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RNA Polymerase I-mediated expression of viral RNA for the rescue of infectious virulent and avirulent Rift Valley fever viruses

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Abstract

Rift Valley fever virus (RVFV, Bunyaviridae, *Phlebovirus*) is a mosquito-transmitted arbovirus that causes human and animal disease in sub-Saharan Africa and was introduced into the Arabian Peninsula in 2000. Here, we describe a method of reverse genetics to recover infectious RVFV from transfected plasmids based on the use of the cellular RNA polymerase I promoter to synthesize viral transcripts. We compared its efficiency with a system using T7 RNA polymerase and found that both are equally efficient for the rescue of RVFV generating titers of approx 10^7 to 10^8 pfu per ml. We used the RNA polymerase I-based system to rescue both attenuated MP12 and virulent ZH548 strains as well as chimeric MP12-ZH548 viruses, and in addition RVFV expressing reporter proteins.

Keywords

bunyavirus; phlebovirus; Rift Valley fever virus; nonstructural protein NSs; expression of reporter protein; reverse genetics; RNA polymerase I

Introduction

Over the past two decades, the ability to genetically manipulate viral genomes has facilitated our understanding of viral pathogenesis and studies on interactions between viral and cellular components. Reverse genetics has now been established to modify infectious viruses for many negative stranded RNA virus types, among the first of which was influenza virus (Luytjes et al., 1989). Subsequently, using a bacteriophage T7 RNA polymerase-driven antigenomic transcription approach, rabies virus (*Mononegavirales*) was successfully rescued (Schnell *et al.*, 1994). Similar methodology was used to recover Bunyamwera orthobunyavirus, which was the first segmented negative stranded RNA viruses to be recovered entirely from cloned cDNA (Bridgen and Elliott, 1996). Whereas the methodology based on expression of T7

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transcripts has been mostly applied to rescue viruses replicating in the cytoplasm, an alternative technique using the cellular DNA dependent RNA polymerase I (Pol I) to transcribe transfected plasmids was developed for influenza and Thogoto orthomyxoviruses (for review, Neumann and Kawaoka, 2004). The choice of method was mostly dictated by the site of replication of the virus, Pol I-based methodology being used for the viruses which have a nuclear replication strategy and the T7-based method being used for those which replicate in the cytoplasm. More recently, this concept has been revisited: it was shown that the T7- and the Pol I-based reverse genetic systems were equally efficient for the recovering lymphocytic choriomeningitis (LCMV) arenavirus which has a cytoplasmic strategy for transcription and replication (Flatz et al., 2006; Sanchez and de la Torre, 2006), and that the T7-promoter driven system could be applied successfully to generate influenza virus which replicates in the nucleus (de Wit et al., 2007). Furthermore, it was also reported that RNA Pol II-mediated expression of cRNA from Borna and measles virus cDNAs greatly improved the recovery of these members of the *Mononegavirales* that replicate in the nucleus and cytoplasm, respectively (Martin *et al.*, 2006).

Within the *Bunyaviridae* family, viruses are classified into five genera, *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Hantavirus* and *Tospovirus* (Nichol *et al.*, 2005; Schmaljohn and Hooper, 2001). Several bunyaviruses such as Rift Valley fever virus (RVFV), La Crosse, Crimean-Congo hemorrhagic fever, and Hantaan viruses are of medical and public health importance. RVFV is an arthropod-borne member of the *Phlebovirus* genus that causes recurrent outbreaks affecting humans and ruminants predominantly in Sub-Saharan Africa, but which spread to the Arabian Peninsula in 2000. During the past two years (2006–2008), RVFV has circulated in East Africa causing serious epidemics in Kenya, Tanzania, Somalia, Sudan and Madagascar. In humans the disease can lead to hemorrhagic fever, encephalitis or retinal vasculitis. In ruminants, it is characterized by massive death of young animals, especially of lambs, abortion and fetal deformities (Flick and Bouloy, 2005; Gerdes, 2004; Swanepoel and Coetzer, 2003).

Bunyaviruses are characterized by possession of a tripartite negative stranded RNA genome (the segments are designated L, M and S), replicate in the cytoplasm of infected cells and mature by budding in the Golgi compartment (Nichol et al., 2006). Rescue of different bunyavirus minigenomes has been demonstrated to be efficient with both T7- and Pol I-driven systems (Blakqori *et al.*, 2003; Dunn *et al.*, 1995; Flick and Pettersson, 2001; Gauliard *et al.*, 2006). Recovery of infectious Bunyamwera, La Crosse and RVF viruses has been reported based on the T7-polymerase methodology (Bird *et al.*, 2008; Bird *et al.*, 2007; Blakqori and Weber, 2005; Bridgen and Elliott, 1996; Bridgen *et al.*, 2001; Gerrard *et al.*, 2007; Ikegami *et al.*, 2006; Lowen *et al.*, 2004), while recently the Pol I-mediated expression of viral cRNA was applied to the rescue of Akabane orthobunyavirus (Ogawa et al., 2007). Since phleboviruses and orthobunyaviruses differ in the coding strategy of their S segments, utilizing an ambisense or classical negative stranded strategy to express the nucleoprotein N and the nonstructural protein NSs, respectively, it was of interest to determine if a Pol Ibased rescue system could also be applied to the phlebovirus RVFV, and to compare it to the T7 system. Thus, we cloned full-length genomic cDNAs of the virulent ZH548 strain and its attenuated derivative MP12 (Caplen *et al.*, 1985) into appropriate vectors. Our data show that the Pol I-driven method represents an efficient alternative for the recovery of RVFV. In addition, we utilized this method to create chimeric RVFV between the attenuated MP12 and the virulent ZH548 strains, and to produce RVFV expressing reporter proteins.

Results and Discussion

Development of a Pol I-driven plasmids for rescue of infectious RVFV

The unidirectional vector pRF108 which contains the murine Pol I promoter and terminator sequences, encompassed between a multiple cloning site allowing the insertion of foreign sequences, was previously used for expression of RVFV minigenomes (Gauliard et al., 2006). In the present work, it served as a vector in which to insert the full length L, M and S cDNAs of the RVFV MP12 strain. The three cDNAs were cloned in the antigenomic orientation with respect to the Pol I promoter. Several L, M and S segment cDNA-containing plasmids were obtained and the inserts sequenced. One S plasmid was selected for the following work because it contained a mutation at nucleotide residue 453 in the NSs sequence changing one codon CAG (Q_{140}) to CTG (L_{140}) compared to the authentic MP12, and thus could be used as a genetic tag.

Since plasmids pTM1-N and pTM1-L expressed N and L proteins that were functional to reconstitute transcription- and replication-competent RNPs from RVFV minigenomes (Gauliard et al., 2006), we used these plasmids to support the rescue of infectious virus in BHK/T7-9 cells that constitutively express T7 RNA polymerase. After cotransfection of the Pol I-driven full length L, M, S plasmids together with the L and N protein expression plasmids, the cell culture medium was collected at various times post transfection and RVFV titrated by plaque assay. A high level of infectious virus was detected at 48 h post transfection and reached a plateau of 5×10^7 pfu/ml at 72 to 96h post transfection (Fig 1A). Once the RVFV rescue protocol was established, production of infectious virus was successful in all subsequent experiments, routinely generating titers of 1×10^7 to 5×10^7 pfu/ml. Titration experiments showed that the optimal amounts of plasmids were 0.5 μg each of pTM1-L and pTM1-N, and 1 μg of each pPol I-L, M and S plasmids. Addition of the L and N expression plasmids was absolutely required: no virus was rescued when one or both of these plasmids were omitted (Table 1). However, expression of the envelope glycoproteins was not necessary for successful rescue (data not shown).

To confirm the identity of the recombinant RVFV MP12 strain (rMP12) recovered from cDNAs, we took advantage of the point mutation inserted serendipitously in the S sequence. Total RNA from infected Vero cells was extracted and RT-PCR was carried out using primers spanning the mutation site. Sequencing of the RT-PCR products indicated that the MP12 virus was derived from the transfected plasmids and not from contaminating authentic virus (Fig 1B). Both plaque formation and growth properties of the rescued virus rMP12 were similar to those of authentic MP12 (not shown).

As an alternative to the use of T7-based plasmids (pTM1-L, pTM1-L) to express the L and N proteins necessary to reconstitute functional RNPs, we assessed conditions in which proteins were expressed under the control of a Pol II promoter. Thus, we cloned the N and L sequences into the plasmid pCAGGS (which contains a chicken β-actin promoter) and subsequently compared rescue efficiency of rMP12 using T7- versus chicken β-actin promoter-driven RVFV L and N expression plasmids. The results are summarized in Table 1: expression of the L and N proteins via pTM1 or pCAGGS gave repeatedly high titers of virus. However, the results obtained with the pCAGGS constructs were 1.7 – 6.2 fold lower, depending on the ratio and amounts of RVFV L and N plasmids tested (Table 1).

Comparison of the Pol I and the T7-driven system for rescue efficiency

To compare the efficiency of the Pol I- and T7-promoter driven systems for RVFV MP12 rescue, we subcloned the full-length L, M and S cDNAs derived from RVFV MP12 in the antigenomic orientation into plasmid pTVT7R(0,0) which contains a truncated version of the

T7 promoter (Johnson *et al.*, 2000). Because T7 transcription efficiency was reported to be enhanced by the inclusion of one or two non-viral Gs at the 5' end of the transcripts (Pattnaik *et al.*, 1992) our constructs were designed to express one additional G at the 5' end of the transcript. The kinetics of rMP12 virus release into the cell culture medium were similar to those observed with the Pol I-promoter driven system (Fig 1A). In this experiment, the protein expression plasmids pTM1-L and pTM1-N were cotransfected with the three pTVT7 plasmids. Table 2 summarizes the results obtained under conditions where either L or N or neither of the proteins was expressed. In contrast to the Pol I-promoter driven system, which required co-expression of the RVFV L and N proteins (Table 1), rescue of RVFV from pTVT7 plasmids was obtained routinely in the absence of the protein expression plasmids (Table 2). In addition, we noticed that whereas expression of N was required if L is expressed, the reciprocal did not apply. Furthermore, co-expression of L and N proteins had no deleterious effect on RVFV production, confirming the results of Ikegami *et al.*(2006) and as reported originally for Bunyamwera virus production (Lowen *et al.*, 2004), but in contrast to the data reported for La Crosse virus (Blakqori and Weber, 2005) and RVFV (Gerrard *et al.*, 2007) in which the authors showed an inhibitory effect. These results suggest that a low but significant L and N protein expression level occurs from the primary transcripts synthesized from the pTVT7 plasmids, as observed by others. The differences observed in the different laboratories might be due to the variable efficacy of the L and N expression or/and to the degree of protein expression ("leakage") from the pT7 plasmids used. We do not explain why the leakage occurs on the T7 but not on the Pol I transcripts although both have the same sequence and lack 5' cap and 3' polyA tails.

To determine if the extra G residue present at the 5' end in the constructs could be eliminated, we generated plasmids with the T7 promoter initiating directly with the consensus A residue of the natural phlebovirus terminal sequence 5'-ACACAAA….. When transfected together with the RVFV L and N expression plasmids, these modified transcription plasmids did not produce any virus or, one experiment out of six, only at a low titer (Table 3). However, this failure is not due to a sequence defect in any of the L, M or S segment constructs, since combinations of one pT7 plasmid initiating with A with the other two initiating with G led to significant virus rescue (Table 3). Rather, this suggests that when the three segments are synthesized under the minimal promoter i.e. without an extra G, the modified promoter produce primary transcripts in limited quantities not sufficient to efficiently initiate the viral cycle. Altogether these data show that the two methods are equally efficient. Each system has advantages and disadvantages: recovering RVFV after transfection of the three T7- driven plasmids is attractive but cells expressing T7 RNA polymerase must be used. In many cases, BSR or BHK21 expressing constitutively the polymerase are used but expression is not always very stable over many passages. On the other hand, rescue of virus using the Pol I technology requires transfection of the two protein expression plasmids and the three pol I based plasmids. However, if proteins are expressed via pol II based plasmids, experiments can be carried out in any cell of murine or hamster origin.

Recovery of virulent ZH548 RVFV and its avirulent .NSs version

Having established a RVFV reverse genetics system for rescuing the attenuated RVFV strain MP12, we adapted the Pol I system to manipulate the genome of the virulent strain ZH548. Recombinant ZH548 (rZH) was recovered under the same conditions as rMP12, with titers ranging from 5×10^7 to 2×10^8 pfu/ml at 72 h post transfection. The growth curve of rZH (Z/Z/Z) was similar to that of authentic ZH548 (Fig 2B).

To study further the established reverse genetics system, we modified the full-length ZH548 S segment cDNA and generated plasmid pPol I-SZHΔNSs in which the NSs sequence was replaced by a short cloning site. Such a plasmid would allow the insertion of mutated NSs

sequences or any foreign sequence. The corresponding RVFV ZHΔNSs mutant (Z/Z/ZΔNSs) was rescued and displayed a growth curve similar to that of ZH548 and rZH (Fig 2B). The plaques formed by rZH and authentic ZH548 viruses appeared similar, whereas the plaques formed by rZHΔNSs were fuzzy with a faint staining inside (Fig 2A) as reported for the recombinant virus MP12ΔNSs (Ikegami, 2006). This suggests that a proportion of infected cells did not die but established persistent infection as previously described for RVFV Clone 13 (Billecocq *et al.*, 1996).

Using the cloning site in pPol I-SZHΔNSs, we inserted either eGFP or humanized firefly luciferase (FLuc) reporter genes in place of NSs. The recombinant viruses rZHΔNSs-GFP and rZHΔNSs-FLuc were generated by cotransfection of the appropriate plasmids. The plaques produced by rZHΔNSs-GFP were similar to those of rZHΔNSs (Fig 2A). Cells infected by rZHΔNSs-GFP were readily visualized as fluorescing green under UV light (Fig 2C). A similar virus created in the RVFV strain ZH501 context has been described very recently by Bird et al (Bird et al., 2008).

The recombinant rZHΔNSs-Fluc was recovered similarly but its titer in the maintenance medium of transfected cells was approx. 100-fold lower than rZHΔNSs. The plaques were fuzzy and much smaller than those formed by rZHΔNSs-GFP (Fig 2A). Despite the low titer, cells infected with rZHΔNSs-FLuc (at passage 0, i.e. virus recovered from the transfected cells) displayed luciferase activity. Luciferase activity increased with time and was dependent on the multiplicity of infection (Fig 2D), indicating that expression of the luciferase could be used to monitor viral replication.

In order to check the stability of the recombinant ZH virus expressing GFP or FLuc, we passaged them and checked their titers and reporter gene expression at each passage. No apparent change in GFP expression was noticed over four passages (Fig 2C), and the viral titers at the different passages remained constant (Fig 3A). In contrast, luciferase activity in cells infected with rZHΔNSs-FLuc virus decreased after the first passage (Fig 3B) and, simultaneously, viral titer increased and plaque morphology change to resemble that of rZHΔNSs (Fig 2A). These data indicate that the GFP sequence is well tolerated while the FLuc sequence appeared unstable when expressed by RVFV. To gain further insight into the mechanism leading to instability, total RNA from cells infected by rZHΔNSs-FLuc at passages 0 and 4 was amplified by RT-PCR using the 5’ and 3’ terminal primers of the S segments. Thus the amplified PCR products represent the complete S segment. Gel analysis of the DNA products (Fig 3C) clearly indicates that a product of the expected size (2,547bp) was obtained from RNA from cells infected by virus of passage 0 (lane 6) but a smaller product (approx. 1200nt) was generated from RNA from cells infected by virus of passage 4 (lane 7); this suggests that a large region, but not the whole sequence, of the FLuc gene was eliminated rapidly during virus replication. This phenomenon was not observed for ZHΔNSs-GFP: the amplification product of S sequence with the expected size (1614 bp) migrated slightly faster (Fig 3B, lane 4) than the original RVFV S segment (1690bp) (lane 2) and remained stable for at least 4 passages (Fig 3B, lane 5). These data show that the NSs sequence could be replaced by certain foreign sequences such as GFP, but probably not by any one, as suggested by the instability of FLuc. The deletion observed in FLuc is reminiscent of what happened to the NSs sequence in the evolution of Clone 13 (Muller *et al.*, 1995). The large size of FLuc (1653 bp) compared to GFP (720bp) or to Renilla Luciferase (936 bp) stably expressed by Ikegami *et al.* via a recombinant RVFV MP12 (Ikegami *et al.*, 2006) and in our laboratory via rZH (unpublished data), may be a factor. However, data published by Bird *et al.* (Bird et al., 2008) who produced a virus expressing a NSs-GFP fusion protein, suggest that a S segment larger than the natural one can be packaged in the particle.

Production of a chimeric RVFV strain ZH548 expressing the NSs protein of RVFV MP12

RVFV strain MP12 was obtained by 12 serial passages of the virulent ZH548 strain in the presence of the mutagen 5-fluorouracil (Caplen *et al.*, 1985). Several mutations accumulated in each segment of the genome, one substitution in the S segment and 7 and 3 respectively in the M and L segments (Vialat et al., 1997). To determine if the mutations in MP12 specifically attenuated each segment of the tripartite genome, Saluzzo and Smith (Saluzzo and Smith, 1990) produced reassortants between a Senegalese strain and MP12. Characterization of the reassortant viruses indicated that each segment of MP12 carried attenuation markers. However, in our hands, the Senegalese strain was insufficiently virulent to kill adult mice (unpublished observations). Therefore, we revisited the existence of attenuation markers in the S segment in particular because it encodes a major virulence factor, NSs (Vialat *et al.*, 2000). The S segment of MP12 contains two mutations in regions which may be involved in virulence, one in the NSs sequence and the other in the intergenic region. We used the reverse genetics methodology to rescue two recombinant RVFV, a reassortant virus possessing the L and M segments of ZH548 and the S segment of MP12 (Z/Z/MP12) and chimeric virus in which just the NSs sequence of MP12 was inserted into the ZH548 backbone, using pPolI-SZHΔNSs in which to clone the MP12 NSs sequence. The reassortant rZH-SMP12 and the chimeric rZH-NSs_{MP12} viruses were recovered and the S segment cDNAs were analyzed by enzymatic digestion using Taq1α restriction enzyme (this has one site in the NSs sequence of MP12 but not ZH548, and one site in N sequence that is present in the two viruses) (Fig 4A). In addition the complete S segment sequences were determined. The biological properties of these recombinant viruses were compared to the wild type rZH and to MP12. When interferon-competent cells were infected with either viruses, synthesis of IFN-β mRNA was blocked by RVFV NSs both from ZH548 or MP12 (Fig 4B). To determine whether the recombinant viruses were virulent, a dose of 10^4 pfu of each virus was inoculated into groups of 12 4–5 week-old mice (Fig 4C). As expected, rZH killed 100% of the animals with a similar survival time as authentic ZH548 as previously reported, whereas all the mice inoculated with the virus completely deleted of NSs survived, confirming the critical role of NSs in virulence (Le May et al., 2008). As already reported, MP12 was non-pathogenic in these animals (Caplen *et al.*, 1985) but when the S segment or only the NSs sequence of MP12 was expressed in the context of ZH548, 80% lethality was observed, but with a survival time significantly longer than for mice inoculated with rZH (Fig 4C). This suggests that the virulence associated with NSs of MP12 is less than that associated with the NSs of ZH548. Of note, mice inoculated with MP12 virus were not killed, indicating that, even though NSs is able to block the IFN-β response, factors other than NSs must also be involved in virulence/attenuation. The thermo-sensitive mutations in the L and/or the glycoproteins (Caplen *et al.*, 1985; Saluzzo and Smith, 1990) contribute to attenuation but the mechanisms are not yet known. In a recently published paper, we showed that inhibition of IFN-β transcription following infection with RVFV strain ZH548 correlates with NSs interacting with the SAP30 subunit of repression complex Sin3A (Le May et al., 2008). We have subsequently shown the NSs of MP12 also interacts with SAP30 using the two-hybrid system (unpublished data). The mouse survival curves clearly indicate that the virulence of rZH-SMP12 or rZH-NSs_{MP12} is reduced compared to ZH548 since not all the animals were killed and those that did die had a longer survival period. This suggests that the one mutation present in NSs of MP12 (V_{160} which is A in ZH548) has an impact on the interaction with 12 SAP30 that the two-hybrid system did not detect or that other cellular partners may be involved in modulating virulence.

In conclusion, the established rescue system is functional to produce RVFV containing specific mutations. The ability to manipulate the genome of a virulent strain like ZH548 is of great interest for future developments for dissecting viral protein functions, for vaccine production and for pathogenicity studies.

Materials and Methods

Viruses and Cells

Stocks of RVFV strains ZH548 and MP12 (Caplen *et al.*, 1985) and recombinant viruses were produced under BSL3 conditions by infecting Vero cells with 0.01 pfu per cell and by harvesting the medium at 72 h p.i.

Vero cells and BF cells (murine fibroblasts derived from Balb/c mice, kindly provided by Stephen Goodbourn, London, United Kingdom) were grown in DMEM supplemented with 10% FCS. Baby hamster kidney cells stably expressing T7 RNA polymerase (BHK/T7-9 cells) (Ito *et al.*, 2003) kindly provided by Naoto Ito (Gifu, Japan) were grown in MEM supplemented with 5 % FCS, and 1x tryptose phosphate broth. The cell culture media were supplemented with 10 IU of penicillin and 10 µg of streptomycin/ml.

Plasmids

Plasmids pTM1-L and pTM1-N have been described (Gauliard et al., 2006) and pCAGGS-L and pCAGGS-N were constructed by inserting into pCAGGS vector the PCR products of L and N synthesized by PCR using pTM1-L and pTM1-N as template.

cDNAs and PCR products corresponding to the M and S segments of MP12 as well as the L, M and S segments of ZH548 were synthesized by RT-PCR from RNA extracted from infected cells. PCR products to the L segment of MP12 was amplified by PCR using plasmid pLc as template (Lopez *et al.*, 1995). The amplification was carried out as a single PCR product using TaKaRa Prime STAR DNA polymerase for the L and S segments and Finnzymes Phusion Taq polymerase for the M segment. They were cloned between the murine pol I RNA polymerase promoter and terminator sequences in pRF108 (Flick and Pettersson, 2001). The three PCR products of MP12 antigenomes were also cloned into pTVT7R(0,0) (Johnson et al., 2000) between the T7 RNA polymerase promoter and terminator sequences using primers containing or lacking an extra G residue at the 5’ end of each segment. Because variations to the consensus sequence were observed in the M plasmids, the mutations were corrected by replacement with MP12 sequence.

To introduce specific mutations in NSs we modified pPol I-SZH in which the NSs coding sequence was replaced by a BbsI cloning site. For this, we synthesized two PCR products from the Pol I-SZH, - one starting from the first nucleotide of the intergenic region (nt 833)) possessing at its 5’ end BbsI and XhoI sites and at its 3’ end the unique BamHI site of Pol I-SZH present in the N ORF and - a second one corresponding to the entire 5’ noncoding region possessing at its 5’ end the PstI site present in the Pol I vector and at its 3’ end BbsI and XhoI sites (Fig. 5). The two PCR products were digested with BamHI and XhoI or PstI and XhoI, and inserted into Pol I-SZH that had been cleaved with BamHI and PstI. This generated plasmid pPolI-SZHΔNSs. We then used this plasmid, linearized at the BbsI site, to insert the eGFP, hFLuc or NSsMP12 sequences which were amplified by PCR from pEGFP-N1 (Clontech), pGL3 (Promega) or pCI-NSsMP12, respectively (Billecocq et al., 2004). The sequences were verified to ensure no unintentional mutations. All sequences of the specific oligonucleotides used for the different constructions are available on request.

Virus rescue

RVFV was rescued by transfecting BHK/T7-9 cells with the expression plasmids pTM1-L (0.5 µg) and pTM1-N (0.5 µg) together with 1 µg each of L, M and S Pol I or T7 plasmids in the presence of FuGENE (Roche) using 3 µl per 1 µg of plasmids for 0.5×10^6 cells. Each rescue was performed in triplicate. After 3 to 5 days, extensive cytopathic effect was normally observed; maintenance medium from the triplicates was pooled at day 5 and stored at −80°C.

Working stocks were prepared by infecting Vero cells at moi of 0.001. This work was carried out under BSL3 conditions at the Pasteur Institute.

Virus titration by plaque assay

Vero cells were infected with serial dilutions of virus and incubated under an overlay consisting of DMEM, 2% FCS, antibiotics and 1% agarose at 37°C. At 4 days p.i., the lytic plaques were counted after staining with a solution of crystal violet (0.2 % in 10% formaldehyde and 20% ethanol).

GFP and Fluc expression analysis

Cells infected by rZHΔNSs-GFP were fixed with 3.7% formaldehyde in PBS and observed under an epifluorescence microscope equipped with specific filter. Cells infected by rZHΔNSs-FLuc were lysed in the Fluc lysis buffer and luciferase activity measured using a specific substrate (Promega) according to the recommendations of the manufacturer.

RNA analysis

Total RNA was extracted by Trizol reagent (Roche) according to the manufacturer's instructions. RT-PCR was done using specific oligonucleotides (available on request) using AMV reverse transciptase (Promega) for the RT and PrimeSTAR (Takara), or Taq (Promega) DNA polymerase for the PCR of S segment, IFN β mRNA and GAPDH mRNA, respectively according to the recommendations of the manufacturers.

Infection of mice

Groups of 12 4- to 5-week old mice, Swiss strain, (Charles River) were injected intraperitoneally with 10^4 pfu per mice under anesthesia under BSL3 conditions

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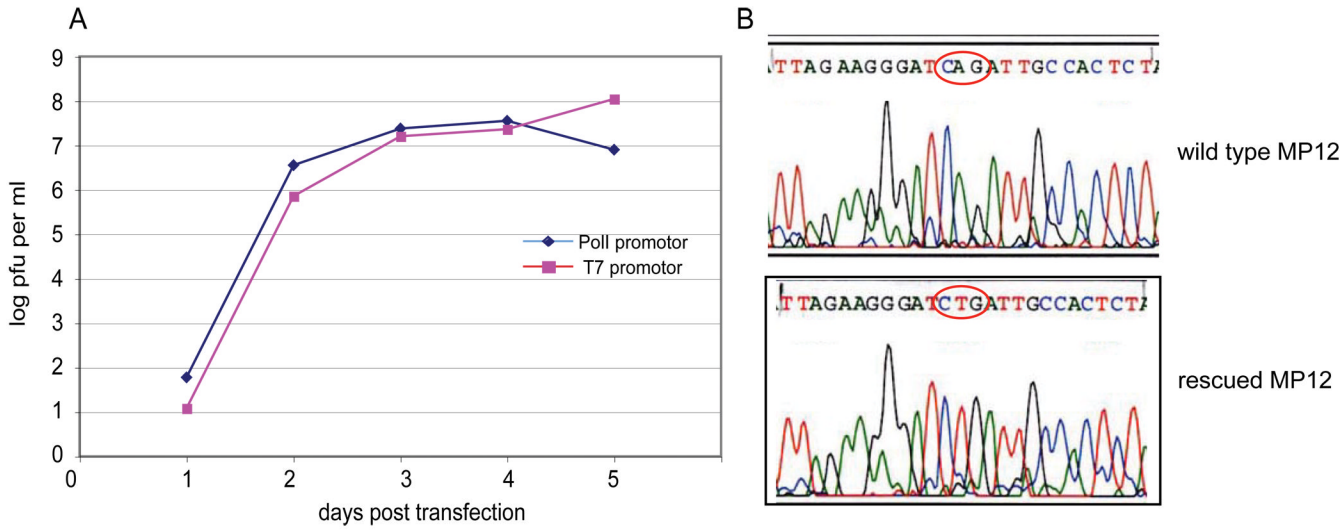

Fig. 1. Production of rMP12 using Pol I- and T7-promoter driven rescue system

A) Kinetics of rMP12 production after transfection of plasmids expressing L, M and S segments under control of Pol I or T7 promotor together with the RVFV L and N expression plasmids pTM1-L and pTM1-N in BHK/T7-9 cells. B) Sequence analysis of the rMP12 S segment compared to authentic MP12 S segment carried out with total RNA extracted from infected cells.

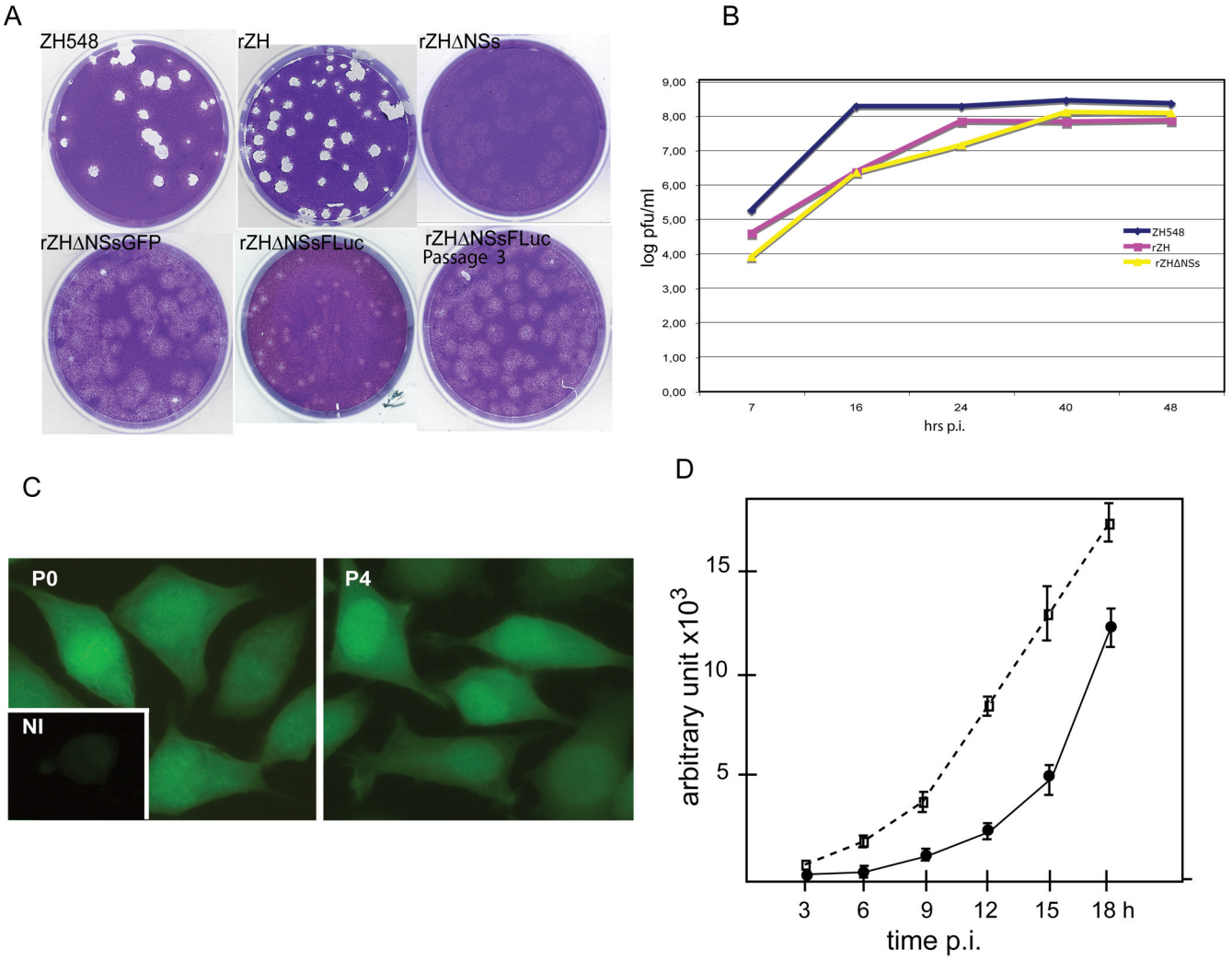

Fig. 2. Properties of rescued recombinant RVFV

A) Plaque assay showing authentic ZH548 and the different recombinant viruses: rZH, rZHΔNSs, rZHΔNSs-GFP and rZHΔNSs-FLuc at passages 0 and 3. B) Growth curves of rZH and rZHΔNSs in comparison to ZH548 after infection of Vero cells at a moi=3. C) Expression of GFP in Vero cells infected with rZHΔNSs-GFP at passage 0 or 4 and in non-infected cells (NI). D) Kinetics of FLuc expression in Vero cells infected with rZHΔNSs-Fluc at a moi= 0.1 or 0.5. Luciferase activity is expressed in arbitrary units per 2.5×10^4 cells. Each data point is the mean of triplicate assay.

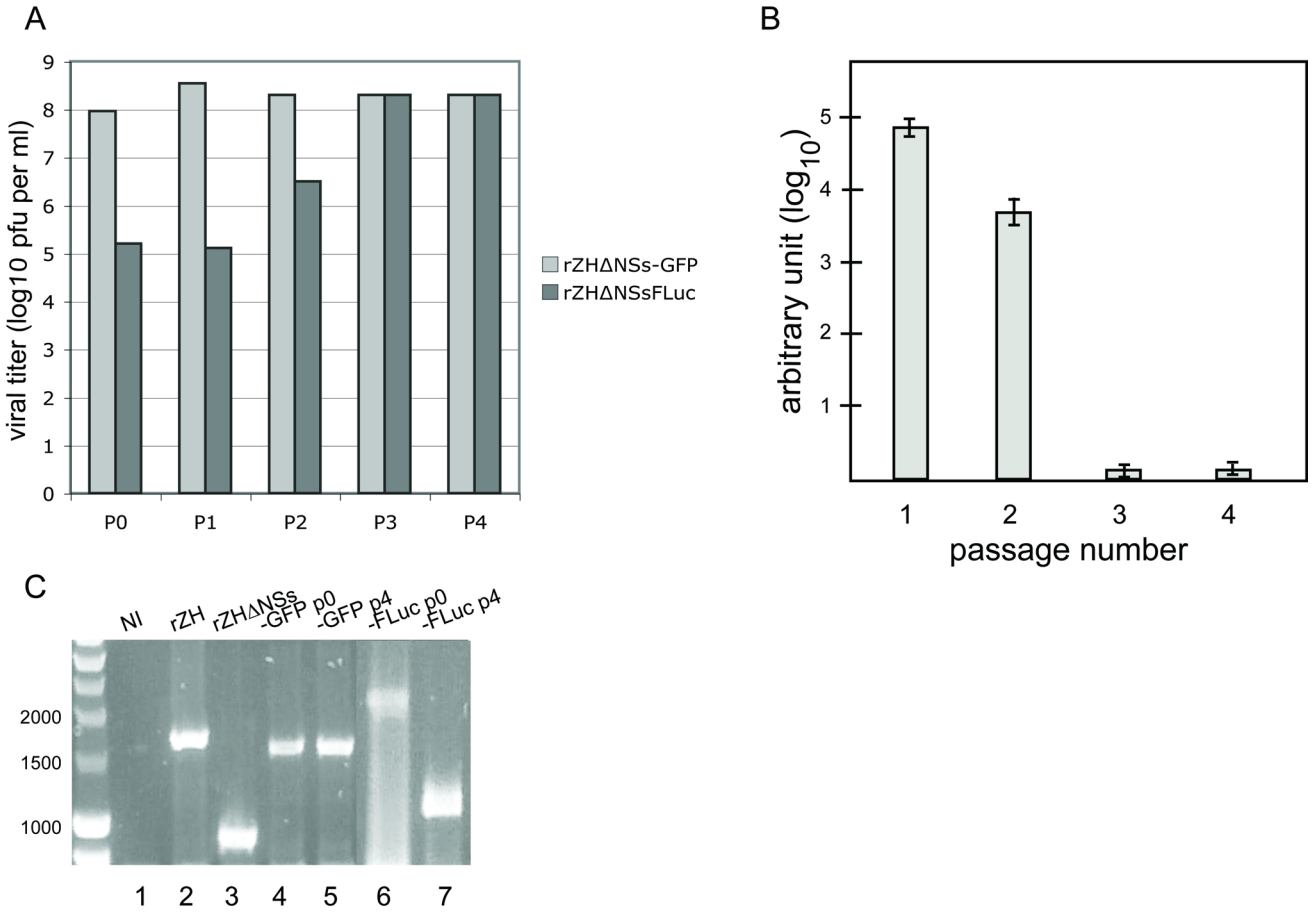

Fig. 3. Analysis of virus stability for foreign gene expression

A) Titer of rZHΔNSs-GFP and rZHΔNSs-Fluc after rescue (P0) or passaged one to four times (P1–P4) on Vero cells at a moi=0.001 and recovered at day 3. B) Luciferase activity in Vero cells infected with different passages of rZHΔNSs-FLuc at a moi=3, expressed in arbitrary units per 2.5×10^4 cells. Mean of triplicate assays. C) Gel analysis of RT-PCR products representing the full-length S segment of rZHΔNSs-GFP and rZHΔNSs-Fluc at P0 and P4. RT-PCR was performed using total RNA extracted at 15h p.i. from Vero cells infected by these viruses or ZH548 and rZHΔNSs.

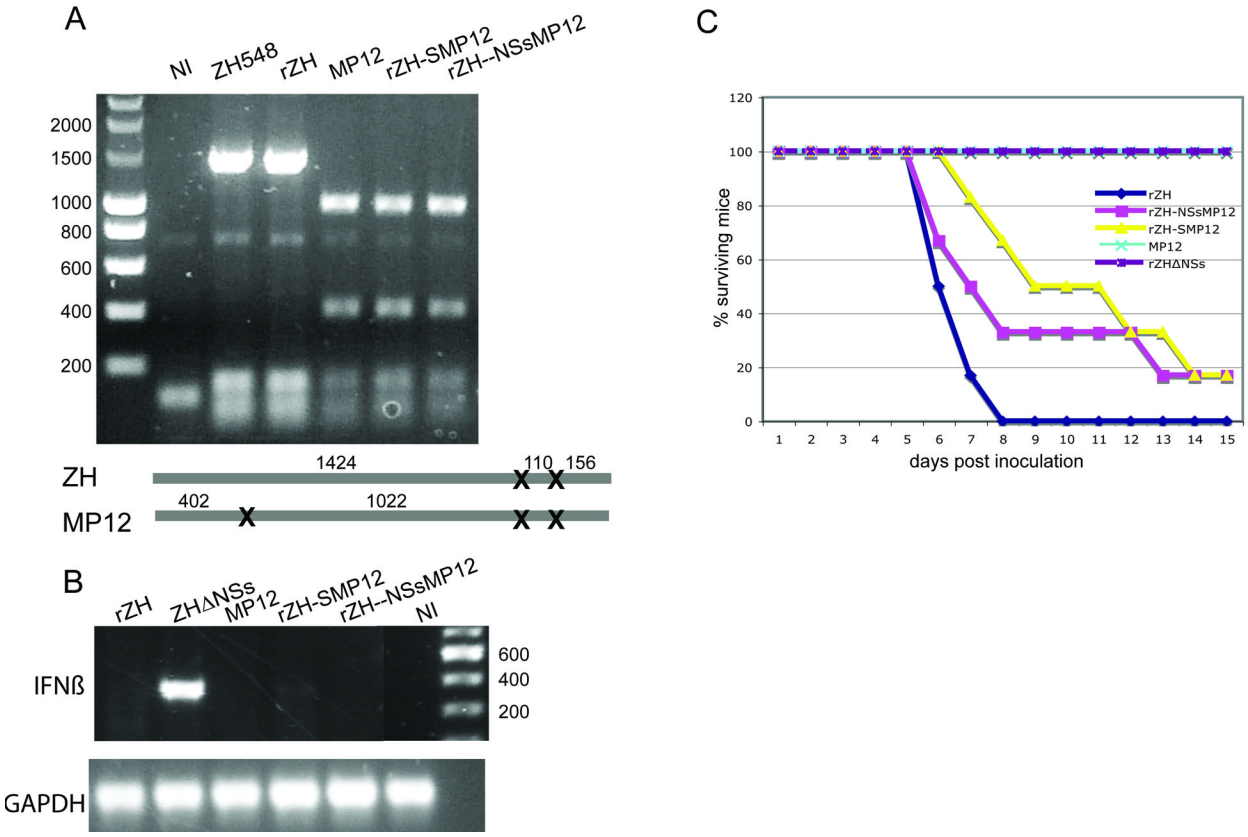

Fig. 4. Analysis of the virulence associated with the NSs of RVFV MP12

A) Characterization of S segment by Taq1α digestion. S segment RT-PCR carried out on total RNA extracted at 15h p.i. from Vero cells infected by ZH548, MP12, rZH, rZH-SMP12 or rZH-NSsMP12 at a moi=3. Schematic representation of the position of Taq1α in the S segment of ZH548 and MP12. The size of digestion products for ZH548 and rZH are indicated. B) Inhibition of the IFN-β mRNA transcription by these viruses except for the rZHΔNSs. IFN-β- and GAPDH-specific RT-PCR performed on total RNA extracted at 8h p.i. from L929 cells infected at a moi=3 by ZH548, MP12, or the recombinant RVFV. C) Survival curves of 4 to 5 week old mice (Swiss) infected with 10^4 pfu of each RVFV. The experiment was carried out using lots of 12 mice.

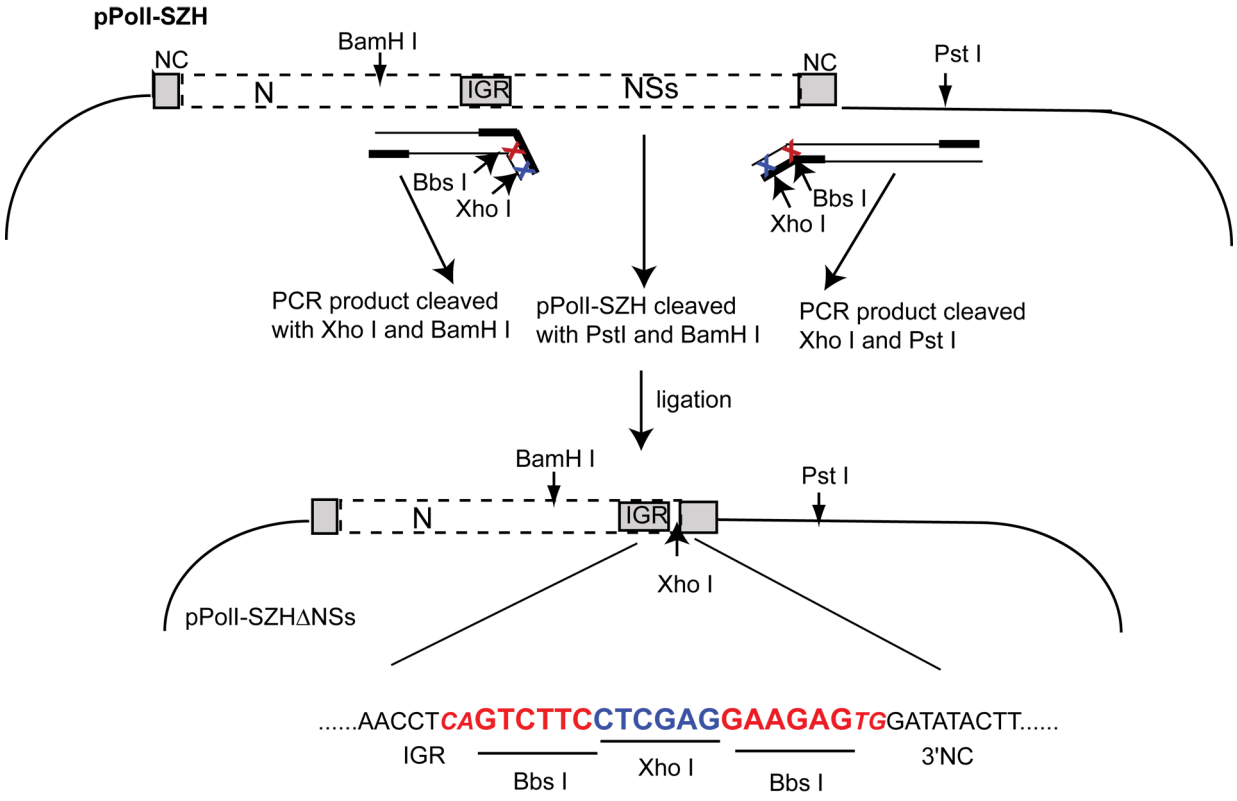

Fig. 5.

Schematic diagram of the pPolI-SZH plasmid and the generation of pPolI-SΔNSs in which the NSs sequence was deleted and replaced by a XhoI/BbsI cloning site. The terminal noncoding (NC) and intergenic (IGR) regions are indicated as well as the restriction sites utilized to generate the constructs.

Table 1

Effects of T7- or Pol II- promoter driven protein expression plasmids on the efficiency of RVFV rescue using Pol I based plasmids for viral antigenome expression.

Expression plasmid				Titer* pfu/ml
T7 promoter		Pol II promoter		
pTM1-L (μg)	pTM1-N (μg)	pCAGGS-L (μg)	pCAGGS-N (μg)	
0.25	0.25			4.0×10^7
0.5	0.5			8.8×10^7
1	1			7.0×10^7
0.5	-			<10
-	0.5			<10
-	-			<10
		0.25	0.25	1.4×10^7
		0.5	0.5	2.4×10^7
		1	1	1.7×10^7
		0.25	0.5	2.2×10^7
		0.25	1	2.9×10^7
		0.5	1	1.6×10^7
		0.5	0.25	5.0×10^7
		1	0.5	1.6×10^7
		1	5	1.4×10^7

* Titer in pfu per ml in the maintenance medium collected at 5d post transfection

Table 2

Effect of various conditions for protein expression using the T7-promoter driven system for antigenome expression after transfection into BHK/T7-9 cells.

Protein expression plasmids		Titer*
pTM1-L (μg)	**pTM1-N (μg)**	**pfu/ml**
0.5	0.5	1.7×10^{7}
0.5	-	<10
-	0.5	8.0×10^{6}
-	-	7.2×10^{7}

* titers in pfu per ml in the maintenance medium collected at 5d post transfection

Table 3

Effect of the presence of an additional G at the 5'end of the viral transcripts on the efficiency of infectious virus rescue

Expression plasmids pTVT7-	Titer*: pfu/ml
gL/gM/gS	$4{,}0\times10^{7}$
L/gM/gS	$2{,}0\times10^{7}$
gL/M/gS	$2{,}4\times10^{6}$
gL/gM/S	$1{,}6\times10^{7}$
L/M/S	$8{,}0\times10^{2}$ n=1, <10 n=5

* titers in pfu per ml in the maintenance medium collected at 5d post transfection