

DOWNREGULATION OF YIELD-REDUCING TRANSGENES EXPRESSED BY POXVIRUS

Technical Field

The present invention relates to the field of viral vectors, particularly to viral vector-based vaccines. More specifically, the present invention relates to a recombinant poxvirus, most preferably a recombinant Modified Vaccinia Virus Ankara (MVA), comprising binding sequences for transcriptional repressors, particularly of the tetracycline repressor (TetR) family, and to transgenic cells expressing the corresponding repressor. The present invention further relates to virus production of said recombinant poxvirus using the transgenic cells as poxvirus producer cells.

Background

A common problem associated with recombinant viral vectors for vaccine or gene therapy purposes is the negative effects that transgene products expressed by such vectors can exert on cellular processes in the producer cells of the vector. This can ultimately lead to impaired yields of a given recombinant viral vector (1). Yield-reducing effects can be the result of expression of a single or of multiple transgenes, or even a combination of transgene products showing no yield-reducing effects when expressed separately. The problem of yield-reducing transgene products decreasing the yields of a viral vector also pertains to vectors based on replication-restricted Modified Vaccinia Virus Ankara (MVA), which was derived from the prototype species *vaccinia virus* (VACV) from the *Orthopoxvirus* genus within the family *Poxviridae*. Impaired replication has for example been observed for an MVA expressing the envelope gene of HIV (2).

MVA-BN® is a well-characterized virus vector isolated from a Modified Vaccinia Virus Ankara (MVA) virus stock. MVA originates from the dermal VACV Ankara strain (Chorioallantois vaccinia virus Ankara, CVA) that is a replicating vaccinia virus (3). By serial propagation of CVA over more than 570 passages on primary chicken embryo fibroblasts (CEFs or CEF cells), the attenuated CVA-derived virus MVA was obtained. This MVA was further passaged

by Bavarian Nordic resulting in a further attenuated MVA strain, MVA-BN[®] (4). MVA-BN[®] lacks approximately 15% of the genome compared to ancestral CVA virus (loss of 31 kb resulting in six major deletion sites). These deletions affect a number of virulence and host range genes, as well as the gene for Type A inclusion bodies. MVA-BN[®] can attach to and enter human cells and can express very efficiently virally encoded genes in the infected human cells. However, assembly and release of progeny virus does not occur in human cells. Therefore, MVA-BN[®] is an important and versatile vaccine vector able to efficiently express antigen-encoding transgenes for use in vaccination approaches that target diseases with hitherto unmet medical need (for example Ebola virus disease (5)). Preparations of MVA-BN[®] and derivatives have been administered to many types of animals and to more than 10.500 human subjects in clinical studies, including immunodeficient individuals, without any serious adverse events.

Common producer cells for the CEF-adapted MVA including MVA-BN[®] are primary CEF cells or a few avian cell lines including the continuous chicken fibroblast cell line DF-1. Mammalian BHK-21 cells (a clone derived from baby hamster kidney cells) are also permissive but produce up to 10-fold lower yields of MVA compared to CEFs (15-17).

In the avian producer cells, the decrease in viral yields of some MVA recombinants expressing yield-reducing transgenes can vary over a wide range resulting in significant reduction of viral yields up to severe replication impairment or even failure to generate particular recombinant MVAs expressing certain transgenes. Impairment of recombinant MVA replication can be triggered by just a single, strongly yield-reducing transgene, but also by the combination of multiple transgenes, which might not appear to be yield-reducing individually upon MVA-mediated expression. However, their minimal yield-reducing effects appear to add up or to even synergize resulting in significantly decreased MVA yields. The failure to generate recombinant MVAs containing certain transgenes might be attributed to the selective disadvantage conferred by a deleterious transgene to the replication process of an MVA recombinant to an extent that its replication is so inefficient that it cannot be successfully selected and isolated from the parental MVA background. In addition, the genetic stability of the transgenic insert or the genome of the viral vector expressing this transgene can be compromised by the expression of deleterious transgenes during virus vector production (1, 2).

Thus, there is a need for improved processes for reproducing recombinant MVA containing yield-reducing transgenes.

Summary of invention

It is an objective of the present invention to provide means and methods for reproducing recombinant poxvirus, most preferably recombinant Modified Vaccinia Virus Ankara (MVA), containing yield-reducing transgenes at increased virus yields.

The problem underlying the invention is solved by the provision of (1) recombinant poxvirus, most preferably recombinant MVA, having inserted a binding sequence for a transcriptional repressor protein between a yield-reducing transgene and its promoter, and (2) transgenic poxvirus producer cells, most preferably MVA producer cells, expressing the transcriptional repressor protein. When said recombinant poxvirus is propagated in said transgenic poxvirus producer cells, transgene expression is downregulated with the result of increased virus yields.

In particular, the invention is defined by the appended claims and by the following aspects and their embodiments.

In one aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, comprising a nucleotide sequence comprising a virus yield-reducing transgene operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.

In another aspect, the invention provides a transcription unit comprising a nucleotide sequence comprising a virus yield-reducing transgene operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.

In a further aspect, the invention provides a process for generating a recombinant poxvirus, most preferably a recombinant MVA, according to the invention, comprising the steps of:

- (1) providing a transcription unit comprising a nucleotide sequence comprising a virus yield-reducing transgene operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene;
- (2) inserting the transcription unit prepared in step (1) into a poxvirus genome, most preferably an MVA genome;
- (3) obtaining the recombinant poxvirus, most preferably the recombinant MVA.

In yet a further aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, produced by a process according to the invention.

In yet a further aspect, the invention provides a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, which is genetically modified to express a transcriptional repressor protein.

In yet a further aspect, the invention provides a process for generating a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, according to the invention, comprising the steps of:

- (1) providing a poxvirus permissive cell, most preferably an MVA permissive cell;
- (2) providing a plasmid which encodes a transcriptional repressor protein;

wherein step (2) may also occur prior to step (1), so long as step (3) can be accomplished;

- (3) transfecting the poxvirus permissive cell, most preferably the MVA permissive cell, of step (1) with the plasmid of step (2);
- (4) obtaining the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell.

In yet a further aspect, the invention provides a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, produced by a process according to the invention.

In yet a further aspect, the invention provides a use of a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, according to the invention for propagating a recombinant poxvirus, most preferably a recombinant MVA, according to the invention, preferably in the production of a poxvirus-based vaccine, most preferably an MVA based vaccine.

In yet another aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, according to the invention which has been propagated using a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, according to the invention.

In yet a further aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, according to the invention and a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, according to the invention, wherein the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus, most preferably the recombinant MVA, is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell, and/or *vice versa*, i.e., wherein the transcriptional repressor protein expressed

by the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell, is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus, most preferably the recombinant MVA.

In yet a further aspect, the invention provides a use of a recombinant poxvirus, most preferably a recombinant MVA, according to the invention and a transgenic poxvirus producer cell, most preferably a transgenic MVA producer cell, according to the invention, wherein the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus, most preferably the recombinant MVA, is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell, and/or *vice versa*, i.e., wherein the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell, is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus, most preferably the recombinant MVA, for propagating the recombinant poxvirus, most preferably the recombinant MVA, preferably in the production of a poxvirus-based vaccine, most preferably an MVA based vaccine.

In yet a further aspect, the invention provides a process for propagating recombinant poxvirus, most preferably recombinant MVA, comprising the steps of:

- (1) providing a recombinant poxvirus, most preferably a recombinant MVA, according to the invention;
 - (2) providing a transgenic cell according to the invention;
- wherein step (2) may also occur prior to step (1), so long as step (3) can be accomplished; wherein the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus, most preferably the recombinant MVA, provided in step (1) is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell, provided in step (2), and/or *vice versa*, i.e., wherein the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, most preferably the transgenic MVA producer cell, provided in step (2) is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus, most preferably the recombinant MVA, provided in step (1);
- (3) infecting the transgenic cell provided in step (2) with the recombinant poxvirus, most preferably the recombinant MVA, provided in step (1);
 - (4) cultivating the transfected cell of step (3) in order to propagate the recombinant poxvirus, most preferably the recombinant MVA;
 - (5) harvesting the recombinant poxvirus, most preferably the recombinant MVA, propagated in step (4).

In yet a further aspect, the invention provides a pharmaceutical composition or a vaccine comprising the recombinant poxvirus, most preferably the recombinant MVA, according to the invention, optionally further comprising a pharmaceutically acceptable carrier or excipient.

In yet a further aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, according to the invention for use as a medicament or a vaccine, preferably for use in the treatment or prevention of a disease.

In yet a further aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, according to the invention for use in the treatment or prevention of an infectious disease or cancer.

In yet a further aspect, the invention provides a recombinant poxvirus, most preferably a recombinant MVA, according to the invention for use in immunotherapy or gene therapy.

In yet a further aspect, the invention provides a use of a recombinant poxvirus, most preferably a recombinant MVA, according to the invention for the manufacture of a medicament or vaccine for use in the treatment or prevention of an infectious disease or cancer, or for the manufacture of a medicament or vaccine for use in immunotherapy or gene therapy.

In yet a further aspect, the invention provides a method of treating or preventing an infectious disease or cancer in a subject, the method comprising administering to the subject a recombinant poxvirus, most preferably a recombinant MVA, according to the invention.

In yet a further aspect, the invention provides a method of immunotherapy or gene therapy in a subject, the method comprising administering to the subject a recombinant poxvirus, most preferably a recombinant MVA, according to the invention.

In yet a further aspect, the invention provides a use of or a method using a transcriptional repressor protein and its corresponding binding sequence for downregulation of a mainly or completely late, or intermediate poxviral promoter driven transgene expression by a recombinant poxvirus, most preferably a recombinant MVA.

These aspects and their embodiments will be described in more detail in connection with the description of invention.

Brief Description of Drawings/Figures

Figure 1 illustrates the design of transgene inserts encoding EGFP or an EBV-derived LMP1/EBNA2 fusion protein and containing the Tet repressor (TetR) binding sequence 2xTetO2 between the promoter and the ORF of the transgene.

Inserts of recombinant MVAs without 2xTetO2 sequence (MVA-mBNbc440, MVA-res034, MVA-res057), with 2xTetO2 sequence linked to EGFP transgene (MVA-res005) or with 2xTetO2 sequence linked to LMP1/EBNA2 transgene either under control of the PrH5m promoter (MVA-res035) or the PrS promoter (MVA-res058). EGFP = enhanced green fluorescent protein; 2xTetO2 = two tandem binding sites of version 2 of the Tet operon (= TetR binding) sequence; LMP1/EBNA2 = fusion protein from latent membrane protein 1 from Epstein-Barr virus (EBV) and the EBV nuclear antigen 2 from EBV; npt II = neomycin phosphotransferase II; gpt = xanthine-guanine phosphoribosyltransferase; mRFP = monomeric red fluorescent protein; PrS = synthetic early/late (mainly late) promoter; PrH5m = early/late (mainly early) promoter; IRES = internal ribosomal entry site; IGR = intergenic region. MVA gene numbers according to MVA genome annotation in Antoine et al., 1998 (18).

Figure 2 shows that a 2xTetO2 sequence inserted between poxvirus promoter and transgene ORF does not affect transgene expression by MVA.

Cells as specified (CCX.2C4, CEF, DF-1, HeLa) not expressing the TetR were seeded into 6-well plates on the day before infection and were infected on day 0 with recombinant MVA-res005 or MVA-mBNbc440 at a multiplicity of infection (MOI) of 5. Cells were harvested 5 and 20 hours post infection (p.i.) by accutase treatment and analyzed by flow cytometry for EGFP fluorescence indicating EGFP expression. EGFP levels were determined as geometric mean fluorescence intensities (GMFI) of EGFP-positive cells among mRFP-positive (i.e., infected) cells.

Figure 3 shows the TetR expression in transgenic DF-1 cell clones as determined by immunoblot.

Immunoblot results are shown for TetR expression by DF-1 clones #59 to #72 (with clone #60 on a separate blot). DF-1 cells were either transfected with 0.2 µg of a plasmid expressing the TetR ("wt DF-1 TetR transf") or were not transfected ("wt DF-1"). DF-1 clones were selected for blasticidin resistance. Cell lysates of clones #59 to #72 were analyzed using an anti-TetR antibody. "tetR" = 23 kDa. The band at approximately 21 kDa is a background band ("bkg") that is also detectable in untreated DF-1 cells but is not detectable after transient plasmid transfection. M = Marker. (*) Clones with only about 50-60% confluency at harvest.

Figure 4 shows the TetR-mediated suppression of PrS-driven EGFP expression by MVA-res005.

DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline ("DOX") or not treated ("No DOX") from day (-1) throughout the experiment to allow EGFP expression (+DOX) or inhibit EGFP expression (no DOX). DF1-TR59 cells were infected with MVA-res005 on day 0 at a MOI of 5. Cells were harvested at the indicated time points after infection by accutase treatment, followed by fixation and permeabilization. MVA-res005 infected cells were analyzed for EGFP fluorescence by flow cytometry.

Figure 5 shows that LMP1/EBNA2 impairs replication of MVA-res035 encoding PrH5m-driven TetR controlled LMP1/EBNA2 in DF-1 cells not expressing the TetR.

Non-transgenic DF-1 cells were seeded into 6-well plates on the day before infection and treated with doxycycline ("DOX") or not treated ("No DOX") from day (-1) throughout the experiment to activate (no DOX) or inactivate (+DOX) the TetR. Cells were infected on day 0 at a MOI of 0.05 with BAC-derived reference MVA (MVA-mBNbc166; referred to as "MVA-WT" in the figure) and MVA-res035 in triplicates per condition. Cells were harvested on day 3 p.i. and lysates were titrated using the standard TCID₅₀ method on CEF cells. One titration per single well was conducted such that each bar represents the results of three biological replicates. Total average yields in 2 ml lysates per well are indicated.

Figure 6 shows the TetR-controlled PrH5m-driven expression of LMP1/EBNA2 by MVA-res035.

DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline ("DOX") or not treated ("No DOX") from day (-1) throughout the experiment to allow LMP1/EBNA2 expression (+DOX) or inhibit LMP1/EBNA2 expression (no DOX). DF1-TR59 cells were infected with MVA-res035 on day 0 at a MOI of 5. Cells were harvested at the indicated time points p.i. by accutase treatment, followed by fixation and permeabilization. MVA-res035 infected cells were stained with an EBNA2-specific antibody followed by incubation with an APC-coupled secondary antibody. Cells were analyzed for allophycocyanin (APC) fluorescence (**A**) and for EGFP fluorescence (**B**) by flow cytometry. The APC GMFI are depicted as GMFI LMP1/EBNA2.

Figure 7 shows the effect of TetR-controlled PrH5m-driven expression of LMP1/EBNA2 on MVA-res035 replication.

DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline ("DOX") or not treated ("no DOX") from day (-1) throughout the experiment to allow

LMP1/EBNA2 expression (+DOX) or to inhibit LMP1/EBNA2 expression (no DOX). DF1-TR59 cells were infected on day 0 at a MOI of 0.05 with BAC-derived reference MVA (MVA-mBNbc166; referred to as “MVA-WT” in the figure) or MVA-res035 in triplicates per condition. Cells were harvested at the indicated time points p.i. and lysates were titrated using the standard TCID₅₀ method on CEF cells. One titration per single well was conducted such that each data point represents the results of three replicates. Total average yields in 2 ml lysates per well are indicated, with the inoculum titer for day 0.

Figure 8 shows the TetR-mediated regulation of early and late expression of EGFP from PrS early/late promoter.

DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline (“DOX”) or not treated (“No DOX”) from day (-1) throughout the experiment to allow EGFP expression (+DOX) or inhibit EGFP expression (no DOX) by MVA-res005. DF1-TR59 cells were infected with MVA-res005 on day 0 at a MOI of 10. A subset of infected cell cultures was treated with 40 µg/ml cytosine arabinoside (“Plus AraC”) from 1 hour before start of infection until the end of the infection period as indicated (**B, D**), while parallelly infected cell cultures remained untreated (“NO AraC”) (**A, C**). Cells were harvested at the indicated time points p.i. by accutase treatment, followed by fixation and permeabilization. MVA-res005 infected cells were analyzed for EGFP (**A, B**) and mRFP (**C, D**) fluorescence by flow cytometry. The mRFP signal was derived from the MVA-encoded mRFP serving as reference transgene. EGFP and mRFP expression levels are shown as GMFI of all cells in the cell gate excluding debris (“live cell gate”).

Figure 9 shows the TetR-controlled PrS-driven expression of LMP1/EBNA2 by MVA-res058.

DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline (“DOX”) or not treated (“No DOX”) from day (-1) throughout the experiment to allow LMP1/EBNA2 expression (+DOX) or inhibit LMP1/EBNA2 expression (no DOX). DF1-TR59 cells were infected with MVA-res058 on day 0 at a MOI of 5. Cells were harvested at the indicated time points p.i. by accutase treatment, followed by fixation and permeabilization. MVA-res058 infected cells were stained with an EBNA2-specific antibody followed by incubation with an APC-coupled secondary antibody. Cells were analyzed for APC (**A**) and EGFP (**B**) fluorescence by flow cytometry. EGFP and EBNA2/LMP1 expression levels are shown as GMFI of all cells in the live cell gate. The APC GMFI are depicted as GMFI LMP1/EBNA2.

Figure 10 shows the effect of TetR-controlled PrS-driven expression of LMP1/EBNA2 on MVA-res058 replication.

DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline ("plus DOX") or not treated ("no DOX") from day (-1) throughout the experiment to allow LMP1/EBNA2 expression (+DOX) or inhibit LMP1/EBNA2 expression (no DOX). DF1-TR59 cells were infected on day 0 at a MOI of 0.05 with BAC-derived reference MVA (MVA-mBNbc166; referred to as "MVA-WT" in the figure) or MVA-res058 in triplicates per condition. Cells were harvested at the indicated time points p.i. and lysates were titrated using the standard TCID₅₀ method with DF1-TR59 cells as substrate. One titration per single well was conducted such that each data point represents the results of three replicates. Average total yields in 2 ml lysates per well are indicated, with the inoculum titer for day 0. Background levels of staining with an anti-EBNA2 antibody was determined using cells infected with the BAC-derived reference MVA and the background LMP1/EBNA2 signal from the live cell gate was subtracted from all GMFI values for LMP1/EBNA2 shown.

Figure 11 shows TetR expression by the quail CCX.2C4-TR16 cell clone in comparison to sister clones.

(A) CCX.2C4 clone TR16 cells (CCX.2C4-TR16) stably transfected with a TetR expressing plasmid were kept under 2 µg/ml blasticidin selection. Cells from the parental CCX.2C4 wildtype ("2C4 wt") cell line and the various clones were collected on the same day and lysed in Laemmli buffer, and aliquots of cell lysates were subjected to immunoblotting using a TetR-specific antibody (MW of TetR = 23 kDa). A lysate of TetR expressing DF1-TR59 cells served as reference. The upper part of the same blot (mol masses of proteins above ca 45 kDa) was developed with an anti-β-Tubulin antibody (MW of β-Tubulin = 55 kDa). The β-tubulin specific signal ("β-tubulin") used for normalization of sample loading, the TetR-specific signal ("tetR") and a background signal ("bkg") that became apparent in cell lysates with the anti-TetR antibody are shown. (B) Stably transfected CCX.2C4-TR16 quail cells expressing the TetR were infected with MVA-BN ("MVA") at a MOI of 5 or mock infected for the indicated times. Cell lysates were analyzed for TetR and β-tubulin expression levels by semi-quantitative immunoblot as described above in (A). The TetR and β-tubulin specific signals were quantified using the ChemiDoc Touch system and ImageQuant software and TetR expression is shown as arbitrary units. The β-tubulin specific signals were used to normalize the TetR signal values shown in (B).

Figure 12 shows the TetR-controlled PrS-driven expression of LMP1/EBNA2 by MVA-res058 in CCX.2C4-TR16 cells.

CCX.2C4-TR16 cells expressing the TetR were seeded into 12-well plates on the day before infection and treated with doxycycline ("DOX") or not treated ("No DOX") from day (-1) throughout the experiment to allow LMP1/EBNA2 expression (+DOX) or inhibit LMP1/EBNA2 expression (no DOX). CCX.2C4-TR16 cells were infected with MVA-res005 (**A**), MVA-res035 (**B**), or MVA-res058 (**C**) in duplicate and with reference MVA (MVA-mBNbc166) in a single well on day 0 at a MOI of 10. Cells were harvested at the indicated time points p.i. by accutase treatment, followed by fixation and permeabilization. Cells infected with MVA-res035 or MVA-res058 were stained with an EBNA2-specific antibody followed by incubation with an APC-coupled secondary antibody. Cells were analyzed for APC and EGFP fluorescence by flow cytometry. EGFP, mRFP and LMP1/EBNA2 expression levels are shown as GMFI of all cells in the live cell gate. For APC levels, the background staining observed with anti-LMP1/EBNA2 antibody in cells infected with the reference MVA was subtracted from all APC GMFI of MVA-res035 or MVA-res058 infected cells. The APC GMFI are depicted as GMFI LMP1/EBNA2.

Figure 13 shows the effect of TetR-controlled PrS-driven expression of LMP1/EBNA2 on MVA-res058 replication in CCX.2C4-TR16 cells.

CCX.2C4-TR16 cells expressing the TetR were seeded into 6-well plates on the day before infection and treated with doxycycline ("plus DOX") or not treated ("no DOX") from day (-1) throughout the experiment to allow LMP1/EBNA2 expression (+DOX) or inhibit LMP1/EBNA2 expression (no DOX). CCX.2C4-TR16 cells were infected on day 0 at a MOI of 0.05 with BAC-derived reference MVA (MVA-mBNbc166; referred to as "MVA-WT" in the figure) or MVA-res058 in triplicates per condition. Cells were harvested at the indicated time points and lysates were titrated using the standard TCID₅₀ method with DF1-TR59 cells (also expressing the TetR) as substrate. One titration per single well was conducted such that each data point represents the results of three replicates. Total yields in 2 ml lysates per well are indicated, with the inoculum titer for day 0.

Brief Description of Sequences

SEQ ID NO: 1	Nucleic acid sequence of TetO2
SEQ ID NO: 2	Nucleic acid sequence of 2xTetO2
SEQ ID NO: 3	Nucleic acid sequence of TetR gene
SEQ ID NO: 4	Amino acid sequence encoded by TetR gene
SEQ ID NO: 5	Nucleic acid sequence encoding LMP1/EBNA2 fusion protein

SEQ ID NO: 6	Amino acid sequence of LMP1/EBNA2 fusion protein
SEQ ID NO: 7	Nucleic acid sequence of EGFP gene
SEQ ID NO: 8	Amino acid sequence encoded by EGFP gene
SEQ ID NO: 9	Nucleic acid sequence encoding gpt-mRFP fusion protein
SEQ ID NO: 10	Nucleic acid encoding npt II
SEQ ID NO: 11	PrS promoter
SEQ ID NO: 12	PrH5m promoter

Detailed Description of Invention

The objective underlying the invention was to improve the productivity of recombinant MVA containing yield-reducing transgenes.

To produce high-titer virus stocks of MVA constructs containing yield-reducing transgenes, downregulating of the expression of such proteins during virus production would be advantageous. We aimed to evaluate whether a member of the tetracycline (Tet) repressor family of proteins (6), the prototypic tetracycline repressor (TetR), when expressed in MVA producer cells might be utilized to downregulate transgene expression during recombinant MVA production. The TetR system is often used for repression of nuclear transcription, but the TetR has no eukaryotic nuclear localization signal (NLS), has an extremely high affinity to its DNA recognition sequence (tetracycline operon or operator, TetO), and should therefore be available in the cytoplasm of eukaryotic cells in sufficient amounts to also regulate genes of MVA or other vaccinia viruses that exclusively replicate in the cytoplasm.

The TetR functions as a homodimer that binds to short palindromic sequence stretches of DNA also called Tet operon sequence or TetR binding sequence (6-8). When these stretches are placed between the promoter and the open reading frame (ORF) of a gene, transcription of this gene will be blocked upon binding of the TetR to this sequence. The addition of an "inducer", in this case the antibiotic tetracycline or its more active derivative doxycycline (DOX), will lead to binding of the inducer to the repressor in turn causing an allosteric response of the repressor. The three-dimensional structure of the repressor will change causing a dramatic reduction in affinity to its target sequence in the DNA. Thus, the TetR will be released from the DNA and transcription of the regulated gene can occur.

The original TetR technology and in particular a fusion protein of modified TetR-derivatives with a herpesviral transactivator protein ("tet-on/tet-off" system) have been widely used to regulate gene expression in experimental systems and in biotechnology applications. The TetR as well as the functionally similar lac repressor have also been used to modulate

expression of native VACV genes to create conditional knockout mutants of replicating vaccinia virus strains for the functional characterization of essential poxviral genes (9, 10). In most cases, the recombinant or mutant vaccinia virus itself was used for expression of the TetR, which is not desirable for vaccine vectors intended to be used in humans. A system based on TetR expression by the commercially available human cell lines T-RExTM-293 and T-RExTM-HeLa with the Tet operon sequence inserted in an essential vaccinia virus gene was described to be able to regulate this essential vaccinia virus genes (11). TetR has also been employed to reduce transgene expression by adenovirus and vaccinia virus-based vectors. In the case of the adenovirus vectors, the goal was to improve the genetic stability of adenovirus vectors expressing certain transgenes, and the study found that TetR-mediated repression of the transgene increased adenovirus vector yields and facilitated the production of recombinant adenoviruses with transgenes that rapidly mutated without TetR regulation (1).

For regulating the expression of transgenes from recombinant replicating vaccinia viruses, an indirect approach of using a lac repressor system for regulating VACV-driven transgene expression has been described, in which bacteriophage T7 RNA polymerase encoded by the recombinant VACV drives the transcription of T7 promoter controlled transgenes from the same recombinant VACV (12, 13). However, this system requires expression of the T7 polymerase as well as the lac repressor from the recombinant VACV or MVA in addition to the transgene to be expressed as vaccine antigen or gene therapy protein. Furthermore, only early promoter driven expression is supported by the system described (12, 13), which therefore forgoes the strong late component of many poxviral promoters used for transgene expression to generate the large quantities of transgene product that are advantageous as vaccine antigen or gene therapy protein. An effect of cell expressed TetR on the late expression was observed for the expression of a neutral transgene (luciferase) by a recombinant replicating vaccinia virus in TetR expressing human HeLa cells (14).

Early poxviral gene expression is detectable within 15-30 min after entry of VACV or MVA into a producer cell and precedes replication of the viral genomic DNA. Early expression can persist into the intermediate and late phases of gene expression that start with the replication of viral DNA at around 2 hours post infection (p.i.) in the case of VACV and MVA. Intermediate and late genes are therefore also termed post-replicative genes. Intermediate expression starts concomitant with DNA replication at 2 hours p.i., and late expression follows as soon as the first late transcription factors encoded by intermediate genes have been synthesized, while intermediate transcription factors are encoded by early genes. Thus, expression of each temporal class of genes is dependent on the synthesis of the preceding temporal gene class, and early transcription factors are encoded by late genes and packaged into virions to start a new replication cycle upon viral entry into the cell. Late gene expression can last over the

complete late phase of the MVA infection cycle from about 2 hours p.i. up to about 24 hours p.i. when the infected cells start to die, and late genes are often expressed at high abundance. Most of the structural proteins of poxviruses that are required in abundant amounts for viral morphogenesis are encoded by late genes. Early, intermediate, or late expression of a poxviral gene is determined by the poxviral promoters, which exist in three corresponding types distinguished by the presence of specific sequence motifs each driving one of the three distinct temporal classes of poxviral genes. Expression of a fourth class of genes termed immediate-early starts very early after infection, but their expression is not a prerequisite for the expression of any other early poxviral gene and these genes are thus defined as immediate-early solely based on their temporal kinetics of expression.

Expression of transgenes from poxviral vectors is depending on poxviral promoters, that belong to either the immediate early, early, intermediate, or late class. Since poxviruses encode their own transcription machinery in the cytoplasm of infected cells, cellular promoters driving RNA polymerase II-mediated gene expression cannot be used. Poxviral promoters used to drive transgene expression have frequently been chosen from a class of combined promoters that initiate expression in the early as well as late phase of viral infection. The synthetic PrS promoter, designed to induce strong transgene expression (24), is a classic example thereof and is widely used in poxviral vectors. Despite the presence of an optimized early promoter motif in the PrS promoter, transgene expression under the control of this promoter occurs predominantly late. However, for best possible induction of T cell responses against a transgene product, early, more preferably even immediate-early expression of the transgene is favorable (28-30) and an early element should therefore also be contained. Hence the PrS promoter is a preferable promoter if both high overall expression and early start of expression for good T cell induction is required. The PrH5m promoter is generally considered to be an early/late promoter, but when the replication of recombinant MVAs expressing a transgene under the PrH5m promoter is arrested in the early phase by araC treatment, only a minor proportion of the transgene expression is blocked, indicating that PrH5m is a mainly early promoter with a minor late component.

On this basis, the approach to solving the problem underlying the invention was to repress expression of yield-reducing transgenes during propagation of the recombinant MVA, while preserving the recombinant MVA's potential to induce transgene-specific immune responses in a vaccine recipient.

To this end, we made use of a bacterial gene regulation system consisting of a transcriptional repressor protein, here the prototypic tetracycline repressor (TetR), and a TetR binding

sequence (also termed tetracycline operon) that is placed between the promoter and the open reading frame (ORF) of the transgene to be downregulated.

Briefly, the invention provides (1) recombinant MVA containing a TetR binding sequence inserted between a yield-reducing transgene and its promoter, and (2) transgenic MVA producer cells expressing the corresponding TetR. When said recombinant MVA is grown on said MVA producer cells, the TetR binds to its binding sequence, thereby repressing expression of the yield-reducing transgene. In contrast, when the recombinant MVA is administered to a vaccine recipient, transgene expression remains unaffected due to the absence of TetR protein in the recipient's body cells.

Here it was demonstrated that both a transgenic chicken DF-1 and a transgenic quail CCX.2C4 cell line expressed sufficient amounts of TetR to efficiently downregulate an MVA-expressed transgene coupled to a TetR binding sequence.

Particularly, the TetR was efficient in downregulating EGFP transgene expression driven by the late part of the strong early/late PrS promoter, while expression from the early part of the PrS promoter was completely unaffected.

Consistent with the finding just described, expression of a strongly yield-reducing transgene encoding LMP1/EBNA2 fusion protein under the mainly early PrH5m promoter was only transiently and moderately downregulated in TetR expressing DF-1 cells, and only a transient increase in viral titers over the course of a growth curve experiment could be achieved.

Importantly, expression of the strongly yield-reducing LMP1/EBNA2 transgene from the mainly late expressing PrS promoter was significantly downregulated in TetR expressing DF-1 and CCX.2C4 cells. As a consequence, the profoundly impaired replication of recombinant MVA expressing LMP1/EBNA2 fusion protein was massively increased by insertion of a TetR binding sequence such that the efficiency of replication of the recombinant MVA containing the LMP1/EBNA2 transgene linked to the TetR binding sequence equaled that of non-recombinant wildtype MVA in TetR expressing DF-1 and CCX.2C4 cells.

It was also demonstrated that downregulation of the LMP1/EBNA2 transgene in TetR expressing cells to 50% or more of the doxycycline induced amounts of this transgene was unexpectedly efficient in compensating the negative effects of the late expressed yield-reducing transgene on viral replication and increased viral yields by at least 10-fold reaching yields similar to those of wildtype MVA without any transgene.

In conclusion, it was demonstrated that linking a binding sequence for the TetR to a transgene expressed by recombinant MVA efficiently downregulates the transgene's expression in MVA

producer cells modified to express the corresponding TetR. For the reason alone that downregulation of expression of the strongly yield-reducing transgene LMP1/EBNA2 was significant but far from complete, and in view of the massive activity of the poxviral promoter used to achieve high amounts of the transgene's product in a vaccine recipient, the largely unimpaired replication and close to wildtype MVA like yields of recombinant MVA containing a TetR-controlled LMP1/EBNA2 transgene was remarkable.

Definitions

It must be noted that, as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a nucleic acid sequence" includes one or more nucleic acid sequences.

As used herein, the conjunctive term "and/or" between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by "and/or", a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or" as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or."

Throughout this specification and the appended claims, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated feature but not the exclusion of any other feature. When used in the context of an aspect or embodiment in the description of the present invention the term "comprising" can be amended and thus replaced with the term "containing" or "including" or when used herein with the term "having." Similarly, any of the aforementioned terms (comprising, containing, including, having), whenever used in the context of an aspect or embodiment in the description of the present invention include, by virtue, the terms "consisting of" or "consisting essentially of," which each denotes specific legal meaning depending on jurisdiction.

When used herein "consisting of" excludes any feature, element, step, or ingredient not specified in the claim. When used herein, "consisting essentially of" does not exclude features, materials or steps that do not materially affect the basic and novel characteristics of the claim.

The term “poxvirus” refers to the family of Poxviridae including the two subfamilies Chordopoxvirinae and Entomopoxvirinae. Members of the Chordopoxvirinae subfamily infect vertebrates. Members of the Entomopoxvirinae subfamily infect insects (i.e., invertebrates). The term “poxvirus” also refers to members of any of the genera of Chordopoxvirinae (e.g., avipox viruses, capripox viruses, leporipox viruses, molluscipox viruses, orthopox viruses, parapox viruses, suipox viruses, and yatapox viruses), including those four that may infect humans (orthopox viruses, parapox viruses, yatapox viruses, and molluscipox viruses). The term “poxvirus” also refers to members of any of the genera of Entomopoxvirinae (e.g., alpha-entomopox viruses, beta-entomopox viruses, and gamma-entomopox viruses). Avipox viruses include canarypox virus, fowlpox virus, mynahpox virus, pigeonpox virus, and quailpox virus. Capripox viruses include sheeppox viruses, goatpox viruses, and lumpy skin disease virus. Leporipox viruses include myxoma virus, Shope fibroma virus (also known as rabbit fibroma virus), hare fibroma virus, and squirrel fibroma virus. Molluscipox viruses include Molluscum contagiosum virus. Orthopox viruses include buffalopox virus, camelpox virus, cowpox virus, ectromelia virus, monkeypox virus, raccoonpox virus, smallpox virus (also known as variola virus), and vaccinia virus. Parapox viruses include bovine papular stomatitis virus, ORF virus, parapoxvirus of New Zealand red deer, and pseudocowpox virus. Suipox viruses include swinepox virus. Yatapox viruses include tanapox virus and yaba monkey tumor virus.

The term “vaccinia virus” refers to both the wild-type vaccinia virus and any of the various attenuated strains or isolates subsequently isolated including, for example, vaccinia virus-Western Reserve, vaccinia virus-Copenhagen, Dryvax (also known as vaccinia virus-Wyeth), ACAM2000, chorioallantois vaccinia virus Ankara (CVA), Modified Vaccinia Virus Ankara (MVA), and MVA of Bavarian Nordic (MVA-BN®).

The term “recombinant poxvirus” as described herein refers to a poxvirus comprising a nucleic acid sequence inserted in its genome, which is not naturally present in the wildtype virus (i.e., is foreign or heterologous to poxvirus). A recombinant poxvirus thus refers to poxvirus made by an artificial combination of two or more segments of nucleic acid sequence of synthetic or semisynthetic origin which does not occur in nature or is linked to another nucleic acid in an arrangement not found in nature. A “recombinant poxvirus” is a genetically engineered or a genetically modified poxvirus.

The term “recombinant MVA” as described herein includes recombinant MVA (e.g., derived from MVA-BN®) which has integrated at least one recombinant nucleic acid, preferably in the form of a transcription unit, in its genome. Recombinant MVA may express heterologous polypeptides or proteins (antigens) upon induction of the regulatory elements e.g., the promoter.

A “transcription unit” as the term is used herein basically includes at least a coding sequence of a gene of interest, e.g., a transgene, and furthermore a promoter operably linked thereto and a terminator.

The term “operably linked” as used herein in respect of a promoter means that the promoter is placed in a position where it can direct transcription of the coding sequence of a gene of interest, e.g., a transgene.

The term “open-reading frame” or “ORF” as used herein, e.g., an ORF of a transgene, means a nucleotide sequence located between a start codon and a stop codon.

The term “transgene” as used herein refers to a foreign or heterologous gene that has been inserted into a poxvirus, e.g., an MVA genome, by genetical engineering.

A “neutral” transgene, in contrast to a “virus yield-reducing” transgene, does not affect or barely affects replication of the recombinant poxvirus, e.g., the recombinant MVA, harboring the transgene and thus does not decrease or barely affects virus yield when the recombinant poxvirus, e.g., the MVA is propagated. Herein, EGFP is described as a prototypic “neutral” transgene.

A “virus yield-reducing” or “yield-reducing” transgene is a transgene the expression of which, or rather an expression product of which, e.g., a transgene protein, negatively affects virus replication of a recombinant poxvirus, e.g., a recombinant MVA, and thereby decreases the virus yield. Herein, LMP1/EBNA2 fusion protein is described as a strongly yield-reducing transgene.

The term “virus yield” or simply “yield” as used herein refers to the production yield of recombinant poxvirus, e.g., recombinant MVA, i.e., the difference in virus quantity before and after a time during which the recombinant poxvirus, e.g., the recombinant MVA, is allowed to propagate. Herein, the virus yield may be determined in terms of 50% of tissue culture infection dose (TCID₅₀) per well.

The term “downregulation” in the context of transgene expression relates to a reduction of or decrease in the amount of a transgene product. This reduction or decrease results from a reduction in the amount of transgene mRNA or from a reduction in the translation of transgene mRNA. Alternatively, “suppression” or “inhibition” of transgene expression may be used.

The terms “early” and “late” as used herein to describe promoter activities and kinetics of transgene expression during the poxvirus, e.g. MVA infectious cycle pertain to the temporal cascade of gene expression observed for all poxviruses. Early, intermediate, and late gene

expression is overlapping but in general, the temporal sequence of the replication phases is as follows: 0-2 hours p.i., early phase; onset of intermediate and late gene expression around 2 hours p.i., with very short interval between intermediate and late phase. The time between 2-4 hours p.i. is the onset of the late phase, while 20 hours represents a late timepoint during the late phase.

The PrS promoter is described as an “early/late” promoter. Since the bulk of the gene expression driven by PrS is occurring in the late phase, we also refer to this promoter as “mainly late”.

The PrH5m promoter is described as an “early/late” promoter, but since the major gene expression driven by this promoter occurs early in the infection cycle, it is herein referred to as a “mainly early” promoter.

The term “Tet operon” or “Tet operator” means a nucleotide binding sequence for a tetracycline repressor (TetR), i.e., a TetR binding sequence.

The Tet operator or TetR binding sequence referred to as “2xTetO2” consists of two copies of the 19-nucleotide Tet operator 2 (TetO2) sequence, 5'-TCCCTATCAGTGATAGAGA-3', separated by a 2-base pair spacer (7, 8). The “2xTetO2” sequence thus contains two copies of TetO2 arranged in tandem. Each 19-nucleotide TetO2 sequence serves as the binding site for two molecules of TetR.

The term “corresponding” when used in the context of a transcriptional repressor protein or a binding sequence for a transcriptional repressor protein means that the repressor protein is capable of binding to its corresponding binding sequence, or that the binding sequence is capable of binding its corresponding repressor protein. For example, if the binding sequence comprises a TetO2 sequence or consists of 2xTetO2, a prototypic TetR may represent the corresponding repressor protein.

Abbreviations

araC	cytosine arabinoside
CCX.2C4	an adherent quail CCX cell line, clone 2C4
CCX.E10	a suspension quail CCX cell line, clone E10
CEF	chicken embryo fibroblast
CMV	cytomegalovirus
DF-1	a continuous cell line derived from chicken embryo fibroblasts
DOX	doxycycline, a more stable tetracycline analogue

EBNA2	Epstein-Barr virus nuclear antigen 2
EBV	Epstein-Barr virus
EGFP	enhanced green fluorescent protein
GMFI	geometric mean fluorescence intensity
gpt	xanthine-guanine phosphoribosyltransferase
IGR	intergenic region
IRES	internal ribosomal entry site
LMP1	latent membrane protein 1 from EBV
min	minutes
MOI	multiplicity of infection
mRFP	monomeric red fluorescent protein
MVA	Modified Vaccinia Virus Ankara
MVA-BN	MVA-BN [®] (Bavarian Nordic)
MVA-wt	MVA wildtype
npt II	neomycin phosphotransferase II
p.i.	post infection
ORF	open reading frame
PrH5m	modified early/late promoter, herein referred to as mainly early promoter
PrS	synthetic vaccinia virus early/late promoter
SFV	Shope fibroma virus
TCID ₅₀	50% of tissue culture infection dose
Tet	tetracycline
TetO2	Tet operator 2
2xTetO2	Tet repressor binding sequence comprising two TetO2 sequences (separated by a 2-nucleotide spacer)
TetR	tetracycline repressor
VACV	vaccinia virus

Embodiments

Aspects and embodiments relating to recombinant poxvirus

In one aspect, provided is a recombinant poxvirus comprising a nucleotide sequence comprising a transgene, preferably a virus yield-reducing transgene, operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.

In one aspect, provided is a recombinant poxvirus comprising a transcription unit comprising a nucleotide sequence comprising a transgene, preferably a virus yield-reducing transgene, operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.

In one embodiment of the recombinant poxvirus, the virus yield-reducing transgene is a transgene reducing the yield of the recombinant poxvirus, preferably the production yield during propagation of the recombinant poxvirus.

In one embodiment of the recombinant poxvirus, the virus yield-reducing transgene is a transgene reducing the yield of the recombinant poxvirus as compared to a poxvirus or a recombinant poxvirus not comprising the yield-reducing transgene.

In one embodiment of the recombinant poxvirus, the virus-yield reducing transgene is a transgene reducing the replication rate of the recombinant poxvirus, preferably as compared to a poxvirus or a recombinant poxvirus not comprising the yield-reducing transgene.

In one embodiment of the recombinant poxvirus, the virus yield-reducing transgene is a transgene the expression of which reduces the yield or the replication rate of the recombinant poxvirus, preferably as compared to a poxvirus or a recombinant poxvirus not comprising the yield-reducing transgene.

In one embodiment of the recombinant poxvirus, the virus yield-reducing transgene is a transgene the expression product (e.g., mRNA, protein) of which reduces the yield or the replication rate of the recombinant poxvirus, preferably as compared to a poxvirus or a recombinant poxvirus not comprising the yield-reducing transgene.

In one embodiment of the recombinant poxvirus, the virus yield-reducing transgene reduces the yield or the replication rate of the recombinant poxvirus by up to about 10%, 30%, 50%, 70%, 80%, 90% or more than 90%.

In one embodiment, the recombinant poxvirus is derived from a member of the Avipoxvirus, Orthopoxvirus or Parapoxvirus genus.

In one embodiment, the member of the Avipoxvirus genus is selected from the group consisting of canarypox virus, fowlpox virus, mynahpox virus, pigeonpox virus, and quailpox virus.

In one embodiment, the member of the Parapoxvirus genus is selected from the group consisting of bovine papular stomatitis virus, ORF virus, parapoxvirus of New Zealand red deer, and pseudocowpox virus.

In one embodiment, the member of the Orthopoxvirus genus is selected from the group consisting of buffalopox virus, camelpox virus, cowpox virus, ectromelia virus, monkeypox virus, raccoonpox virus, smallpox virus (variola virus), or vaccinia virus (VACV).

In a preferred embodiment, the recombinant poxvirus is a recombinant vaccinia virus.

In one embodiment, the recombinant vaccinia virus is derived from a wild-type vaccinia virus or an attenuated vaccinia virus strain, preferably from vaccinia virus-Western Reserve, vaccinia virus-Copenhagen, Dryvax (vaccinia virus-Wyeth), ACAM2000, chorioallantois vaccinia virus Ankara (CVA), and Modified Vaccinia Virus Ankara (MVA).

In one embodiment, the recombinant poxvirus is derived from an attenuated poxvirus vector selected from the group consisting of ALVAC (a canarypox virus-based vector), NYVAC (a vaccinia virus vector) and MVA.

In a particularly preferred embodiment, the recombinant poxvirus is recombinant MVA.

In one embodiment, the recombinant poxvirus is recombinant MVA and the nucleotide sequence (i.e., the nucleotide sequence comprising a transgene, preferably a virus yield-reducing transgene, operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene) is inserted into an MVA genome at an intergenic region (IGR) selected from the group consisting of IGR 44/45, 51/52, 64/65, 88/89, and 148/149.

In a preferred embodiment, the recombinant poxvirus is recombinant MVA, and the IGR is IGR 44/45 or IGR 51/52, more preferably is IGR 51/52.

In one embodiment, the recombinant poxvirus is propagated using a poxvirus producer cell expressing a transcriptional repressor protein capable of binding to the binding sequence (i.e., the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus).

In one embodiment, the recombinant poxvirus is propagated using a poxvirus producer cell expressing the transcriptional repressor protein (i.e., the transcriptional repressor protein which the recombinant poxvirus comprises a binding sequence for).

In one embodiment, the recombinant poxvirus is propagated using a transgenic poxvirus producer cell being genetically modified to express the transcriptional repressor protein (i.e., the transcriptional repressor protein which the recombinant poxvirus comprises a binding sequence for).

In one embodiment, the recombinant poxvirus is recombinant MVA derived from an MVA or an MVA derivative having the capability of reproductive replication *in vitro* in chicken embryo fibroblast (CEF) cells, but no capability of reproductive replication in the human keratinocyte cell line HaCaT, the human bone osteosarcoma cell line 143B, the human embryo kidney cell line 293, and the human cervix adenocarcinoma cell line HeLa.

In one embodiment, the recombinant poxvirus is recombinant MVA derived from MVA-BN® as deposited at the European Collection of Animal Cell cultures (ECACC) under accession number V00083008 on 30 August 2000.

Aspects and embodiments relating to a transcription unit

In one aspect, provided is a transcription unit comprising a nucleotide sequence comprising a transgene, preferably a virus yield-reducing transgene, operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.

In one embodiment of the transcription unit, the virus yield-reducing transgene is a transgene reducing the yield of a recombinant poxvirus comprising the transcription unit, preferably the production yield during propagation of the recombinant poxvirus.

In one embodiment of the transcription unit, the virus yield-reducing transgene is a transgene reducing the yield of a recombinant poxvirus comprising the transcription unit, as compared to a poxvirus or a recombinant poxvirus not comprising the transcription unit.

In one embodiment of the transcription unit, the virus-yield reducing transgene is a transgene reducing the replication rate of a recombinant poxvirus comprising the transcription unit, preferably as compared to a poxvirus or recombinant poxvirus not comprising the transcription unit.

In one embodiment of the transcription unit, the virus yield-reducing transgene is a transgene the expression of which reduces the yield or the replication rate of a recombinant poxvirus comprising the transcription unit, preferably as compared to a poxvirus or recombinant poxvirus not comprising the transcription unit.

In one embodiment of the transcription unit, the virus yield-reducing transgene is a transgene the expression product (e.g., mRNA, protein) of which reduces the yield or the replication rate of a recombinant poxvirus comprising the transcription unit, preferably as compared to a poxvirus or recombinant poxvirus not comprising the transcription unit.

In one embodiment of the transcription unit, the virus yield-reducing transgene reduces the yield or the replication rate of the recombinant poxvirus comprising the transcription unit by up to about 10%, 30%, 50%, 70%, 80%, 90% or more than 90%.

In one embodiment, the transcription unit is functionally insertable or inserted into a poxviral genome.

In one embodiment of the transcription unit, the recombinant poxvirus preferably is a recombinant vaccinia virus, most preferably is a recombinant Modified Vaccinia Virus Ankara (MVA).

In one embodiment, the poxvirus is MVA and the transcription unit is functionally insertable or inserted into an MVA genome at an intergenic region (IGR) selected from the group consisting of IGR 44/45, 51/52, 64/65, 88/89, and 148/149.

In a preferred embodiment, the poxvirus is MVA and the IGR is IGR 44/45 or IGR 51/52, more preferably is IGR 51/52.

In one embodiment, the poxvirus is MVA and the transcription unit is functionally insertable or inserted into an MVA or an MVA derivative having the capability of reproductive replication *in vitro* in chicken embryo fibroblasts (CEF) cells, but no capability of reproductive replication in the human keratinocyte cell line HaCaT, the human bone osteosarcoma cell line 143B, the human embryo kidney cell line 293, and the human cervix adenocarcinoma cell line HeLa.

In one embodiment, the poxvirus is MVA and the transcription unit is functionally insertable or inserted into MVA-BN® as deposited at the European Collection of Animal Cell cultures (ECACC) under accession number V00083008 on 30 August 2000 or a derivative thereof.

Embodiments relating to a poxviral promoter

The following embodiments may relate to any aspect of the invention.

In one embodiment, the poxviral promoter is an early/late promoter or an intermediate promoter.

In a preferred embodiment, the poxviral promoter is a mainly or completely late promoter.

In one embodiment, the mainly or completely late promoter is selected from the group consisting of promoters Pr11, Pr7,5, PrSSL, PrATI, and PrS.

In a particularly preferred embodiment, the poxviral promoter is a mainly late promoter.

In one embodiment, the mainly late promoter is PrS.

In another preferred embodiment, expression of a transgene, preferably a virus-yield reducing transgene, comprised by a recombinant poxvirus or a transcription unit is driven by the late part of an early/late promoter operably linked to the transgene, for example the late part of PrS promoter.

In one embodiment, the poxviral promoter is a mainly early promoter.

In one embodiment, the mainly early promoter is selected from the group consisting of promoters Pr13.5long/Pr13.5 (29), PrHyb, Pr1328, and PrH5m, preferably is PrH5m.

Embodiments relating to a binding sequence for a transcriptional repressor protein

The following embodiments may relate to any aspect of the invention.

In one embodiment, the binding sequence for a transcriptional repressor protein is capable of binding the transcriptional repressor protein.

In one embodiment, the binding sequence is capable of binding a transcriptional repressor protein of the tetracycline repressor (TetR) or lactose repressor (LacR) family.

In one embodiment, the binding sequence is capable of binding a transcriptional repressor protein of the TetR family, preferably is capable of binding TetR or a prototypic TetR, for example from *E. coli*.

In one embodiment, binding a transcriptional repressor protein is a reversible binding.

In one embodiment, the binding sequence comprises two copies of a Tet operator 2 (TetO2) nucleotide sequence, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

In one embodiment, the binding sequence comprises two copies of a Tet operator 2 (TetO2) nucleotide sequence arranged in tandem and separated by a 2-base spacer, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

In one embodiment, the binding sequence for a transcriptional repressor protein comprises or consists of a nucleic acid sequence according to SEQ ID NO: 2.

In one embodiment, the amount of TetR protein generated in a transgenic poxvirus producer cell is sufficient to downregulate transgene expression for at least 2, 4, 10, 20, 22, or 24 hours after infection of the transgenic cell with recombinant poxvirus comprising a yield-reducing transgene linked to a binding sequence for the TetR protein.

Embodiments relating to a transgene

The following embodiments may relate to any aspect of the invention.

In one embodiment, the transgene encodes a protein or peptide, preferably a protein or peptide comprising one or more antigenic determinants, more preferably a proteinaceous or peptidic antigen.

In one embodiment, the transgene encodes a protein or antigen selected from the group consisting of a viral, bacterial, fungal, plant, parasite, non-human animal, and human protein or antigen, or an antigenic part thereof.

In one embodiment, the transgene encodes a viral antigen, or an antigenic part thereof.

In one embodiment, the transgene encodes a disease-associated antigen or protein.

In one embodiment, the disease-associated antigen or protein is a vaccine antigen or a gene therapy protein.

In one embodiment, the disease-associated antigen is derived from a virus selected from the group consisting of alpha-virus, adenovirus, Coxsackievirus, Crimean-Congo hemorrhagic fever virus, cytomegalovirus (CMV), dengue virus, Ebola virus, Epstein-Barr virus (EBV), Eastern, Western or Venezuelan equine encephalitis virus (EEV), Guanarito virus, herpes simplex virus-type 1 (HSV-1), herpes simplex virus-type 2 (HSV-2), human herpesvirus-type 8 (HHV-8), hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), human immunodeficiency virus (HIV), influenza virus, Junin virus, Lassa virus, Machupo virus, Marburg virus, measles virus, human metapneumovirus, mumps virus, Norwalk virus, human papillomavirus (HPV), parainfluenza virus, parvovirus, poliovirus, rabies virus, respiratory syncytial virus (RSV), rhinovirus, rotavirus, rubella virus, Sabia virus, severe acute respiratory syndrome virus 2 (SARS-CoV-2), middle east respiratory syndrome coronavirus (MERS-CoV), varicella zoster virus, variola virus, West Nile virus, and yellow fever virus.

In one embodiment, the disease-associated antigen is derived from Epstein-Barr virus (EBV), or an antigenic part thereof, preferably selected from the group consisting of EBV proteins BLLF1a/b (gp350/220), BALF4 (gB, gp110), BXLF2 (gH, gp85), BKRF2 (gL, gp25), BZLF2

(gp42), BILF2 (gp78), BDLF3 (gp150), BBRF3 (gM), BLRF1 (gN), BMRF2, EBNA1, EBNA2, EBNA3, LMP1, LMP2, BRLF1 or BZLF1 protein.

In one embodiment, the transgene encodes a LMP1/EBNA2 fusion protein.

In one embodiment, the transgene encodes a tumor specific antigen (TSA) or a tumor associated antigen (TAA), or an antigenic part thereof.

Aspects and embodiments relating to a transgenic poxvirus producer cell

In one aspect, provided is a transgenic poxvirus producer cell that is genetically modified to express a transcriptional repressor protein.

In one embodiment, the transgenic poxvirus producer cell is genetically modified to constitutively or in a regulated manner express a transcriptional repressor protein.

In one embodiment of the transgenic poxvirus producer cell, the transcriptional repressor protein is embodied as described below under "Embodiments relating to a transcriptional repressor protein".

In one embodiment, the transgenic poxvirus producer cell was generated by stable transfection of a poxvirus permissive cell with a plasmid encoding a transcriptional repressor protein.

In one embodiment, the transgenic poxvirus producer cell is derived from a poxvirus permissive cell.

In one embodiment, the transgenic poxvirus producer cell, or the poxvirus permissive cell, is a eukaryotic poxvirus producer cell.

In one embodiment, the transgenic poxvirus producer cell, or the poxvirus permissive cell, is a cell of a cell line, preferably of a continuous or permanent cell line.

In one embodiment, the cell line is an adherent or suspension cell line.

In one embodiment, the poxvirus permissive cell is an avian cell or is derived therefrom.

In one embodiment, the poxvirus permissive cell is a chicken, duck, or quail cell or is derived therefrom.

In one embodiment, the transgenic poxvirus producer cell preferably is a transgenic vaccinia virus producer cell, most preferably is a transgenic MVA producer cell.

In one embodiment, the poxvirus permissive cell preferably is a vaccinia virus permissive cell, most preferably is an MVA permissive cell.

In one embodiment, the poxvirus permissive cell is a cell of chicken DF-1 cell line.

In one embodiment, the transgenic poxvirus producer cell is a cell of a cell clone derived from chicken DF-1 cell line.

In one embodiment, the transgenic poxvirus producer cell is a cell of cell clone DF1-TR59.

In one embodiment, the poxvirus permissive cell is a cell of a quail CCX cell line, preferably of quail CCX.2C4 or CCX.E10 cell line.

In one embodiment, the transgenic poxvirus producer cell is a cell of a cell clone derived from quail CCX.2C4 or CCX.E10 cell line, preferably from CCX.2C4 cell line.

In one embodiment, the transgenic poxvirus producer cell is a cell of cell clone CCX.2C4-TR16.

Other poxvirus, vaccinia virus or MVA permissive cell lines such as QOR2/E11, a continuous avian cell line from quail embryo (Kraus B, von Fircks S, Feigl S et al. 2011. Avian cell line – Technology for large scale vaccine production. BMC Proc 5: P51.), or AGE.CR1-plX (available from ProBiogen), or EB66 (duck embryonic stem cell-derived line, available from Valneva), or BHK-21 (baby hamster kidney cell line) may be also considered for preparation of the transgenic MVA producer cell.

In one embodiment, the poxvirus producer cell comprises a recombinant poxvirus as described above under “Aspects and embodiments relating to recombinant poxvirus”.

Embodiments relating to a transcriptional repressor protein

The following embodiments may relate to any aspect of the invention.

In one embodiment, the transcriptional repressor protein is capable of binding to the binding sequence for a transcriptional repressor protein comprised by a recombinant poxvirus.

In one embodiment, binding a transcriptional repressor protein is a reversible binding.

In one embodiment, the transcriptional repressor protein is one of the tetracycline repressor (TetR) or lactose repressor (LacR) family.

In one embodiment, the transcriptional repressor protein is a one of the TetR family, preferably is a TetR or a prototypic TetR, for example from *E. coli*.

In one embodiment, the transcriptional repressor protein is capable of binding to a nucleotide sequence comprising two copies of a Tet operator 2 (TetO2) nucleotide sequence, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

In one embodiment, the transcriptional repressor protein is capable of binding to a nucleotide sequence comprising two copies of a Tet operator 2 (TetO2) nucleotide sequence arranged in tandem and separated by a 2-base spacer, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

In one embodiment, the transcriptional repressor protein is capable of binding to a nucleotide sequence comprising or consisting of a nucleic acid sequence according to SEQ ID NO: 2.

Embodiments relating to the interrelation between transcriptional repressor protein and corresponding binding sequence

The following embodiments may relate to any aspect of the invention.

In one embodiment, binding of the transcriptional repressor protein to a corresponding binding sequence for a transcriptional repressor protein downregulates expression of the transgene by the recombinant poxvirus, preferably as compared to a poxvirus or recombinant poxvirus not comprising the binding sequence for a transcriptional repressor.

In one embodiment, binding of the transcriptional repressor protein to a corresponding binding sequence for a transcriptional repressor protein decreases expression of the transgene by the recombinant poxvirus by at least 40% or 60%, more preferably by 70-90%, most preferably by at least 90%, preferably as compared to a poxvirus or recombinant poxvirus not comprising the binding sequence for a transcriptional repressor.

In one embodiment, binding of the transcriptional repressor protein to a corresponding binding sequence for a transcriptional repressor protein increases replication of a recombinant poxvirus by at least 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, or 60-fold.

In one embodiment, binding of the transcriptional repressor protein to a corresponding binding sequence for a transcriptional repressor protein increases viral yield of a recombinant poxvirus by at least 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, 30-fold, or 40-fold.

In one embodiment, the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, and/or *vice versa*, i.e., the transcriptional repressor protein expressed by the transgenic poxvirus producer cell is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus.

In one embodiment, the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus (which is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell) comprises two copies of a Tet operator 2 (TetO2) nucleotide sequence, preferably arranged in tandem and separated by a 2-base spacer, and the transcriptional repressor protein expressed by the transgenic poxvirus producer cell (which transcriptional repressor protein is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus) is a TetR or a prototypic TetR,

In one embodiment, the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus (which is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell) comprises two copies of a TetO2 nucleotide sequence according to SEQ ID NO: 1, preferably arranged in tandem and separated by a 2-base spacer, and the transcriptional repressor protein expressed by the transgenic poxvirus producer cell (which transcriptional repressor protein is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus) is a TetR or a prototypic TetR.

In one embodiment, the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus (which is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell) comprises or consists of a nucleotide sequence according to SEQ ID NO: 2, and the transcriptional repressor protein expressed by the transgenic poxvirus producer cell (which transcriptional repressor protein is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus) is a TetR or a prototypic TetR.

Embodiments relating to the interrelation between recombinant poxvirus and transgenic poxvirus producer cell

The following embodiments may relate to any aspect of the invention.

In one embodiment, the recombinant poxvirus is recombinant vaccinia virus, and the transgenic poxvirus producer cell is a vaccinia virus producer cell.

In one embodiment, the recombinant poxvirus is recombinant MVA, and the transgenic poxvirus producer cell is an MVA producer cell.

Aspects and embodiments relating to medical uses

In one aspect, provided is a recombinant poxvirus according to the invention for use in the treatment or prevention of an infectious disease or cancer.

In one aspect, provided is a use of a recombinant poxvirus according to the invention for the manufacture of a medicament or vaccine for use in the treatment or prevention of an infectious disease or cancer.

In one aspect, provided is a method of treating or preventing an infectious disease or cancer in a subject, the method comprising administering to the subject a recombinant poxvirus according to the invention.

In one embodiment, the infectious disease is selected from the group consisting of diseases the causative agent of which is a virus, a bacterium, a fungus, or a parasite.

In a preferred embodiment, the infectious disease is a viral disease.

In one embodiment, the causative agent of the viral disease is selected from the group consisting of alpha-virus, adenovirus, Coxsackievirus, Crimean-Congo hemorrhagic fever virus, cytomegalovirus (CMV), dengue virus, Ebola virus, Epstein-Barr virus (EBV), Eastern, Western or Venezuelan equine encephalitis virus (EEV), Guanarito virus, herpes simplex virus-type 1 (HSV-1), herpes simplex virus-type 2 (HSV-2), human herpesvirus-type 8 (HHV-8), hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), human immunodeficiency virus (HIV), influenza virus, Junin virus, Lassa virus, Machupo virus, Marburg virus, measles virus, human metapneumovirus, mumps virus, Norwalk virus, human papillomavirus (HPV), parainfluenza virus, parvovirus, poliovirus, rabies virus, respiratory syncytial virus (RSV), rhinovirus, rotavirus, rubella virus, Sabia virus, severe acute respiratory syndrome virus 2 (SARS-CoV-2), middle east respiratory syndrome coronavirus (MERS-CoV), varicella zoster virus, variola virus, West Nile virus, and yellow fever virus.

In one embodiment, the viral disease is Epstein-Barr virus (EBV) infection.

Aspects and embodiments relating to a transcriptional repressor protein and corresponding binding sequence for downregulation of poxviral transgene expression

In one aspect, provided is a use of or a method using a transcriptional repressor protein and its corresponding binding sequence for control or downregulation of a mainly or completely late, or an intermediate poxviral promoter driven transgene expression by a recombinant poxvirus.

In one embodiment, transgene expression is driven by a mainly or completely late promoter, preferably by a mainly late promoter.

In one embodiment, transgene expression is driven by PrS promoter.

In one embodiment, transgene expression is driven by the late part of an early/late promoter, preferably by the late part of PrS promoter.

In one embodiment, the transcriptional repressor protein is one of the tetracycline repressor (TetR) or lactose repressor (LacR) family.

In one embodiment, the transcriptional repressor protein is a one of the TetR family, preferably is a TetR or a prototypic TetR, for example from *E. coli*.

In one embodiment, the corresponding binding sequence comprises two copies of a Tet operator 2 (TetO2) nucleotide sequence, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

In one embodiment, the corresponding binding sequence comprises two copies of a Tet operator 2 (TetO2) nucleotide sequence arranged in tandem and separated by a 2-base spacer, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

In one embodiment, the corresponding binding sequence for a transcriptional repressor protein comprises or consists of a nucleic acid sequence according to SEQ ID NO: 2.

In one embodiment, the transgene is a virus yield-reducing transgene.

In one embodiment, the recombinant poxvirus preferably is a recombinant vaccinia virus, most preferably is a recombinant MVA.

Further description

Modified Vaccinia Virus Ankara (MVA)

In the past, MVA was generated by 516 serial passages on chicken embryo fibroblasts of the Ankara strain of vaccinia virus (CVA) (for review see Mayr A et al. 1975. Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. Infection 3:6-14). This virus was renamed from CVA to MVA at passage 570 to account for its substantially altered properties. MVA was subjected to further passages up to a passage number of over 570. As a consequence of these long-term passages, the genome of the resulting MVA virus had about 31 kilobases of its genomic sequence deleted and, therefore, was described as highly host cell restricted for replication to avian cells (19). It was shown in a variety of animal

models that the resulting MVA was significantly avirulent compared to the fully replication competent starting material (20).

An MVA useful in the practice of the present invention includes MVA-572 (deposited as ECACC V94012707 on 27 January 1994); MVA-575 (deposited as ECACC V00120707 on 7 December 2000), MVA-I721 (referenced in (4)), NIH clone 1 (deposited as ATCC® PTA-5095 on 27 March 2003) and MVA-BN (deposited at the European Collection of Cell Cultures (ECACC) under number V00083008 on 30 August 2000).

More preferably the MVA used in accordance with the present invention includes MVA-BN and MVA-BN derivatives. MVA-BN has been described in WO 02/042480. "MVA-BN derivatives" refer to any virus exhibiting essentially the same replication characteristics as MVA-BN, as described herein, but exhibiting differences in one or more parts of their genomes.

MVA-BN, as well as MVA-BN derivatives, is replication incompetent, meaning a failure to reproductively replicate *in vivo* and *in vitro*. More specifically *in vitro*, MVA-BN or MVA-BN derivatives have been described as being capable of reproductive replication in chicken embryo fibroblasts (CEF), but not capable of reproductive replication in the human keratinocyte cell line HaCaT (21), the human bone osteosarcoma cell line 143B (ECACC Deposit No. 91112502), the human embryo kidney cell line 293 (ECACC Deposit No. 85120602), and the human cervix adenocarcinoma cell line HeLa (ATCC Deposit No. CCL-2). Additionally, MVA-BN or MVA-BN derivatives have a virus amplification ratio at least two-fold less, more preferably three-fold less than MVA-575 in HeLa cells and HaCaT cell lines. Tests and assay for these properties of MVA-BN and MVA-BN derivatives are described in WO 02/42480 and WO 03/048184.

The term "not capable of reproductive replication" in human cell lines *in vitro* as described above is, for example, described in WO 02/42480, which also teaches how to obtain MVA having the desired properties as mentioned above. The term applies to a virus that has a virus amplification ratio *in vitro* at 4 days after infection of less than 1 using the assays described in WO 02/42480 or US 6,761,893.

Exemplary generation of a recombinant MVA virus

For the generation of a recombinant MVA as disclosed herein, different methods may be applicable. The DNA sequence to be inserted into the virus can be placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA sequence to be inserted can be ligated to a promoter. The

promoter-gene linkage can be positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of poxvirus DNA containing a non-essential locus. The resulting plasmid construct can be amplified by propagation within *E. coli* bacteria and isolated. The isolated plasmid containing the DNA gene sequence to be inserted can be transfected into a cell culture, *e.g.*, of chicken embryo fibroblasts (CEFs), at the same time the culture is infected with MVA. Recombination between homologous MVA viral DNA in the plasmid and the viral genome, respectively, can generate an MVA modified by the presence of foreign (heterologous) DNA sequences.

A cell of a suitable cell culture as, *e.g.*, CEF cells, can be infected with a MVA virus. The infected cell can be, subsequently, transfected with a first plasmid vector comprising a foreign or heterologous gene or genes, such as one or more of the nucleic acids provided herein, preferably under the transcriptional control of a poxvirus expression control element. As explained above, the plasmid vector also comprises sequences capable of directing the insertion of the exogenous sequence into a selected part of the MVA viral genome. Optionally, the plasmid vector also contains a cassette comprising a marker and/or selection gene operably linked to a poxvirus promoter. The use of selection or marker cassettes simplifies the identification and isolation of the generated recombinant MVA. However, a recombinant poxvirus can also be identified by PCR technology. Subsequently, a further cell can be infected with the recombinant MVA obtained as described above and transfected with a second vector comprising a second foreign or heterologous gene or genes. In case, this gene shall be introduced into a different insertion site of the poxvirus genome, the second vector also differs in the poxvirus-homologous sequences directing the integration of the second foreign gene or genes into the genome of the poxvirus. After homologous recombination has occurred, the recombinant virus comprising two or more foreign or heterologous genes can be isolated. For introducing additional foreign genes into the recombinant virus, the steps of infection and transfection can be repeated by using the recombinant virus isolated in previous steps for infection and by using a further vector comprising a further foreign gene or genes for transfection. There are ample of other techniques known to generate recombinant MVA.

The practice of the invention will employ, if not otherwise specified, conventional techniques of immunology, molecular biology, microbiology, cell biology, and recombinant technology, which are all within the skill of the art. See *e.g.*, Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd edition, 1989; *Current Protocols in Molecular Biology*, Ausubel FM, et al., eds, 1987; the series *Methods in Enzymology* (Academic Press, Inc.); *PCR2: A Practical Approach*, MacPherson MJ, Hams BD, Taylor GR, eds, 1995; *Antibodies: A Laboratory Manual*, Harlow and Lane, eds, 1988.

EXAMPLES

The following examples serve to further illustrate the disclosure. They should not be understood as limiting the invention, the scope of which is determined by the appended claims.

EXAMPLE 1: Materials and methods

1.1 Viruses and cells

The recombinant MVAs described herein were derived from MVA-BN[®] (Modified Vaccinia Virus Ankara of Bavarian Nordic; herein also referred to as "MVA-BN"), namely either derived from MVA-BN seed stock (in the case of MVA-res005) or from a bacterial artificial chromosome (BAC) clone constructed from MVA-BN (all other recombinant MVAs). MVA-BN wildtype (MVA-wt) and MVA-BN recombinants were propagated on primary chicken embryo fibroblast (CEF) cells or DF-1 cells (a continuous cell line of chicken embryo fibroblasts) or transgenic DF1-TR59 cells. Recombinants were titrated on CEF cells or DF1-TR59 cells using the TCID₅₀ method. Shope fibroma virus (SFV) as a helper virus for MVA-BN-BAC reactivation was obtained from ATCC (VR-364) and was propagated and titrated on rabbit cornea SIRC cells.

Primary CEF cells were prepared from 11-day old embryonated chicken eggs. CEF cells were cultured in VP-SFM medium (Thermo Fisher Scientific) supplemented with 1% gentamycin and 4 mM L-glutamine for transfection and virus stock production, or in DMEM supplemented with 10% fetal calf serum (FCS) for replication analysis and virus titration. Chicken DF-1 cells were obtained from ATCC.

The chicken DF-1 cell line as well as a quail cell line, namely CCX.2C4, were used for the generation of transgenic cell lines expressing the tetracycline repressor (TetR). Such cell lines could also be used as producer cells for recombinant MVAs. We recently had developed CCX.2C4 cells, a permanent adherent cell line derived from quail embryo, and the suspension quail cell line CCX.E10, that both allow efficient propagation of MVA-wt as well as of recombinant MVAs. Both cell lines are obtainable from Nuvonis. The CCX.2C4 cell line allows satisfactory replication and yields of MVA-wt and forms sufficiently homogenous monolayers to allow focus- or plaque-based generation and selection of recombinant or otherwise mutated MVA clones.

1.2 BAC recombineering and reactivation of infectious recombinant MVA

Construction of the MVA-BN-BACs has been described previously (22). Briefly, the inserted BAC cassette contains miniF plasmid sequences derived from plasmid pMBO131 (23) for maintenance in *E. coli*. The BAC cassette was inserted in the intergenic region between the MVA orthologues of vaccinia virus (VACV)-Copenhagen genes I3L and I4L (MVA064L/MVA065L). All recombinant MVA constructs described herein were generated using variations of the BAC technology except MVA-res005, which was generated using classical homologous recombination between MVA-BN and a transfer plasmid.

For the generation of MVA-mBNbc440 (see Fig. 1), the originally contained neomycin-phosphotransferase npt II-IRES-EGFP marker cassette within the BAC cassette was replaced by a bacterial tetracycline expression cassette to remove the enhanced green fluorescence protein (EGFP) gene from the BAC backbone. This enabled insertion and analysis of an EGFP transgene at another insertion site.

MVA-mBNbc440 was generated by linear exchange mutagenesis of the MVA-BN-BAC in *E. coli* as previously described (22), using the MVA-BN-BAC with the npt II-IRES-EGFP-deleted BAC cassette as a starting construct. The EGFP ORF under control of the synthetic poxviral early/late promoter PrS (24) without a 2xTetO2 operon sequence was inserted together with a PrS driven gpt-mRFP-fusion gene in intergenic region (IGR) 044/045. For the generation of MVA-res005 the EGFP gene containing the 2xTetO2 operon sequence between the PrS promoter and the EGFP ORF in MVA-res005 was inserted together with the PrS-gpt-mRFP cassette (see Fig. 1) in the IGR between genes MVA044L/MVA045L (F14L and F15L in VACV). Here, MVA-BN was used as a parent, and classical homologous recombination with a co-transfected transfer plasmid was employed for MVA-res005 generation. For the generation of MVA-res034, MVA-res035, MVA-res057, and MVA-res058 (see Fig. 1), MVA-BN-BACs were modified by deletion of an essential gene in the MVA-BN-BAC and recombinant MVAs were then generated by homologous recombination and simultaneous reactivation of the modified MVA-BN-BAC using the essential gene as positive selective marker and SFV as helper virus.

For simultaneous recombination and reactivation of infectious recombinant MVA from MVA-BN-BACs, 10^6 DF1-TR59 cells (see Example 4.1) were transfected with 3 μ g of BAC DNA using FuGENE® HD transfection reagent (Promega) and 60 min later infected with SFV to provide the necessary helper functions. Recombinant MVA-res034, MVA-res035, MVA-res057, and MVA-res058 still contained the BAC cassette in IGR 64/65, which harbors a PrS-driven npt II-IRES-EGFP expression cassette (see Fig. 1), that was used as marker for infected cells. Reactivated virus was isolated by further passaging in CEF cells (MVA-

mBNbc440) or in DF1-TR59 cells (MVA-res034, MVAres035, MVA-res057, MVA-res058), and helper virus was eliminated as previously described by passaging of the newly generated recombinant MVA on avian cells that are not permissive for SFV (22).

A BAC-derived MVA (MVA-mBNbc166) also harboring the BAC cassette with the identical EGFP expression cassette as in MVA-res034, MVA-res035, MVA-res057, and MVA-res058 but without other inserts served as reference MVA (not shown in Fig. 1). MVA-mBNbc166 had previously been shown to behave like MVA-wt (22) and therefore is sometimes referred to herein as “MVA-wt”.

Recombinant MVA viral stocks were produced in DF-1 or DF1-TR59 cells and were titrated on CEF cells or DF1-TR59 cells using the TCID₅₀ method as described (3).

1.3 Flow cytometry analysis of transgene expression by recombinant MVA

Cell culture monolayers were washed with phosphate-buffered saline (PBS) and harvested by trypsinization to prepare single cell suspensions. For analysis of EGFP and monomeric red fluorescent protein (mRFP) expression (MVA-res005, MVA-mBNbc440), cells were resuspended in PBS-FACS (2% FCS, 0.1% NaN₃) and directly analyzed by flow cytometry using an LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc.). For flow cytometric analysis of LMP1/EBNA2 expression (MVA-res035, MVA-res058), cells were stained with an antibody against EBNA2 (abcam ab90543, 1:500) and a secondary allophycocyanin (APC)-coupled anti-mouse antibody (F(ab')₂ fragment Goat Anti-Mouse IgG (H+L), Jackson Immuno Research, 115-136-146, 1:500).

1.4 Viral replication analysis

For analysis of multicycle virus replication, confluent monolayers in 6-well cell culture plates were infected with sonicated virus dilutions at the indicated multiplicities of infection (MOIs) in 500 µl of DMEM without FCS. After 60 min of adsorption at 37°C and 5% CO₂, the inoculum was aspirated, cells were washed once with DMEM and were further incubated at 37°C and 5% CO₂ in DMEM/2% FCS. Cells plus supernatant were harvested at the indicated points in time, freeze-thawed three times and sonicated before titration. MVA yields were determined on CEF cells using the TCID₅₀ titration method as described (3).

1.5 Generation of transgenic chicken DF-1 and quail CCX.2C4 cells expressing the TetR

Transgenic DF-1 cells expressing the TetR were generated by transfection of wildtype DF-1 cells with a plasmid expressing the TetR gene from a eukaryotic cytomegalovirus (CMV)

promoter and containing a blasticidin selection cassette to select for stable insertion of the plasmid in the transfected cells with the antibiotic blasticidin.

Cloning of the cell lines was accomplished by seeding 5 cells per well of a 96-well plate of a transfected DF-1 cell pool selected with 5 µg/ml blasticidin for 21 days. Expression of TetR in the transfected DF-1 cells was determined by immunoblot using a TetR specific antibody (see Example 1.6). See also Example 4.1.

A TetR expressing quail cell line was generated by transfection of wildtype CCX.2C4 cells with the same TetR expression plasmid used for the generation of transgenic DF-1 cells as described above. Cloning of the cell lines was accomplished by seeding 5 cells per well of a 96-well plate of a transfected CCX.2C4 cell pool selected with 5 µg/ml blasticidin for 8 weeks. Again, expression of TetR in the transfected CCX.2C4 cells was determined by immunoblot using a TetR specific antibody. See also Example 7.1.

1.6 Immunoblot analysis of TetR protein expressed by transgenic cells

Cells were seeded on the day before infection in 12-well tissue culture plates. Infections were performed as previously described (25). At predefined times after infection cells were washed with cold phosphate-buffered saline (PBS) and lysed in 200 µl 1X Laemmli loading buffer (65 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.1% bromophenol blue, beta-mercaptoethanol [35 µl/ml]) for 5 min at room temperature followed by 3 min sonication, and the lysates were subsequently heated to 95°C for 5 min. Lysates were centrifuged at 18,000 x g for 1 min to remove cell debris.

Soluble proteins in cell lysates were separated on precast SDS-polyacrylamide gels (MiniProtein TGX, 10%, Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes using a Trans-Blot Turbo blotting system (Bio-Rad) and Trans-Blot Turbo transfer packs (Bio-Rad). Membranes were blocked using 5% bovine serum albumin (BSA, Carl Roth) in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 and 0.1% NaN₃, and were incubated with the primary antibodies listed below (diluted in blocking buffer) overnight with shaking at 4°C. Membranes were washed between steps with TBS containing 0.1% Tween-20 four times (20-40 min in total) and incubated for 1 hour with shaking at room temperature with secondary antibodies coupled to horseradish peroxidase and directed against murine IgG. The secondary antibodies had been diluted in TBS containing 5% skim milk powder (VWR International). Bands were visualized by enhanced chemiluminescence (ECL) using two different substrate reagents, SuperSignal West Pico (Thermo Fisher Scientific) as the standard reagent and Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences) in 1:10 dilution for detection with

high sensitivity. Signals were recorded using the ChemiDoc Touch System and Image Lab™ Software (Bio-Rad) for image analysis and quantification.

The following primary antibodies were used in immunoblot analysis: anti-TetR (MoBiTec, TET02, mouse; 1:500; expected to detect a signal at 23 kDa which is the calculated molecular weight of the protein of 207 amino acids encoded by the TetR gene), and anti-mouse β -tubulin (Sigma-Aldrich, T7816, mouse; 1:40000, expected to detect a signal at a molecular mass of 55 kDa).

EXAMPLE 2: Design of recombinant MVAs with TetR regulatable transgenes

A number of recombinant MVAs was generated containing different transgenes that were used either as prototypic examples of “neutral” transgenes (i.e., transgenes not affecting the replication and yields of the respective recombinant MVAs) or as examples of transgenes significantly reducing the yields of the respective recombinant MVA. Examples used in the experiments described herein comprise (1) EGFP as a prototypic “neutral” transgene and (2) a fusion protein of modified versions of the LMP1 and EBNA2 proteins (LMP1/EBNA2) derived from Epstein-Barr virus (EBV) as a yield-reducing transgene.

A first set of two recombinant MVAs with the “neutral” EGFP transgene was generated in which the 2xTetO2 sequence (7, 8) that binds the TetR with high affinity was inserted between the EGFP gene and the early/late PrS promoter (MVA-res005) or was omitted (MVA-mBNbc440) (see Fig. 1). A second transcription unit encoding an mRFP fluorescent marker under the same PrS promoter was co-inserted (Fig. 1) into these recombinant MVAs to allow convenient flow cytometric and microscopic monitoring of infection independent of potential downregulation of EGFP.

For generation of recombinant MVA harboring the LMP1/EBNA2 transgene either under control of the PrH5m promoter (MVA-res034, MVA-res035; see Fig. 1) or the PrS promoter (MVA-res057, MVA-res058; see Fig. 1), see Example 4.3 and 6.1 below, respectively.

EXAMPLE 3: The 2xTetO2 sequence does not impair MVA-mediated EGFP transgene expression.

To evaluate a potential impact of the 2xTetO2 sequence inserted between the poxviral promoter, in this case PrS, and the transgene ORF on transgene expression, EGFP levels (i.e., expression of a neutral transgene) produced by MVA-res005 (see Fig. 1) were analyzed. Infection of the two MVA permissive cell lines DF-1 and CCX.2C4, permissive primary CEFs

as well as of the non-permissive HeLa cell line demonstrated that the levels of EGFP (determined as geometric mean fluorescence intensities = GMFI) were highly similar in cells infected with either MVA-mBNbc440 or MVA-res005 (Fig. 2). MVA-res005 contained a 2xTetO2 sequence between PrS promoter and EGFP transgene while MVA-mBNbc440 did not (see Fig. 1).

Thus, the 2xTetO2 sequence between promoter and ORF did not impair transcription efficiency of EGFP, at least not under the PrS promoter. The data also indicated that the levels of a stable protein like EGFP, the expression of which was controlled by the mainly late poxviral PrS promoter, strongly increased by about 7 to 10-fold from 5 hours p.i. in the initial late phase until 20 hours p.i. in the advanced late phase of the MVA infection cycle (Fig. 2), confirming that the PrS promoter is highly active into the late phase of MVA replication.

EXAMPLE 4: Downregulation of EGFP or LMP1/EBNA2 transgene expression in TetR expressing transgenic DF-1 cells

To enable downregulation of poxviral transgenes using the TetR system, we generated a transgenic chicken fibroblast DF-1 cell line (26, 27) permanently expressing the TetR. DF-1 cells are highly permissive for MVA. The new transgenic DF-1 cell line (referred to as DF1-TR59, see Example 4.1) constitutively expressed the TetR. MVA-mediated expression of the transgene linked to the 2xTetO2 sequence would therefore be “off” without addition of tetracycline or its analogue doxycycline in these cells. In cells not expressing the TetR, which does also apply to the body cells of a vaccinated individual, the transgene would be readily produced since no TetR is present. For *in vitro* test purposes in TetR expressing cells, transgene expression can be switched on by addition of tetracycline or doxycycline.

4.1 Generation of DF1-TR59 cells

As described in Example 1.5, wildtype DF-1 cells were transfected with a plasmid expressing TetR from a eukaryotic CMV promoter and containing a blasticidin selection cassette.

A total of 38 stably transfected blasticidin-resistant DF-1 cell clones were obtained. Of those stably transfected DF-1 cells, 15 clones were expressing intermediate to high levels of the TetR as determined by immunoblot with a TetR specific antibody (see Example 1.6). A lysate of DF-1 cells that had been transiently transfected with the TetR expression plasmid for 24 hours served as reference for achievable TetR levels. Eleven of the 15 cell clones expressing intermediate to high TetR levels showed satisfactory propagation properties. Clone DF1-TR59 with a high level of TetR expression (Fig. 3) was selected for further development.

4.2 Downregulation of PrS-driven EGFP transgene expression

One day after seeding, DF1-TR59 cells expressing the TetR were infected with MVA-res005 harboring the EGFP gene linked to the 2xTetO2 sequence (see Fig. 1) at a high multiplicity of infection (MOI = 5). Cells were treated with doxycycline or not treated from the day of seeding throughout the experiment. No doxycycline allowed TetR-mediated downregulation of EGFP expression while the addition of doxycycline inactivated the TetR. Cells were harvested at the indicated time points after infection (see Fig. 4) by accutase treatment, followed by fixation and permeabilization. Cells were analyzed for EGFP fluorescence by flow cytometry and the geometric mean fluorescence intensities (GMFI) were determined as a measure of EGFP expression levels.

As shown in Fig. 4, the addition of doxycycline (DOX) provided for a distinct EGFP expression, while without DOX the EGFP expression was very low. Thus, highly efficient downregulation of EGFP in MVA-res005 infected DF1-TR59 cells demonstrated that the TetR expressed in DF1-TR59 cells was functional and was able to substantially downmodulate transgene expression from a very strong poxviral promoter (i.e., PrS). In addition, these results showed that sufficient TetR was produced in DF1-TR59 cells under conditions of an MVA infection to downregulate a TetR-controlled transgene for 24 hours after infection, which is the time period after which MVA-infected cells will usually start to undergo cell death.

4.3 Recombinant MVA encoding an LMP1/EBNA2 fusion protein

We generated a recombinant MVA expressing a fusion protein termed LMP1/EBNA2 encoded by the two fused Epstein-Barr virus (EBV) derived genes LMP1 and EBNA2 under the control of the poxviral early/late PrH5m promoter.

Recombinant MVA harboring the LMP1/EBNA2 transgene inserted in IGR 51/52 under control of the PrH5m promoter with insertion of the 2xTetO2 sequence (MVA-res035; see Fig. 1) was generated using DF1-TR59 cells expressing the TetR. EGFP expression from the npt II-IRES-EGFP cassette within the BAC cassette in IGR 64/65 served as internal infection control for these recombinants.

MVA-res035 was generated in DF1-TR59 cells expressing the TetR to facilitate the generation of this recombinant with the TetR regulatable LMP1/EBNA2 transgene. MVA-res034 encoding PrH5m-driven LMP1/EBNA2 but lacking the TetR binding site (i.e., 2xTetO2 sequence) between promoter and transgene (see Fig. 1) could not be rescued as recombinant virus in DF1-TR59 cells, indicating that LMP1/EBNA2 is a yield-reducing transgene that can also perturb the generation of recombinant MVA with uncontrolled expression of this transgene.

4.4 LMP1/EBNA2 is a yield-reducing transgene in MVA

Wildtype DF-1 cells are permissive for MVA and produce similar MVA yields like primary CEF cells. When DF-1 cells were infected with MVA-res035 at a low MOI, we observed a significant 38-fold reduction in yields compared to MVA-mBNbc166 ("MVA-wt") on day 3 p.i. (Fig. 5, left). Yields of MVA-mBNbc166 and MVA-res035 were not affected by doxycycline treatment over the three-day course of infection (Fig. 5, compare left and right). These results demonstrate that LMP1/EBNA2 is a yield-reducing transgene in recombinant MVA, and that doxycycline treatment has no impact on the MVA infection cycle *per se*.

4.5 Downregulation of PrH5m-driven expression of LMP1/EBNA2 transgene

For quantitative analysis of LMP1/EBNA2 transgene downregulation, DF1-TR59 cells were seeded into 6-well plates on the day before infection and treated with doxycycline (DOX) to inactivate the TetR or were not treated (no DOX) from the day of seeding (day (-1)) throughout the experiment. DF1-TR59 cells were infected with recombinant MVA-res035 on day 0 at a MOI of 5. Cells were harvested at the indicated time points after infection (see Fig. 6) by accutase treatment, followed by fixation and permeabilization. MVA-res035 infected cells were stained with an EBNA2-specific antibody for the detection of transgene expression by flow cytometry (see Example 1.3).

In Fig. 6, LMP1/EBNA2 and EGFP expression levels are shown as geometric mean fluorescence intensities (GMFI) of live cells. Here, we conducted a time kinetics of LMP1/EBNA2 expression by MVA-res035 through analyzing LMP1/EBNA2 protein levels at 2, 4, 6, and 24 hours post infection (p.i.). LMP1/EBNA2 levels were highly similar with and without inactivation of the TetR by doxycycline at 2 hours p.i. (Fig. 6A). At 4 hours and 6 hours p.i., the expression levels of LMP1/EBNA2 were slightly higher under doxycycline treatment compared to the "no DOX" condition, while the levels were again highly similar at 24 hours p.i. (Fig. 6A).

Analysis of expression of the unregulated reference transgene EGFP co-encoded by MVA-res035 indicated that cells treated or not treated with doxycycline had almost identical EGFP expression levels (Fig. 6B), also confirming that all cells had been infected with very similar efficiency. Thus, the moderate differences in LMP1/EBNA2 levels (Fig. 6A) are most likely indeed a result of TetR-mediated downregulation of LMP1/EBNA2 expression.

The data shown in Fig. 6A indicate a transient and very moderate downregulation of LMP1/EBNA2 expression by the active TetR in the starting period of the late phase of the MVA-res035 infectious cycle around 4-6 hours .

It is known that early poxviral transcription occurs in the viral core. This might impair access of the cytoplasmic TetR to the viral genomic DNA still encapsidated in the core during early transcription though TetR is a small protein. Thus, the specific mode of early poxviral transcription might prevent its regulation by the TetR. After uncoating of the incoming poxviral core in the cytoplasm, the viral DNA is released, and intermediate and late transcription occur from free cytoplasmic poxviral genomic DNA, where cytoplasmic TetR could bind. Indeed, a more thorough evaluation of the expression kinetics of the PrH5m promoter showed that the nominally early/late PrH5m promoter drives predominantly early expression and has a very moderate late activity. Thus, our results showing moderate downregulation at the onset of intermediate and late gene expression around 2-4 hours p.i. would be consistent with a TetR effect restricted to the late MVA replication phase.

4.6 Effect of PrH5m-driven LMP1/EBNA2 transgene downregulation on recombinant MVA replication

A viral multi-step growth curve analysis of MVA-res035 in DF1-TR59 cells expressing the TetR was conducted with and without doxycycline (see Fig. 7). Virus infection was started at an MOI of 0.05 in DF1-TR59 cells. MVA-res035 with or without doxycycline reached maximum yields that were around 1.5 logs lower than those obtained with MVA-mBNbc166 ("MVA-wt"). There was only a slight increase in yields on days 1 and 2 p.i. when the TetR was active ("no DOX"). Yields of MVA-res035 on the final day 3 of analysis were again highly similar independent of a doxycycline treatment. Thus, an active TetR exerted only a slight and temporary beneficial effect on the yields of MVA-res035. It can be assumed that the slight and transient TetR-mediated downregulation of LMP1/EBNA2 levels (see Fig. 6A) allowed for a slightly higher peak titer of MVA-res035 that occurred earlier (day 2 p.i. vs day 3 p.i.) than with unhindered LMP1/EBNA2 expression under doxycycline treatment condition (Fig. 7).

The results confirm that LMP1/EBNA2 is a yield-reducing transgene strongly impairing the replication kinetics and maximum yields of the respective recombinant MVA-res035. The slight downregulation of the PrH5m-driven LMP1/EBNA2 expression by TetR had only a small beneficial effect on MVA-res035 replication kinetics and yields.

EXAMPLE 5: Effect of TetR on early and late EGFP transgene expression

To confirm that the TetR is suitable for controlling MVA-driven late gene expression but less so or not suitable to decrease early MVA-driven gene expression, we analyzed expression of EGFP under the early/late PrS promoter in both the early and late phase.

Treatment of MVA-res005 infected DF1-TR59 cells with cytosine arabinoside (araC) and/or doxycycline was used to discern TetR effects on early versus late expression. A time kinetic experiment revealed that at 2 hours p.i., which is the end of the early phase, no difference in EGFP expression was detectable with and without doxycycline treatment (Fig. 8A). Addition of doxycycline to the MVA-res005 infected DF1-TR59 cells (switching off the TetR) increased EGFP expression earliest at 4 hours p.i. compared to the “no DOX” condition (i.e., repressed state) (Fig. 8A). Late in the MVA replication cycle at 24 h p.i. (i.e., when the late phase had proceeded for 22 hours), PrS-driven EGFP expression was clearly downregulated to 3.6% without doxycycline (i.e., repressed state) (Fig. 8A).

Arresting virus replication in the early phase by araC treatment significantly reduced overall EGFP expression (Fig. 8B), confirming that most of the expressed EGFP under the PrS promoter is derived from late transcription from this promoter. No difference in EGFP expression levels with or without doxycycline treatment was discernible under araC (Fig. 8B), indicating that the TetR does not affect the very moderate gene expression driven by the early component of the early/late PrS promoter.

Expression of the PrS-driven mRFP reference transgene that was also encoded by MVA-res005 and not linked to a 2xTetO2 sequence (see Fig. 1) remained completely unaffected by an active TetR (“no DOX”) as evidenced by comparison with an inactive TetR under doxycycline (Fig. 8C). AraC treatment strongly reduced the maximal mRFP expression driven by the PrS promoter and kept it at levels similar to those in the early phase (i.e., up to 2 hours p.i.) (Fig. 8D), confirming that PrS is mainly driving late expression. There was no difference between doxycycline treated and untreated cells regarding mRFP expression (Fig. 8D), what was expected due to the lack of a 2xTetO2 sequence linked to the mRFP ORF.

In conclusion, the TetR has no effect on transgene expression from the early part of an early/late poxviral promoter such as PrS, but efficiently downregulates expression from the late portion of the promoter. Thus, the TetR system is most suitable for the downregulation of mainly or completely late promoter driven poxviral transgene expression.

To summarize, the data from both the PrH5m and PrS-driven transgene expression demonstrated that the TetR system had no downregulation activity on early transgene expression by recombinant MVAs, while it was highly effective in downregulating late expression. The data showing significant suppression of PrS-driven EGFP expression by TetR also indicated that even expression from very strong poxviral late promoters could effectively be downregulated. It is anticipated that gene expression from intermediate poxviral promoters will also be efficiently inhibited by cellularly expressed TetR, since intermediate transcripts are also generated from free cytosolic viral DNA after disintegration of the viral core.

EXAMPLE 6: TetR-mediated regulation of the yield-reducing LMP1/EBNA2 transgene driven by the mainly late PrS promoter

6.1 Downregulation of PrS-driven expression of LMP1/EBNA2 transgene

Recombinant MVA-res058 with the yield-reducing transgene LMP1/EBNA2 under the mainly late PrS promoter and with a 2xTetO2 sequence between promoter and LMP1/EBNA2 ORF (see Fig. 1) was generated using the TetR expressing DF1-TR59 cells to evaluate whether efficient reduction of the bulk of transgene expression in the late phase would achieve significantly higher yields of a recombinant MVA expressing a yield-reducing transgene mainly late.

It was also attempted to generate a reference recombinant MVA containing the LMP1/EBNA2 ORF under the PrS promoter without a TetR binding site (MVA-res057, see Fig. 1). As with MVA-res034 containing the LMP1/EBNA2 ORF under the PrH5m promoter and lacking a 2xTetO2 sequence (see Example 4.3), the few and small viral foci obtained after MVA-res057 recombination/reactivation did not sufficiently propagate to produce viral stocks and the respective recombinants died out in passages 2-3 after recombination/reactivation, corroborating our results with MVA-res034. These findings are most likely explained by the detrimental effects of unhindered LMP1/EBNA2 expression on MVA replication. In contrast, MVA-res058 with the 2xTetO2 sequence linked PrS-LMP1/EBNA2 gene sequence could be readily generated using DF1-TR59 cells that expressed TetR throughout the generation and propagation process of MVA-res058.

PrS-driven LMP1/EBNA2 transgene expression by MVA-res058 in DF1-TR59 cells with and without active TetR ("no DOX" condition or under doxycycline treatment) was analyzed by flow cytometry. The LMP1/EBNA2 signal in DF1-TR59 cells without doxycycline at 24 hours after infection with MVA-res058 was downregulated to 14% compared to the LMP1/EBNA2 signal in the presence of doxycycline (Fig. 9A). Downregulation of the LMP1/EBNA2 signal in DF1-TR59 cells in the absence of doxycycline was even more effective at earlier times of infection at 6 hours p.i. (7% remaining LMP1/EBNA2 signal, see Fig. 9A).

MVA-res058 also expressed EGFP under the PrS promoter as a reference marker of infection and gene expression (see Fig. 1). Analysis of the expression levels at 6 hours and 24 hours p.i. showed that EGFP expression in cells not treated with doxycycline was at both time points not lower than that in the presence of doxycycline (Fig. 9B), confirming that the lower LMP1/EBNA2 signal shown in Fig. 9A was not due to lower infection or gene expression efficiency. To the contrary, reference EGFP expression by MVA-res058 in DF1-TR59 cells incubated without doxycycline, i.e., under conditions of repressed LMP1/EBNA2 expression,

was even significantly higher at 24 hours p.i. than under doxycycline treatment (Fig. 9B). This can possibly be explained by the suppression of negative effects of LMP1/EBNA2 expression under the “no DOX” condition on expression of other viral genes including the EGFP marker gene co-expressed by MVA-res058.

In conclusion, the TetR in DF1-TR59 cells was able to downmodulate PrS-driven LMP1-EBNA2 expression by approximately 85-90% of the LMP1-EBNA2 expression levels obtained when the TetR was inactivated by doxycycline.

6.2 Effect of PrS-driven LMP1/EBNA2 transgene downregulation on recombinant MVA replication

A growth curve experiment evaluating replication of MVA-res058 with and without doxycycline in DF1-TR59 cells was carried out. As shown in Fig. 10, MVA-res058 expressing LMP1/EBNA2 from the PrS promoter under doxycycline treatment reached a peak yield in DF1-TR59 cells on day 2 p.i. of only 3.2×10^6 TCID₅₀ per well. This confirmed that the replication of a recombinant MVA expressing LMP1/EBNA2 under the mainly late PrS promoter was also severely impaired, very similar to what was observed for MVA-res035 with PrH5m promoter driven LMP1/EBNA2 expression (see Example 4.6).

MVA-res058 yielded 35-fold higher virus peak titers in DF1-TR59 cells when expression of LMP1/EBNA2 was suppressed by the TetR (“no DOX” condition) compared to LMP1/EBNA2 expression by MVA-res058 in the presence of doxycycline (Fig. 10). The average difference in MVA-res058 replication over days 1-3 p.i. when comparing TetR repressed (“no DOX”) and un-repressed LMP1/EBNA2 expression was 57-fold. Notably, MVA-res058 replication in DF1-TR59 cells without doxycycline (i.e., repressed LMP1/EBNA2 expression) was almost indistinguishable from replication of MVA-mBNbc166 (“MVA-wt”) with or without doxycycline (Fig. 10).

Significantly, the approximately 85% efficiency of downregulation of LMP1/EBNA2 expression (see Example 6.1), clearly representing an incomplete downregulation of transgene expression, was sufficient to allow an almost unhindered replication of MVA-res058 in DF1-TR59 cells expressing the TetR.

EXAMPLE 7: Downregulation of LMP1/EBNA2 transgene expression in TetR expressing transgenic quail CCX.2C4 cells**7.1 Generation of CCX.2C4-TR16 cells**

As described in Example 1.5, a TetR expressing CCX.2C4 clone was generated by transfection of adherently growing CCX.2C4 cells with a plasmid expressing the TetR from a eukaryotic CMV promoter and containing a blasticidin selection cassette.

A total of 37 stably transfected CCX.2C4 cell clones were obtained that were blasticidin-resistant. Expression of TetR was determined by immunoblot using a TetR specific antibody and the blots were quantified using the luminescence based ChemiDoc Touch system (see Example 1.6). From the 37 clones analyzed, six showed a TetR-specific signal that was equal or higher than the respective signal observed in DF1-TR59 cells (Fig. 11A). Clone CCX.2C4-TR16 with a high level of TetR expression ("2C4-TR16" in Fig. 11A) was selected for further cell banking and development.

The impact of MVA-wt (MVA-BN wildtype) infection on expression of the TetR in CCX.2C4-TR16 cells was evaluated by immunoblot analysis in a time kinetic experiment (Fig. 11B). The signal intensities of the TetR-specific bands were analyzed by using the ImageQuant software and normalized to the levels of the housekeeping protein β -tubulin. The levels of TetR expression varied to some extent over the course of infection. Clearly, however, TetR levels were not reduced in CCX.2C4-TR16 cells by infection with MVA over a 24-hours course of infection, but rather slightly increased (Fig. 11B). Either cellular transcription specifically of the TetR gene under the CMV promoter integrated in the cellular genome is not significantly affected, or the TetR protein is very stable such that a decreasing transcription during infection would not alter the TetR protein levels over the course of 24 hours of MVA infection.

7.2 Downregulation of LMP1/EBNA2 transgene expression in CCX.2C4-TR16 cells

Expression of the yield-reducing LMP1/EBNA2 transgene was analyzed using the newly generated adherent transgenic cell line termed CCX.2C4-TR16 that expressed the TetR (see Example 7.1). LMP1/EBNA2 expression under the mainly early PrH5m promoter (MVA-res035, see Fig. 1) and under the mainly late PrS promoter (MVA-res058, see Fig. 1) was evaluated. Expression of the neutral EGFP (MVA-res035, MVA-res058, see Fig. 1) or mRFP transgene (MVA-res005, see Fig.1) under the PrS promoter in CCX.2C4-TR16 cells served as reference.

Without doxycycline treatment (i.e., TetR was active), EGFP expression from the PrS promoter in MVA-res005 was downregulated (Fig. 12A, left) in quail CCX.22C4 cells at 24 h p.i., while expression of the co-encoded PrS-mRFP gene was unaffected (Fig. 12A, right).

No reduction of LMP1/EBNA2 transgene expression by an active TetR ("no DOX" condition) was observed in CCX.2C4-TR16 cells infected with MVA-res035 (Fig. 12B, left), in which the LMP1/EBNA2 transgene was expressed under the mainly early PrH5m promoter. Expression of the co-encoded neutral EGFP gene was unaffected (Fig. 12B, right).

With MVA-res058, 51% of remaining LMP1/EBNA2 expression was observed at 24 hours p.i. under repressing conditions ("no DOX") compared to LMP1/EBNA2 expression in the presence of doxycycline (Fig. 12C, left). At 6 hours p.i., TetR-mediated downregulation was even more efficient with 27% remaining LMP1/EBNA2 expression (Fig. 12C, left). Expression levels of the EGFP marker gene not regulated by TetR in MVA-res058 was not reduced in TetR expressing CCX.2C4-TR16 cells independent of doxycycline treatment (Fig. 12C, right). To the contrary, EGFP expression under the "no DOX" condition was even higher than that after doxycycline treatment (Fig. 12C, right), as previously observed with MVA-res058 grown in DF1-TR59 cells (see Example 6.1), likely reflecting the positive effects of suppressed expression of the yield-reducing LMP1/EBNA2 transgene in CCX.2C4-TR16 cells. An experiment evaluating reference MVA-mBNbc166 also harboring the BAC cassette with the identical EGFP expression cassette in CCX.2C4-TR16 cells showed very similar EGFP expression in doxycycline-treated vs untreated cells, supporting the conclusion that potential direct effects of doxycycline treatment on the CCX.2C4-TR16 cells *per se* apart from deactivating the TetR were not responsible for the observed differences in transgene expression.

Thus, expression of the TetR in the transgenic CCX.2C4-TR16 cells was able to downmodulate the mainly late expressed yield-reducing LMP1/EBNA2 transgene until the end of the MVA infectious cycle.

7.3 Effect of LMP1/EBNA2 transgene downregulation on recombinant MVA replication in CCX.2C4-TR16 cells

The effect of TetR-mediated downregulation of LMP1/EBNA2 expression on the replication of MVA-res058 in CCX.2C4-TR16 cells was determined in a multistep growth curve experiment (Fig. 13). BAC-derived MVA-mBNbc166 served as reference. When LMP1/EBNA2 expression was rendered possible by adding doxycycline to the infected CCX.2C4-TR16 cells, MVA-res058 did not replicate at all; titers of viral output 1-3 days after infection were even lower than the titer of the inoculum (Fig. 13). In contrast, when the TetR was active ("no DOX" condition), MVA-res058 replicated to levels similar to those of the reference MVA ("MVA-wt")

in Fig. 13). This finding was remarkable insofar as TetR-mediated downregulation of LMP1/EBNA2 expression had been shown to be rather moderate over the course of the infectious cycle of MVA-res058 in TetR expressing CCX.2C4-TR16 cells, as described above (see Example 7.2).

Doxycycline treatment again had no significant impact on the replication of reference MVA-mBNbc166 ("MVA-wt"; Fig. 13). Thus, doxycycline treatment as such did not affect MVA replication and was not responsible for the strongly decreased replication of MVA-res058 when LMP1/EBNA2 transgene expression was not suppressed, confirming the results with MVA-res058 in TetR expressing DF1-TR59 cells (see Example 6.2, Fig. 10).

Thus, despite an apparently moderate effect of TetR-mediated downregulation on LMP1/EBNA2 expression, this moderate downregulation was sufficient for almost completely restoring replication of MVA-res058 to levels observed for reference MVA-mBNbc166.

These findings show that downregulating MVA-driven transgene expression to variable degrees from strongly to even moderately has the potential to exert very dramatic effects on final yields of the respective recombinant MVA. Since strong expression of a transgene that impairs viral replication is also a strong counterselective factor for the retention of the transgene, it is likely that the respective recombinant virus will quickly accumulate mutations that will lower the expression of the respective transgene or inactivate its yield-reducing activity by point mutations or deletions. Thus, the genetic stability of a recombinant MVA with a yield-reducing transgene will likely also be strongly increased by transgene downregulation during passaging and/or production.

Final remark: Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.) are hereby incorporated by reference in their entirety. To the extent, the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

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Sequences

SEQ ID NO: 1 Nucleic acid sequence of TetO2

TCCCTATCAGTGATAGAGA

SEQ ID NO: 2 Nucleic acid sequence of 2xTetO2

TCCCTATCAGTGATAGAGATATCCCTATCAGTGATAGAGA

SEQ ID NO: 3 Nucleic acid sequence of TetR gene

ATGTCTAGATTAGATAAAAGTAAAGTGATTAACAGCGCATTAGAGCTGCTTAATGAGGTC
GGAATCGAAGGTTTAACAACCCGTAAACTCGCCCAGAAGCTAGGTGTAGAGCAGCCTA
CATTGTATTGGCATGTAAAAAATAAGCGGGCTTTGCTCGACGCCTTAGCCATTGAGATG
TTAGATAGGCACCATACTCACTTTTGCCCTTTAGAAGGGGAAAAGCTGGCAAGATTTTTTA
CGTAATAACGCTAAAAGTTTTAGATGTGCTTTACTAAGTCATCGCGATGGAGCAAAAGTA
CATTTAGGTACACGGCCTACAGAAAAACAGTATGAAACTCTCGAAAATCAATTAGCCTTT
TTATGCCAACAAAGGTTTTTCACTAGAGAATGCATTATATGCACTCAGCGCTGTGGGGCA
TTTTACTTTAGGTTGCGTATTGGAAGATCAAGAGCATCAAGTCGCTAAAGAAGAAAGGG
AAACACCTACTACTGATAGTATGCCGCCATTATTACGACAAGCTATCGAATTATTTGATC
ACCAAGGTGCAGAGCCAGCCTTCTTATTCGGCCTTGAATTGATCATATGCGGATTAGAA
AAACAACCTAAATGTGAAAGTGGGTCCGCGTACAGCGGATCCCGGGAATTCAGATCTTA
TTAA

SEQ ID NO: 4 Amino acid sequence encoded by TetR gene

MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAIEMLDL
HHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYETLENQLAFLCQ
QGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSMPPLLRQAIELFDHQGAE
PAFLFGLLELIICGLEKQLKCESGSAYS SRSREFRSY

SEQ ID NO: 5 Nucleic acid sequence encoding LMP1/EBNA2 fusion protein

ATGGAACACGACCTGGAAAGAGGTCCACCTGGACCTAGAAGGCCTCCTAGAGGACCTC
CTCTGAGCAGCTCTCTGGGACTGGCACTCCTTCTGCTACTGCTGGCTCTCCTGTTCTG
GCTGTACATCGTGATGAGCGACTGGACAGGTGGAGCCCTGCTGGTGCTGTACTCCTTT
GCCCTGATGCTGATTATCATTATCCTGATCATCTTCATCTTCCGACGGGACCTGCTGTG
CCCTCTGGGAGCACTGTGTATCCTACTGCTTATGATCACACTTCTGCTAATCGCTCTGT
GGAACCTGCACGGACAGGCTCTGTTTCTGGGCATCGTGCTGTTTCATATTCGGCTGTCT
GCTGGTGCTGGGCATCTGGATCTACCTGCTGGAAATGCTGTGGAGGCTGGGTGCCAC
AATCTGGCAGCTGCTGGCTTTCTTCTGGCCTTCTTTCTGGACCTGATCCTGCTGATTA
TTGCCCTGTACCTGCAGCAGAACTGGTGGACACTGCTGGTGGATCTGCTGTGGCTGCT
GCTGTTCTCTAGCCATCCTGATCTGGATGTACTACACGGACAGAGACATTAGATGAAC
ACCATCATCCCACTTCTACCTGGCTCTGCATGGAGGCCAGACCTACCACCTGATCGT
GGACACAGACAGCCTGGGCAATCCAGCCTGAGCGTGATCCCTAGCAACCCATATCAG
GAACAGCTGAGCGACACACCACTGATCCCTCTGACCATCTTCGTTGGCGAGAACACCG
GAGTGCCACCTCCACCACCTCCACAGAGAAGAGATGCCTGGACCCAGGAACCCAGCC

CACTGGACAGAGATCCTCTGGGCTATGATGTGGGTACGGACCTCTGGCCAGTGCCAT
GAGGATGCTGTGGATGGCCAACTACATCGTGAGGCAGAGCAGAGGCGACAGAGGCCT
GATTCTGCCTCAGGGACCTCAGACAGCTCCTCAGGCTAGACTGGTGCAGCCTCATGTT
CCTCCACTGAGGCCTACCGCTCCTACCATCTGAGCCCACTGTCCCAGCCCAGACTGA
CCCCTCCTCAGCCACTGATGATGCCTCCTAGACTTGAGCTGGAACCTACTCCTCCTACA
CCACTGCCACCAGCCACACTGACAGTGCCTCCCAGACCCACAAGACCCACCACCTTAC
CTCCAACGCCTCTGCTGACTGTGCTGCAGAGGCCAACCGAGCTGCAGCCTACACCTAG
CCCACCTAGAATGCATCTGCCAGTGTGTCACGTGCCCCGACCAGTCTATGCACCCTCTG
ACCCACCAGAGCACACCCAACGACCCTGATAGCAGAGCAAGAGCTCCTGAGCCTAGAA
GTCCCACCGTGTTCTACAACATCCCACCTATGCCGCTCCCTCCATCTCAGCTGCCTCCT
CCTGCTGCTCCTGCTCAGCCTCCTCCTGGCGTGATCAACGATCAGCAGCTGCACCATC
TGCCCAGCGGTCTCCATGGTGGCCACCTATTTGTGACCCTCCTCAGCCCAGCAAGAC
CCAGGCCAGAGCAGAAAGAGCAGAGACAAGCAGAGAGAAAGCCTGGAGGTCCTTGAGAG
ACCCGAGCCTAATACCAGCAGTCCCAGCATGCCTGAGCTGAGCCCTGTGCTGGGACTG
CACCAGGGACAGGGTGCTGGCGATTCTCCTACACCAGGACCTTCTAATGCAGCTCCTG
TGTGCAGAAACAGCCACACAGCCACTCCCAACGTGTCACCAATTCACGAGCCTGAGAG
CCACAACAGCCCAGAGGCTCCTATCCTGTTCCCAGACGACTGGTATCCTCCCAGC

SEQ ID NO: 6

Amino acid sequence of LMP1/EBNA2 fusion protein

MEHDLERGPPGPRRPPRGPPPLSSSLGLALLLLLLLALLFWLYIVMSDWTGGALLVLYSFALML
IIIIIFIFRRDLLCPLGALCILLMITLLLIALLWNLHGQALFLGIVLFIFGCLLVLGIIWYLLLEMLWR
LGATIWQLLAFFLAFFLDLILLIALLYLQQNWWTLLVDLLWLLFLAILIWMYYHGRHSDEHH
HPTFYALHGGQTYHLIVDTDSLGNPSLSVIPSNPYQEQLSDTPLIPLTIFVGENTGVPPPPP
PQRRDAWTQEPSPLDRDPLGYDVGHGPLASAMRMLWMANYIVRQSRGDRGLILPQGPQT
APQARLVQPHVPLRPTAPTILSPLSQPRLTPPQPLMMPRLELEPTPTPLPPATLTVPPR
PTRPTTLPTPLLTVLQRPTLQPTSPPRMHLVLPDQSMHPLTHQSTPNPDPSRARA
PEPRSPTVFYNIPPMPLPPSQLPPAAPAQPPPGVINDQQLHHLPSGPPWWPPICDPPQPS
KTQGQSRKSRDKQRKPGGPWRPEPNTSSPSMPELSPVLGLHQGGGAGDSPTPGPSNAA
PVCNRNHSHTATPNVSPHIEPESHNSPEAPILFPDDWYPPS

SEQ ID NO: 7

Nucleic acid sequence of EGFP gene

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTTCGAGCTG
GACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGC
CACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC
TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCACTGCTTCAGCCGCTACCCC
GACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGG
AGCGCACCATCTTCTTCAAGGACGACGGCACTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGA
CGGCAACATCCTGGGGCACAAGCTGGAGTACAACACAACAGCCACAACGTCTATATC
ATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAGTCAAGATCCGCCACAACATCG
AGGACGGCAGCGTGACGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACG
GCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAG
ACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGA
TCACTCTCGGCATGGACGAGCTGTACAAG

SEQ ID NO: 8

Amino acid sequence encoded by EGFP gene

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTL
VTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLV
NRIELKGIDFKEDGNILGHKLEYNNSHNVIYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQ
QNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELK

SEQ ID NO: 9

Nucleic acid sequence encoding gpt-mRFP fusion protein

ATGAGCGAAAAATACATCGTCACCTGGGACATGTTGCAGATCCATGCACGTAAACTCGCA
AGCCGACTGATGCCTTCTGAACAATGGAAGGCATTATTGCCGTAAGCCGTGGCGGTCT
GGTACCGGGTGCCTTACTGGCGCGTGAAGTGGGTATTCTGTCATGTCGATACCGTTTGT
TTCCAGCTACGATCACGACAACCAGCGCGAGCTTAAAGTGTGAAACGCGCAGAAGG
CGATGGCGAAGGCTTCATCGTTATTGATGACCTGGTGGATACCGGTGGTACTGCGGTG
CGATTCGTGAAATGTATCCAAAAGCGCACTTTGTCAACCATCTTCGAAAACCGGCTGGT
CGTCCGCTGGTTGATGACTATGTTGTTGATATCCCGCAAGATACCTGGATTGAACAGCCG
TGGGATATGGGCGTCGTATTCTGCCCGCAATCTCCGGTCGCGGGCCGCGCCCTCCT
CCGAGGACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAA
CGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCC
AGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCTGCCCTTCGCCTGGGACATCC
TGTCCCTCAGTTCAGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCGACATCCC
CGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAGTTC
GAGGACGGCGGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGACGGCGAGTTC
ATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCGTAATGCAGA
AGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGGATGTACCCGAGGACGGCGCCC
TGAAGGGCGAGATCAAGATGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCCG
AGGTCAAGACCACCTACATGGCCAAGAAGCCCGTGACGCTGCCCGGCGCCTACAAGAC
CGACATCAAGCTGGACATCACCTCCACAACGAGGACTACACCATCGTGGAACAGTACG
AGCGCGCCGAGGGCCGCCACTCCACCGGCGCCTAA

SEQ ID NO: 10

Nucleic acid encoding npt II

ATGGGATCGGCCATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGG
AGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGT
GTTCCGGCTGTCAGCGCAGGGGCGCCCGTTCTTTTTGTCAAGACCGACCTGTCCGGT
GCCCTGAATGAACTGCAGGACGAGGACGCGCGGCTATCGTGGCTGGCCACGACGGGC
GTTCTTGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTAT
TGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGT
ATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCAT
TCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCT
TGTCGATCAGGATGATCTGGACGAAGACATCAGGGGCTCGCGCCAGCCGAAGTTC
GCCAGGCTCAAGGCGCGCATGCCGACGCGGATGATCTCGTGTGACCCATGGCGAT
GCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTATCGACTGTGG
CCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCT
GAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCGTGCTTTACGGTATCGCCGCTC
CCGATTGCGAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA

SEQ ID NO: 11 PrS promoter

AAAAATTGAA ATTTTATTTT TTTTTTTTGG AATATAAATA

SEQ ID NO: 12 PrH5m promoter

TACTTAAAAA TTGAAAATAA ATACAAAGGT TCTTGAGGGT TGTGTAAAT
TGAAAGCGAG AAATAATCAT AAATAATTC ATTATCGCGA TATCCGTTAA
GTTTGTATCG TA

Claims

1. A recombinant poxvirus comprising a nucleotide sequence comprising a virus yield-reducing transgene operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.
2. A transcription unit comprising a nucleotide sequence comprising a virus yield-reducing transgene operably linked to a poxviral promoter, the nucleotide sequence further comprising a binding sequence for a transcriptional repressor protein, which binding sequence is located between the poxviral promoter and an open reading frame (ORF) of the transgene.
3. The recombinant poxvirus of claim 1, or the transcription unit of claim 2, wherein the poxviral promoter is a mainly or completely late promoter, or an intermediate promoter.
4. The recombinant poxvirus of claim 3, or the transcription unit of claim 3, wherein the mainly or completely late promoter is selected from the group consisting of promoters Pr11, Pr7,5, PrSSL, PrATI, and PrS, preferably the poxviral promoter is mainly late promoter PrS.
5. The recombinant poxvirus of claim 1, or the transcription unit of claim 2, wherein the poxviral promoter is a mainly early promoter, preferably selected from the group consisting of promoters Pr13.5long/Pr13.5, PrHyb, Pr1328, and PrH5m, more preferably the poxviral promoter is mainly early promoter PrH5m.
6. The recombinant poxvirus of anyone of claims 1 and 3 to 5, or the transcription unit of anyone of claims 2 to 5, wherein the transcriptional repressor protein is one of the tetracycline repressor (TetR) or lactose repressor (LacR) family, preferably of the tetracycline repressor (TetR) family, more preferably the transcriptional repressor protein is a tetracycline repressor (TetR) protein.
7. The recombinant poxvirus of anyone of claims 1 and 3 to 6, or the transcription unit of anyone of claims 2 to 6, wherein the binding sequence for a transcriptional repressor protein comprises two copies of a Tet operator 2 (TetO2) nucleotide sequence

arranged in tandem and separated by a 2-base spacer, wherein the TetO2 nucleotide sequence preferably is according to SEQ ID NO: 1.

8. The recombinant poxvirus of anyone of claims 1 and 3 to 7, or the transcription unit of anyone of claims 2 to 7, wherein the binding sequence for a transcriptional repressor protein comprises or consists of a nucleic acid sequence according to SEQ ID NO: 2.
9. The recombinant poxvirus of anyone of claims 1 and 3 to 8, or the transcription unit of anyone of claims 2 to 8, wherein the yield-reducing transgene encodes a disease-associated antigen, preferably an antigen associated with an infectious disease or cancer.
10. The recombinant poxvirus of claim 9, or the transcription unit of claim 9, wherein the disease-associated antigen is derived from Epstein-Barr virus (EBV), or an antigenic part thereof, preferably selected from the group consisting of EBV proteins BLLF1a/b (gp350/220), BALF4 (gB, gp110), BXLF2 (gH, gp85), BKRF2 (gL, gp25), BZLF2 (gp42), BILF2 (gp78), BDLF3 (gp150), BBRF3 (gM), BLRF1 (gN), BMRF2, EBNA1, EBNA2, EBNA3, LMP1, LMP2, BRLF1 or BZLF1 protein.
11. The recombinant poxvirus of anyone of claims 1 and 3 to 10, which is a recombinant vaccinia virus, preferably is a recombinant Modified Vaccinia Virus Ankara (MVA).
12. A transgenic poxvirus producer cell which is derived from a continuous poxvirus permissive cell line, wherein the transgenic cell is genetically modified to express a transcriptional repressor protein.
13. The transgenic poxvirus producer cell of claim 12, wherein the continuous poxvirus permissive cell line is an avian cell line, preferably is a chicken, duck, or quail cell line, more preferably is chicken DF-1 or quail CCX cell line.
14. The transgenic poxvirus producer cell of claim 12 or 13, which is a vaccinia virus producer cell, preferably is an MVA producer cell.
15. The transgenic poxvirus producer cell of anyone of claims 12 to 14, wherein the transcriptional repressor protein is one of the tetracycline repressor (TetR) or lactose repressor (LacR) family, preferably of the tetracycline repressor (TetR) family, more preferably the transcriptional repressor protein is a tetracycline repressor (TetR) protein.
16. Use of a transgenic poxvirus producer cell of anyone of claims 12 to 15 for propagating a recombinant poxvirus of anyone of claims 1 and 3 to 11, wherein the binding

sequence for a transcriptional repressor protein comprised by the recombinant poxvirus is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, and wherein the transcriptional repressor protein expressed by the transgenic poxvirus producer cell is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus.

17. A recombinant poxvirus of anyone of claims 1 and 3 to 11, which has been propagated in a transgenic poxvirus producer cell of anyone of claims 12 to 15, wherein the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell, and wherein the transcriptional repressor protein expressed by the transgenic poxvirus producer cell is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus.
18. A process for propagating recombinant poxvirus, comprising the steps of:
 - (1) providing a recombinant poxvirus of anyone of claims 1 and 3 to 11;
 - (2) providing a transgenic poxvirus producer cell of anyone of claims 12 to 15;
 - (3) infecting the transgenic cell provided in step (2) with the recombinant poxvirus provided in step (1);
 - (4) cultivating the transfected cell of step (3) in order to propagate the recombinant poxvirus;
 - (5) harvesting the recombinant poxvirus propagated in step (4);wherein the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus provided in step (1) is capable of binding the transcriptional repressor protein expressed by the transgenic poxvirus producer cell provided in step (2), and wherein the transcriptional repressor protein expressed by the transgenic poxvirus producer cell provided in step (2) is capable of binding to the binding sequence for a transcriptional repressor protein comprised by the recombinant poxvirus provided in step (1).
19. A pharmaceutical composition or a vaccine comprising the recombinant poxvirus of anyone of claims 1 and 3 to 11, optionally further comprising a pharmaceutically acceptable carrier or excipient.
20. A recombinant poxvirus of anyone of claims 1 and 3 to 11 for use in the treatment or prevention of an infectious disease or cancer.

21. The recombinant poxvirus of claim 20, wherein the infectious disease is Epstein-Barr virus (EBV) infection.
22. Use of a transcriptional repressor protein and its corresponding binding sequence for control of a mainly or completely late, or an intermediate poxviral promoter driven transgene expression by a recombinant poxvirus, preferably a recombinant vaccinia virus, more preferably a recombinant MVA.