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# Expression of fluorescent proteins within the repeat long region of the Marek's disease virus genome allows direct identification of infected cells while retaining full pathogenicity

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ABSTRACT

Marek's disease virus (MDV) is a lymphotropic alphaherpesvirus and causes Marek's disease (MD) in chickens. RLORF4 is an MDV-specific gene located in the repeat long (RL) regions of the genome and is directly involved in attenuation. In this report, we generated recombinant (r)MDVs in which eGFP or mRFP was inserted in-frame of the 3′ end of the RLORF4 gene. *In vitro* growth was unaffected and infected cells could be identified by using fluorescent microscopy. Interestingly, though inserted in-frame with RLORF4, eGFP and mRFP were expressed alone, confirming mRNA expression and splicing within the RL of MDV is complex. *In vivo*, rMDVs expressing mRFP or eGFP caused tumors similar to wild-type MDV. Fluorescent protein expression could be seen in spleen, tumor, and feather follicle epithelial cells. These results show that expression of fluorescent proteins within the RL region results in fluorescent rMDVs that still maintains full pathogenicity in the chicken.



## 1. Introduction

Marek's disease (MD) in chickens is caused by the *Gallid herpesvirus* type 2 (GaHV-2), better known as Marek's disease virus (MDV). MDV is a member of the *Mardivirus* genus in the subfamily of *Alphaherpesvirinae* within the family *Herpesviridae* (ICTV, 2012). The most prominent sign of MD is the development of solid lymphomas in the viscera and other organs (Calnek, 2001). According to our current understanding, infection begins through the respiratory route by inhalation of cell-free virus or infected cells in chickens. The virus initially infects B lymphocytes in the lung (Baaten et al., 2009) and/or is taken up by pulmonary macrophages or dendritic cells and transported to lymphoid organs, where primary cytolytic infection occurs in B lymphocytes. MDV then infects T lymphocytes where it can establish latency, predominantly in activated CD4+ T cells, which can also undergo oncogenic transformation ultimately resulting in lymphoma formation. Irrespective of the transformation event, migrating lymphocytes transport MDV to feather follicle (FF) epithelial (FFE) cells in the skin. A major difficulty in studying MD pathogenesis has been the inability to generate fluorescently tagged recombinant (r)MDV that would allow the direct visualization of infected cells *in vivo* and *ex vivo*. Former attempts at generating fluorescent protein fusions with MDV proteins or insertion of fluorescent proteins under the control of independent promoters into the virus genome have mostly resulted in attenuated virus, loss of fluorescent protein expression over time, or the recombinant viruses were never tested *in vivo* (Anobile et al., 2006; Chbab et al., 2009; Denesvre et al., 2007; Dienglewicz and Parcells, 1999; Jarosinski et al., 2012; Mao et al., 2013; Parcells et al., 2001; Prigge et al., 2004; Remy et al., 2013). Additionally, these attempts have resulted in fluorescent protein expression almost strictly during the lytic phase of viral replication, thereby limiting their use in latency or transformation studies. Most recently, fusion of enhanced green fluorescent protein (eGFP) or monomeric red fluorescent protein (mRFP) to the C-terminus of the MDV UL47 gene encoding VP13/14 protein resulted in fully pathogenic recombinant rMDVs; however its usefulness for *in vitro* and *in vivo* lymphocyte infections was limited since VP13/14eGFP was expressed at very low levels (Jarosinski et al., 2012). Therefore, we sought to generate and test alternative rMDVs expressing fluorescent proteins. In this report, the MDV-specific protein, RLORF4, was targeted.

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**Table 1**
Primers used for generation of recombinant Marek's disease virus (rMDV).

| Primer Name | Direction[a] | Sequence (5′ → 3′)[b] |
|---|---|---|
| RLORF4eGFP | Forward | ATGCAAACTCCTGTTCATTTTAGTGTTTCGATGCTA**ATGGTGAGCAAGGGCGAGGAG** |
| | Reverse | CCGTGAGATGCATTTTGTTTATTGAAAATTTCCATTCGATGGGGCATAAATTA**CTTGTACAGCTCGTCCATGCCG** |
| RLORF4mRFP | Forward | ATGCAAACTCCTGTTCATTTTAGTGTTTCGATGCTA**ATGGCCTCCTCCGAGGACG** |
| | Reverse | GTTTATTGAAAATTTCCATTCGATGGGGCATAAATTA**CAAGGCGCCGGTGGAGTG** |

[a] Directionality of the primer.
[b] Bold nucleotides indicate priming sites within the mutagenesis template plasmids pEP-eGFP-in and pEP-mRFP-in, respectively.

RLORF4 has been shown to be directly involved in attenuation of MDV since following attenuation through serial passage of MDV *in vitro*; RLORF4 is systematically deleted (Jarosinski et al., 2003; Spatz et al., 2008; Spatz and Silva, 2007). Originally identified as a hypothetical reading frame within the repeat long (RL) regions of MDV, the mRNA of RLORF4 was identified during both lytic and latent infection (Jarosinski et al., 2003) as a single transcript encoding a 142 amino acids protein in wild-type strains. Further studies confirmed its expression at the protein level during lytic replication (Liu et al., 2006). Common among genes expressed within the RL regions of MDV, multiple mRNA splice variants have been identified between RLORF4, the viral interleukin 8 (vIL-8) and the MDV oncoprotein, Meq (Jarosinski and Schat, 2007); however, the functional importance of these transcripts is currently not known. Further experiments confirmed that deletion of RLORF4 from virulent MDV resulted in highly attenuated virus *in vitro* and *in vivo* (Jarosinski et al., 2005). Deletion of both copies of RLORF4 in the virulent RB-1B bacterial artificial chromosome (BAC) clone resulted in plaques sizes almost twice as large as wild-type virus, thereby allowing a direct functional readout prior to testing *in vivo*. With this in mind, we generated rMDVs in which eGFP or mRFP was inserted in-frame of the 3′ end of RLORF4, predicted to generate tagged RLORF4 proteins. *In vitro* growth of rMDVs was unaffected and infected cells could be easily identified by direct analysis using fluorescent microscopy. *In vivo*, rMDVs expressing fluorescent proteins caused tumors similar to wild-type MDV and was expressed in spleen, tumor, and FFE cells. Most cells within tumors were negative for eGFP or mRFP expression using immunofluorescence assays. In summary, the data presented here show that fluorescent proteins inserted at the 3′ end of the RLORF4 gene results in fully pathogenic rMDVs that can be visualized *in vitro*, *in vivo*, and *ex vivo* using fluorescent microscopy.

## 2. Materials and methods

### 2.1. Generation of eGFP or mRFP-tagged rMDVs

Coding sequences of the eGFP or mRFP genes were inserted in frame at the 3′ end of RLORF4 by two-step Red-mediated mutagenesis (Tischer et al., 2010) in a modified version of the pRB-1B infectious BAC clone that only contained a single RL region (Engel et al., 2012). Briefly, the eGFP-I-*Sce*I-*aphAI* and mRFP-I-*Sce*I-*aphAI* cassettes were amplified from pEP-EGFP-in and pEP-mRFP-in (Tischer et al., 2006), respectively, and used for mutagenesis in GS1783 *Escherichia coli* cells. All clones were confirmed by restriction fragment length polymorphism (RFLP) analysis, analytical PCR, and DNA sequencing. Oligonucleotides used for mutagenesis are shown in Table 1. Primers used for sequencing have been previously published (Jarosinski et al., 2003; Jarosinski and Schat, 2007).

### 2.2. Cell cultures and viruses

Chick embryo cell (CEC) cultures were prepared from 11-day-old specific-pathogen-free (SPF) embryos following standard methods (Schat and Sellers, 2008) and maintained in minimal Eagle's media (MEM) supplemented with 0.2–4% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). DF-1 cells, a spontaneously immortalized chicken fibroblast cell line, were obtained from the American Type Culture Collection (CRL-12203) and grown in ATCC-formulated Dulbecco's modified Eagle's media (DMEM) supplement with 10% FBS and antibiotics. DF-1-Cre cells have been previously published (Niikura et al., 2011) and were kindly provided by Masahiro Niikura (USDA-ARS-ADOL). DF-1-Cre cells were grown in a 1:1 mixture of Leibovitz's L-15 and McCoy 5A (LM) media (both from Life Technologies, Inc.) supplemented with 10% FBS and antibiotics, and maintained in 50 μg/ml Zeocin (Life Technologies, Inc.). CEC cultures and DF-1-Cre cells were incubated at 38 °C under a 5% $CO_2$ atmosphere. Recombinant viruses were reconstituted by transfecting DF-1-Cre cells using previously described methods (Schumacher et al., 2000), which efficiently removes the mini-F BAC sequences from the viral genome, then passaged directly into CEC cultures. All rMDVs were used at ≤5 passages for *in vitro* and *in vivo* studies.

Tumor cells were obtained from vRLORF4_mRFP-induced tumors using standard methods (Calnek et al., 1978, 1981). Briefly, tumor tissue from vRLORF4_mRFP-infected animals was harvested and sieved through a cell strainer before lymphocytes were isolated using Histopaque®-1077 (Sigma–Aldrich) density gradient centrifugation. Purified cells were cultivated *ex vivo* at $10^6$ cells/ml in LM media and modified according to Hahn as described earlier (Calnek et al., 1981). The medium was supplemented with 10% FBS and 8% chicken serum at 41 °C under a 5% $CO_2$ atmosphere.

### 2.3. Measurement of plaque areas

Plaque areas were measured as previously described (Jarosinski et al., 2005). Briefly, CEC cultures were seeded in 6-well dishes and infected with 100 plaque forming units (PFU) of each virus per well. After 5 days, cells were washed once with phosphate buffered saline (PBS), fixed and permeabilized with PFA buffer (2% paraformaldehyde, 0.1% Triton X-100) for 15 min and washed twice with PBS. Immunofluorescence assays were performed using anti-MDV chicken sera and goat anti-chicken IgY-Alexa Fluor 488 or 568 secondary antibodies. Digital images of 30 individual plaques were obtained using a Nikon Eclipse-Ti-E inverted fluorescent microscope. Plaque sizes were measured using ImageJ (Abramoff et al., 2004) version 1.41o software (rsb.info.nih.gov/ij) and plaque area means were determined for each virus. Significant differences in mean plaque areas were determined using Student's *t* tests.

### 2.4. Fluorescent microscopy

CEC cultures were infected with vRB-1B, vRLORF4_mRFP, or vRLORF4_eGFP on sterile glass coverslips in 24-well dishes at 50 PFU per coverslip. At 5 days pi, cells were fixed and permeabilized with PFA buffer for 15 min and then washed twice with PBS. Tissues were collected from MDV-infected chickens and snap-frozen in Tissue