyn-pp65-IEfusion-MVA was serially passaged five times, and even after

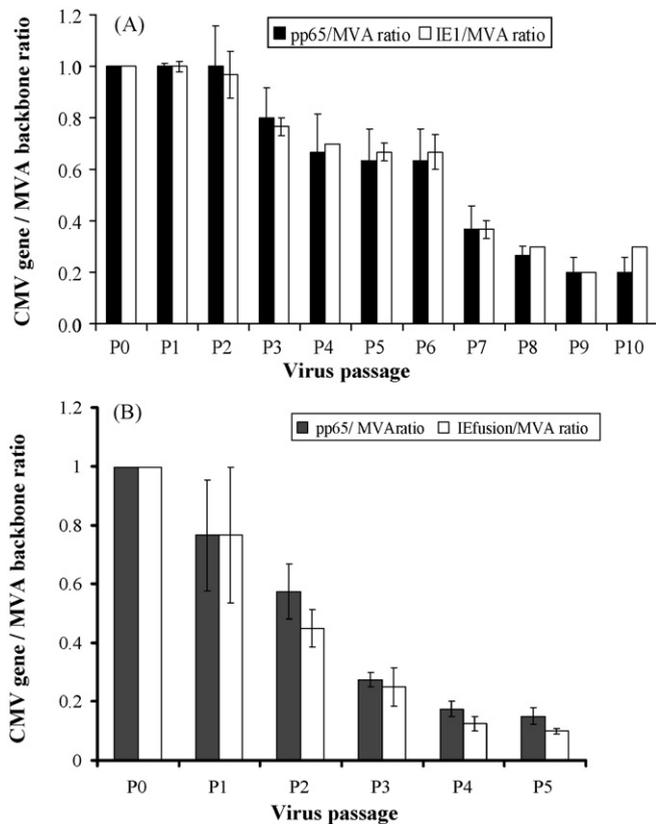


Fig. 4. Genetic stability of pSyn-pp65-IE1/e4-MVA serial passages and pSyn-pp65-IEfusion-MVA determined by qPCR: (A) pSyn-pp65-IE1/e4-MVA genomic DNA was extracted as described in Section 2. pSC11 plasmid containing CMV genes (pp65, IE1/e4 and IE2/e5) was used to prepare absolute standards. The qPCRs were performed using primers specific for pp65, IE1/e4 and TK gene. The copy numbers for pp65 gene, IE1 gene and MVA backbone copies were calculated using ABI software (SDS3.2) and the genetic stability of the mH5-pp65-IEfusion-MVA was determined by computing the ratio of the pp65 gene insert and the MVA backbone or the ratio of the IE1/e4 gene insert and the MVA backbone as indicated in Y-axis. The ratio at passage 1 is normalized to 1 and each consecutive passage was normalized based on passage 1. The qPCR for each DNA sample were performed for three times independently in duplicates and average ratio and error bar shown in the figure represented three independent determinants. (B) pSyn-pp65-IEfusion-MVA viral genomic DNA was extracted and qPCR was performed using pp65, IEfusion and TK specific primers as described in Section 2. The copy numbers for pp65 gene, IEfusion gene and MVA backbone were analyzed using ABI software (SDS3.2) and the genetic stability of the mH5-pp65-IEfusion-MVA was determined by computing the ratio of the pp65 gene insert and the MVA backbone or the ratio of the IEfusion gene insert and the MVA backbone. The ratios at passage 1 for pp65 and IE1/e4 gene were normalized to 1. The qPCR for each DNA sample were performed for three times independently in duplicates and average ratio and error bar shown in the figure represented three independent determinants.

a single passage, evidence of instability was observed (Fig. 4B). Only 10% of the original levels of CMV-pp65 and IEfusion insert sequences were detected by qPCR after 5 passages, which demonstrates an unexpected decrease in stability (Fig. 4B). This result highlights that different combination of genes (pp65 and IE1/e4 and pp65 and IEfusion) result in pronounced genetic instability using the pSyn promoter, suggesting that the genes themselves are not the main contributor to genetic instability compared to the activity of the pSyn promoter.

3.7. Construction of mH5-pp65-MVA and measurement of genetic stability

It was hypothesized that the instability of pSyn-pp65-IE1/e4-MVA and pSyn-pp65-IEfusion-MVA is due to the properties of pSyn promoters. The pSyn promoter was optimized for high level pro-

tein expression. It was designed to be highly active by combining several early and late promoter elements, but is dominated by its late stage promoter activity [15]. To improve genetic stability, the pSyn promoter was replaced with the mH5 promoter which stimulates a greater proportion of its transcriptional activity at an earlier stage of the virus life cycle (Fig. 5A) [16,23]. rMVA was generated using shuttle plasmids that had the mH5 promoter directing the transcription of the CMV-pp65 gene (data not shown). Quantification by qPCR revealed no significant changes in the ratio of CMV insert gene/MVA backbone genomic copy number during 10 serial passages of a virus using the mH5 promoter directing recombinant protein expression (Fig. 5B).

3.8. Genetic stability of rMVA expressing CMV-pp65 and IEfusion under mH5 promoter control

We proceeded to construct a single rMVA simultaneously expressing both CMV-pp65 and IEfusion proteins using dual mH5 promoters by evaluating two different strategies. In the 1st strategy, an MVA expressing all three antigens was constructed by targeting a single integration site (del II) with a plasmid shuttle vector that had tandem mH5 promoters in opposing orientation (data not shown). It could not be stably prepared, possibly due to intramolecular homologous recombination, presumably initiated by the identical mH5 promoter copies (data not shown). In the second strategy, the CMV-pp65 and IEfusion genes were inserted at two separate sites in MVA (del II located at 149,261 and del III located at 20,625 of the MVA genome) to prevent deletional recombination mediated by the two identical copies of the mH5 promoter. A schematic picture of the structure of this rMVA and the insertion sites are shown in Fig. 5A. This virus was successfully constructed, and passed 10 times in a similar manner as was done for the pSyn viruses (Fig. 4A and B). The passages were conducted on both BHK-21 (Fig. 5C) and CEF (Fig. 5D). The genetic stability was evaluated by qPCR using 3 primer pairs specific for the CMV-pp65 and IEfusion genes, and the MVA viral genomic backbone, respectively. The qPCR results for both CMV antigens are computed as a ratio to the viral genomic MVA backbone (Fig. 5C). Excellent stability was found for both CMV gene inserts at del II and III integration sites with almost 100% of each gene copy number maintained after 10 passages compared to P0 (Fig. 5C). A similar result was found with virus passaged on CEF, using the CMV-pp65 and the MVA backbone sequences as targets for qPCR (Fig. 5D). These results suggest that the choice of cell line is a minor or insignificant component to the findings of enhanced stability of MVA viruses using the mH5 promoter.

Since target sequences measured by qPCR represent a small region (0.2–0.3 kbp) of CMV-pp65 (1.7 kb) and IEfusion gene (2.9 kb) insertion, the results may not represent focused regions of instability throughout the entire length of both genes. To exclude this possibility, several additional pairs of primers targeting different regions of CMV-pp65 and IEfusion gene were designed. It was found that the ratio of CMV-pp65 or IE1 or IEfusion compared to the MVA genomic DNA backbone was similar throughout the length of each insert gene (data not shown).

3.9. Minimal change in immunogenicity of mH5-pp65-IEfusion-MVA after serial passage

We evaluated if genetic stability of mH5-pp65-IEfusion-MVA after 10 multiple passages translated to equivalent immunogenicity at passage P1 and P7. We assessed the capacity of both the P1 and P7 passage viral stocks to support vigorous amplification of a memory T cell response after exposure of human PBMC to MVA vaccines [42]. Equivalent immunogenicity of both P7 and P1 passages ($p = NS$ by Student t -test) (Fig. 6A) was observed. The qualitative differences between T cell subsets in PBMC from four healthy volunteers

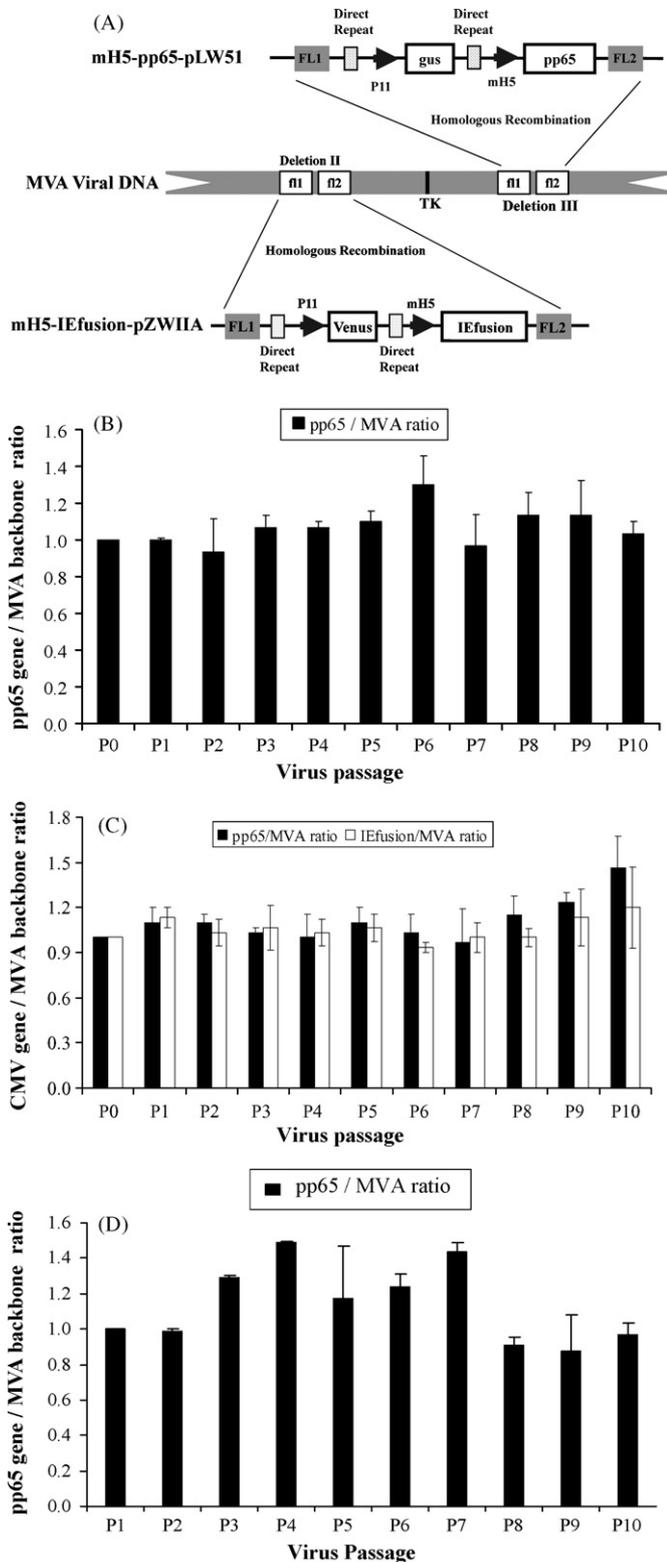


Fig. 5. Schematic representation of mH5-pp65-IEfusion-MVA and qPCR to determine genetic stability of 10 serial passages of mH5-pp65-MVA and mH5-pp65-IEfusion-MVA. (A) Schematic representation of mH5-pp65-IEfusion-MVA. (B) qPCR to determine genetic stability of 10 serial passages of mH5-pp65-MVA. mH5-pp65-MVA viral genomic DNA was extracted and qPCR was performed using pp65, and TK specific primers as described in Section 2. The copy numbers for pp65 gene and MVA backbone were analyzed using ABI software (SDS3.2) and the genetic stability of the mH5-pp65-IEfusion-MVA was determined by computing the ratio of the pp65 gene insert and the MVA backbone. The ratios at passage 1 were normalized to 1. The qPCR for each DNA sample were performed for three times independently in duplicates and average ratio and error bar shown in the figure represented three

Table 2

Early and late activities of mH5 and pSyn promoters as measured by quantitative western blot. BHK-21 cells were infected at MOI=5 with either mH5-pp65-MVA and pSyn-pp65-MVA in the presence or absence of Ara-C. The infected cells were harvested at 24 h post-infection and lysed in SDS-PAGE loading buffer. The blots were incubated first with purified mAb 28-103 against pp65 and mAb against β -tubulin, and then with HRP-labeled goat anti-mouse Ab. pp65 protein expression were measured by scanning blots using Typhoon 9410 workstation and analyzing using ImageQuant software. β -tubulin is used as internal control for each lane. Numbers shown are relative levels of signal strength using an arbitrary scale.

Promoter	Insert gene	pp65 expression		pp65 expression ratio (-Ara-C/+Ara-C)
		+Ara-C	-Ara-C	
mH5	pp65	0.90	2.25	(2.25/0.90)
pSyn	pp65	0.13	2.23	(2.23/0.13)

stimulated by individual CMV antigens are not altered after 7 passages. There was also no significant difference ($p > 0.5$, paired t test) in the response of HHDII mice immunized with the mH5-pp65-IEfusion MVA virus stocks at passages P1 and P7 similar in design to experiments shown in Fig. 3. Very high levels of CMV pp65-specific, IE1-specific and IE2-specific IFN- γ ⁺CD8⁺ T cells were found, confirming the equivalence of P1 and P7 viral passages at eliciting high-level immunogenicity in all immunized mice (Fig. 6B).

3.10. Early expression of CMV-pp65 is stronger under control of mH5 promoter than pSyn promoter while late expression levels are similar

We hypothesized that the stronger early transcriptional activity of the mH5 promoter compared to the pSyn promoter could contribute to improved genetic stability of rMVA while maintaining immunogenicity. Ara-C (cytosine β -D-arabinofuranoside) is a deoxycytidine analog which incorporates into DNA and inhibits DNA replication by forming cleavage complexes with topoisomerase I resulting in DNA fragmentation [44]. It is a selective inhibitor of DNA synthesis that does not affect RNA synthesis in mammalian cells [45] and so can be used to distinguish early and late protein expression in cells and the timing of transcriptional activation of the mH5 and pSyn promoters. Quantitative WB employing β -tubulin was used as an internal standard to compare CMV-pp65 expression levels in lysates from cells infected with either mH5-pp65-MVA or with pSyn-pp65-MVA in the absence or presence of Ara-C. In the absence of Ara-C, similar CMV-pp65 protein expression levels were observed in both mH5-pp65-MVA-infected and in pSyn-pp65-MVA-infected cells. However, in the presence of Ara-C, there was a remarkable 7-fold higher level of CMV-pp65 expression in cells infected with mH5-pp65-MVA versus cells infected with pSyn-pp65-MVA. Early CMV-pp65 expression from mH5-pp65-MVA accounted for 40% of total CMV-pp65, while early CMV-pp65 expression in pSyn-pp65-MVA constituted only 6% of total CMV-pp65 protein (Table 2). The effect of Ara-C on the pSyn and mH5 promoters is consistent with earlier studies [15,16].

independent determinants. (C) qPCR to determine genetic stability of 10 serial passages of mH5-pp65-IEfusion-MVA. mH5-pp65-IEfusion-MVA genomic DNA was extracted and qPCR was performed using pp65, IEfusion and TK specific primers as described in Section 2. The copy numbers for pp65 gene, IEfusion gene and MVA backbone were analyzed using ABI software (SDS3.2) and the genetic stability of the mH5-pp65-IEfusion-MVA was determined by computing the ratio of the pp65 gene insert and the MVA backbone or the ratio of the IEfusion gene insert and the MVA backbone. The ratios at passage 1 for pp65 and IE1/e4 gene were normalized to 1. (D) Similar to (C) except 10 serial passages were conducted on CEF and shown are results computed using pp65 and TK-specific primers. The qPCR for each DNA sample were performed for three times independently in duplicates and the ratios and error bar shown in the figure presented average of three independent determinants.

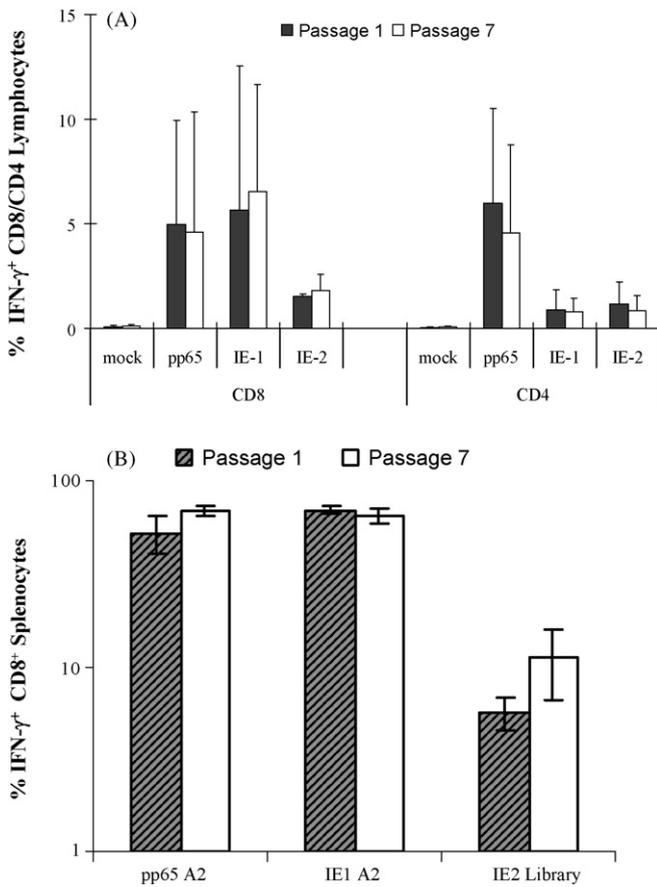


Fig. 6. Immunogenicity of mH5-pp65-IEfusion-MVA of passage 1 and 7 using human PBMC and in HHD II mice (HLA A2.1). (A) Immunogenicity of mH5-pp65-IEfusion-MVA of passages 1 and 7 using human PBMC. PBMCs from healthy donors who were *ex vivo* positive responders to CMV antigens [29] were incubated with antigen presenting cells infected with either passage 1 or passage 7 of mH5-pp65-IEfusion-MVA for 7 days followed by overnight incubation with diluent (mock), pp65, IE1 or IE2 peptide libraries in the presence of brefeldin A. Cells were then harvested and stained with anti-human CD8 or CD4 mAb, permeabilized and stained with anti-human IFN- γ mAb and evaluated by flow cytometry. Average percentages of IFN- γ producing CD8 or CD4 lymphocytes are shown ($N=4$). Error bars represent standard deviation. (B) Immunogenicity of mH5-pp65-mH5-IEfusion-MVA of passages 1 and 7 in HHD II mice (HLA A2.1). Splenocytes from HHD II mice immunized with mH5-pp65-IEfusion-MVA from passage 1 (P1) or passage 7 (P7) were subjected to IVS separately with either pp65A2, IE1A2 peptides or IE2 peptide library loaded HLA-A*0201 EBV-lymphoblastoid cells (LCL) derived from a healthy CMV positive volunteer [39] for 8 days. After IVS, the splenocytes were incubated with mock A2, pp65A2, IE1A2 peptides or IE2 peptide library overnight and harvested for ICC described in Section 2. Average levels of CD8+ T-cell IFN- γ production specific for the CMV pp65A2, IE1A2 epitopes or IE2 peptide library shown (x-axis) for all immunized mice. IFN- γ production to mock stimulated cells during the ICS procedure was subtracted. Error bars represent the SEM for all immunized mice.

3.11. Pulse-chase analysis reveals equal protein stability of CMV-pp65 antigen under the control of either pSyn or mH5 promoters

We explored alternative explanations for the profound difference in stability of MVA viruses that utilize the pSyn or mH5 promoter originally demonstrated by a reduction of specific signal from the CMV-pp65 and IE1/e4 protein bands (Figs. 1 and 2). The reduction in expression might be explained by differential protein stability when the pSyn promoter is used, rather than timing of expression we proposed based on experiments shown in Table 1. Consequently, we used an approach of metabolic radio-labeling of MVA-infected CEF (Fig. 7) and BHK-21 cells (data not shown), followed by cold chase to measure the disappearance of radio-labeled CMV-pp65 protein, which is a measure of its stability to degradation. The approach closely followed our previous work using VV expressing CMV-pp65 [42]. We utilized 3 time points of cold chase through 10 h, as this time frame is sufficient to measure differences in protein stability based on prior work with CMV-pp65. The infection conditions of CEF and BHK-21 cells closely followed our analysis of protein expression in Figs. 1 and 2. The change in labeled CMV-pp65 is limited over the 1st 4 h of chase, with only a minimal decline at the 10 h time point for both promoter constructs (Fig. 7). The pattern of CMV-pp65 expression and stability is equivalent when either the mH5 or pSyn constructs were evaluated. The specificity of the recognition of radiolabeled CMV-pp65 is shown by the absence of an equivalent CMV-pp65-specific radiolabeled band in the Gus-MVA infected control lane. Similar to the prior study using VV expressing CMV-pp65, two closely juxtaposed bands are found after IP with mAb 28–103. Based on the differences in the CMV-pp65 decay profile with the non-specific band in the prior study, the lower band reflects the bona-fide protein, while the upper band is likely a contaminant. Moreover, examining the same extracts using an isotype control mAb shows absolute specificity for the pp65 protein (data not shown). We conclude that the choice of promoter does not dramatically influence the degradation rate of the CMV-pp65 antigen. Consequently, protein stability is likely not a factor in determining the stability characteristics of both MVA expressing CMV-pp65.

4. Discussion

The purpose of this study is to determine the relationship of protein expression and stability, vector stability, and immunogenicity of rMVA viral stocks after sequential passage. rMVA expressing foreign antigen genes are being evaluated as clinical vaccine candidates for both infectious disease and cancer [8,10–13]. The genetic stability of the recombinant virus is a major concern for viral vector based vaccines intended for clinical investigation, because they

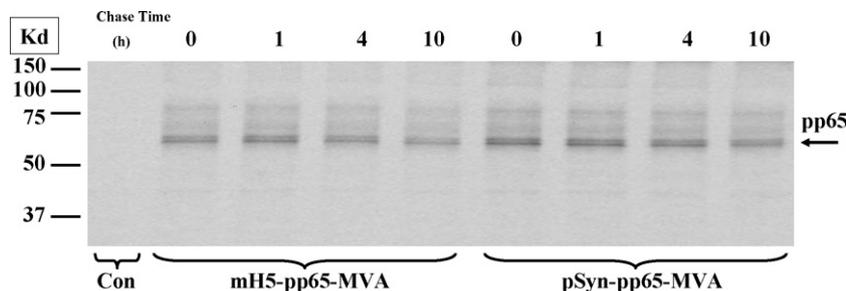


Fig. 7. Metabolic radio-labeling of CMV-pp65 detected by immunoprecipitation after viral infection of CEF. mH5-pp65-MVA (lanes 2–5) and pSyn-pp65-MVA (lanes 6–9) viruses were used to infect primary CEF plated on 60 mm TC dishes at an MOI of 10 for 1 h, followed by depletion of intracellular stores of Met + Cys for 1 h, and labeled with ^{35}S [Met + Cys] for an additional 30 min. Excess unlabeled Met + Cys was diluted into fresh medium, and further incubation times are indicated in hours (0, 1, 4 and 10) above the gel profile. At the conclusion of the “chase” period, cell lysates were made and immunoprecipitation was conducted as described in Section 2. The CMV-pp65 antigen detected by the mAb 28–103 is indicated by an arrow to the right and adjacent to the gel profile. The 1st lane at the far left (Con) represents a control CEF culture that was radio-labeled after infection with a Gus-MVA virus which expresses the α -glucuronidase bacterial marker without CMV-pp65 [27].

must be amplified multiple times to reach the scale needed for cGMP manufacturing process [22,23]. The vector must retain its potency to fulfill expectations of regulatory agencies including FDA that require the manufacturing process not irrevocably alter the virus structure or the potency of the vaccine.

In this study, the stability of pSyn-pp65-IE1/e4-MVA by serial passage was investigated. Our data showed that CMV-pp65 and IE1/e4 protein expression levels decreased significantly during the latter passages, especially after P6. The reduction of CMV-pp65 and IE1 expression was likely caused by genetic changes in rMVA that result in non-expressing variants which lost the entire gene expression cassette during sequential passage. In addition, it was found that later passages of pSyn-containing viruses had reduced immunogenicity measured using the IFN- γ assay in HHD II mice. We attribute the reduced immunogenicity to the population of viruses having fewer clones still expressing CMV-pp65 and IE1 compared to the earlier passages. This is consistent with previous reports that *in vitro* expression levels of recombinant antigens in MVA vaccines specific for HIV are correlated with immunogenicity [46]. Other examples of viral vectors in which stability through passage is influenced by the recombinant insert have been observed. These examples include coronaviruses [47], West Nile Virus replicons [48], potexvirus [49] and the Lister strain, another poxvirus member [50]. Therefore, we evaluated the genetic stability of rMVA expressing CMV viral genes using the alternative mH5 promoter. It was found that the mH5 promoter can improve genetic stability of insert genes in rMVA while maintaining potent immunogenicity during extended virus passage.

Genetic stability of a recombinant virus can be assessed by several means, including WB detection of foreign protein expression levels at each passage or by immunostaining virus plaques and calculating the percentage of foreign protein producing foci before and after serial passage [18,21,46,47]. Although these methods are technically simple, they are labor intensive. The qPCR method we developed to measure genetic stability is sensitive, high-throughput, and reproducible. A prior approach using the Taq-Man assay also can be adapted for stability testing [51]. Genetic stability as defined by computing the ratio of the foreign protein gene and MVA genomic backbone gene copies at each passage correlated with foreign insert protein expression levels at each passage (data not shown). qPCR was used to demonstrate that pSyn-pp65-IE1/e4-MVA was unstable, as only 70% of CMV-pp65 and IE1/e4 insert gene copies remained after 10 passages. pSyn-pp65-IEfusion-MVA was even more highly unstable, such that only 10% of the CMV-pp65 and IEfusion gene copies remained after five consecutive passages.

The molecular mechanism for genetic instability of rMVA using pSyn promoter and improved genetic stability using mH5 promoter has yet to be fully investigated. The cause for the instability of rMVA might be due to high protein expression levels leading to toxicity of the gene products to the cells which are infected by the rMVA, since pSyn promoter is optimized for the highest attainable levels of transcriptional activity [15,22]. However, metabolic radiolabeling, followed by cold chase established the equivalent stability of the CMV-pp65 antigen expressed from either the mH5 or pSyn promoter construct before passage. Our conclusions must be tempered by the fact we cannot completely exclude that properties of the inserts may have contributed to vector genetic instability in unpredictable ways. For instance, the combination of CMV-pp65 and IE1/e4 genes using a single integration site was only evaluated using the pSyn promoter, and not fully reconstructed using the mH5 promoter. However, we did observe even greater instability when the IEfusion gene replaced the IE1/e4 sequence in the context of the pSyn promoter. In contrast, we have not observed marked instability of any of the gene products we examined in this report in the context of the mH5 promoter, although the combi-

nation of genes and insertion sites are not identically comparable to the pSyn series. Similar problems have been noted by others in regards to HIV-Env and the measles virus F protein expressed in MVA [21,52]. In both cases, toxicity of the expressed protein contributed to genetic instability of the rMVA expressing them [22]. It has been reported that rMVA expressing hemagglutinin-neuraminidase (HN) glycoproteins under control of the vaccinia pSyn promoter replicate poorly due to toxic levels of the gene product [16]. The rMVA expressing PIV3 F and HN genes under control of pSyn replicates poorly whereas rMVA expressing both genes under control of mH5 promoter can replicate to high titer in CEF cells due to less expression of PIV3 and HN [16]. Genetic stability of rMVA was enhanced by reducing expression levels of HN glycoproteins.

Previous reports suggested that rMVA instability is related to the genomic position of the inserted foreign protein gene. As an example, rMVA in which HIV-1 tat and gag-pol genes were inserted into an intergenic region of MVA showed no loss of stability or expression after many passages [18,23]. We have recently explored the expression and genetic stability of the intergenic region 3 (IGR3) site by inserting the identical CMV-pp65 gene cassette that includes the mH5 promoter as shown in Fig. 5A into that region of the MVA genome. We obtained excellent expression levels and stability rivaling what we found as shown in Fig. 5B. Our conclusion is that promoter qualities of early timing and moderate expression levels are critical for vector stability. However, protein toxicity is probably the most important factor, while several integration sites impart similar levels of stability to the rMVA recombinant virus.

We found that rMVA expressing CMV viral proteins using pSyn promoter can grow and replicate to high titer in both BHK-21 and CEF cells (data not shown). The quantitative WB results indicated that CMV-pp65 protein expression levels are similar in promoter cassettes driven by either pSyn or mH5. This suggests that instability of rMVA using pSyn promoter is not associated with CMV-pp65 protein expression levels and that genetic stability of rMVA using mH5 promoter is not improved by reduction of CMV-pp65 protein expression levels. We cannot exclude that a component of the instability maybe imparted by the choice of cell line in selected cases, since we did not directly compare every construct in both cell lines. In those cases that we compared stability in both BHK-21 and CEF, no substantial differences were found attributable to the cell line. Results of the quantitative WB indicated that early expression of CMV-pp65 is seven-fold stronger under control of mH5 compared to the pSyn promoter. Consequently, the timing of expression, not its magnitude can be hypothesized as a mechanism to explain the increased genetic stability of rMVA using mH5 promoter. In this report a select number of gene products were investigated, therefore the conclusion drawn from our results can only be generalized to other gene products when they are evaluated in the same manner as we conducted these evaluations. However, this new finding does not exclude other mechanisms by which the mH5 promoter enhances the genetic stability of rMVA such as decreasing late expression of toxic proteins [16]. The mH5 promoter should be an important consideration in vaccine design to improve genetic stability while maintaining immunogenicity of rMVA developed for clinical application [23]. These recommendations and several others have been recently published based upon experience with HIV antigens inserted into deletion sites and intergenic regions [22,23].

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