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Protocols

Construction and characterization of an infectious molecular clone of maedi-visna virus that expresses green fluorescent protein

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The construction of a molecular clone of maedi-visna virus (MVV) expressing the enhanced green fluorescent protein (EGFP) is described. The *egfp* gene was inserted into the gene for dUTPase since it has been shown that dUTPase is dispensable for MVV replication both in vitro and in vivo. MVV-egfp is infectious and EGFP expression is stable over at least six passages. This fluorescent virus will be a useful tool for monitoring MVV infections.

1. Introduction

Maedi-visna virus (MVV) belongs to the lentivirus subgroup of retroviruses, causing slowly progressive interstitial pneumonia and encephalitis in sheep (Sigurdsson et al., 1957). The lentivirus group comprises equine infectious anemia virus (EIAV), bovine immune deficiency virus (BIV), feline immunodeficiency virus (FIV) and the small ruminant lentiviruses, caprine arthritis encephalitis virus (CAEV) and MVV as well as the simian and human immunodeficiency viruses (SIV and HIV). The genomes of lentiviruses contain three structural genes: *gag*, *pol* and *env*, and various numbers of accessory genes. MVV and HIV-1 have three accessory genes in common, *rev*, *vif* and *tat*, but the latter probably provides a function analogous to *vpr* in HIV-1 (Villet et al., 2003).

A subset of the lentiviruses, namely EIAV, FIV, CAEV, and MVV contain the enzyme dUTPase. The dUTPase gene is located in the *pol* reading frame in these viruses, and dUTPase-deficient mutants of EIAV, FIV, CAEV and MVV have been shown to replicate normally in some cell lines, but the replication of EIAV, FIV and CAEV in primary cells is delayed (Lichtenstein et al., 1995; Petursson et al., 1998; Threadgill et al., 1993; Turelli et al., 1996; Wagaman et al., 1993). dUTPase of MVV was shown to be dispensable for infectivity in primary cells and in vivo (Petursson et al., 1998).

The introduction of a label that can be detected in living cells has been used widely in HIV and SIV research. The enhanced green fluorescent protein (EGFP) is the most commonly used marker (Alexander et al., 1999; Lee et al., 1997). An attempt to introduce EGFP into MVV has been reported, but fluorescence was not detected in infected cells (Zhang et al., 2003). In the present study the construction and characterization of an infectious molecular clone of MVV with the *egfp* gene fused in the dUTPase (*du*) ORF is described. Cells infected with this virus fluoresce and the marker appears stable through multiple passages. This virus will be a useful tool for analyzing the host–cell interactions and pathogenicity of MVV.

2. Materials and methods

2.1. Plasmid construction

The MVV molecular clone KV1772 (GenBank: S55323.1) (Andresson et al., 1993) is split between two plasmids, p8XSp5-RK1 and p67r (Skraban et al., 1999). Plasmid pKV1772-egfp was constructed by making use of an MfeI restriction site at position 3873 in the *du* gene of plasmid p8XSp5-RK1 (Skraban et al., 1999). The *egfp* gene was amplified from pEGFP-N3 plasmid with primers that were designed to contain the MfeI site and omitting the stop codon of the *egfp* gene (forward primer 5′-GATAG*CAATTG*ACATGGTGAGCAAGGGCGA-3′; and reverse primer 5′-CAAGT*CAATTG*CCTTGTACAGCTCGTCCAT-3′, the MfeI sites are shown in italic letters) yielding a 742 bp PCR product. The PCR

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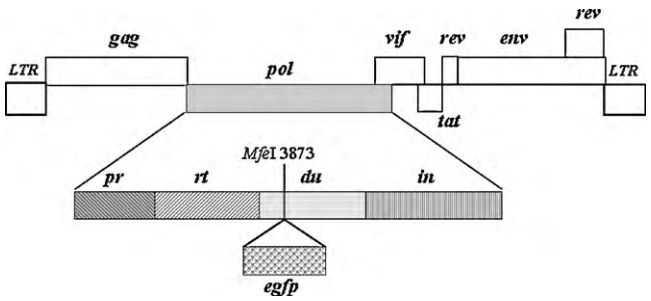

Fig. 1. Schematic representation of the pKV1772-egfp construction. The egfp sequence was inserted at an MfeI site in the *du* gene.

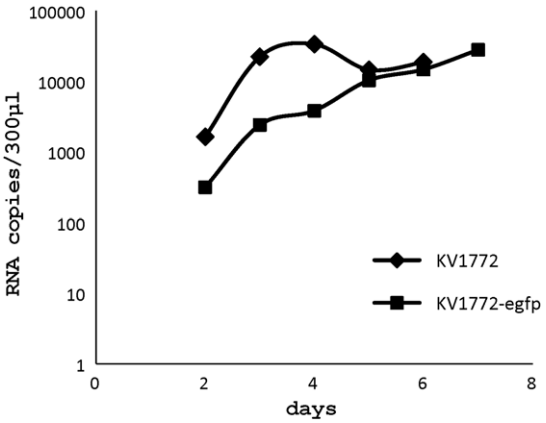

Fig. 2. Kinetics of KV1772-egfp replication compared to parent KV1772 in SCP cells as measured by Taqman-based real-time PCR

product was first cut with MfeI and cloned into a pUC plasmid containing a $BamHI_{1563}$–$BamHI_{4587}$ fragment of p8XSp5-RK1, and then the $BamHI_{1563}$–$BamHI_{4587}$ fragment containing the *egfp* gene was cloned back into p8XSp5-RK1. The correct insertion of the *egfp* gene was confirmed by sequencing. Primers on both sides of the insert used for detecting the insert and for sequencing were: forward primer 5′-CACAAGGGGATTCCTCAAAA-3′ and reverse primer 5′-GCTTTTGGTCCCTATCATGG-3′.

2.2. Virus, cells, titration and transfections

The MVV molecular clone KV1772 was propagated in sheep choroid plexus (SCP) cells that were cultivated in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, California) containing 1% or 10% lamb serum as described previously (Skraban et al., 1999).

$TCID_{50}$ (tissue culture infectious $dose_{50}$) was determined by endpoint titration in 96-well flat-bottomed tissue culture plates. Tenfold dilutions of virus were inoculated in quadruplicate onto monolayers of SCP cells and cytopathic effects were determined after 2–4 weeks of incubation. The infectivity titres were calculated by the Reed–Muench method.

For transfections, equimolar quantities of the two plasmids containing the viral genome, a total of 6 μg, were cut with XbaI and ligated. Transfections of SCP cells were carried out with Lipofectamine 2000 (Invitrogen) in T25 tissue culture flasks as specified by the manufacturer.

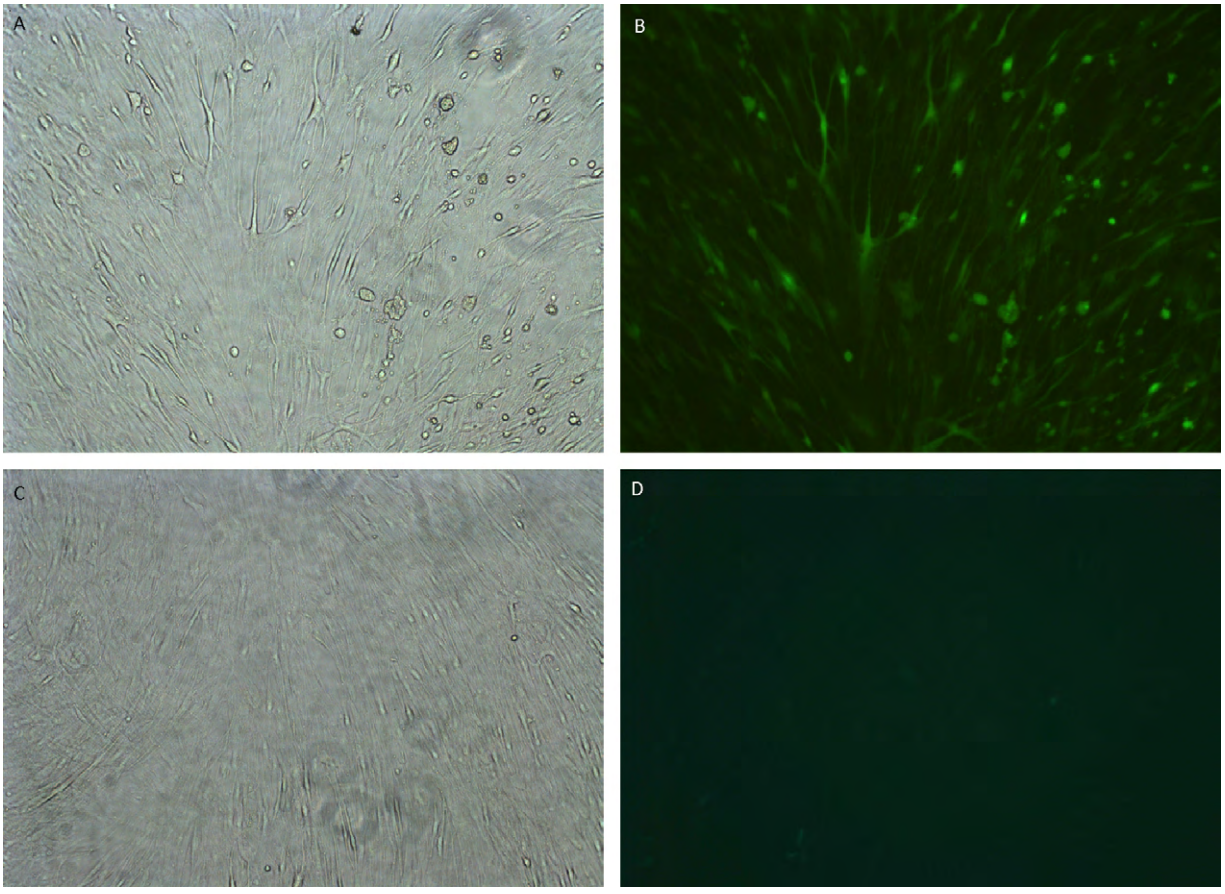

Fig. 3. Phase contrast microscopy of CPE in SCP cells at the end of the growth experiments. KV1772-egfp (A) and KV1772 (C). Same cells visualized by fluorescence microscopy (B and D). Cells infected with KV1772-egfp were fluorescent whereas cells infected with KV1772 were not.

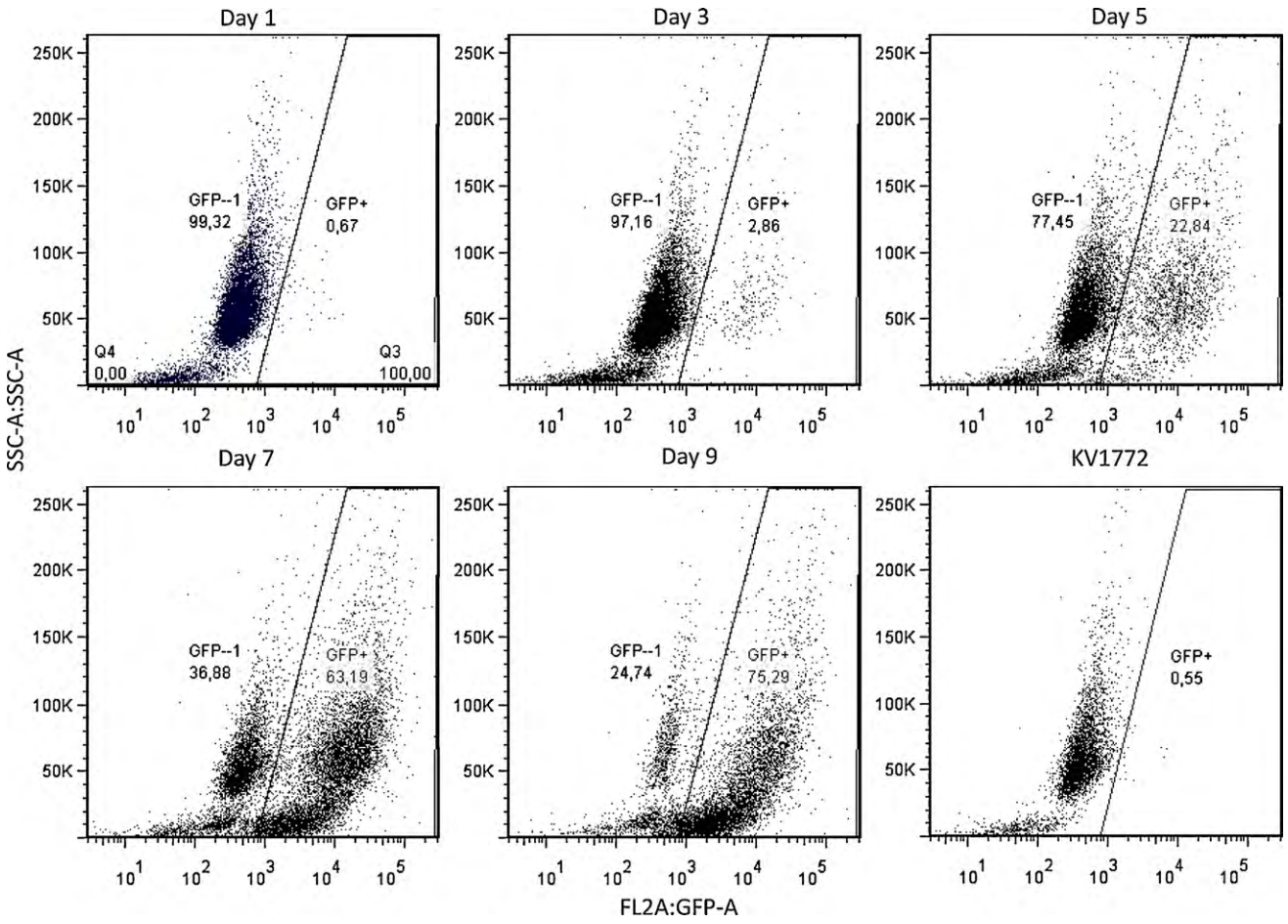

Fig. 4. FACScan analysis of SCP cells infected with 6th passage of KV1772-egfp virus after 1, 3, 5, 7 and 9 days of infection. Cells taken at day 7 of infection with KV1772 were used as a negative control.

2.3. RT assay

Viral particles from 200 μl of cell-free supernatants from infected cells were pelleted at 14,000 rpm for 1 h in a microfuge. The pelleted virus was resuspended in TNE (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA) containing 0.1% Triton X-100. RT activity was assayed on a poly(A) template, adding oligo-dT primer and dTTP. The resulting RNA–DNA heteroduplexes were detected by PicoGreen reagent as specified by the manufacturer (Molecular Probes Inc., Eugene, Oregon).

2.4. Real-time PCR assay

Viral particles from 300 μl of cell-free supernatants from infected cells were pelleted at 14,000 rpm for 1 h in a microfuge. The pellet was dissolved in 10 μl TNE (10 mM Tris pH 7.5; 100 mM NaCl; 1 mM EDTA) with 0.1% Triton X-100. This lysate was used for generating cDNA using RevertAid M-MuLV reverse transcriptase (Fermentas International Inc., Ontario, Canada) and a primer from the *gag* gene (V-1818 5′-CGG GGTACCTTACAACATAGGGGGCGCGG-3′). Real-time PCR was carried out in a final volume of 20 μl.

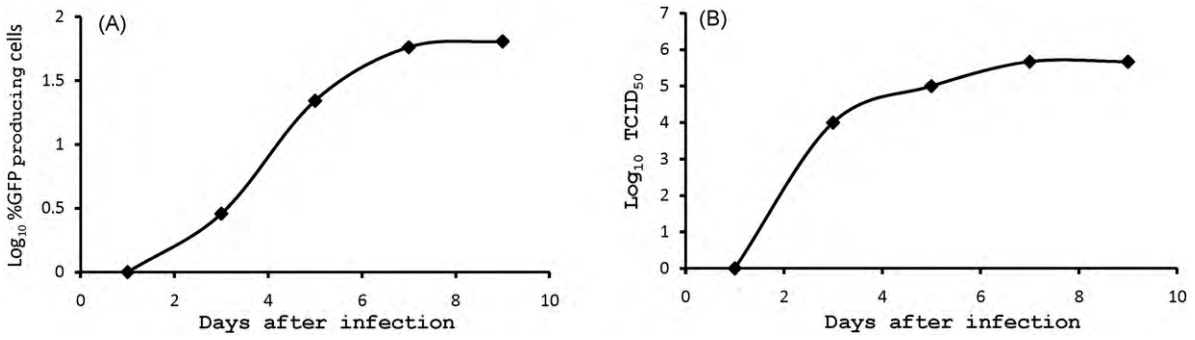

Fig. 5. Data from Fig. 4 (A) compared to $TCID_{50}$ of the respective supernatants (B).

The primers and Taqman probe were as follows: Forward primer: V1636 5′-TAAATCAAAAGTGTTATAATTGTGGGA-3′, reverse primer: V-1719: 5′-TCCCACAATGATGGCATATTA TTC-3′, Taqman probe: V1665Taqman 5′-FAM-CCAGGACATCTCGCAAGA CAGTGTAGACA-BHQ-1-3′. Calibration curves were derived by running 10-fold dilutions of specific cDNA over the range of 6×10–6×10^7 copies. Each assay included duplicate wells for each dilution of calibration DNA and for each cDNA sample.

2.5. FACScan analysis

The samples were fixed in PBS with 2% paraformaldehyde and the amount of EGFP expressing cells was determined using a MACSQuant flow cytometer (Miltenyi Biotec, Germany) with a 488-nm argon ion laser and 525/50 nm band-pass filter. Data were analysed using FlowJo v7.5.5 software (Tree Star, Ashland, Oregon). For each sample, 10,000 events were counted.

3. Results

The *egfp* gene was inserted into the gene for dUTPase (DU) since it had previously been shown that dUTPase is dispensable for MVV replication both in vitro and in vivo (Petursson et al., 1998; Turelli et al., 1996). An MfeI site in the *du* gene was used to insert the *egfp* gene in frame with the *du* sequence resulting in a construct called pKV1772-egfp, with EGFP embedded in dUTPase as shown in Fig. 1.

Primary SCP cells were transfected with this construct and analysed by fluorescent microscopy and flow cytometry yielding fluorescent cells. To test the replication properties of the virus, the supernatant from the transfected cells was used to infect fresh SCP cells. Virus from third passage in SCP cells was used for a replication experiment, where SCP cells were inoculated with equal amounts of KV1772-egfp and KV1772 as determined by RT activity. A multiplicity of infection (m.o.i.) of 1 was used. The m.o.i. was estimated by using a titrated stock of KV1772 as a standard and determining the correlation between RT activity and $TCID_{50}$. As shown in Fig. 2, the virus containing the *egfp* gene was fully infectious and replicated to the same RT titre in SCP cells as the parent KV1772 virus, albeit with slightly slower kinetics. At day 6 the $TCID_{50}$ titre of KV1772 was 10^7 $TCID_{50}$/ml whereas that of KV1772-egfp was 2×10^6 $TCID_{50}$/ml. Typical MVV induced cytopathic lesions were observed with the cells becoming slightly rounded and less transparent than normal cells and with processes stretching out, ending with multinuclear giant cells appearing and cell death. At the end of the experiment the infected cells were examined by fluorescence microscopy. The cells infected with KV1772-egfp were brightly fluorescent, while cells infected with KV1772 were not (Fig. 3). To further test the stability of the egfp sequence, virus that had been passaged six times in SCP cells was seeded onto SCP cells in 12-well plates and after 1, 3, 5, 7, 9 days cells were examined by FACScan and at the same time the $TCID_{50}$ of the virus in the supernatants were determined. The titre of the virus went up to 5×10^5 $TCID_{50}$/ml and at that time more than 70% of the cells were producing EGFP (Fig. 4). There was good correlation between measurements of $TCID_{50}$ and EGFP although titration was more sensitive in the lowest titres (Fig. 5). The EGFP marker can thus be used for monitoring replication of this virus. It appears that the 720 bp increase in genome size of the virus did not have a deleterious effect on replication.

4. Discussion

A number of studies where reporter genes have been added to the whole genome of HIV-1 or SIV have found that the resulting virus was attenuated in replication and/or the inserted gene was unstable. This may either be due to the disturbance of normal activity of the gene where the marker gene was inserted, or to size constraints of the proviral genome (Brown et al., 2005; Muller et al., 2004; Terwilliger et al., 1989). MVV seems to tolerate an increase in genome size better than HIV-1, where viruses with reporter genes added to the whole genome were reported to replicate with considerable slower kinetics than wild type (Terwilliger et al., 1989).

An earlier attempt to clone the *egfp* gene into the *du* gene of MVV has been reported (Zhang et al., 2003). The resulting virus was replication competent, but cells infected with this virus did not fluoresce, however. In that study most of the *du* sequence was deleted and the *egfp* gene was cloned at the *du* locus in the *pol* gene. The authors speculated that since the Pol proteins are expressed at a lower level than the structural proteins in lentiviruses, the level of expression might not be high enough for detection. Our results show that this is not the case.

CAEV with the *egfp* gene inserted into the *tat* region was recently reported (Mselli-Lakhal et al., 2006). This virus expressed EGFP, but the expression could not be maintained over passages, however (Mselli-Lakhal et al., 2007).

In summary, a fully infectious, fluorescent MVV derivative has been generated. The EGFP marker is stable over at least 6 passages in SCP cells. This virus should be useful for rapid detection of infected cells in studies of cell tropism and pathogenicity in vitro and in vivo.

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