

Generation and characterization of a recombinant Rift Valley fever virus expressing a V5 epitope-tagged RNA-dependent RNA polymerase

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The viral RNA-dependent RNA polymerase (RdRp; L protein) of Rift Valley fever virus (RVFV; family *Bunyaviridae*) is a 238 kDa protein that is crucial for the life cycle of the virus, as it catalyses both transcription of viral mRNAs and replication of the tripartite genome. Despite its importance, little is known about the intracellular distribution of the polymerase or its other roles during infection, primarily because of lack of specific antibodies that recognize L protein. To begin to address these questions we investigated whether the RVFV (MP12 strain) polymerase could tolerate insertion of the V5 epitope, as has been previously demonstrated for the Bunyamwera virus L protein. Insertion of the 14 aa epitope into the polymerase sequence at aa 1852 resulted in a polymerase that retained functionality in a minigenome assay, and we were able to rescue recombinant viruses that expressed the modified L protein by reverse genetics. The L protein could be detected in infected cells by Western blotting with anti-V5 antibodies. Examination of recombinant virus-infected cells by immunofluorescence revealed a punctate perinuclear or cytoplasmic distribution of the polymerase that co-localized with the nucleocapsid protein. The generation of RVFV expressing a tagged RdRp will allow detailed examination of the role of the viral polymerase in the virus life cycle.

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INTRODUCTION

Rift Valley fever virus (RVFV) is a mosquito-borne pathogen of both livestock and humans that is found primarily in sub-Saharan Africa and the Arabian Peninsula. In ruminants, particularly sheep and cattle, RVFV disease is characterized by fetal deformities, abortion and high rates of mortality among young animals (Smithburn, 1949). RVFV is predominantly transmitted by mosquitoes and the virus has been isolated from more than 40 species in nature, while under laboratory conditions many different arthropods are capable of transmitting the virus (Turell *et al.*, 2008 and references therein). RVFV is a member of the genus *Phlebovirus* in the family *Bunyaviridae*. The virus contains a tripartite ssRNA genome comprising two negative-sense segments and one ambisense segment (Bouloy & Weber, 2010). The small (S) segment (approx. 1.7 kb) encodes the nucleocapsid (N) protein and a non-structural protein (NSs) in an ambisense manner. The N protein is translated from a subgenomic mRNA that is transcribed from the genomic RNA, while NSs is translated from a subgenomic mRNA that is transcribed from the antigenomic (replicative-intermediate) RNA. The medium (M) segment (approx. 3.8 kb) encodes four proteins in a single ORF: the virion envelope glycoproteins Gn and Gc, and two other proteins called NSm1 and NSm2 (Gerrard *et al.*, 2007; Won *et al.*, 2006). The large (L) segment

(approx. 6.4 kb) encodes the viral RNA-dependent RNA polymerase (RdRp or L protein). The RNA segments are encapsidated by N protein to form ribonucleoprotein (RNP) complexes, the functional templates for viral RNA synthesis (Ortín & Parra, 2006).

The RVFV L protein is 2092 aa long (237.7 kDa) and associates with the RNP to catalyse viral transcription and replication (Lopez *et al.*, 1995). Comparison of bunyavirus L protein sequences with those of other RNA polymerases enabled the definition of four amino acid motifs, called A to D, that comprise the 'polymerase module' that is conserved in all RNA polymerases (Delarue *et al.*, 1990; Poch *et al.*, 1989). These are located in the middle of the bunyavirus polymerase. Subsequently, a fifth motif upstream of motif A, termed pre-motif A, was identified and shown to be a feature of all RdRp, while a motif downstream of motif D, termed E, was found to be conserved in all segmented virus RdRp (Muller *et al.*, 1994). A further conserved region downstream of motif E has been described that is specific for bunyavirus RdRp (Aquino *et al.*, 2003). Conserved motif C contains the actual catalytic domain for nucleotide polymerization, and for all segmented negative-sense RNA viruses this is characterized by the conserved tripeptide sequence SDD (Jin & Elliott, 1992). Bunyavirus mRNA synthesis is primed by oligonucleotides cleaved from the 5' ends of host

mRNAs in a process known as cap-snatching (Patterson *et al.*, 1984) and the N-terminal domain of bunyavirus L proteins contains a conserved PD-(D/E)xK nuclease motif (Reguera *et al.*, 2010).

Through the use of transient transfection of cDNAs expressing N-terminal or C-terminal externally tagged RVFV L proteins, Zamoto-Niikura *et al.* (2009) reported that L forms oligomers, and they also identified regions of the protein responsible for both inter- and intramolecular interactions. Furthermore, the importance of L oligomerization for viral RNA synthesis was demonstrated in a minigenome assay (Zamoto-Niikura *et al.*, 2009). However, these authors were unable to produce an infectious virus expressing a tagged L protein.

Previously we utilized reverse-genetic technology (Bridgen & Elliott, 1996; Lowen *et al.*, 2004) to generate recombinant Bunyamwera virus expressing L protein tagged internally within the C-terminal domain with the 14 aa V5 epitope derived from parainfluenza virus 5 (Shi & Elliott, 2009; Southern *et al.*, 1991). Here we demonstrate that RVFV RdRp can also tolerate introduction of the epitope into its C terminus and retain functionality in a minigenome assay, and furthermore that infectious virus expressing a tagged L protein can be produced. We provide insight into the intracellular location of the L protein in infected cells and describe its co-localization with the nucleocapsid protein in the cytoplasm.

RESULTS

Generation of a V5-tagged RVFV RdRp

Comparison of the amino acid sequences of bunyavirus L proteins from viruses in all five genera of the family *Bunyaviridae* reveals little sequence similarity, apart from the short motifs described above. In particular, there is no obvious similarity between the C-terminal domain of Bunyamwera virus L, where the V5 epitope was successfully inserted, and the C-terminal end of RVFV L protein. Therefore, we compared the amino acid sequences of three phlebovirus L proteins (RVFV, Uukuniemi virus and Toscana virus) and, along with bioinformatic predictions of the RVFV L protein structure using criteria previously described (Shi & Elliott, 2009), we identified three potential insertion sites at aa 1750, 1794 and 1852. Accordingly, the sequence encoding the V5 epitope was inserted into the L segment cDNA, by using a PCR mutagenesis approach, in pTM1- or pTVT7-based plasmids for viral protein expression and genomic RNA expression, respectively (Fig. 1).

The functionality of the recombinant L proteins was assessed by using a minigenome assay comprising an S-segment reporter RNA in which the coding sequence of the NSs protein had been replaced with that of *Renilla* luciferase (Ren). The construct pTVT7-GSdelNSs:Ren contains anti-genomic sense cDNA to the modified S

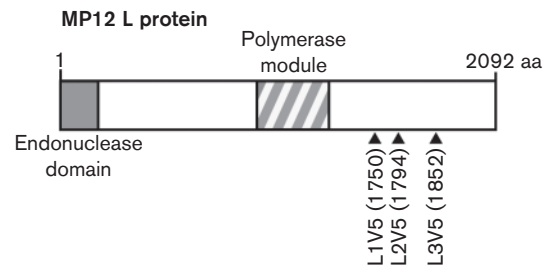


Fig. 1. Schematic representation of the Rift Valley fever virus L protein and V5-epitope insertion sites. The 14 aa V5 epitope (GKPIPPLLGLDST) was inserted into the L protein coding sequence in both pTM1-L and pTVT7-GL by using PCR mutagenesis at the sites indicated (▲). The modified proteins were designated L1V5–L3V5. The grey area represents the putative endonuclease domain (Reguera *et al.*, 2010) and the diagonally shaded box the polymerase module defined by Muller *et al.* (1994).

segment under the control of the T7 promoter, and hence the reporter gene would be in the negative-sense orientation in the T7-driven RNA transcript. *Renilla* luciferase activity will only be detected if a functional viral polymerase is present in the system to transcribe the RNA and generate the appropriate subgenomic mRNA. Our minigenome system is thus similar to those previously reported for RVFV (Gauliard *et al.*, 2006; Habjan *et al.*, 2009; Ikegami *et al.*, 2005; Näslund *et al.*, 2009; Zamoto-Niikura *et al.*, 2009).

The minigenome-encoding plasmid was transfected into BSR-T7/5 cells along with plasmids expressing N protein (pTM1-N) and either wild-type (wt) (pTM1-L) or mutant (pTM1-L1V5 to pTM1-L3V5) polymerases. As shown in Fig. 2(a), insertion of the V5-epitope tag at aa 1750 (L1V5) or aa 1794 (L2V5) within the C terminus of the polymerase abrogated activity in the minigenome assay. However, L protein with the epitope inserted further towards the C terminus of the protein at aa 1852 (L3V5) was still functional, showing 24% of the activity of the wt polymerase. Varying the amounts of L-expressing plasmid DNA transfected (from 50 ng to 2 µg) did not result in detectable *Renilla* luciferase activity in pTM1-L1V5- or pTM1-L2V5-transfected cells, while the luciferase activity produced in wt L- or L3V5-transfected cells was maximal with 100 ng of DNA (data not shown). None of the insertions seemed to affect expression of the mutant polymerases, as similar-sized bands were detected by Western blotting with anti-V5 antibodies (Fig. 2b).

Effect of MP12 NSs protein on polymerase activity

Work by Ikegami *et al.* (2005) showed that expression of the RVFV ZH501 strain NSs protein in a minigenome assay enhanced RNA replication and transcription, as measured by an increase in reporter gene activity. Therefore, in an

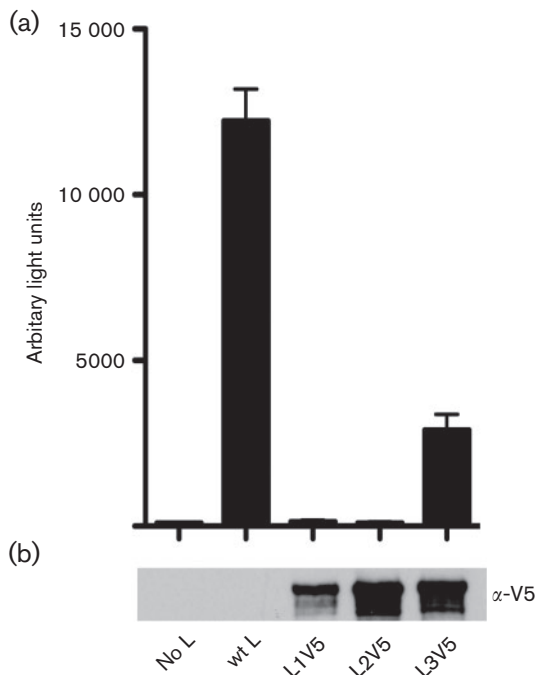


Fig. 2. Activity of V5-tagged L protein in the minigenome assay. (a) Luciferase activity. BSR-T7/5 cells were transfected with pTVT7-GSdelNSs:Ren, pTM1-N and either wt pTM1-L or V5-tagged variants. *Renilla* luciferase activity was measured 24 h post-transfection. No L, Negative control without L cDNA (empty pTM1 vector used); wt L, pTM1-L; L1V5, pTM1-L1V5; L2V5, pTM1-L2V5; L3V5, pTM1-L3V5. (b) Western blot analysis of transfected BSR-T7/5 cells. Cell extracts were prepared in parallel with those for the minigenome assay, and proteins fractionated on a 4–12% NuPage gel (Invitrogen). After transfer to a nylon membrane, the blot was probed with an anti-V5 antibody to detect L protein.

attempt to improve the activity of the L3V5 polymerase in our assay, varying amounts of the expression plasmid pTM1-NSs_{MP12} (from 1 ng to 1 µg) were transfected into BSR-T7/5 cells along with the other constructs, as described above. In contrast to the data reported by Ikegami *et al.* (2005), our results showed that increasing the amount of NSs protein decreased the level of reporter gene activity in cells expressing either wt or L3V5 mutant polymerases. At the highest amount of co-transfected pTM1-NSs_{MP12} (1 µg), *Renilla* luciferase was inhibited by 98% and 91% in wt L- or L3V5-transfected cells, respectively (Fig. 3).

Rescue of recombinant RVFV expressing a V5-tagged polymerase

The three pTVT7-based constructs expressing tagged L proteins were then used in the reverse genetics system (Billecocq *et al.*, 2008; Brennan *et al.*, 2011) to determine whether viable viruses could be rescued. In agreement with the results seen in the minigenome assay, no viruses with

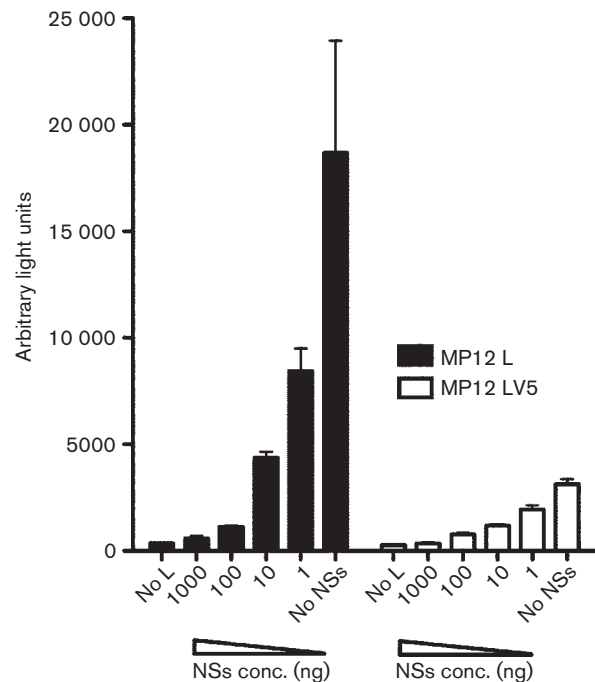


Fig. 3. Effect of MP12 NSs protein on polymerase activity in the minigenome assay. BSR-T7/5 cells were transfected with pTVT7-GSdelNSs:Ren, pTM1-N, either wt pTM1-L or pTM1-L3V5, and the indicated amount of pTM1-NSs_{MP12}. *Renilla* luciferase activity was measured 24 h post-transfection. Black and white bars represent experiments with wt L or L3V5 protein, respectively. No L, Negative control without wt L cDNA (empty pTM1 vector used); No NSs, positive control reaction.

RNA segments derived from the constructs pTVT7-GL1V5 or pTVT7-GL2V5 were recovered (data not shown). However, a virus designated rMP12L3V5 was successfully rescued by using the construct pTVT7-GL3V5. The passage 1 (p1) stock of this virus had a titre of 2.68×10^7 p.f.u. ml⁻¹ but showed a smaller plaque phenotype than that of the parental virus, which was also generated by reverse genetics and designated rMP12 (Fig. 4a). We were also able to rescue a recombinant two-segmented genome virus, designated r2segMP12L3V5, which expresses epitope-tagged L protein. This is based on the recently described two-segmented virus (r2segMP12) in which the NSs gene is replaced by that of the glycoproteins, and consequently the virus lacks an M segment (Brennan *et al.*, 2011). Both viruses expressed L protein, which was clearly detected by Western blotting with anti-V5 antibodies (Fig. 4b). r2segMP12L3V5 was severely attenuated with respect to rMP12, r2segMP12 or rMP12L3V5, and produced extremely small pinpoint plaques (Fig. 4c). Therefore, to determine the titre of the virus, a focus-forming assay was performed by using an anti-N antibody. A p1 stock of r2segMP12L3V5 was generated and had a titre of 7.5×10^5 focus-forming units ml⁻¹.

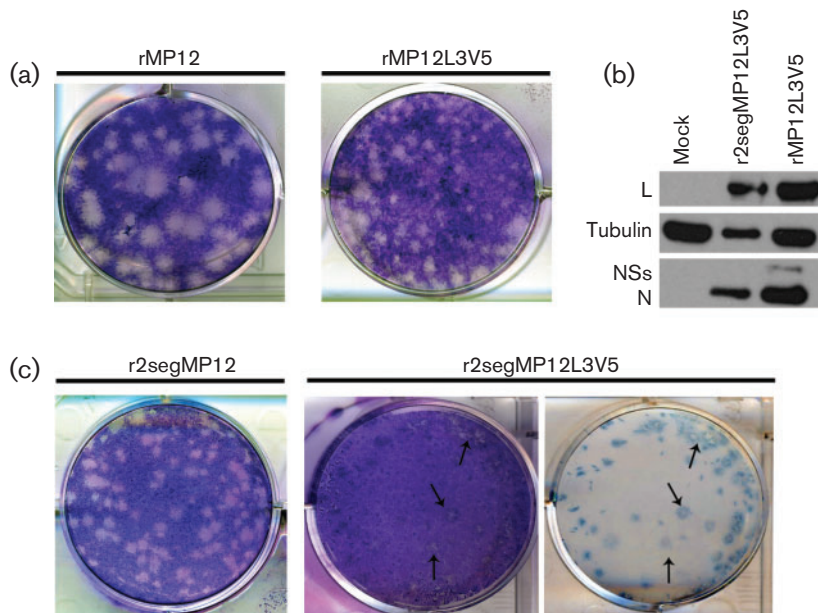


Fig. 4. Characterization of recombinant viruses. (a) Comparison of plaque sizes of rMP12 and rMP12L3V5 on BHK-21 cells. Cell monolayers were fixed at 96 h p.i. with 4% paraformaldehyde and stained with Giemsa solution. (b) Western blot analysis of S and L segment-encoded proteins from rMP12L3V5- and r2segMP12L3V5-infected cells. Cell extracts were fractionated on 4–12% NuPage gels and blots were probed with anti-V5, anti-N, anti-NSs and anti-tubulin antibodies as indicated. (c) Comparison of plaque sizes of r2segMP12 and r2segMP12L3V5 on BHK-21 cells. Cell monolayers were fixed at 96 h p.i. with 4% paraformaldehyde and stained with Giemsa solution. Immunostaining, with anti-N antibody, of viral foci produced by r2segMP12L3V5 on BHK-21 cells is shown on the right.

Growth properties of rMP12L3V5

The replication of the three-segmented genome tagged virus was compared with rMP12 in BHK-21, Vero E6 and *Aedes albopictus* C6/36 cells, each infected at an m.o.i. of 1 (Fig. 5). At different times post-infection (p.i.) the tissue-culture supernatants were harvested and virus titres determined by plaque assay in BHK-21 cells. In all cell lines the replication of the tagged virus, rMP12L3V5, was attenuated by 10- to 100-fold compared with that of rMP12. Peak titres of 2.95×10^7 and 2.3×10^8 p.f.u. ml⁻¹ in BHK-21 cells (Fig. 5a), 8×10^6 and 1×10^8 p.f.u. ml⁻¹ in Vero E6 cells (Fig. 5b), and 1.5×10^7 and 3.95×10^8 p.f.u. ml⁻¹ in C6/36 cells (Fig. 5c) were recorded for rMP12L3V5 and rMP12, respectively.

The cell monolayers were also harvested to monitor the expression of the N, NSs and L proteins by Western blotting. In all cell lines infected with rMP12 or rMP12L3V5, N and NSs expression was first detectable by 12 h p.i., except for NSs in C6/36 cells, which was delayed until 20 h p.i. The levels of N and NSs proteins synthesized in rMP12L3V5-infected cells were less than those seen in rMP12-infected cells, reflecting the reduced growth observed for the tagged virus. The L protein was readily detected by the anti-V5 antibody in rMP12L3V5-infected cells: by 12 h p.i. in both mammalian cell lines and by 20 h p.i. in C6/36 cells. The overall accumulation of L protein was greatest in infected BHK-21 cells (Fig. 5). In addition, rMP12L3V5 appeared genetically stable, in that tagged L protein was detected by Western blotting in cells infected with virus that had been serially passaged three times.

Cellular localization of N protein and polymerase during infection

The intracellular localization of the RVFV polymerase was determined by immunofluorescence imaging. Cells

on coverslips were infected with rMP12L3V5 at an m.o.i. of 1, fixed at 24 h p.i., and stained with anti-N and anti-V5 antibodies. Both proteins were detected in the cytoplasm of infected cells and showed an overall punctate pattern of staining (Fig. 6), with some areas of intense staining in the perinuclear region. These may represent the viral factories, as reported for Bunyamwera virus (Salanueva *et al.*, 2003). There was a high degree of co-localization of the two immunofluorescent signals, which is indicative of interaction between L and N proteins. No obvious co-localization of L with the Golgi marker protein GM130 was apparent when infected cells were co-stained with anti-V5 and anti-GM130 antibodies (data not shown).

DISCUSSION

The RdRp of negative-sense RNA viruses plays a crucial role in the virus life cycle, including replication of the viral genome and transcription of viral mRNAs (Ortín & Parra, 2006). As with other bunyaviruses, detailed study of the RVFV L protein has been impeded by the lack of antibodies that can detect the authentic L protein in infected cells by techniques such as Western blotting or immunofluorescence. In addition, the lack of marked shut-off of host cell protein synthesis during RVFV infection renders visualization of the polymerase in infected cell lysates extremely difficult. Previously, we showed that lack of monospecific antibodies could be overcome by inserting the V5 epitope, against which high-affinity antibodies are available, into the Bunyamwera virus L protein, thus allowing convenient detection of the protein in infected cells (Shi & Elliott, 2009). Here, we investigated whether a virus in a different genus of the family *Bunyaviridae* could also be modified by introduction of the same tag.

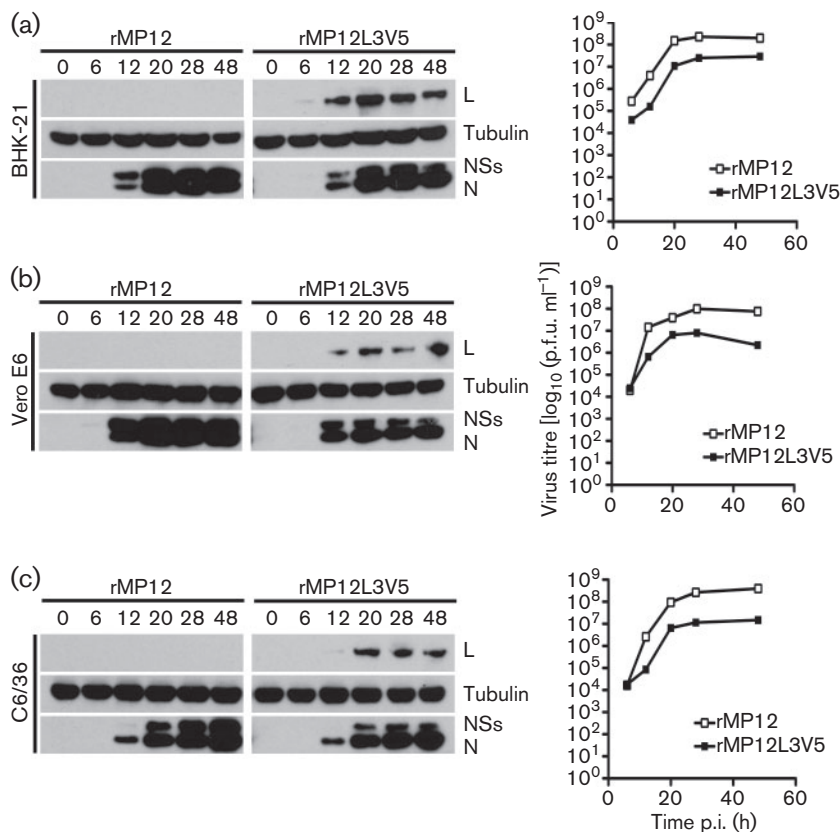


Fig. 5. Growth properties of recombinant viruses. Cells were infected with rMP12 or rMP12L3V5 (m.o.i. of 1). Western blot analysis of viral proteins and corresponding growth curves from infected BHK-21 (a), Vero E6 (b) and C6/36 (c) cells are shown. Cell extracts were prepared at the time points indicated, proteins were fractionated on 4–12% NuPage gels and blots were probed with anti-N, anti-NSs, anti-V5 to detect L, and anti-tubulin antibodies, as indicated. Viral growth-curve samples were titrated by plaque assay on BHK-21 cells.

Little is known about the domain structure of bunyavirus polymerases, and overall the L proteins of viruses in different genera show only limited regions of amino acid identity. Muller *et al.* (1994) described a region in the centre of the RVFV L protein, aa 918–1193, to contain the polymerase module composed of six motifs, Pre-A and A–E. These motifs are conserved across both bunyaviruses and arenaviruses, and mutation of certain conserved

residues in these motifs in the Bunyamwera virus L protein was shown to abolish polymerase activity (Jin & Elliott, 1992). A motif downstream of E was reported as being conserved among bunyaviruses, though no function can yet be ascribed to it (Aquino *et al.*, 2003). An ovarian tumour protease domain is found at the N terminus of Crimean–Congo hemorrhagic fever virus L protein (Honig *et al.*, 2004; Kinsella *et al.*, 2004) and appears to be a unique

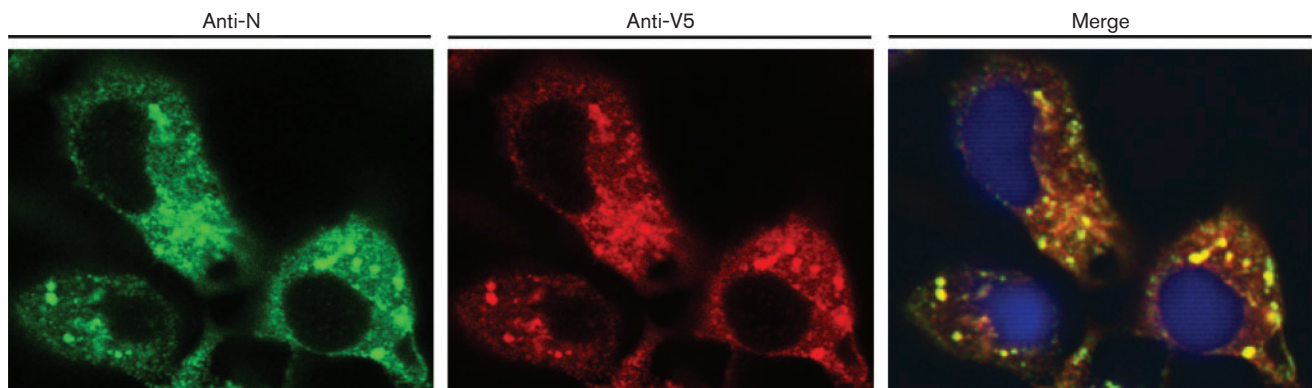


Fig. 6. Intracellular localization of viral N and V5-tagged L proteins in rMP12L3V5-infected cells. BSR-T7/5 cells were infected with rMP12L3V5 (m.o.i. of 1) and at 24 h.p.i. cells were fixed with 4% paraformaldehyde and co-stained with anti-N and anti-V5 antibodies, while nuclei were stained with DAPI. Cells were examined with a Zeiss LSM confocal microscope.

feature of the L proteins of nairoviruses, which are substantially larger than the L proteins of other bunyaviruses. More recently, a region at the very N terminus (aa 1–180) of the La Crosse virus polymerase was shown to have a metal binding and divalent cation-dependent nuclease activity analogous to that of influenza virus endonuclease (Reguera *et al.*, 2010). The endonuclease domain of the influenza virus polymerase is responsible for mediating the cap-snatching mechanism utilized to attach cellular 5' cap structures to viral mRNAs (Plotch *et al.*, 1981). Reguera *et al.* (2010) reported that other bunyavirus polymerases, including that of RVFV, contain this endonuclease domain. The C-terminal domains of bunyavirus L proteins are substantially more variable in sequence, and our accumulated experience suggests that the C-terminal domain is a suitable target for manipulation to insert foreign epitopes while maintaining polymerase function. In addition, Bergeron *et al.* (2010) introduced the V5-epitope at the N terminus of the Crimean–Congo hemorrhagic fever virus L protein and showed that the modified L protein retained polymerase activity in a minireplicon assay.

Insertion of the V5 epitope at aa 1750 (L1V5), 1794 (L2V5) and 1852 (L3V5) within the RVFV L protein did not appear to disrupt the expression or stability of the protein, but only L3V5 retained polymerase activity (Fig. 2). The loss of polymerase activity by L1V5 and L2V5 suggests disruption of a functional domain, or that insertion of the V5 epitope into the C terminus could have affected the ability of the protein to interact both inter- and intramolecularly with the N or C termini of (other) L proteins, as described by Zamoto-Niikura *et al.* (2009); indeed, these authors showed that oligomerization of L was required for efficient polymerase activity.

The L3V5 polymerase showed 24% of the activity of wt in the minigenome assay. To try to improve this activity, the viral NSs protein was co-expressed in the system, as Ikegami *et al.* (2005) had reported that the NSs protein of the virulent RVFV strain ZH501 enhanced reporter-gene expression in an analogous minigenome assay. However, results from our experiment demonstrated that the transfection of MP12 NSs protein actually dramatically inhibited the activity of both wt and mutant polymerases, with 1 µg of co-transfected NSs-expressing plasmid reducing reporter-gene activity by >90% (Fig. 3). These results are in accord with previous reports that the NSs proteins of Bunyamwera and La Crosse orthobunyaviruses also inhibit viral RNA synthesis in a minigenome system (Blakqori *et al.*, 2003; Weber *et al.*, 2001). The difference between our results and those of Ikegami and colleagues may reflect differences in the experimental details, such as types of cells used for the assay or the way in which T7 polymerase is expressed; this requires further investigation.

We were able to rescue a recombinant virus expressing V5 epitope-tagged L protein, based on the L3V5 construct. Comparison of the growth kinetics of the tagged virus

revealed that its replication was modestly attenuated in the mammalian and insect cell lines tested, and the virus produced a smaller plaque phenotype than that of the parental rMP12 virus (Fig. 4). We also produced a two-segmented genome version of the L-tagged virus, based on our recent creation of a virus, r2segMP12, which expresses the viral glycoproteins from the NSs locus in the S segment. This virus thus lacks the M segment but is genetically stable (Brennan *et al.*, 2011). r2segMP12 is attenuated compared with its three-segmented genome parent and forms small plaques; insertion of the V5 epitope into the L protein of r2segMP12 further attenuated it, and the virus produced pinpoint sized plaques. Additional characterization of this recombinant virus is ongoing.

By using immunofluorescence analysis, we showed that the L protein had a punctate cytoplasmic distribution in infected cells (Fig. 6). At 24 h p.i., large foci of co-localizing N and L proteins were detected in the perinuclear region, which we suggest represent late-stage RVFV replication complexes, similar to those seen with other bunyaviruses such as Bunyamwera virus (Fontana *et al.*, 2008; Shi & Elliott, 2009), Tula hantavirus (Kukkonen *et al.*, 2004) and Crimean–Congo hemorrhagic fever nairovirus (Bergeron *et al.*, 2010). The recombinant RVFV will allow further detailed investigation of L protein synthesis, distribution and interactions by using a variety of experimental techniques. In addition, it will be of interest to determine whether the epitope is immunogenic in an infected animal, as this could lead to ways of tagging live-attenuated vaccine strains of RVFV to differentiate vaccinated animals from those that have been naturally infected.

METHODS

Cells. Vero E6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. BSR-T7/5 cells (Buchholz *et al.*, 1999), which stably express T7 RNA polymerase, were provided by K. K. Conzelmann (Max-von-Pettenkofer Institut, Munich, Germany) and were grown in Glasgow minimal essential medium (GMEM) supplemented with 10% FCS and 1 mg G418 ml⁻¹. BHK-21 cells were grown in GMEM supplemented with 10% tryptose phosphate broth (TPB) and 10% newborn calf serum (NCS). All mammalian cell lines were grown at 37 °C in a 5% CO₂ atmosphere unless otherwise stated. The *A. albopictus*-derived cell line C6/36 (Igarashi, 1978) was maintained in Leibovitz's L-15 medium supplemented with 10% FCS and 8% TPB. These cells were incubated at 28 °C in the absence of CO₂.

Antibody production. The coding sequences for RVFV MP12 N or NSs proteins were amplified from plasmid pTVT7-GS and cloned into pDEST14 (Invitrogen) with an N-terminal hexahistidine (6-His) tag sequence and a tobacco etch virus (TEV) protease site (for removal of the 6-His tag) to generate plasmids p14RVFN and p14RVFNs respectively. Proteins were expressed in *Escherichia coli* BL-21 CodonPlus cells (Stratagene) following induction with IPTG (1 mM final concentration) at 20 °C for 16 h with shaking. Recombinant proteins were purified by binding to Ni-NTA resin, eluted with 200 mM imidazole, 0.3 M NaCl, 0.1 M Tris/HCl pH 8.0, and concentrated into PBS (pH 7.3) containing 5% glycerol by using Vivaspinn columns (10 000 MW Cut-off; Sartorius). Purified proteins

were used to generate polyclonal rabbit antisera commercially (Eurogentec).

Plasmids. Plasmids for the recovery of RVFV have been described previously (Billecocq *et al.*, 2008) and were provided by A. Billecocq and M. Bouloy, Pasteur Institute, Paris, France. pTM1-L, pTM1-N and pTM1-NS_{MP12} contain the RVFV strain MP12 L, N and NSs ORFs under the control of the T7 promoter and encephalomyocarditis virus internal ribosome entry site sequence. pTVT7-GS, pTVT7-GM and pTVT7-GL contain full-length cDNAs to the RVFV strain MP12 antigenome segments flanked by T7 promoter and hepatitis delta ribozyme sequences. pTVT7-GS was modified to replace the NSs coding sequence with a sequence for three restriction enzyme sites, *PmlI*, *KpnI* and *SpeI*. For this, an outward PCR product was generated comprising nt 819 of the NSs coding sequence to nt 18 of the virus 3' UTR, thereby excising the NSs coding sequence. The PCR product incorporated *KpnI* and *SpeI* sites at its 3' end and *KpnI* and *PmlI* sites at its 5' end. It was digested with *KpnI* and self-ligated to create plasmid pTVT7-GSdelNSs-*KpnI*. The coding sequence for *Renilla* luciferase was amplified by PCR from pRL-CMV (Promega) to contain *SpeI* and *PmlI* restriction enzyme sites, and directionally cloned into appropriately digested pTVT7-GSdelNSs-*KpnI* to generate pTVT7-GSdelNSs:Ren. pTM1-FF-Luc expresses *Photinus* (firefly) luciferase and was used as a transfection control (Weber *et al.*, 2002).

The coding sequence for the V5 epitope (GKPIPPLLGLDST; Southern *et al.*, 1991) was inserted into various sites within the MP12 L protein-encoding region in both pTVT7-GL and pTM1-L, as shown in Fig. 1, by using a PCR mutagenesis approach (Shi & Elliott, 2009). Six V5-tagged MP12 L protein mutants were constructed, three originating from pTVT7-GL (designated pTVT7-GL1V5 to pTVT7-GL3V5) and three based on pTM1-L (pTM1-L1V5 to pTM1-L3V5). All constructs were verified by nucleotide sequencing to ensure that no other mutations had occurred during the cloning process. Details of molecular cloning, primers and PCRs are available upon request.

RVFV reporter segment assay. Briefly, BSR-T7/5 cells were transfected with pTM1-N (0.5 µg) and pTM1-L (0.5 µg), or one of the mutant L protein cDNAs cloned into the pTM1 vector (0.5 µg), together with the reporter plasmid, pTVT7-GSdelNSs:Ren (1.0 µg) and pTM1-FF-Luc (0.05 µg) as a transfection control. At 24 h post-transfection, *Renilla* and firefly luciferase activities were measured by using a Dual-Luciferase Assay kit (Promega) according to the manufacturer's protocol.

Generation of recombinant viruses from cDNA. Recombinant RVFV was generated by transfecting 7×10^5 BSR-T7/5 cells with expression plasmids pTM1-L (0.5 µg) and pTM1-N (0.5 µg) to deliver the N and L proteins, together with 1 µg of each pTVT7-based plasmid expressing the viral genomic segments as appropriate, using 3 µl Lipofectamine 2000 (Invitrogen) ($\mu\text{g DNA}^{-1}$). After 5–7 days, when extensive cytopathic effects were observed, the virus-containing supernatants were collected and stored at -80°C . Stocks of recombinant viruses were grown in BHK-21 cells at 33°C by infecting them at an m.o.i. of 0.01 and harvesting the culture medium at 72 h p.i. All experiments with infectious virus were conducted under containment level 3 conditions.

Virus growth curves. BHK-21, Vero E6 or *A. albopictus* C6/36 cells were infected with each virus at an m.o.i. of 1. One hour p.i., the inoculum was removed and the cells washed with PBS to remove unattached viruses. At the indicated time points, the supernatant fluid was collected and virus titrated by plaque assay on BHK-21 cells.

Virus titration by plaque assay or immunostaining. BHK-21 cells were infected with serial dilutions of virus and incubated under an overlay consisting of GMEM supplemented with 2% NCS and 0.6%

Avicel (FMC BioPolymer) (Matrosovich *et al.*, 2006) at 37°C for 4 days. Cell monolayers were fixed with 4% paraformaldehyde and plaques were visualized by Giemsa staining. For immunostaining, after fixation the cell monolayer was permeabilized with 0.1% Triton X-100 in PBS, saturated in blocking buffer (PBS containing 2% FCS) before staining with anti-N antibodies, followed by HRP-conjugated secondary antibody. To visualize the foci, TrueBlue peroxidase substrate (InSight Biotechnology) was applied to the monolayer until foci were detected.

Western blotting. Mammalian and insect cells were infected as described above. At various time points after infection cell lysates were prepared, and equal amounts of cell extract were separated on a 4–12% SDS-PAGE gel (Invitrogen). Proteins were transferred to a Hybond-C Extra membrane (Amersham), and the membrane was blocked in saturation buffer for 1 h (PBS containing 5% non-fat dried milk powder and 0.1% Tween 20). The membrane was reacted with rabbit anti-N and anti-NSs polyclonal antibodies, and mouse anti-V5 (Serotec) and anti-tubulin (Sigma) mAbs. This was followed by incubation with either HRP-conjugated anti-rabbit (Cell Signaling Technology) or anti-mouse (Sigma) antibodies. Visualization of detected proteins was achieved by using SuperSignal WestPico chemiluminescent substrate (Pierce).

Indirect immunofluorescent staining. BSR-T7/5 cells were grown on glass coverslips (13 mm diameter), infected with recombinant viruses and fixed at 24 h p.i. with 4% paraformaldehyde in PBS. After permeabilization with 0.1% Triton X-100 in PBS, the cells were reacted with specific primary antibodies, followed by secondary antibody conjugates. Localization of the fluorescently labelled proteins was examined at various times p.i. by using a Zeiss LSM confocal microscope.

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