Video Article

Using Reverse Genetics to Manipulate the NSs Gene of the Rift Valley Fever Virus MP-12 Strain to Improve Vaccine Safety and Efficacy

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Abstract

Rift Valley fever virus (RVFV), which causes hemorrhagic fever, neurological disorders or blindness in humans, and a high rate abortion and fetal malformation in ruminants[1], has been classified as a HHS/USDA overlap select agent and a risk group 3 pathogen. It belongs to the genus *Phlebovirus* in the family *Bunyaviridae* and is one of the most virulent members of this family. Several reverse genetics systems for the RVFV MP-12 vaccine strain[2,3] as well as wild-type RVFV strains [4-6], including ZH548 and ZH501, have been developed since 2006. The MP-12 strain (which is a risk group 2 pathogen and a non-select agent) is highly attenuated by several mutations in its M- and L-segments, but still carries virulent S-segment RNA[3], which encodes a functional virulence factor, NSs. The rMP12-C13type (C13type) carrying 69% in-frame deletion of NSs ORF lacks all the known NSs functions, while it replicates as efficient as does MP-12 in VeroE6 cells lacking type-I IFN. NSs induces a shut-off of host transcription including interferon (IFN)-beta mRNA[7,8] and promotes degradation of double-stranded RNA-dependent protein kinase (PKR) at the post-translational level.[9,10] IFN-beta is transcriptionally upregulated by interferon regulatory factor 3 (IRF-3), NF-kB and activator protein-1 (AP-1), and the binding of IFN-beta to IFN-alpha/beta receptor (IFNAR) stimulates the transcription of IFN-alpha genes or other interferon stimulated genes (ISGs)[11], which induces host antiviral activities, whereas host transcription suppression including IFN-beta gene by NSs prevents the gene upregulations of those ISGs in response to viral replication although IRF-3, NF-kB and activator protein-1 (AP-1) can be activated by RVFV7. . Thus, NSs is an excellent target to further attenuate MP-12, and to enhance host innate immune responses by abolishing the IFN-beta suppression function. Here, we describe a protocol for generating a recombinant MP-12 encoding mutated NSs, and provide an example of a screening method to identify NSs mutants lacking the function to suppress IFN-beta mRNA synthesis. In addition to its essential role in innate immunity, type-I IFN is important for the maturation of dendritic cells and the induction of an adaptive immune response [12-14]. Thus, NSs mutants inducing type-I IFN are further attenuated, but at the same time are more efficient at stimulating host immune responses than wild-type MP-12, which makes them ideal candidates for vaccination approaches.

Video Link

The video component of this article can be found at http://www.jove.com/video/3400/

Protocol

1. Recovery of recombinant MP-12 encoding NSs mutation(s) from plasmid DNAs[2]

1. Spread baby hamster kidney (BHK)/T7-9 cells[15], which stably express T7 RNA polymerase, into 6-cm dishes in Minimum Essential Medium (MEM)-alpha (Invitrogen, Cat# 32561037) containing 10% fetal bovine serum (FBS), Penicillin-Streptomycin (Penicillin:100 U/ml, Streptomycin: 100 µg/ml) (Invitrogen, Cat#15140122), and 600 µg/ml of hygromycin B (Cellgro, Cat#30-240-CR).

   * The efficiency of viral recovery is higher in 6-cm dishes than in 35-mm dishes. BHK/T7-9 cells with low passage level support higher rates of recovery. Alternatively, other BHK cell lines that stably express T7 RNA polymerase could be used[4,5,16,17].
2. When cells have reached 70-80% confluency, replace the culture supernatant with fresh MEM-alpha containing 10% FBS and Penicillin-Streptomycin (not containing hygromycin B).

   * Cells should be transfected within 1 hour after replacing the medium to avoid the loss of T7 RNA polymerase expression.
3. For the recovery of RVFV, a set of plasmids encoding viral genomic RNAs for full-length viral RNA expression, and a second set encoding viral gene open reading frames for viral protein expression (**Figures 1 and 2**) are required. Prepare a mixture of the following plasmids2 (**Figure 2**) in a 1.5 ml tube:
   1. pProT7-S(+) with mutation(s) in the NSs gene (2 mg): This plasmid encodes the anti-viral-sense (positive-sense) full-length RVFV MP-12 S-segment flanked by the T7 promoter and the hepatitis delta virus (HDV) ribozyme sequence.
   2. pProT7-M(+) (2 mg): This plasmid encodes the anti-viral-sense (positive-sense) full-length RVFV MP-12 M-segment flanked by the T7 promoter and the HDV ribozyme sequence.
   3. pProT7-L(+) (2 mg): This plasmid encodes anti-viral-sense (positive-sense) full-length RVFV MP-12 L-segment flanked by the T7 promoter and the HDV ribozyme sequence.

4. pT7-IRES-vN (2 mg): This plasmid encodes the RVFV MP-12 N open reading frame (ORF) downstream of the T7 promoter and an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES).
5. pT7-IRES-vL (1 mg): This plasmid encodes the RVFV MP-12 L ORF downstream of the T7 promoter and an EMCV IRES.
6. pCAGGS-vG (1 mg): This plasmid encodes RVFV MP-12 M ORF downstream of the chicken beta-actin promoter.

   * The addition of pT7-IRES-vN, pT7-IRES-vL and pCAGGS-vG is not essential for the recovery of MP-12, but it enhances the efficiency of rescue[2]. Authors experienced poor expression of Gn/Gc by using pT7-IRES plasmid, probably due to the lack of leaky scanning of AUGs by ribosomes. Therefore, we constructed pCAGGS-vG for cap-dependent Gn/Gc expression.

4. Add 30 ml of TransIT-LT1 (Mirus, Cat#MIR2300) to 385 ml of Opti-MEM (Invitrogen, Cat# 31985070) in a 1.5 ml tube, and vortex briefly.
5. After 5 min incubation at room temperature, slowly add the Opti-MEM containing liposomes to the plasmid mixture from step 1.3 to, mix gently by pipetting and incubate for 15 min at room temperature.
6. Add the mixture of liposome and plasmids to the culture medium of the BHK/T7-9 cells from step 1.2 drop by drop (**Figure 3**).
7. Incubate the transfected cells at 37°C in an incubator with 5% CO_2 for 24 h, and replace the culture supernatant with fresh MEM-alpha containing 10% FBS and Penicillin-Streptomycin (not containing hygromycin B).
8. Incubate the cells at 37°C in an incubator with 5% CO_2 for 4 additional days (incubate for 5 days total), and collect the culture supernatants into a 15 ml tube.

   * The cytopathic effect (CPE) observed here does not necessarily reflect the result of successful viral recovery, because the transfection induces cell death which appears similar to CPE caused by viral RNA replication or viral protein synthesis.
9. Centrifuge the supernatants at 2,200 xg at 4°C for 5 min.

   * The purpose of this step is to pellet down the cellular debris from viral stock. An aerosol-tight centrifuge bucket is recommended for increased safety.
10. Transfer the supernatants into screw-cap 5 ml cryotubes, and store the passage 0 (P0) virus stock at -80°C for further use.

2. Amplification of P0 virus

1. The P0 samples often contain insufficient viral titer for downstream experiments[2]. An amplification step in VeroE6 cells, which is a clone of African green monkey kidney (Vero) cells lacking the IFN-alpha/beta genes[18,19], increases viral titer up to maximum level. Alternatively, other cells lacking type-I IFN responses such as Hec1B cells[20] or MEF cells from IFNAR1-knockout mice[21] might be used for this step. Spread VeroE6 cells into 10-cm dishes in Dulbecco's modified minimum essential medium (DMEM) (Invitrogen, Cat# 11965092) containing 10% FBS, Penicillin-Streptomycin (Penicillin:100 U/ml, Streptomycin: 100 mg/ml), and incubate at 37°C in an incubator with 5% CO_2 until they reach 80% confluency.

   * Recombinant MP-12 strains encoding mutant NSs often fail to replicate efficiently in type-I IFN-competent cells.
2. Mix 300 ml of P0 samples with 2.7 ml of DMEM with 10% FBS and Penicillin-Streptomycin. Remove culture medium from the VeroE6 cells from step 2.1 and replace with the diluted P0 sample. Incubate at 37°C for 1 h in an incubator with 5% CO_2.
3. Remove the inocula and add 10 ml of DMEM with 10% FBS and Penicillin-Streptomycin to each dish.
4. Incubate at 37°C for 3 to 4 days until CPE of VeroE6 cells becomes apparent.

   * Disruption of monolayer occurs during MP-12 infection, while recombinant MP-12 lacking NSs, such as rMP12-C13type (C13type) (**Figure 4**), does not disrupt the monolayer, but a number of dead floating cells appear 2 to 3 days after infection.
5. Harvest the supernatant at 3 to 4 dpi, as described in sections 1.9) and 1.10), and designate the samples as E6P1.

3. Titration of recombinant MP-12 by plaque assay

1. Spread VeroE6 cells into 6-well plates.

   * Duplicate analysis per sample is more reliable than single analysis.
2. When VeroE6 cells have grown to 80% confluency, prepare 10-fold serial dilutions of virus samples in DMEM with 10% FBS and Penicillin-Streptomycin up to 10-6 as follows:

   - 10 µl of E6P1 sample + 990 ml of DMEM with 10% FBS and Penicillin-Streptomycin (10^{-2} dilution)
   - 100 µl of 10^{-2} sample + 900 ml of DMEM with 10% FBS and Penicillin-Streptomycin (10^{-3} dilution)
   - 100 µl of 10^{-3} sample + 900 ml of DMEM with 10% FBS and Penicillin-Streptomycin (10^{-4} dilution)
   - 100 µl of 10^{-4} sample + 900 ml of DMEM with 10% FBS and Penicillin-Streptomycin (10-5 dilution)

3. Aspirate medium from the 6-well plate from step 3.1 and add 400 µl of each dilution (from step 3.2) into the wells (**Figure 3**).
4. Incubate at 37°C for 1 h in an incubator with 5% CO_2.
5. During the incubation, prepare two 15 ml tubes for the agar-overlay as follows:

   Tube A (keep in 42°C water bath): 7 ml of 1.2% noble agar (VWR, Cat#101170-362) in water

   Tube B (keep in 37°C water bath): 7 ml of Modified Eagle Medium (MEM 2x) (Invitrogen, Cat# 11935046) containing 10% FBS, Penicillin-Streptomycin (Penicillin: 100 U/ml, Streptomycin: 100 µg/ml), and 10% Tryptose phosphate broth (MP biomedicals, Cat#1682149).
6. After 1 h incubation, remove the viral inocula, and immediately add 2 ml per well of a 1:1 mixture of tube A and tube B (from step 3.5).

   * Take care to add the overlay immediately as drying up of wells causes the death of uninfected cells.
7. Incubate the plates at 37°C for 3 days in an incubator with 5% CO_2.

8. Prepare tube A and tube B again as described in step 3.5. Prepare also 500 µl of 0.33% neutral red solution (Sigma Aldrich, Cat#N2889-100ML) per plate which is also kept at 37°C water bath.
9. Mix tube A, tube B and 500 ml (final conc. 0.011%) of neutral red solution and add 2 ml of mixture per well.

   * The amount of neutral red solution to be added varies by the lot of neutral red solution, and initial optimization is required. Long-term storage of 0.33% neutral red solution causes precipitation. In such cases, the precipitate can be completely dissolved by incubation at 55°C for 10 min followed by vigorous shaking. The use of precipitated neutral red solution results in weak staining of cells, while re-dissolved neutral red solution stains cells well.
10. Incubate the plate for 16 h (or overnight) at 37°C in an incubator with 5% CO_2.
11. Count the number of plaques in the well which contains 10 to 100 plaques per well. Calculate the number of plaque forming units/ml. For example, if we observe 28 plaques in the wells inoculated with the 10^{-5} dilutions, 28 (# of plaques) x (1 ml/0.4 ml) x 10^5 (dilution) = 7.0 x 10^6 plaque forming units (pfu)/ml (**Figures 5**).

4. Screening of NSs mutants lacking the type-I IFN suppression function

1. Spread C57/WT MEF cells (InvivoGen, Cat#mef-c57wt), which encode a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-kB and IRF-3/7 (**Figure 6**), into 12-well plates. The cells are maintained in DMEM with 10% FBS, Penicillin-Streptomycin (Penicillin: 100 U/ml, Streptomycin: 100 µg/ml), Blasticidin S (3 mg/ml), and Zeocin (100 µg/ml).
2. When cells become sub-confluent (80%), cells are mock-infected or infected with MP-12 or recombinant MP-12 encoding NSs mutations at multiplicity of infection (moi) of 3 or 0.1 (see sections 2 and 3, amount of each inoculum should be 300 µl). At 1 h post infection, remove the inocula, and add 1 ml per well of DMEM with 10% FBS, Penicillin-Streptomycin (Penicillin: 100 U/ml, Streptomycin: 100 µg/ml) (Blasticidin and Zeocin are not added at this time).
3. At 14 h post infection, collect culture supernatants. Add 200 µl of QUANTI-Blue (InvivoGen, Cat # rep-qb1) and 50 µl of each sample (in triplicate) to wells of a 96-well plate. Seal and incubate the plate at 37°C for 1 h.

   * Both MP-12 and recombinant MP-12 lacking NSs clearly induce host translational suppression in IFN-alpha/beta competent cells including 293 cells, MRC-5 cells and mouse embryonic fibroblast (MEF) cells after 14 hours post infection at high moi. On the other hand, IFN-beta mRNA or ISG56 mRNA accumulates abundantly at 7 to 8 hours post infection in type-I IFN competent cells. Thus, we chose 14 hours post infection to collect the supernatants to see the accumulation of SEAP induced by innate immune responses.
4. Read the OD values at 650 nm using a plate reader (**Figure 7**).

   * The results are consistent with the data obtained by Northern blot using an RNA probe specific to mouse ISG56 mRNA, which shows up-regulation of ISG56 mRNA in the absence of NSs expression (**Figure 8**).

   * It should be noted that the SEAP activity is determined by the abundance of proteins which could be affected by host translation activity. A relative level of SEAP might not be high compared to the increased level of mRNA because SEAP cannot be synthesized even in the presence of SEAP mRNA if cellular translation is suppressed. Northern blot is a more straightforward assay and a more accurate way to evaluate the amount of mRNA induced by the lack of NSs functions in infected cells than the SEAP reporter assay. However, the SEAP reporter system is more rapid than Northern blot and hence useful for rapid screening of NSs mutants potentially lacking host transcription suppression function.

5. Representative Results:

The reverse genetics system consistently generated viable recombinant MP-12 viruses with titers higher than 1 x 10^6 pfu/ml. C13type virus lacking NSs functions formed large turbid plaques, while MP-12 formed clear plaques of various sizes[2] (Figure 5). Mock-infected C57/WT MEF cells or those infected with MP-12 did not increase the level of SEAP in culture supernatant compared to mock-infected cells, while the culture supernatant of C57/WT MEF cells infected with C13type contained an increased level of SEAP by 14 hours post infection (hpi) (Figure 7). These results are consistent with those obtained by Northern blot using an RNA probe specific to mouse ISG56 mRNA (Figure 8).

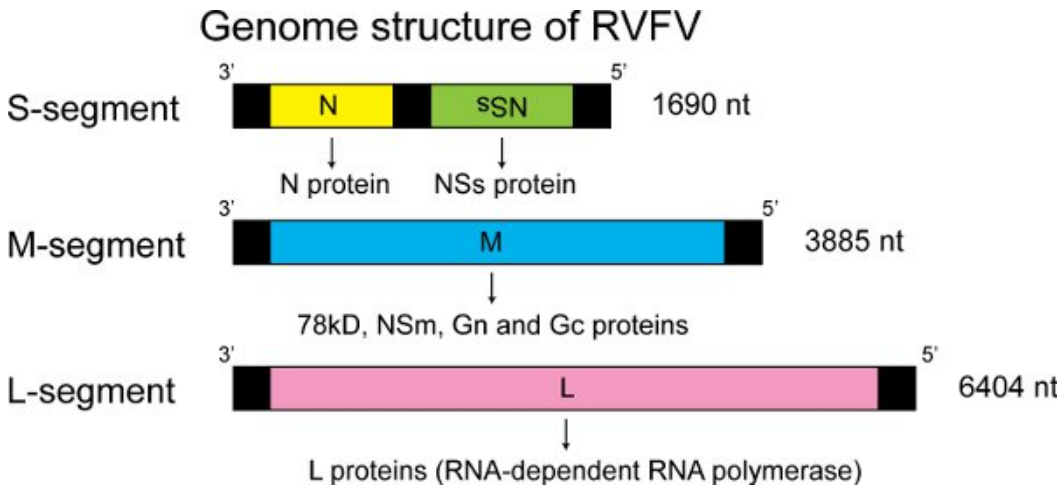

Figure 1. Genome structure of RVFV

RVFV has a tripartite negative-sense or ambisense RNA genome named S-, M-, and L-segment. S-segment encodes N and NSs genes in an ambisense manner. N mRNA is synthesized from viral-sense (negative-sense) S-segment, while NSs mRNA is synthesized from anti-viral-sense

(positive-sense) S-segment. M-segment encodes a single M mRNA and synthesizes the78kD, NSm, Gn or Gc proteins by leaky scanning of several AUGs at 5'region of M mRNA, followed by their co-translational cleavage[22,23]. L-segment encodes the L protein. Both N and L proteins are essential for viral transcription and replication, while Gn and Gc are the viral envelope proteins. NSs and NSm proteins are nonstructural proteins, which are not incorporated into virus particles.

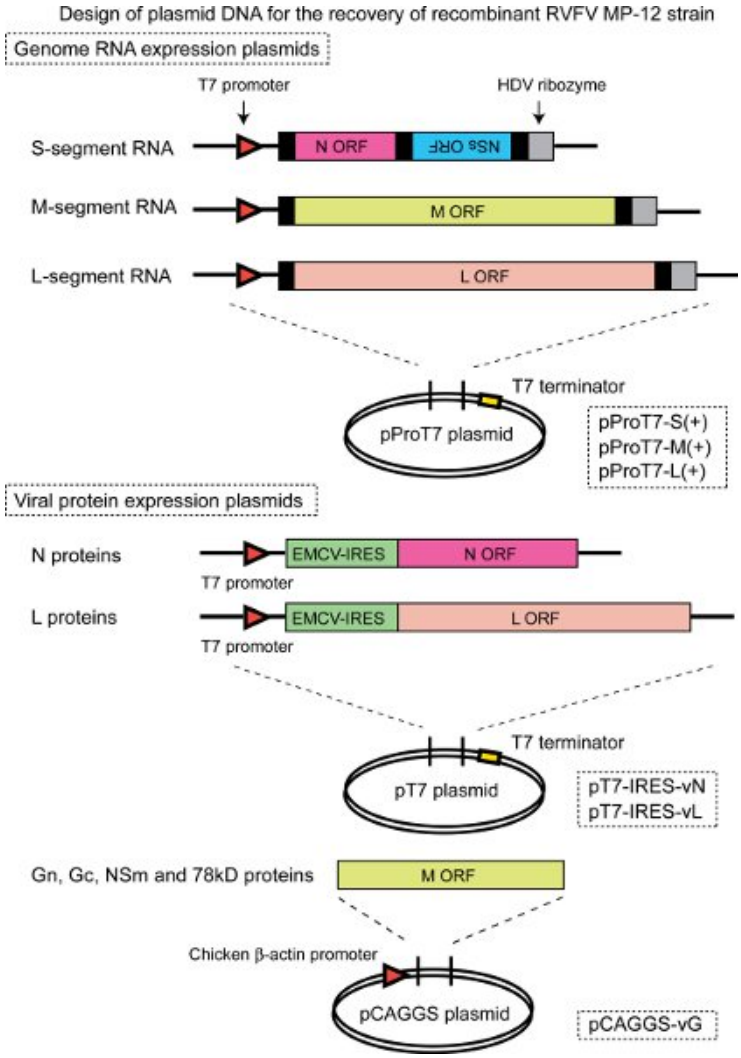

Figure 2. . Design of plasmid DNA for the recovery of recombinant RVFV MP-12 strain

The cDNA encoding full-length anti-viral-sense S-, M-, or L-segment are cloneddownstream of T7 promoter and upstream of hepatitis delta virus (HDV) ribozyme sequences, designated as pProT7-S(+), pProT7-M(+), or pProT7-L(+), respectively[2]. T7 RNA polymerase expressed in BHK/T7-9 cells transcribes the RNA encoding full-length S-, M-, or L-segment with precise genome 3' end. The open reading frame (ORF) of N or L proteins are cloned under encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), which are designated as pT7-IRES-vN or pT7-IRES-vL, respectively, to allow the uncapped T7 RNA transcript to be recognized by ribosomes in cap-independent manner. The M ORF is cloned under chicken b-actin promoter of pCAGGS plasmid[24], which is designated as pCAGGS-vG, to allow the synthesis of 78kD, NSm, Gn and Gc proteins, which are generated from different AUGs by leaky scanning[23]. Both N and L proteins are required for initiating transcription or RNA replication, while the pT7-IRES-vN and pT7-IRES-vL are not essential for the recovery of recombinant MP-12[2], probably due to the presumable leaky expression of Pol-II-driven capped RNA transcripts encoding N-ORF and L-ORF from pProT7-S(+) and pProT7-L(+), respectively.

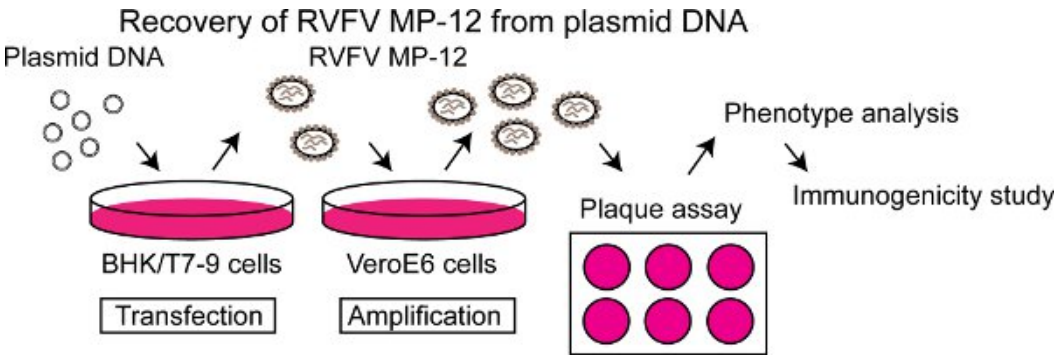

Figure 3. Recovery of RVFV MP-12 from plasmid DNA

Transfection of BHK/T7-9 cells with pProT7-S(+), pProT7-M(+), pProT7-L(+), pT7-IRES-vN, pT7-IRES-vL and pCAGGS-vG plasmids (Fig.1) generates infectious recombinant RVFV MP-12 strain in culture supernatants. The supernatant at 5 day post transfection is collected, and passaged into fresh Vero E6 cells for viral amplification. Typically, more than 1 x 10^6 pfu/ml of virus can be recovered at 3 to 4 days post infection. The amplified virus (E6P1 virus) is titrated by using plaque assay with Vero E6 cells and used for phenotype analysis and immunogenicity studies.

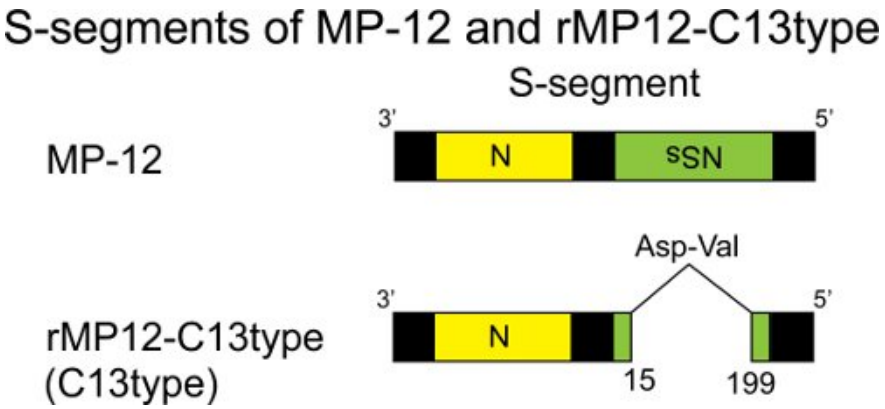

Figure 4. S-segment of MP-12 and rMP12-C13type

The comparison of MP-12 and rMP12-C13type (C13type) S-segments. Compared to NSs of MP-12 strain, the NSs ORF of C13type is truncated by 69%, and is identical to that of naturally isolated clone 13 strain[2,25].

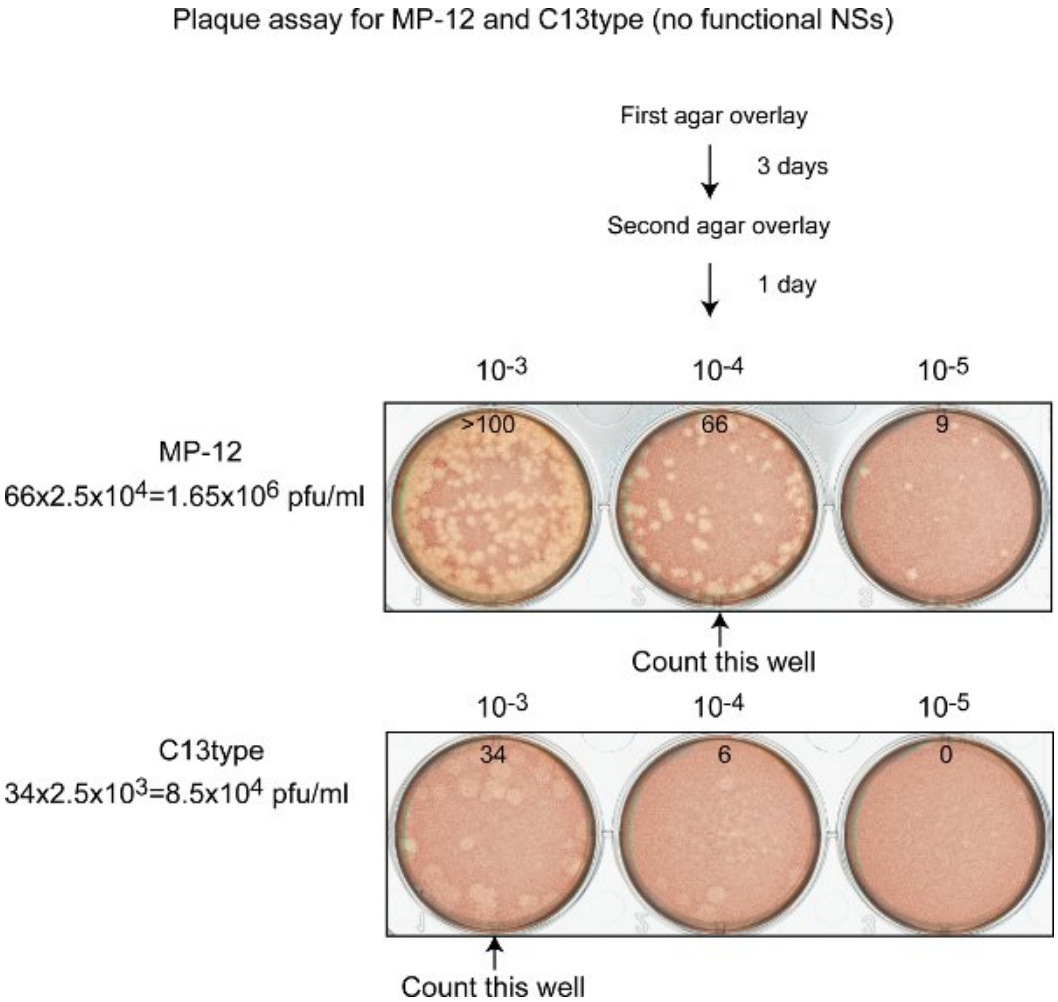

Figure 5. Plaque assay for MP-12 (functional NSs) and C13type (non-functional NSs)

The monolayer of Vero E6 cells in a 6-well plate is used for plaque assay. After 3 days incubation with 0.6% agar overlay, the second agar overlay containing neutral red solution is added. Then, plaques are counted at day 4 post infection. MP-12 forms clear plaques of varied sizes, while C13type forms large turbid plaques (or foci). The well with 10 to 100 plaques should be used for counting.

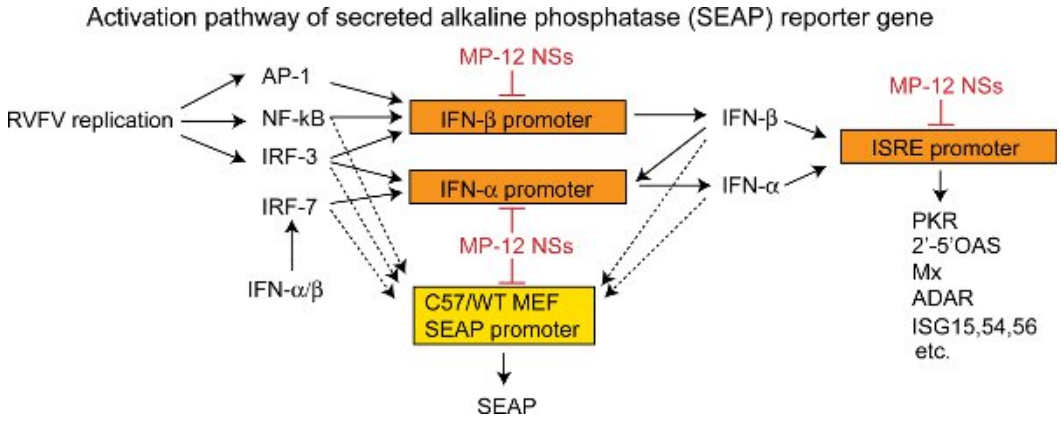

Figure 6. Activation pathway of secreted alkaline phosphatase (SEAP) reporter gene in C57/WT MEF cells

Interferon-beta promoter includes the binding sequences of AP-1, NF-kB and IRF-3. RVFV replication activates AP-1, NF-kB and IRF-3[7,11]. However, NSs inhibit the release of repressor complex from the IFN-beta promoter even after the binding of those transcription factors, thus suppressing the synthesis of IFN-beta mRNA[26]. Furthermore, NSs sequesters TFIIH p44 subunits8 and also promotes degradation of TFIIH p62 subunits[27], thus inducing a general host transcription suppression including IFN-alpha gene and genes under the ISRE promoter. C13type or other NSs mutants lacking IFN-beta suppression function induce IFN-beta synthesis, which in turn activates the IFN-alpha promoter and the interferon-sensitive response element (ISRE) promoter. IRF-7 is then transcriptionally upregulated by IFN-alpha/beta stimulation and further upregulated by IFN-alpha in support of IRF-3[28,29]. In this assay, C57/WT MEF cells encode secreted alkaline phosphatase (SEAP) at the downstream of an artificial binding sequence of NF-kB, IRF-3 and IRF-7. Thus, the NSs mutants lacking IFN-beta suppression function upregulate SEAP whose secretion is then measured.

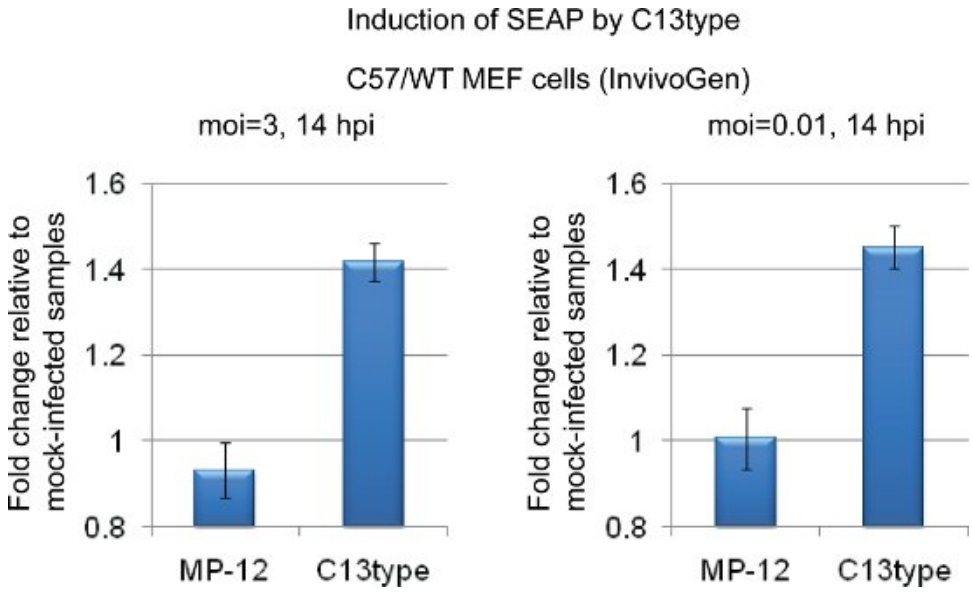

Figure 7. Induction of SEAP by C13type

C57/WT MEF cells (InvivoGen) were mock-infected or infected with MP-12 or rMP12-C13type (C13type) at moi of 3 (left panel) or 0.01 (right panel). Culture supernatants (50 µl) at 14 hpi were mixed with 200 µl of QUANTI-Blue (InvivoGen) substrate in 96 well plate and the OD values at 650 nm were measured after 1 h incubation at 37°C by a plate reader. The relative increases of SEAP to mock-infected cells are shown. The data represent the mean +/- standard deviation of three independent experiments. The culture supernatant from C13type-infected cells shows increased SEAP, suggesting the lack of host transcription suppression by NSs.

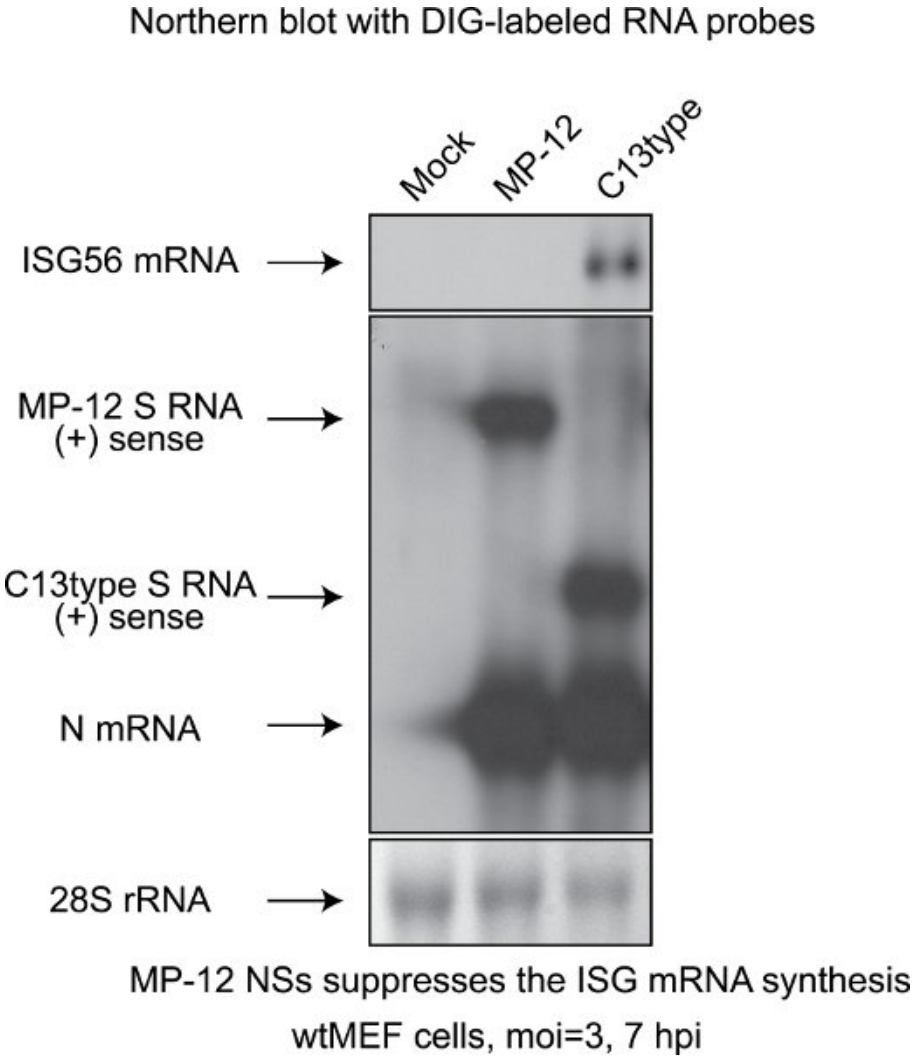

Figure 8. Northern blot with DIG-labeled RNA probe

Wild-type mouse embryonic fibroblast (MEF) cells were mock-infected or infected with MP-12 or rMP12-C13type (C13type) at moi of 3. Total RNA was collected at 7 hpi by using Trizol (Invitrogen), and Northern blot was performed by using digoxigenin-labeled RNA probe specific to mouse endogenous ISG56 mRNA or MP-12 N mRNA/anti-viral-sense S-segment[2,30]. For making the probes for mouse endogenous ISG56 mRNA or RVFV N mRNA/anti-viral-sense S-segment, the PCR fragments amplified by the primer set of KpnmISG56F (GGG TGG TAC CGC TCC ACT TTC AGA GCC TTC GCA AAG CAG) and HindmISG56 (TAC AAA GCT TAT GGG AGA GAA TGC TGA TGG TGA CCA GG) for ISG56 mRNA or KpnNF (AGT TGG TAC CAT GGA CAA CTA TCA AGA GCT TGC G) and HindNR (GGG CAA GCT TTT AGG CTG CTG TCT TGT AAG) for RVFV N mRNA/anti-viral-sense S-segment were digested with *Kpn*I and *Hind*III, and ligated into pSPT18 plasmid (Roche. Then RNA probes labeled with digoxigenin were synthesized by using DIG RNA Labeling Kit (SP6/T7) (Roche, Cat#1 175 025). The 28S rRNA level of each sample is also shown as loading control. Wild-type MEF cells infected with C13type induced ISG56 mRNA synthesis, while those infected with MP-12 did not induce it, suggesting the lack of host transcription suppression in C13type-infected cells. The data is consistent with those obtained by SEAP assay in Figure 6.

Discussion

Reverse genetics systems for RVFV have been developed by several groups by utilizing T7 promoter[2,4,5] or mouse[3] or human[4] pol-I promoter. In this manuscript, we describe a protocol to generate recombinant RVFV MP-12 strains by using BHK/T7-9 cells[15] that stably express T7 RNA polymerase. The efficiency of viral recovery varied depending on the condition of BHK/T7-9 cells, the amount of plasmids, the number of transfected cells and so on. We always amplify the P0 virus in Vero E6 cells to obtain high titer virus stocks for experiments. Type-I IFN-competent cells such as human lung diploid (MRC-5) cells could be used for viral amplification only when the NSs function supported an efficient viral replication by inhibiting antiviral activities including type-I IFN or PKR.

A plaque assay is a quite useful method for titrating MP-12 and the NSs mutants. As a general precaution for the plaque assay, cells should be immediately added with overlay at the appropriate temperature after the removal of viral inocula. Crystal violet staining is not suitable for detecting C13type virus plaques, because C13type virus does not disrupt the monolayer of Vero E6 cells[2]. On the other hand, neutral red staining could successfully detect plaques formed by C13type virus. The strength of cellular staining with neutral red is determined by the relative incorporation of neutral red dye into live cells. Thus, it is likely that C13type-infected cells have a reduced metabolic activity which leads to decreased incorporation of neutral red dye compared to that of uninfected cells, and which forms a weak staining focus. The required amount of neutral red varies by the lot of neutral red solution. Long-term storage of neutral red solution often generates precipitates. This could be prevented by heat-dissolving of neutral red solution at 55°C for 10 min; however, it affects the staining of cells.

It is important to use viruses with accurate viral titer in experiments. The titer in a viral stock could be decreased by media with pH below 6.5[31] which is indicated by yellowish color of phenol red in the viral stock, long-term storage, repeated freeze-thaw cycles and so on. Thus, it is important to use fresh viral stock for animal vaccination or titrate the stock again just before experiment.

RVFV encode two characterized nonstructural proteins; NSs and NSm. The RVFV lacking NSs is significantly attenuated in animals[25,32], while the RVFV lacking NSm is still highly virulent in rats[33]. Thus, NSs plays a major role as virulence factor in the pathogenesis. NSs is a multifunctional protein which induces 1) suppression of host general transcription[8], 2) suppression of IFN-beta mRNA synthesis[26] and 3) degradation of dsRNA-dependent protein kinase (PKR)[9,10]. The parental MP-12 strain could be further attenuated by introducing truncation or mutations into NSs. On the other hand, it is also important to maintain the immunogenicity of such mutant MP-12. Type-I IFN plays a role for the maturation of dendritic cells, which is important for the differentiations of T cells and B cells[12-14]. Various NSs mutants lacking IFN-beta suppression function, which maintain full or partial NSs functions, are useful to analyze the impact of the NSs-mediated IFN-beta suppression function on the immunogenicity and safety of vaccine candidates. It is known that cells infected with RVFV wt ZH548 strain activate NF-kB, IRF-3, and AP-1 (**Figure 6**), while IFN-beta mRNA synthesis is suppressed by NSs at transcriptional level[7]. Thus, MP-12, which expresses fully functional NSs[3], inhibits the synthesis of NF-kB/IRF-3/7-inducible SEAP reporter gene mRNA at transcriptional level, while recombinant MP-12 lacking NSs functions such as C13type could induce the SEAP mRNA synthesis (**Figure 7**). To correctly understand the result of immunogenicity of such mutants, the phenotypes should be further characterized in cell culture system. For example, the viral replication kinetics should be tested in type-I IFN competent cells such as human lung diploid MRC-5 cells or mouse embryonic fibroblast (MEF) cells. The PKR degradation function can be tested by Western blot with antibodies specific to human or mouse PKR. IFN-beta mRNA induction by NSs mutation should be confirmed by Northern blot with digoxigenin-labeled RNA probe specific to human or mouse IFN-beta mRNA. Host general transcription suppression can be tested by analyzing the incorporation of a uridine analog into nascent mRNA. Eventually, the immunogenicity and safety of candidate MP-12 NSs mutants should be tested in mice. Typically, 1 x 10^5 pfu of the candidate vaccine diluted in PBS is given subcutaneously and sera collected from the retro-orbital sinus at day 30 (or 42) and day 180 are tested for the presence of neutralizing antibodies by plaque reduction neutralizing test ($PRNT_{80}$) and total IgG against RVFV N and Gn/Gc by IgG ELISA coated with purified recombinant viral proteins. The efficacy of vaccine candidates can be tested by challenging mice at 42 days post immunization with wild-type ZH501 (e.g.; i.p., 1 x 10^3 pfu) at animal biosafety level (BSL)4 facility or enhanced BSL3+ facility in the U.S.A. The reverse genetics approach is a powerful tool to design and generate safe and immunogenic live-attenuated RVFV vaccines.

Disclosures

We have nothing to disclose.

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