Virus Research 181 (2014) 43–52

Contents lists available at ScienceDirect

Virus Research

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

# Antibiotic-dependent expression of early transcription factor subunits leads to stringent control of vaccinia virus replication

Caitlin J. Hagen [1], Allison Titong, Ethan A. Sarnoski, Paulo H. Verardi *

*Department of Pathobiology and Veterinary Science and Center of Excellence for Vaccine Research, College of Agriculture and Natural Resources, University of Connecticut, Storrs, CT 06269, United States*

ARTICLE INFO

*Article history:*
Received 21 July 2013
Received in revised form
12 December 2013
Accepted 17 December 2013
Available online 3 January 2014

*Keywords:*
Vaccinia virus
Viral vectors
Safety
Vaccines and therapeutic vectors
Antibiotics
Vaccinia early transcription factor

ABSTRACT

The use of vaccinia virus (VACV) as the vaccine against variola virus resulted in the eradication of smallpox. VACV has since been used in the development of recombinant vaccine and therapeutic vectors, but complications associated with uncontrolled viral replication have constrained its use as a live viral vector. We propose to improve the safety of VACV as a live-replicating vector by using elements of the *tet* operon to control the transcription of genes that are essential for viral growth. Poxviruses encode all enzymes and factors necessary for their replication within the host cell cytoplasm. One essential VACV factor is the vaccinia early transcription factor (VETF) packaged into the viral core. This heterodimeric protein is required for expression of early VACV genes. VETF is composed of a large subunit encoded by the A7L gene and a small subunit encoded by the D6R gene. Two recombinant VACVs were generated in which either the A7L or D6R gene was placed under the control of *tet* operon elements to allow their transcription, and therefore viral replication, to be dependent on tetracycline antibiotics such as doxycycline. In the absence of inducers, no plaques were produced but abortively infected cells could be identified by expression of a reporter gene. In the presence of doxycycline, both recombinant viruses replicated indistinguishably from the wild-type strain. This stringent control of VACV replication can be used for the development of safer, next-generation VACV vaccines and therapeutic vectors. Such replication-inducible VACVs would only replicate when administered with tetracycline antibiotics, and if adverse events were to occur, treatment would be as simple as antibiotic cessation.



## 1. Introduction

Vaccinia virus (VACV) is the prototype member of the genus *Orthopoxvirus* within the family *Poxviridae*. This genus also includes cowpox virus, monkeypox virus, and most notably variola virus, the causative agent of smallpox. Immunization of humans with VACV was used to successfully eradicate naturally occurring smallpox worldwide (Wehrle, 1980). Since then, VACV has been utilized as a viral vector for the development of recombinant vaccines for humans and animals, as well as cancer immunotherapies and oncolytic therapies. In addition, there is still significant interest in the development of next-generation smallpox vaccines to be used in case of a bioterrorist event or the emergence of other orthopoxvirus threats. However, the safety of live VACV vectors is always a concern, as uncontrolled replication can result in complications from vaccination or therapeutic use. Although VACV has not been associated with any disease, it can cause mild to severe complications that include accidental infection, generalized vaccinia, eczema vaccinatum, progressive vaccinia, and post-vaccinial encephalitis (Casey et al., 2005; Fenner et al., 1988; Fulginiti et al., 2003; Henderson et al., 1999; Lane and Goldstein, 2003a). The occurrence of complications has been correlated with pre-existing conditions such as atopic dermatitis, cardiac disease, and immunosuppression due to infection (e.g., HIV/AIDS) or drug therapy. Consequently, individuals with such conditions or with contacts that have these conditions are contraindicated for vaccination or treatment with replication-competent VACVs (Kemper et al., 2002; Lane and Goldstein, 2003b).

A number of strategies have been attempted to enhance the safety of VACV vectors, including the selection of natural strains

*Abbreviations:* VACV, vaccinia virus; VETF, vaccinia early transcription factor; TET, tetracycline; DOX, doxycycline; ATC, anhydrotetracycline; DPI, days post-infection.

* Corresponding author at: Department of Pathobiology and Veterinary Science, University of Connecticut, 61 N Eagleville Road, Storrs, CT 06269-3089, United States. Tel.: +1 860 486 3420; fax: +1 860 486 2794.
*E-mail address:* paulo.verardi@uconn.edu (P.H. Verardi).
[1] Present address: Novartis Vaccines and Diagnostics, Cambridge, MA, United States.

0168-1702 
http://dx.doi.org/10.1016/j.virusres.2013.12.033

of lower virulence such as those derived from the New York City Board of Health (NYCBH) strain, the development of highly attenuated strains such as modified vaccinia Ankara (MVA), NYVAC, and LC16m8 (Drexler et al., 1998; Kenner et al., 2006; Tartaglia et al., 1992), inactivation of virulence factors such as the thymidine kinase gene (Buller et al., 1985), deletion of immunomodulatory and other non-essential viral genes (Legrand et al., 2004; Verardi et al., 2001), and expression of attenuating genes such as cytokines (Flexner et al., 1987; Giavedoni et al., 1992; Grigg et al., 2013; Legrand et al., 2005; Ramshaw et al., 1987). However, these safety improvements can lead to a reduction in the effectiveness of the vectors. For example, vaccine efficacy is typically compromised with attenuation, next-generation smallpox vaccines must provide immunogenicity in clinical trials equivalent to current licensed vaccines such as ACAM2000 (a clone derived from NYCBH) (Greenberg and Kennedy, 2008), the effectiveness of oncolytic VACV vectors is contingent upon the replication-competence of the vector, and expression of cytokines can lead to unforeseen immune activation and complications (Bakacs et al., 2012; Tisoncik et al., 2012).

Here we propose an approach to generate replication-inducible VACV vectors that are significantly safer, yet replicate to the same levels as their parental strains, and therefore maintain their full immunogenic and oncolytic potential. These replication-inducible VACV vectors, based on elements of the transposon Tn*10* operon that confers tetracycline (TET) resistance in bacteria, would replicate only in the presence of tetracyclines. Tetracyclines such as TET and doxycycline (DOX) are commonly used broad-spectrum antibiotics that inhibit translation in gram-positive, gram-negative, and atypical bacteria by binding to the 30S bacterial ribosome subunit (Chopra and Roberts, 2001). In the *tet* operon, the Tet repressor (TetR) is unable to bind to *tet* operators in the presence of tetracyclines, allowing transcription of the efflux gene that confers resistance to tetracyclines (Hillen and Berens, 1994). The *tet* operon has been adapted to a variety of organisms for inducible gene expression, including prokaryotes, yeast, insect, plant, and mammalian cells, and transgenic organisms (Bertram and Hillen, 2008; Faryar and Gatz, 1992; Gatz and Quail, 1988; Stebbins et al., 2001; Stieger et al., 2009; Yao et al., 1998; Zhu et al., 2002). The *tet* system has also been adapted to control gene expression in a number of viruses (Cunningham et al., 2010; Gall et al., 2007; Hedengren-Olcott and Hruby, 2004; Yamaguchi et al., 2012), and in some instances for conditional replication of herpesviruses, adenoviruses, and retroviruses (Legrand et al., 2012; Manoussaka et al., 2013; Yao and Eriksson, 1999; Yao et al., 2010; Zhang et al., 2009). In particular, inducible VACVs have been generated by expressing the TetR gene (*tetR*) constitutively and inserting a *tet* operator element ($O_2$) immediately after the transcriptional start sites of VACV genes, enabling their expression to be regulated by tetracyclines (Traktman et al., 2000; Unger and Traktman, 2004). Using this system, inducible genes are expressed at minimal levels in the absence of inducer and at high levels in the presence of TET or DOX, without any apparent effect on VACV replication (Grigg et al., 2013; Traktman et al., 2000). Therefore, we propose to improve the safety of VACV as a live-replicating vector by using elements of the *tet* operon to control the transcription of genes that are essential for viral growth. These replication-inducible VACVs would replicate to wild-type levels in the presence of low concentrations of tetracyclines and would be unable to replicate in the absence of these antibiotics, thus allowing treatment of adverse events resulting from uncontrolled replication of the virus to be as simple as antibiotic cessation.

The vaccinia early transcription factor (VETF) is a heterodimeric protein required for the expression of early VACV genes (Broyles et al., 1988). VETF is composed of an 82 kDa large subunit encoded by the A7L gene and a 70 kDa small subunit encoded by the D6R gene (Broyles and Fesler, 1990; Gershon and Moss, 1990). The VACV A7L gene is referred to as A8L in some literature; currently the A8 open reading frame is considered to be A8R and to encode a subunit of an intermediate transcription factor (Sanz and Moss, 1999). The VETF subunits are produced late in infection and packaged into virions to be used in the next round of replication. VETF provides early promoter specificity by binding to early promoters and recruiting the RNA polymerase (Baldick et al., 1994; Li and Broyles, 1993b). A7 interacts with the core region of the early promoter while D6 interacts with the region downstream from the transcriptional start site (Cassetti and Moss, 1996). D6 also contains the DNA-dependent ATPase activity of the transcription factor (Li and Broyles, 1993a). Both D6R and A7L genes have been shown to be essential for VACV replication (Hu et al., 1996, 1998; Li et al., 1994; Yang and Moss, 2009). Thus, we used elements of the *tet* operon to design and construct VACVs that inducibly express the D6R or A7L genes. We evaluated and characterized the growth properties of these VACVs and showed that these viruses fail to replicate in the absence of tetracyclines, but replicate indistinguishably from wild-type VACV in the presence of tetracycline antibiotics.

## 2. Materials and methods

### 2.1. Cells and viruses

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). African green monkey BS-C-1 (CCL-26) and human HeLa-S3 (CCL-2.2) cells were grown in Dulbecco's modified Eagle medium (D-MEM; Life Technologies, Gaithersburg, MD) supplemented with 10% tetracycline-tested fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). All cells were grown at 37 °C in 5% $CO_2$. The L-variant of VACV strain Western Reserve (WR) was obtained from ATCC (VR-2035) and a clone (9.2.4.8) derived by sequential plaque purification was used to generate the recombinant viruses in this study. High-titer stocks of VACV were obtained in HeLa-S3 cells and titered in BS-C-1 cells.

### 2.2. Construction of the VACV transfer vectors

The schematic representation of the VACV transfer vector backbone used for the generation of the recombinant VACVs is shown in Fig. 1C and D. The two transfer vectors were generated in multiple steps by a combination of DNA synthesis (DNA2.0, Menlo Park, CA), PCR cloning, and standard subcloning, using engineered restriction endonuclease sites (not shown) to facilitate construction. The *gpt*-EGFP fusion gene for combined xanthine–guanine phosphoribosyl transferase (*gpt*) selection and enhanced green fluorescent protein (EGFP) screening was developed by DNA synthesis of the *Escherichia coli gpt* gene (based on the sequence in plasmid pMSG, GenBank: U13860) and the EGFP gene (based on the sequence in plasmid pEGFP-1, GenBank: U55761), using a previously developed strategy (Cao and Upton, 1997). The *tetR* gene (based on GenBank: X00694) was synthesized with an internal VACV early transcriptional termination sequence (TTTTTNT) removed from the middle of the gene (Leu codon at position 358 changed from TTA to CTT) to ensure early gene expression. The tetR and *gpt*-EGFP genes were placed under back-to-back $P_{E/L}$ synthetic promoters (sequence TATTTATATTCCAAAAAAAAAAAATAAAATTTCAATTTTTAACT-GCAGTTAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATA) (Chakrabarti et al., 1997). The transfer vectors also contained the putative D6R promoter region or a modified $P_{11}$ late VACV promoter with a *tet* operator ($O_2$) (Hillen and Berens, 1994) placed immediately after the late transcriptional initiator element sequences, as shown in Table 1. Each cassette was surrounded by 600 bp of VACV genomic sequences to the left and to the right of the insertion points shown in Fig. 1B (based on GenBank: NC_006998) to direct homologous recombination and insertion

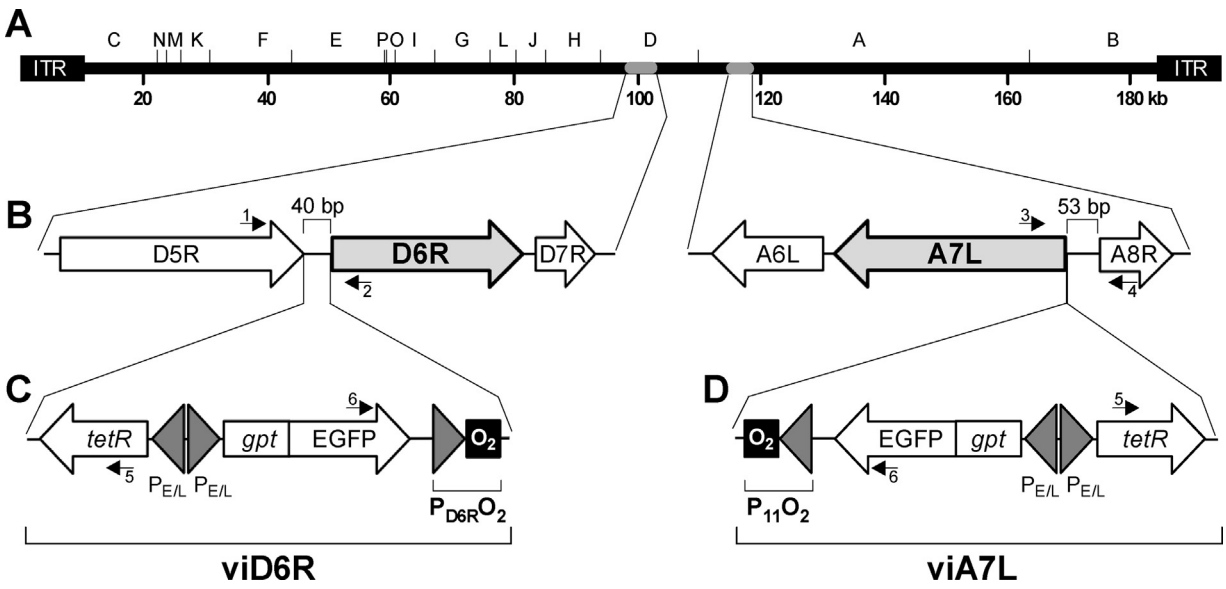


**Fig. 1.** Schematic representation of the genomic organization of the VACVs inducibly expressing D6R (viD6R) or A7L (viA7L). (A) Genome of the WR strain of VACV showing *Hin*dIII restriction fragments A through P. (B) Schematic detail of the D6R, A7L, and adjacent genes within the *Hin*dIII D and A fragments of VACV WR. (C and D) Cassettes containing the D6R promoter ($P_{D6R}$) or the F17R ($P_{11}$) promoter followed by the *tet* operator ($O_2$) were inserted upstream of the D6R or A7L genes to generate the recombinant VACVs viD6R (C) or viA7L (D). The cassettes also contain the *tetR* gene and the *gpt*-EGFP fusion gene under back-to-back synthetic early/late VACV promoters ($P_{E/L}$). Expression of TetR allows transcription from the *tet* operator-controlled promoters only in the presence of tetracyclines. Expression of the *gpt*-EGFP fusion gene allows simultaneous selection of recombinant VACVs in the presence of mycophenolic acid and screening for green fluorescent cells or plaques. Arrows with numbers indicate primers (Table 2) used to amplify specific genomic regions for characterization of the viruses. ITR, inverted terminal repeat. Panels B–D are not drawn to scale.

of the cassettes within the appropriate genomic locations. All plasmids were sequenced after synthesis or PCR cloning to confirm sequence identity.

### 2.3. Generation of VACVs and preparation of high-titer stocks

Recombinant VACVs were generated by standard homologous recombination after transfection of the transfer vectors with FuGENE HD transfection reagent (Promega, Madison, WI) into BS-C-1 cell monolayers infected 2 h previously with VACV WR clone 9.2.4.8. at a multiplicity of infection (MOI) of 0.05. Recombinant *gpt*-positive VACVs were plaque purified from transfection lysates in BS-C-1 cells using selection medium (25 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine) (Falkner and Moss, 1988) in the presence of DOX (1 μg/ml). Green fluorescent plaques were visualized under a Carl Zeiss Axio Observer D1 inverted fluorescence microscope (Oberkochen, Germany) using an XF100-2 (EGFP) filter (Omega Optical, Brattleboro, VT). All VACVs were plaque purified at least four times to eliminate contamination with the parental virus. High-titer stocks were generated by infecting HeLa S3 cells with the VACVs at an MOI of 0.1 in the presence of DOX (1 μg/ml). Infected cells were harvested 4 days post-infection (DPI) by centrifugation at 300 × *g* for 10 min and resuspension in D-MEM without tetracyclines. Cells were then lysed by freezing and thawing, sonicated, and trypsinized. Finally, cell lysates were clarified to remove contaminating cell debris by a second round of sonication and centrifugation at 500 × *g* for 10 min.

### 2.4. Analysis of recombinant VACV stability and purity

To detect any residual parental VACV after plaque purification, the expression of the EGFP gene was confirmed by fluorescence microscopy. Briefly, plaque assays were performed on BS-C-1 cell monolayers in 6-well plates in the absence or presence of DOX (1 μg/ml) using high-titer stocks. After 2 days of incubation at 37 °C, plaques were analyzed by both brightfield and fluorescence microscopy to detect any EGFP-negative plaques that could be present and would represent unstable recombinants or recombinants needing further plaque purification. The genomic organization of each recombinant VACV around the insertion site was checked by PCR analysis of viral DNA purified using a small-scale method employing micrococcal nuclease (Lai and Chu, 1991).

**Table 1**
Sequence of the natural D6R and A7L promoters and the *tet* operator-controlled promoters used to generate the inducible VACVs.

| Promoter[a] | Sequence[b] |
|---|---|
| D6R natural | ATATATGCTCATATATTTATAGAAGATATCACATATC TAAATG |
| $P_{D6R}O_2$ | ATATATGCTCATATATTTATAGAAGATATCACATATC TAAATA TCCCTATCAGTGATAGAGA CGGCCG**ATG** |
| A7L natural | TGTAAGACTTACATCATCGGTAGTAGATTTTCACTTTACCCCACGATA TAAATA**TG** |
| $P_{11}O_2$ | ATATAGTAGAATTTCATTTTGTTTTTTTCTATGCTA TAAATA TCCCTATCAGTGATAGAGA CGGCCG**ATG** |

[a] The putative natural promoters are defined as the intergenic regions upstream from the D6R and A7L genes. The $O_2$-controlled promoters are based on the natural D6R promoter or the F17R ($P_{11}$) promoter.
[b] The promoter sequences are shown with the putative late transcriptional initiator element sequences boxed, the start codons bolded, and the $O_2$ operator sequences underlined.

**Table 2**
Primers used to amplify wild-type and recombinant VACV genomic regions.

| Primer | Gene | Sequence (5′–3′)[a] |
|---|---|---|
| 1 | D5R | CCTACACCCGAAGAGATTCC |
| 2 | D6R | AAGCTACCAAGGCGAACAAC |
| 3 | A7L | ATTGAGTCATTCGAAGCGTC |
| 4 | A8R | TGGTATCCGAGTTTTTCTTG |
| 5 | *tetR* | GACGCCTTAGCCATTGAGAT |
| 6 | EGFP | ACAACCACTACCTGAGCACC |
| 7 | I8R (forward) | ATTTTCCAATTCCGTAGGTAAACGA |
| 8 | I8R (reverse) | TGATCATGCTCATGAACTTCGTCTA |
| 9 | D6R (forward) | ATCTAACGGCCGATGAATACCGGAAT |
| 10 | D6R (reverse) | TCAGTTGCTAGCTTATGGAGAAGATA |
| 11 | A7L (forward) | ATAAATCGGCCGATGCGATATATAGT |
| 12 | A7L (reverse) | AGTTGTGCTAGCTTAATTTATTTGTG |

[a] Engineered restriction endonuclease sites are underlined.

The primer sequences used are shown in Table 2 and their relative locations in Fig. 1B–D. The primer combinations used for PCR analysis included 1–2, 1–5, and 2–6 for the D6R recombinant VACV, as well as 3–4, 3–6, and 4–5 for the A7L recombinant VACV. As a positive control for VACV DNA, primers 7 and 8 were used to amplify a region of the I8R gene (VACWR077).

### 2.5. The effect of DOX on plaque formation

The ability of the VACVs to replicate in the absence or presence of inducer (DOX) was investigated by standard plaque assay. Briefly, near-confluent BS-C-1 cell monolayers in 12-well plates were infected with the VACVs at approximately 40 plaque-forming units (PFU)/well in the absence or presence of 1 μg/ml DOX and incubated at 37 °C for 2, 7, or 14 days. Cells were stained and fixed in 0.5% crystal violet/20% ethanol and isolated viral plaques were imaged with a digital camera or an inverted microscope.

For the analysis of plaque formation by fluorescence microscopy, near-confluent BS-C-1 cell monolayers in six-well plates were infected with the VACVs at 50 PFU/well in the absence or presence of 1 μg/ml DOX. Plaques and infected cells were imaged at 2, 4, 6, 8, 10, and 12 DPI. In a subset of wells infected with the VACVs in the absence of inducers, DOX was added at 2, 4, 6, 8, or 10 DPI and any plaques that formed were imaged 2 days later.

### 2.6. Transient complementation assays

Plasmids expressing the D6R or A7L genes under the VACV $P_{11}$ late promoter ($pP_{11}$-D6R and $pP_{11}$-A7L, respectively) were generated by PCR cloning with primers 9–10 (D6R gene) or 11–12 (A7L gene) (Table 2). Forward primers included an *Eag*I engineered restriction endonuclease sequence preceding the start codon and reverse primers included an *Nhe*I sequence after the stop codon to allow directional cloning into a synthetic plasmid designed to contain only the $P_{11}$ promoter sequence (DNA2.0). The identity of the cloned fragments was confirmed by restriction enzyme analysis.

Near confluent BS-C-1 cell monolayers in 24-well plates were infected with viD6R or viA7L at 100 PFU/well for 1 h in the absence of tetracyclines and transfected (FuGENE HD) with 0.5 μg of plasmid $pP_{11}$-D6R or $pP_{11}$-A7L. Cells were incubated at 37 °C and plaque formation was checked 2 DPI by brightfield and fluorescence imaging. Additionally, triplicate BS-C-1 cell monolayers in 24-well plates were infected with viD6R or viA7L at an MOI of 0.01 for 1 h in the absence of DOX and then transfected (FuGENE HD) with 0.5 μg of plasmid $pP_{11}$-D6R or $pP_{11}$-A7L. Cells were collected immediately (to determine input virus) or incubated at 37 °C for 48 h in the absence or presence of 100 ng/ml of DOX. Briefly, supernatants were collected, centrifuged (to pellet detached cells), and discarded. Cells in each well were resuspended in 0.5 ml of D-MEM, scraped, and added to the pellet of detached cells, containing mostly intracellular mature virions (IMV). Virus yield was determined by plaque assay on BS-C-1 cells in the presence of 1 μg/ml of DOX, in duplicate.

### 2.7. Effect of tetracyclines on plaque size

The size of the plaques formed by the VACVs in the absence or presence of different tetracyclines was investigated by plaque assay. Briefly, near-confluent BS-C-1 cell monolayers in 12-well plates were infected with the VACVs at 30 PFU/well in the absence or presence of 0.1, 1, 10, 100, or 1000 ng/ml DOX, TET, or anhydrotetracycline (ATC) and incubated at 37 °C for 36 h. Cells were stained and fixed in 0.5% crystal violet/20% ethanol and the radius of isolated plaques was measured under an inverted microscope with measurement-capable software (AxioVision 4.8.1, Carl Zeiss).

### 2.8. Effect of DOX on viral growth kinetics

Viral growth kinetics in the absence or presence of DOX were determined by generating growth curves at a low MOI. Briefly, triplicate monolayers of near-confluent BS-C-1 cells (seeded in the absence or presence of 1 μg/ml of DOX) were infected with the VACVs at an MOI of 0.01 for 1 h in 12-well plates. Virus replication was determined as previously described (Verardi et al., 2001) in the presence or absence of DOX (1 μg/ml). For each time point, virus yield was determined by plaque assay on BS-C-1 cells in the presence of 1 μg/ml of DOX, in duplicate.

### 2.9. Statistical analyses and image processing

Statistical analyses were performed with GraphPad Prism v. 6.0c (GraphPad Software, La Jolla, CA). Images were processed in Adobe Photoshop CS6 (Adobe Systems, San Jose, CA) with no manipulations other than for contrast.

## 3. Results

### 3.1. Design of tet-responsive late promoters

Replication-inducible recombinant VACVs were designed by expressing the *tetR* gene under a constitutive VACV promoter and incorporating *tet* operator sequences immediately downstream from promoters directing the expression of the late A7L or D6R genes (Fig. 1). Late VACV promoter sequences consist of an A/T-rich stretch of approximately 20 bp, a 6 bp spacer region, and a highly conserved TAAAT(A/G) transcriptional initiator element (Davison and Moss, 1989). The D6R gene has an upstream intergenic region of 40 bp that is expected to contain only the late promoter for the D6R gene, since the preceding gene (D5R) also transcribes to the right (Fig. 1B). Therefore, this natural sequence was used to engineer a *tet*-responsive (inducible) promoter for the D6R gene ($P_{D6R}O_2$). The *tet* operator ($O_2$) sequence was inserted after the identified putative late transcriptional initiator element sequence TAAATG, which was changed to TAAATA to ensure translation initiation only from the downstream D6R open reading frame (Fig. 1C and Table 1).

The intergenic region upstream from the A7L gene is 53 bp and likely contains promoters for both the A7L and the A8R gene, since A8R is transcribed in the opposite direction (Fig. 1B). It was not possible to accurately identify and separate the sequences for these two promoters, so the well-characterized late promoter for the F17R gene ($P_{11}$), which has been used successfully as a *lac*-responsive promoter (Fuerst et al., 1989), was used to control the transcription of the A7L gene (Fig. 1 and Table 1). However, this entire intergenic region was kept in the viral genome to ensure transcription of the A8R gene, and the putative transcriptional initiator element

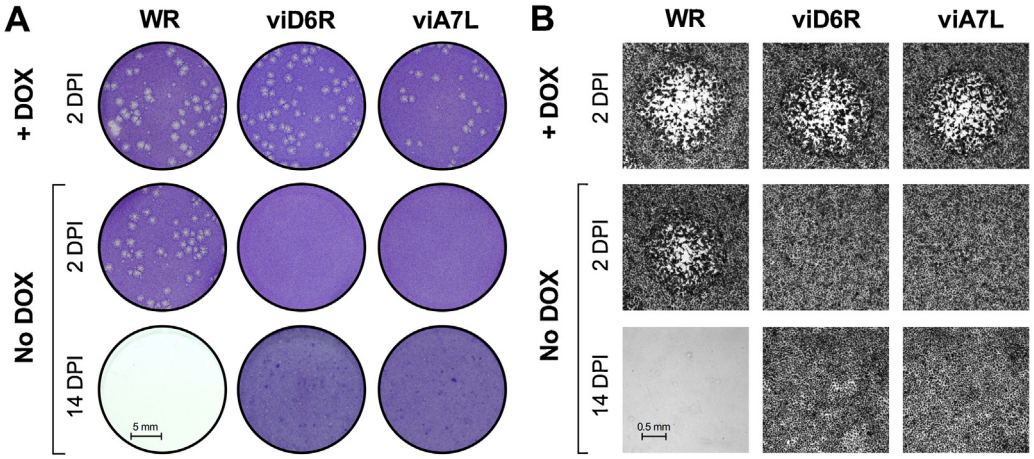


**Fig. 2.** VACVs inducibly expressing D6R or A7L form plaques only in the presence of DOX. BS-C-1 cell monolayers were infected with the indicated VACVs at approximately 40 PFU/well in the absence or presence of DOX (1 μg/ml) and cells were stained with crystal violet 2 or 14 DPI. (A) Image of representative wells showing the plaque phenotypes. (B) Representative brightfield microscopy images of stained cells showing plaques, when present. WR refers to VACV WR clone 9.2.4.8 (parental strain). Data are representative of two separate experiments.

sequence of the natural A7L promoter (Table 1) was changed from TAAATA to TAAGGA to prevent transcription initiation from that site. The resulting recombinant VACVs (viD6R for VACV inducible D6R and viA7L for VACV inducible A7L) were expected to replicate only in the presence of tetracyclines.

### 3.2. Construction of recombinant viruses

A series of cloning steps were used to build the transfer vectors based on existing plasmids, designed synthetic DNA sequences, and PCR cloning. The final transfer vectors contained: (1) the selectable *E. coli gpt* gene and the screening marker EGFP gene as a fusion gene (*gpt*-EGFP) under control of a synthetic strong early/late VACV promoter $P_{E/L}$ (Chakrabarti et al., 1997); (2) the repressor gene *tetR* under another (back-to-back) $P_{E/L}$ promoter; (3) a *tet*-responsive promoter ($P_{D6R}O_2$ or $P_{11}O_2$) to control the expression of the target gene (D6R or A7L, respectively); and (4) left and right recombination sequences (the first 600 bp to the left and to the right of the insertion points shown in Fig. 1B) to direct the precise insertion of the genetic elements contained in each cassette (Fig. 1C and D) by homologous recombination. The recombinant viruses viD6R and viA7L were successfully constructed and plaque purified in the presence of DOX. High-titer stocks did not develop EGFP-negative plaques that would represent unstable recombinant VACVs or residual parental (wild-type) virus (data not shown). In addition, PCR analysis of viral DNA purified from high-titer stocks with multiple primers spanning the regions of interest (Fig. 1B and C and Table 2) confirmed the overall genetic organization of these regions in each recombinant VACV (data not shown).

### 3.3. viD6R and viA7L form plaques only in the presence of DOX

The ability of viD6R and viA7L to replicate in the absence or presence of inducer was first investigated by performing standard plaque assays in BS-C-1 cells, either in the absence or presence of DOX (1 μg/ml), followed by crystal violet staining 2, 7, or 14 DPI. Isolated plaques formed 2 DPI in the presence of DOX were typical (Fig. 2, top row) and identical to the wild-type WR plaques formed in the absence of DOX (Fig. 2, middle row). However, no plaques could be detected 2 DPI for viD6R and viA7L in the absence of DOX (Fig. 2, middle row). Moreover, plaques were not observed in viD6R and viA7L wells even 7 (data not shown) or 14 DPI, when the entire well infected with WR displayed cytopathic effects (Fig. 2, bottom row). Small foci of darker stained cells could be observed 14 DPI (Fig. 2, bottom row), but they were likely due to the overgrowth of the cell monolayer as they were also present in uninfected control wells (not shown).

### 3.4. viD6R and viA7L produce abortive infections in the absence of DOX

Plaque formation in BS-C-1 cells by viD6R and viA7L was also investigated in unfixed cells by brightfield and fluorescence microscopy. Under fluorescence microscopy, only single EGFP-positive cells could be detected in the absence of DOX (Fig. 3A). Under high magnification, these cells were typical and there was no evidence of EGFP expression in the neighboring cells (Fig. 3B). The frequency of these cells corresponded roughly to the number of plaques obtained in the presence of DOX, where EGFP-positive plaques were observed (Fig. 3C). Taken together, the observations are indicative of abortive infections. In addition, detection of high levels of EGFP expression in these abortively infected cells suggests that late gene expression from the $P_{E/L}$ promoter was not compromised in the absence of DOX. When DOX was added to these abortively infected cells 2, 4, or 6 DPI, replication was allowed to resume and plaques were visible 2 days later (Fig. 3D shows results for addition of DOX 48 h post-infection), indicating that transcription of the D6R or A7L genes was sufficient to allow the resumption and completion of the replication cycle. However, plaques were not detected when DOX was added 8 or 10 DPI (not shown).

### 3.5. Transient complementation allows replication of viD6R and viA7L in the absence of DOX

To confirm that replication of viD6R and viA7L was dependent on the expression of the D6R or A7L genes, respectively, a transient complementation assay was performed in the absence of DOX. BS-C-1 cells were infected with viD6R or viA7L and transfected with plasmids expressing the D6R or A7L genes constitutively under the VACV $P_{11}$ promoter ($pP_{11}$-D6R or $pP_{11}$-A7L). Only single abortively infected cells could be observed 2 DPI in viD6R- or viA7L-infected wells without plasmids (Fig. 4A and B, left panels). Wells infected with viD6R and transfected with $pP_{11}$-A7L also displayed similar numbers of abortively infected cells (Fig. 4A, right panels). However, when transfected with $pP_{11}$-D6R, plaques could be detected (Fig. 4A, middle panels). Similarly, plaques were formed in wells infected with viA7L only when transfected with the plasmid constitutively expressing the A7L gene (Fig. 4B, right panels).

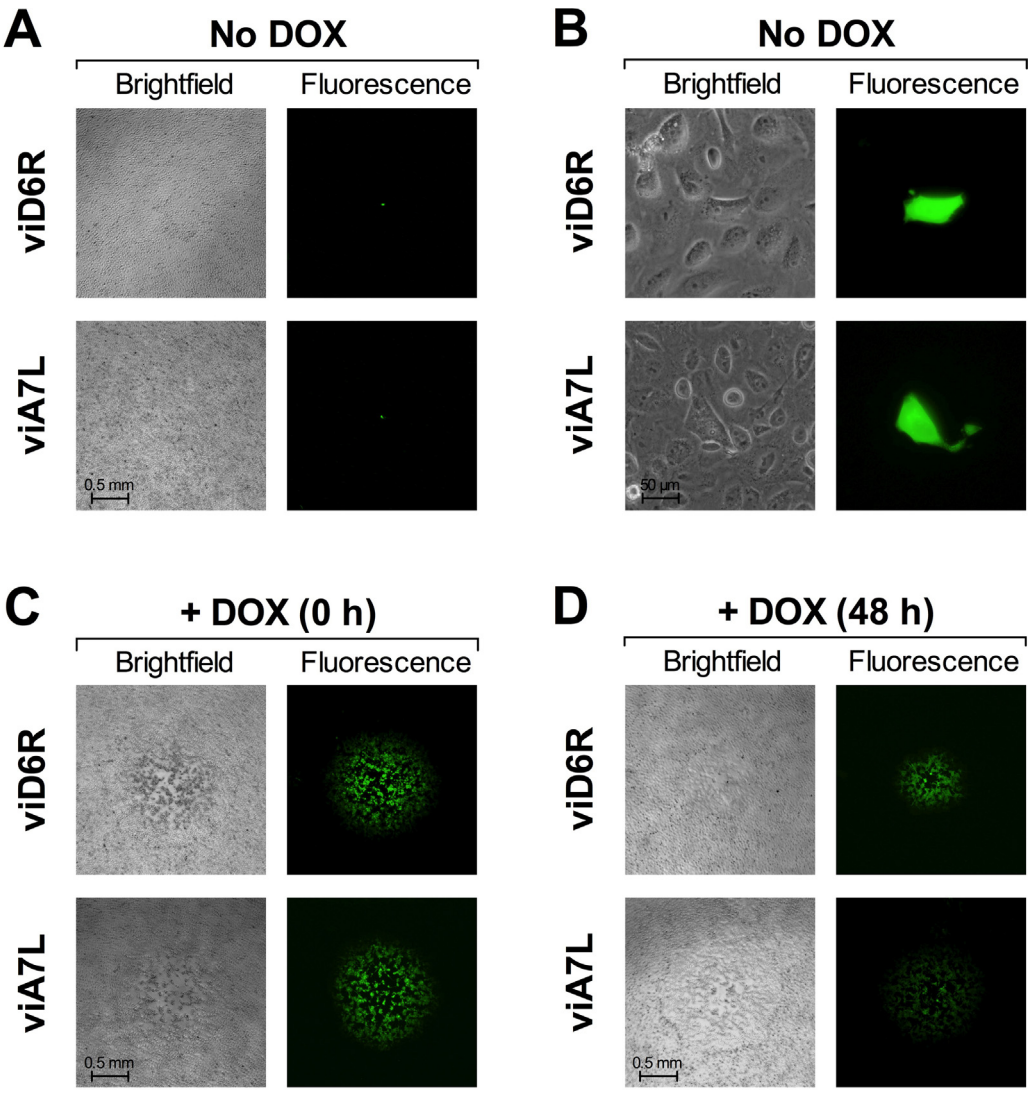


**Fig. 3.** VACVs inducibly expressing D6R or A7L produce abortive infections in the absence of DOX. BS-C-1 cell monolayers were infected with the indicated VACVs at 50 PFU/well in the absence or presence of DOX (1 μg/ml) and cells were imaged by brightfield (phase) or fluorescence microscopy. In the absence of DOX only single EGFP-positive cells were observed 2 DPI (A), and under higher magnification, EGFP expression was contained to single cells and was the only indication of infection, suggesting abortive infections. When DOX was added at the time of infection (C) or 48 h after infection (D), plaques were visible 2 days later (2 DPI in C and 4 DPI in D). Data are representative of two separate experiments.

Complementation was also assessed by measuring virus yield in the absence or presence of the complementing plasmids. Cells infected with viD6R in the absence of DOX and transfected with $pP_{11}$-D6R yielded a four-log increase in virus titers when compared to cells transfected with the non-complementing plasmid ($pP_{11}$-A7L) or with no plasmid, although the yield was not as high as that obtained with viD6R grown in the presence of DOX (Fig. 4C). A similar finding was observed with cells infected with viA7L in the presence of the complementing plasmid $pP_{11}$-A7L (Fig. 4D).

### 3.6. viD6R and viA7L plaque sizes are indistinguishable from wild-type (WR) VACV in the presence of different tetracyclines

The size of the plaques formed by WR, viD6R, and viA7L in the absence or presence of multiple concentrations of three different tetracyclines was analyzed by plaque assay in BS-C-1 cells. The radius of plaques formed by WR in the presence of the three tetracyclines was not affected by the type or dose of tetracycline tested (Fig. 5). In the presence of 0.1 ng/ml of DOX, viD6R formed small plaques, while no plaques were observed with viA7L. At 1 ng/ml of DOX, both inducible viruses formed plaques that were significantly smaller than WR plaques. However, the size of plaques formed at ≥10 ng/ml of DOX were indistinguishable from plaques formed by WR. In the presence of TET, plaques were formed only at ≥10 ng/ml, and at 100 ng/ml there was a small but statistically significant difference between viD6R and WR plaques, but not between viA7L and WR plaques. This shows that the system is less inducible with TET, which is in agreement with the fact that TetR binds TET less strongly than DOX (Degenkolb et al., 1991). In the presence of ATC, a tetracycline derivative that exhibits minimal antibiotic activity, plaque sizes at each concentration tested were similar to DOX, despite the fact that ATC binds TetR about 5-fold more strongly than DOX (Degenkolb et al., 1991). At 10 ng/ml of ATC, there was a small but statistically significant difference between viD6R plaques and WR plaques, and at 0.1 ng/ml of ATC, many abortive infections were observed (data not shown) and the plaques that formed were smaller than those formed at 0.1 ng/ml of DOX, indicating that DOX was a better inducer in this system. In brief, no plaques were observed in the absence of tetracyclines and plaque formation occurred in as little as 0.1 ng/ml (viD6R) or 1 ng/ml (viA7L) of

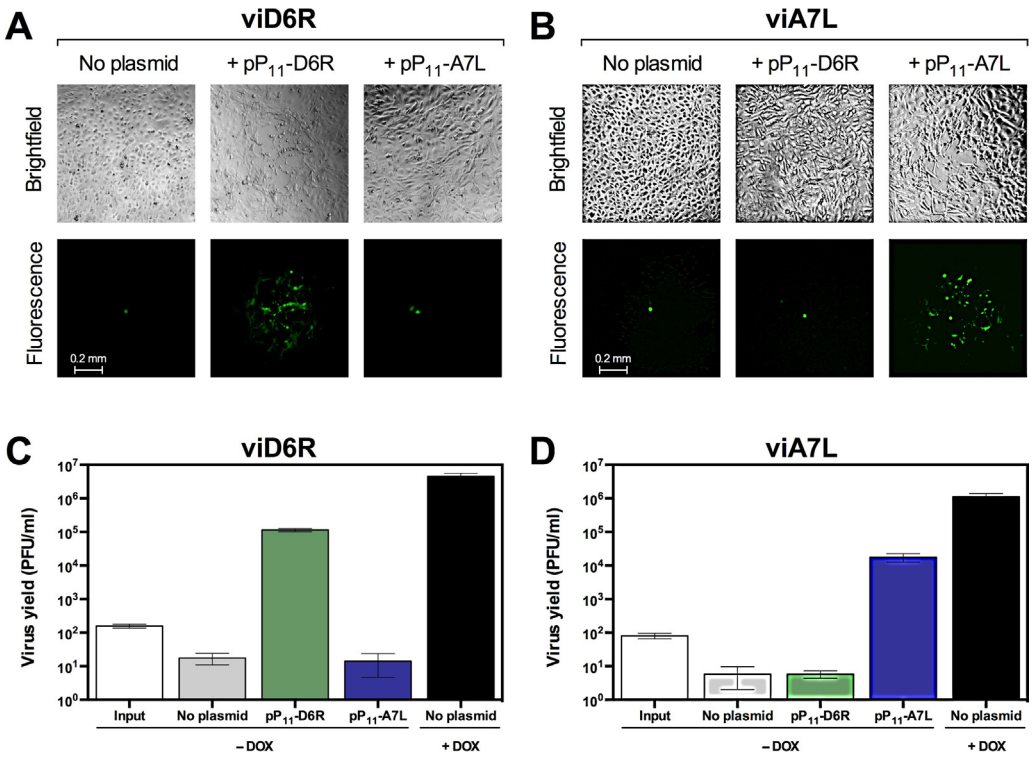


**Fig. 4.** Transient complementation allows replication of VACVs inducibly expressing D6R or A7L in the absence of DOX. BS-C-1 cell monolayers were infected with viD6R (A and C) or viA7L (B and D) at 100 PFU/well (A and B) or an MOI of 0.01 (C and D) in the absence of DOX and transfected with plasmids expressing the D6R ($pP_{11}$-D6R) or A7L ($pP_{11}$-A7L) genes under the constitutive VACV $P_{11}$ promoter. Plaque formation was checked 2 DPI by brightfield (phase) and fluorescence imaging (A and B). Virus yield was determined 2 DPI by plaque assay on BS-C-1 cells in the presence of 1 μg/ml DOX (C and D), with input referring to initial titer determined immediately after infection. The data shown represent the mean viral yields from triplicate samples assayed in duplicate. Error bars indicate standard deviation. Data are representative of two separate experiments.

DOX. Most importantly, viD6R and viA7L plaque sizes were indistinguishable from WR at ≥10 ng/ml of DOX.

### 3.7. viD6R and viA7L replicate indistinguishably from wild-type (WR) VACV in the presence of DOX

The kinetics of viral replication in vitro were determined in BS-C-1 cells infected with the VACVs at a low MOI (0.01) in the absence or presence of DOX (1 μg/ml). As expected, the wild-type WR strain replicated equally in the absence or presence of DOX (Fig. 6). Additionally, viD6R and viA7L replicated indistinguishably from WR in the presence of DOX. In the absence of DOX, virus yield by viA7L was below the input level at all time points. However, viD6R yield was very small but detectable (close to the input level at 0 h post-infection), despite the fact that only abortive infections were detected (Figs. 3 and 4). This suggests that a few mature intracellular viral particles were formed in the absence of tetracyclines with viD6R, but not viA7L.

## 4. Discussion

VACV is a promising vector for the development of recombinant vaccines for humans and animals, as well as cancer immunotherapies and oncolytic therapies (Verardi et al., 2012). VACV was used as a vaccine for the eradication of smallpox and a recombinant VACV vaccine that expresses the rabies virus glycoprotein (V-RG) has been used successfully as an oral vaccine to control sylvatic rabies in Europe and North America (Pastoret and Brochier, 1996). Additionally, multiple clinical trials for animal and human vaccines, immunotherapies, and oncolytic therapies based on replication-competent VACV vectors are underway (Verardi et al., 2012). Nonetheless, safety is always a concern due to potential serious adverse events that can result from uncontrolled VACV replication. While replication-defective VACVs such as MVA and NYVAC address safety issues, they are not as immunogenic as their replication-competent counterparts and unsuitable as oncolytic vectors. We therefore used elements of the *tet* operon to develop replication-inducible VACV vectors that replicate to wild-type levels only in the presence of tetracycline antibiotics, thus allowing control over VACV replication.

As an initial step toward the development of these replication-inducible vectors, recombinant VACVs were designed to control the expression of the genes encoding the two subunits of VETF, the early transcription factor required for virus replication. In viD6R, the $O_2$ operator sequence was placed immediately downstream from the transcriptional initiator element of the D6R promoter (Fig. 1 and Table 1). In viA7L, a late $P_{11}$ promoter with an $O_2$ operator sequence was used to control A7L gene expression, thus preserving native expression of the A8R gene from its putative promoter (Fig. 1). In addition, the *tetR* gene was constitutively expressed in both viruses using a strong synthetic early/late VACV promoter ($P_{E/L}$) (Fig. 1). We showed that viD6R and viA7L do not form plaques in the absence of DOX, even when cells were incubated for 2 weeks after infection (Fig. 2). Singly infected cells could be detected by expression of the EGFP reporter gene (Fig. 3A and B) 2 DPI, but were less evident or not detectable 4 DPI. The neighboring cells did not show any evidence of infection such as cytopathic effects or EGFP expression (Fig. 3B). High levels of EGFP expression in these abortively infected cells suggest that gene expression from the $P_{E/L}$ promoter was not compromised in the absence of DOX, in agreement with previous findings that newly synthetized VETF is not required for early or late gene expression (Hu et al., 1996, 1998). However, plaques were formed in the presence of DOX (Figs. 2 and 3C) even when DOX was added 2 (Fig. 3D), 4, or 6 DPI, indicating that the virus growth cycle

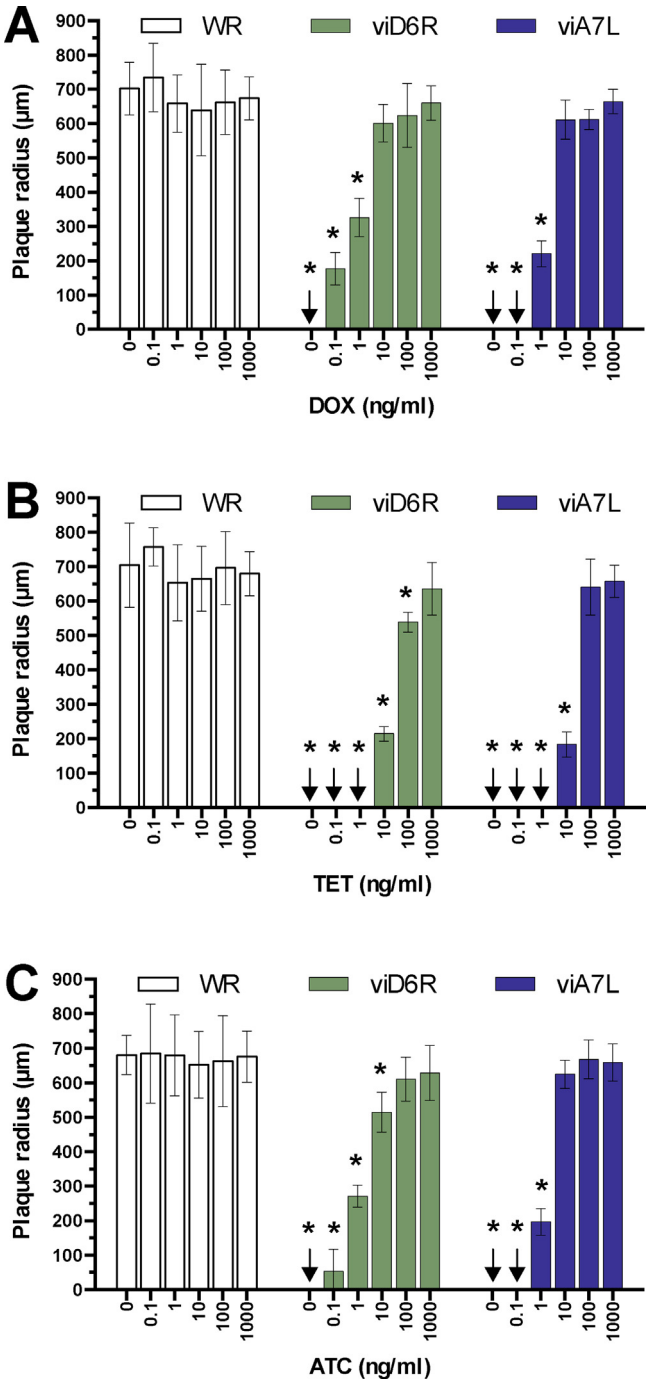


**Fig. 5.** Effect of tetracyclines on the plaque sizes of VACVs inducibly expressing D6R or A7L. BS-C-1 cell monolayers were infected with the VACVs at 30 PFU/well in the absence or presence of multiple concentrations of DOX (A), TET (B), or ATC (C). At 36 h post-infection cells were stained with crystal violet and the size (radius) of approximately 20 representative plaques was measured. Error bars indicate standard deviation. Arrows indicate absence of plaques. The symbol * indicates statistically significant differences ($P \leq 0.05$, two-way ANOVA followed by Tukey's multiple comparisons test) when comparing WR vs. viD6R or viA7L at a given tetracycline concentration. Data are representative of two separate experiments.

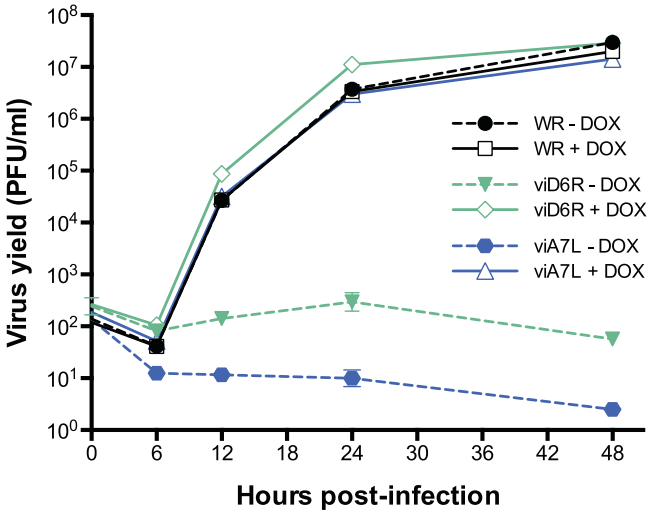


**Fig. 6.** VACVs inducibly expressing D6R or A7L replicate indistinguishably from wild-type (WR) VACV in the presence of DOX. BS-C-1 cells were infected with the indicated VACVs at a low MOI (0.01) in the absence or presence of DOX (1 μg/ml) and at multiple time points the intracellular viral fractions were collected and titered on BS-C-1 cells in the presence of DOX. The data shown represent the mean viral yields from triplicate samples assayed in duplicate. Error bars indicate standard deviation.

resumed once VETF expression was allowed. This is in agreement with a likely role of VETF in virion morphogenesis (Hu et al., 1996, 1998). Replication did not resume when DOX was added 8 or 10 DPI (not shown), possibly because the abortively infected cells or the virus life cycle were compromised this late after infection. In that regard, EGFP expression in abortively infected cells faded rather quickly between 2 and 4 DPI (not shown), indicating that expression of VACV genes was not sustained after 2 DPI.

Transient complementation assays confirmed that in the absence of DOX, viD6R and viA7L plaque formation and replication was dependent on expression of the D6R or A7L genes, respectively (Fig. 4). In addition, plaque formation was induced by different tetracyclines, including DOX, TET, and ATC, with DOX and ATC allowing plaque formation at lower concentrations (Fig. 5). Plaques were observed in as little as 0.1 ng/ml of DOX for viD6R and 1 ng/ml of DOX for viA7L, and at ≥10 ng/ml of DOX, the size of plaques formed by viD6R and viA7L were indistinguishable from plaques formed by the wild-type (WR) VACV. Most importantly, the replication-inducible viruses replicated in cell culture to the same levels as the parental wild-type WR VACV in the presence of DOX (Fig. 6).

The function of the D6R gene has been studied with temperature-sensitive mutants (Li et al., 1994). In addition, inducible D6R and A7L viruses have been generated using *lac* operon elements in a system where the bacteriophage T7 RNA polymerase is expressed under a $P_{11}$ promoter controlled by a *lac* operator element, and the D6R or A7L genes are placed under T7 promoters also controlled by a *lac* operator element (Hu et al., 1996, 1998; Yang and Moss, 2009). In this system, addition of isopropyl-β-D-thiogalactoside (IPTG) leads to T7 RNA polymerase expression and D6R or A7L transcription. Using this system, viruses inducibly expressing D6R or A7L were shown to replicate only in the presence of IPTG, although about 10-fold less efficiently than wild-type (WR virus) (Hu et al., 1996, 1998). In addition, small plaques could be detected with prolonged incubation (Hu et al., 1996), likely indicating some level of leaky expression of the inducible gene. Interestingly, viD6R was unable to produce plaques in the absence of DOX, but the virus titer of viD6R in the absence of DOX increased slightly at 12 and 24 h post-infection. However, yield was negligible when compared to viD6R grown in the presence of DOX (Fig. 6), making viD6R an excellent replication-inducible vector. These results are similar to those generated using the *lac* operon/T7 RNA polymerase system to control the expression of the D6R gene, where in the absence of inducer (IPTG) no plaques formed but the virus titer increased slightly (Hu et al., 1996).

Replication-inducible VACVs based on the inducible expression of the D6R or A7L genes with tetracycline antibiotics

have the potential to be used for the development of safer recombinant vaccines and therapeutics. Future development would require the choice of an appropriate strain of VACV (such as ACAM2000) and the generation of recombinant viruses without screening and selectable markers (e.g., by transient dominant selection) (Falkner and Moss, 1990; Papin et al., 2011). One example would be next-generation smallpox vaccines for non-emergency vaccination of military personnel and first-responders. One of the problems of the current licensed smallpox vaccines is that VACV can inadvertently be transferred to contacts. One such case occurred in 2007 when a child developed a life-threatening case of eczema vaccinatum through contact with a military smallpox vaccinee (Centers for Disease Control and Prevention, 2007). If a replication-inducible version of the vaccine had been used, inadvertent inoculation of contacts would have been avoided altogether as the virus would be unable to replicate in individuals not undergoing tetracycline antibiotic therapy. Another problem is that despite medical screening prior to vaccination, complications can result from unforeseen or unanticipated contraindications, such as the case of an apparently healthy military smallpox vaccinee that developed progressive vaccinia due to chemotherapy to control a cancer diagnosed only after vaccination (Lederman et al., 2012). If a replication-inducible version of the vaccine had been used, the simple withdrawal from tetracycline antibiotic treatment would likely have led to the clearance of VACV. Finally, replication-inducible VACVs would serve as safer oncolytic vectors, since oncolytic therapy could be tightly controlled by administration and withdrawal of antibiotics.

## Acknowledgements

This work was supported by University of Connecticut startup funds and a University of Connecticut Research Foundation (UCRF) grant awarded to PHV. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article. The authors are grateful to Caitlin O'Connell, Brittany Jasperse, John Sopronyi, and Minh Phan for their assistance and helpful discussions.

## References

Bakacs, T., Mehrishi, J.N., Moss, R.W., 2012. Ipilimumab (Yervoy) and the TGN1412 catastrophe. Immunobiology 217 (6), 583–589.
Baldick Jr., C.J., Cassetti, M.C., Harris, N., Moss, B., 1994. Ordered assembly of a functional preinitiation transcription complex, containing vaccinia virus early transcription factor and RNA polymerase, on an immobilized template,. Journal of Virology 68 (9), 6052–6056.
Bertram, R., Hillen, W., 2008. The application of Tet repressor in prokaryotic gene regulation and expression. Microbial Biotechnology 1 (1), 2–16.
Broyles, S.S., Fesler, B.S., 1990. Vaccinia virus gene encoding a component of the viral early transcription factor. Journal of Virology 64 (4), 1523–1529.
Broyles, S.S., Yuen, L., Shuman, S., Moss, B., 1988. Purification of a factor required for transcription of vaccinia virus early genes. Journal of Biological Chemistry 263 (22), 10754–10760.
Buller, R.M., Smith, G.L., Cremer, K., Notkins, A.L., Moss, B., 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317 (6040), 813–815.
Cao, J.X., Upton, C., 1997. gpt–gus fusion gene for selection and marker in recombinant poxviruses. BioTechniques 22 (2), 276–278.
Casey, C.G., Iskander, J.K., Roper, M.H., Mast, E.E., Wen, X.J., Torok, T.J., Chapman, L.E., Swerdlow, D.L., Morgan, J., Heffelfinger, J.D., Vitek, C., Reef, S.E., Hasbrouck, L.M., Damon, I., Neff, L., Vellozzi, C., McCauley, M., Strikas, R.A., Mootrey, G., 2005. Adverse events associated with smallpox vaccination in the United States, January–October 2003. JAMA 294 (21), 2734–2743.
Cassetti, M.A., Moss, B., 1996. Interaction of the 82-kDa subunit of the vaccinia virus early transcription factor heterodimer with the promoter core sequence directs downstream DNA binding of the 70-kDa subunit. Proceedings of the National Academy of Sciences of the United States of America 93 (15), 7540–7545.
Centers for Disease Control and Prevention, 2007. Household transmission of vaccinia virus from contact with a military smallpox vaccinee—Illinois and Indiana, 2007. MMWR 56 (19), 478–481.
Chakrabarti, S., Sisler, J.R., Moss, B., 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. BioTechniques 23 (6), 1094–1097.
Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews 65 (2), 232–260, second page, table of contents.
Cunningham, P.T., Lloyd, M.L., Harvey, N.L., Williams, E., Hardy, C.M., Redwood, A.J., Lawson, M.A., Shellam, G.R., 2010. Promoter control over foreign antigen expression in a murine cytomegalovirus vaccine vector. Vaccine 29 (1), 141–151.
Davison, A.J., Moss, B., 1989. Structure of vaccinia virus early promoters. Journal of Molecular Biology 210 (4), 749–769.
Degenkolb, J., Takahashi, M., Ellestad, G.A., Hillen, W., 1991. Structural requirements of tetracycline-Tet repressor interaction: determination of equilibrium binding constants for tetracycline analogs with the Tet repressor. Antimicrobial Agents and Chemotherapy 35 (8), 1591–1595.
Drexler, I., Heller, K., Wahren, B., Erfle, V., Sutter, G., 1998. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. Journal of General Virology 79 (Pt 2), 347–352.
Falkner, F.G., Moss, B., 1988. *Escherichia coli* gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. Journal of Virology 62 (6), 1849–1854.
Falkner, F.G., Moss, B., 1990. Transient dominant selection of recombinant vaccinia viruses. Journal of Virology 64 (6), 3108–3111.
Faryar, K., Gatz, C., 1992. Construction of a tetracycline-inducible promoter in Schizosaccharomyces pombe. Current Genetics 21 (4–5), 345–349.
Fenner, F., Henderson, D.A., Arita, I., Jeek, Z., Ladnyi, I.D., 1988. Smallpox and its Eradication. World Health Organization, Geneva, Switzerland, pp. 1460 pp.
Flexner, C., Hugin, A., Moss, B., 1987. Prevention of vaccinia virus infection in immunodeficient mice by vector-directed IL-2 expression. Nature 330 (6145), 259–262.
Fuerst, T.R., Fernandez, M.P., Moss, B., 1989. Transfer of the inducible lac repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector. Proceedings of the National Academy of Sciences of the United States of America 86 (8), 2549–2553.
Fulginiti, V.A., Papier, A., Lane, J.M., Neff, J.M., Henderson, D.A., 2003. Smallpox vaccination: a review. Part II: Adverse events. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 37 (2), 251–271.
Gall, J.G., Lizonova, A., EttyReddy, D., McVey, D., Zuber, M., Kovesdi, I., Aughtman, B., King, C.R., Brough, D.E., 2007. Rescue and production of vaccine and therapeutic adenovirus vectors expressing inhibitory transgenes. Molecular Biotechnology 35 (3), 263–273.
Gatz, C., Quail, P.H., 1988. Tn10-encoded tet repressor can regulate an operator-containing plant promoter. Proceedings of the National Academy of Sciences of the United States of America 85 (5), 1394–1397.
Gershon, P.D., Moss, B., 1990. Early transcription factor subunits are encoded by vaccinia virus late genes. Proceedings of the National Academy of Sciences of the United States of America 87 (11), 4401–4405.
Giavedoni, L.D., Jones, L., Gardner, M.B., Gibson, H.L., Ng, C.T., Barr, P.J., Yilma, T., 1992. Vaccinia virus recombinants expressing chimeric proteins of human immunodeficiency virus and gamma interferon are attenuated for nude mice. Proceedings of the National Academy of Sciences of the United States of America 89 (8), 3409–3413.
Greenberg, R.N., Kennedy, J.S., 2008. ACAM2000: a newly licensed cell culture-based live vaccinia smallpox vaccine. Expert Opinion on Investigational Drugs 17 (4), 555–564.
Grigg, P., Titong, A., Jones, L.A., Yilma, T.D., Verardi, P.H., 2013. Safety mechanism assisted by the repressor of tetracycline (SMART) vaccinia virus vectors for vaccines and therapeutics. Proceedings of the National Academy of Sciences of the United States of America 110 (38), 15407–15412.
Hedengren-Olcott, M., Hruby, D.E., 2004. Conditional expression of vaccinia virus genes in mammalian cell lines expressing the tetracycline repressor. Journal of Virological Methods 120 (1), 9–12.
Henderson, D.A., Inglesby, T.V., Bartlett, J.G., Ascher, M.S., Eitzen, E., Jahrling, P.B., Hauer, J., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T., Russell, P.K., Tonat, K., 1999. Smallpox as a biological weapon: medical and public health management Working Group on Civilian Biodefense. JAMA 281 (22), 2127–2137.
Hillen, W., Berens, C., 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annual Review of Microbiology 48, 345–369.
Hu, X., Carroll, L.J., Wolffe, E.J., Moss, B., 1996. De novo synthesis of the early transcription factor 70-kilodalton subunit is required for morphogenesis of vaccinia virions. Journal of Virology 70 (11), 7669–7677.
Hu, X., Wolffe, E.J., Weisberg, A.S., Carroll, L.J., Moss, B., 1998. Repression of the A8L gene, encoding the early transcription factor 82-kilodalton subunit, inhibits morphogenesis of vaccinia virions. Journal of Virology 72 (1), 104–112.
Kemper, A.R., Davis, M.M., Freed, G.L., 2002. Expected adverse events in a mass smallpox vaccination campaign. Effective Clinical Practice 5 (2), 84–90.
Kenner, J., Cameron, F., Empig, C., Jobes, D.V., Gurwith, M., 2006. LC16m8: an attenuated smallpox vaccine. Vaccine 24 (47–48), 7009–7022.
Lai, A.C., Chu, Y., 1991. A rapid method for screening vaccinia virus recombinants. BioTechniques 10 (5), 564–565.
Lane, J.M., Goldstein, J., 2003a. Adverse events occurring after smallpox vaccination. Seminars in Pediatric Infections Diseases 14 (3), 189–195.
Lane, J.M., Goldstein, J., 2003b. Evaluation of 21st-century risks of smallpox vaccination and policy options. Annals of Internal Medicine 138 (6), 488–493.
Lederman, E.R., Davidson, W., Groff, H.L., Smith, S.K., Warkentien, T., Li, Y., Wilkins, K.A., Karem, K.L., Akondy, R.S., Ahmed, R., Frace, M., Shieh, W.J., Zaki, S., Hruby, D.E., Painter, W.P., Bergman, K.L., Cohen, J.I., Damon, I.K., 2012.

Progressive vaccinia: case description and laboratory-guided therapy with vaccinia immune globulin, ST-246, and CMX001. Journal of Infectious Diseases 206 (9), 1372–1385.

Legrand, F.A., Verardi, P.H., Chan, K.S., Peng, Y., Jones, L.A., Yilma, T.D., 2005. Vaccinia viruses with a serpin gene deletion and expressing IFN-gamma induce potent immune responses without detectable replication in vivo. Proceedings of the National Academy of Sciences of the United States of America 102 (8), 2940–2945.

Legrand, F.A., Verardi, P.H., Jones, L.A., Chan, K.S., Peng, Y., Yilma, T.D., 2004. Induction of potent humoral and cell-mediated immune responses by attenuated vaccinia virus vectors with deleted serpin genes. Journal of Virology 78 (6), 2770–2779.

Legrand, N., van der Velden, G.J., Ho Tsong Fang, R., Douaisi, M., Weijer, K., Das, A.T., Blom, B., Uittenbogaart, C.H., Berkhout, B., Centlivre, M., 2012. A doxycycline-dependent human immunodeficiency virus type 1 replicates in vivo without inducing CD4+ T-cell depletion. Journal of General Virology 93 (Pt 9), 2017–2027.

Li, J., Broyles, S.S., 1993a. The DNA-dependent ATPase activity of vaccinia virus early gene transcription factor is essential for its transcription activation function. Journal of Biological Chemistry 268 (27), 20016–20021.

Li, J., Broyles, S.S., 1993b. Recruitment of vaccinia virus RNA polymerase to an early gene promoter by the viral early transcription factor. Journal of Biological Chemistry 268 (4), 2773–2780.

Li, J., Pennington, M.J., Broyles, S.S., 1994. Temperature-sensitive mutations in the gene encoding the small subunit of the vaccinia virus early transcription factor impair promoter binding, transcription activation, and packaging of multiple virion components. Journal of Virology 68 (4), 2605–2614.

Manoussaka, M.S., Berry, N., Ferguson, D., Stebbings, R., Robinson, M., Ham, C., Page, M., Li, B., Das, A.T., Berkhout, B., Almond, N., Cranage, M.P., 2013. Conditionally live attenuated SIV upregulates global T effector memory cell frequency under replication permissive conditions. Retrovirology 10 (1), 59.

Papin, J.F., Verardi, P.H., Jones, L.A., Monge-Navarro, F., Brault, A.C., Holbrook, M.R., Worthy, M.N., Freiberg, A.N., Yilma, T.D., 2011. Recombinant Rift valley fever vaccines induce protective levels of antibody in baboons and resistance to lethal challenge in mice. Proceedings of the National Academy of Sciences of the United States of America 108 (36), 14926–14931.

Pastoret, P.P., Brochier, B., 1996. The development and use of a vaccinia-rabies recombinant oral vaccine for the control of wildlife rabies; a link between Jenner and Pasteur. Epidemiology and Infection 116 (3), 235–240.

Ramshaw, I.A., Andrew, M.E., Phillips, S.M., Boyle, D.B., Coupar, B.E., 1987. Recovery of immunodeficient mice from a vaccinia virus/IL-2 recombinant infection. Nature 329 (6139), 545–546.

Sanz, P., Moss, B., 1999. Identification of a transcription factor, encoded by two vaccinia virus early genes, that regulates the intermediate stage of viral gene expression. Proceedings of the National Academy of Sciences of the United States of America 96 (6), 2692–2697.

Stebbins, M.J., Urlinger, S., Byrne, G., Bello, B., Hillen, W., Yin, J.C., 2001. Tetracycline-inducible systems for Drosophila. Proceedings of the National Academy of Sciences of the United States of America 98 (19), 10775–10780.

Stieger, K., Belbellaa, B., Le Guiner, C., Moullier, P., Rolling, F., 2009. In vivo gene regulation using tetracycline-regulatable systems. Advanced Drug Delivery Reviews 61 (7–8), 527–541.

Tartaglia, J., Perkus, M.E., Taylor, J., Norton, E.K., Audonnet, J.C., Cox, W.I., Davis, S.W., van der Hoeven, J., Meignier, B., Riviere, M., et al., 1992. NYVAC: a highly attenuated strain of vaccinia virus. Virology 188 (1), 217–232.

Tisoncik, J.R., Korth, M.J., Simmons, C.P., Farrar, J., Martin, T.R., Katze, M.G., 2012. Into the eye of the cytokine storm. Microbiology and Molecular Biology Reviews 76 (1), 16–32.

Traktman, P., Liu, K., DeMasi, J., Rollins, R., Jesty, S., Unger, B., 2000. Elucidating the essential role of the A14 phosphoprotein in vaccinia virus morphogenesis: construction and characterization of a tetracycline-inducible recombinant. Journal of Virology 74 (8), 3682–3695.

Unger, B., Traktman, P., 2004. Vaccinia virus morphogenesis: A13 phosphoprotein is required for assembly of mature virions. Journal of Virology 78 (16), 8885–8901.

Verardi, P.H., Jones, L.A., Aziz, F.H., Ahmad, S., Yilma, T.D., 2001. Vaccinia virus vectors with an inactivated gamma interferon receptor homolog gene (B8R) are attenuated In vivo without a concomitant reduction in immunogenicity. Journal of Virology 75 (1), 11–18.

Verardi, P.H., Titong, A., Hagen, C.J., 2012. A vaccinia virus renaissance: new vaccine and immunotherapeutic uses after smallpox eradication. Human Vaccines and Immunotherapeutics 8 (7), 961–970.

Wehrle, P.F., 1980. A reality in our time—certification of the global eradication of smallpox. Journal of Infectious Diseases 142 (4), 636–638.

Yamaguchi, T., Hamanaka, S., Kamiya, A., Okabe, M., Kawarai, M., Wakiyama, Y., Umino, A., Hayama, T., Sato, H., Lee, Y.S., Kato-Itoh, M., Masaki, H., Kobayashi, T., Yamazaki, S., Nakauchi, H., 2012. Development of an all-in-one inducible lentiviral vector for gene specific analysis of reprogramming. PLoS ONE 7 (7), e41007.

Yang, Z., Moss, B., 2009. Interaction of the vaccinia virus RNA polymerase-associated 94-kilodalton protein with the early transcription factor. Journal of Virology 83 (23), 12018–12026.

Yao, F., Eriksson, E., 1999. A novel tetracycline-inducible viral replication switch. Human Gene Therapy 10 (3), 419–427.

Yao, F., Murakami, N., Bleiziffer, O., Zhang, P., Akhrameyeva, N.V., Xu, X., Brans, R., 2010. Development of a regulatable oncolytic herpes simplex virus type 1 recombinant virus for tumor therapy. Journal of Virology 84 (16), 8163–8171.

Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C., Eriksson, E., 1998. Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Human Gene Therapy 9 (13), 1939–1950.

Zhang, H., Takayama, K., Zhang, L., Uchino, J., Harada, A., Harada, T., Hisasue, J., Nakagaki, N., Zhou, C., Nakanishi, Y., 2009. Tetracycline-inducible promoter-based conditionally replicative adenoviruses for the control of viral replication. Cancer Gene Therapy 16 (5), 415–422.

Zhu, Z., Zheng, T., Lee, C.G., Homer, R.J., Elias, J.A., 2002. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Seminars in Cell and Developmental Biology 13 (2), 121–128.