

Rescue of Infectious Rift Valley Fever Virus Entirely from cDNA, Analysis of Virus Lacking the NSs Gene, and Expression of a Foreign Gene

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Rift Valley fever virus (RVFV) (genus *Phlebovirus*, family *Bunyaviridae*) has a tripartite negative-strand genome, causes a mosquito-borne disease that is endemic in sub-Saharan African countries and that also causes large epidemics among humans and livestock. Furthermore, it is a bioterrorist threat and poses a risk for introduction to other areas. In spite of its danger, neither veterinary nor human vaccines are available. We established a T7 RNA polymerase-driven reverse genetics system to rescue infectious clones of RVFV MP-12 strain entirely from cDNA, the first for any phlebovirus. Expression of viral structural proteins from the protein expression plasmids was not required for virus rescue, whereas NSs protein expression abolished virus rescue. Mutants of MP-12 partially or completely lacking the NSs open reading frame were viable. These NSs deletion mutants replicated efficiently in Vero and 293 cells, but not in MRC-5 cells. In the latter cell line, accumulation of beta interferon mRNA occurred after infection by these NSs deletion mutants, but not after infection by MP-12. The NSs deletion mutants formed larger plaques than MP-12 did in Vero E6 cells and failed to shut off host protein synthesis in Vero cells. An MP-12 mutant carrying a luciferase gene in place of the NSs gene replicated as efficiently as MP-12 did, produced enzymatically active luciferase during replication, and stably retained the luciferase gene after 10 virus passages, representing the first demonstration of foreign gene expression in any bunyavirus. This reverse genetics system can be used to study the molecular virology of RVFV, assess current vaccine candidates, produce new vaccines, and incorporate marker genes into animal vaccines.

Rift Valley fever virus (RVFV) causes a disease in sub-Saharan Africa that is endemic and has emerged in explosive mosquito-borne epidemics resulting in massive economic loss in herds of sheep and cattle, but also causing hemorrhagic fever, encephalitis, retinal vasculitis, and lesser disease in humans. In addition to the epidemics in sub-Saharan Africa, RVFV has been exported to Egypt on multiple occasions, particularly in 1977 when thousands of human infections occurred (18). After a particularly large epidemic in Africa in 1997 to 1998, the virus traveled to Egypt and the Arabian peninsula, menacing further spread (23, 25). The possibilities of introduction in many different countries and of its use as a bioterrorist agent (17) demand the availability of effective protective measures for humans and domestic animals.

It is likely that the disease can be controlled only by an effective live attenuated vaccine for livestock, and certainly the control activities will necessitate protection of humans, most likely by vaccination (16). Available livestock vaccines are unsatisfactory either because of fetal pathology or lack of immunogenicity, and modern usage requires the presence of markers to identify vaccinated animals to distinguish them from animals after natural infection. The basis of attenuation of the single viable human vaccine candidate is unknown (6, 24), which hampers further development. A lack of understanding of the molecular virology of members of the family *Bunyaviridae*

and of its medically important genus *Phlebovirus* is a major barrier to further vaccine development. The ability to recover RVFV from DNA constructs would permit rapid progress in all these areas.

Reverse genetics has been established for the members of several RNA virus families, but the *Arenaviridae* and *Bunyaviridae* families have been recalcitrant. Among the family *Bunyaviridae*, only Bunyamwera virus (BUN) (4) and La Crosse virus (LAC) (2) belonging to the genus *Orthobunyavirus* have been successfully recovered from cDNA. No viruses from the other four *Bunyaviridae* genera have been recovered. The *Phlebovirus* genus, in particular, has a number of important human and animal pathogens, is poorly understood at the molecular level, and has a replication strategy that, unlike other members of *Bunyaviridae* but resembling arenaviruses, utilizes an ambisense coding strategy (22). We report the development of a reverse genetics system for RVFV, which will allow us to proceed rapidly in these areas and in particular will advance our knowledge of its molecular virology.

MATERIALS AND METHODS

Media, cells, and viruses. Vero, Vero E6, 293 (human embryonic kidney), and MRC-5 (human diploid fibroblast) cells were maintained in Dulbecco's modified minimum essential medium (DMEM) (Invitrogen, Carlsbad, Calif.) containing 10% fetal calf serum (FCS). BHK-21 cells and BHK/T7-9 cells which express T7 RNA polymerase (10) were grown in MEM-alpha (Invitrogen) containing 10% FCS. Penicillin (100 U/ml) and streptomycin (100 µg/ml) (Invitrogen) were added to the media. BHK/T7-9 cells were selected in medium containing 600 µg/ml hygromycin (Cellgro). RVFV vaccine strain MP-12 was grown in BHK-21 cells, and infectivity was assayed by plaques in Vero E6 cells.

Plasmids. RVFV MP-12 strain full-length S, M, and L segments were cloned between KpnI and NotI sites of the pPro-T7 plasmid which originated from

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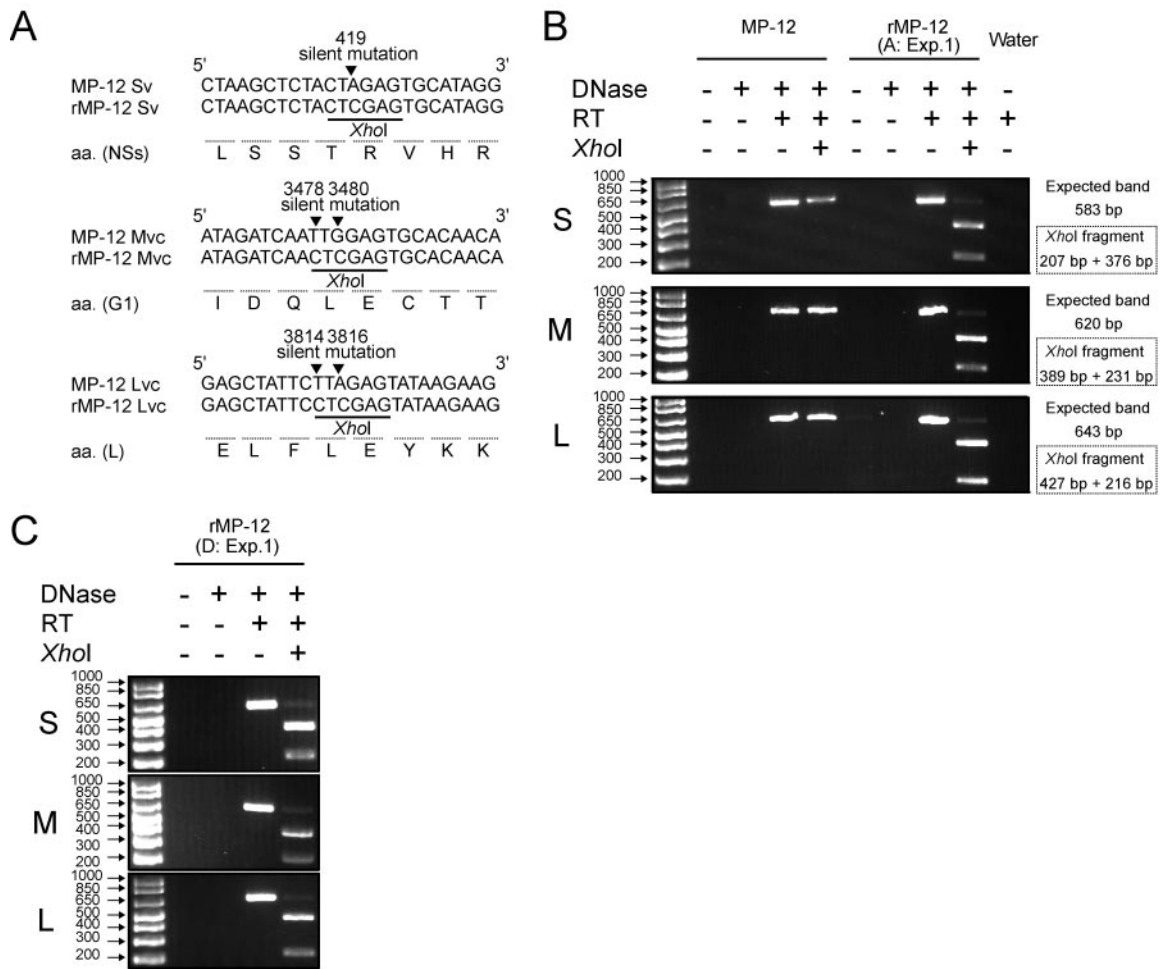


FIG. 1. Introduction of gene marker into S, M, and L segments. (A) Alignment of nucleotide and amino acid (aa.) sequences. Introduced XhoI sites on virus-sense S (Sv), anti-virus-sense M (Mvc), and anti-virus-sense L (Lvc) segments were underlined. Mutation positions were shown as arrowheads. (B) Demonstration of the XhoI marker in rMP-12 RNA. Viral RNA was extracted from culture supernatants of Vero E6 cells infected with MP-12 and rMP-12 in the experiment listed in Table 1, plasmid combination A, experiment 1 (A: Exp.1). After the RNA was digested with DNase I at 37°C for 1 h, PCR was performed with (+) and without (–) the reverse transcription step (RT). Water was used as a negative control. Digestion with XhoI was performed at 37°C for 2 h. The expected sizes of XhoI-digested fragments are shown to the right of the gels. The positions of molecular size markers are shown to the left of the gels. (C) Demonstration of XhoI marker in rMP-12 recovered without using protein expression plasmids in the experiment listed in Table 1, plasmid combination D, experiment 1 (D: Exp.1).

pSinRep5 (Invitrogen) to express full-length anti-virus-sense segments, resulting in pPro-T7-S(+), pPro-T7-M(+), and pPro-T7-L(+). A XhoI site was introduced into each one of the S, M, and L sequences by site-directed mutagenesis. The pPro-T7-S(–), pPro-T7-M(–), and pPro-T7-L(–) plasmids were constructed using a similar strategy. The pPro-T7-S(+)-C13 plasmid was made by introducing two AatII sites into pPro-T7-S(+), digesting with AatII, and self-ligation (see Fig. 2B). The pPro-T7-S(+)-NSdel plasmid was made by introducing SpeI and HpaI near the ends of the NSs open reading frame (ORF), digesting with SpeI and HpaI, filling in by T4 DNA polymerase, and self-ligation (see Fig. 2B). We also made pPro-T7-S(+)-rLuc by inserting a *Renilla* luciferase ORF between the SpeI and HpaI sites of pPro-T7-S(+) (see Fig. 3A). The pT7-IRES-vN, pT7-IRES-vNSs, and pT7-IRES-vL plasmids expressing the N, NSs, and L protein of MP-12 strain, respectively, were constructed as reported previously (9). The pCAGGS-vG plasmid, which expresses 78-kDa, NSm, G2, and G1 proteins, was made by introducing an EcoRI site upstream of the first ATG codon of the ORF in pPro-T7-M(+), and a XhoI site was introduced downstream of the stop codon. The EcoRI-XhoI fragment was cloned into the multicloning site of pCAGGS plasmid.

Virus rescue. Subconfluent monolayers of BHK/T7-9 cells in 60-mm dishes were cotransfected with pPro-T7-S(+), pPro-T7-M(+), pPro-T7-L(+), pT7-IRES-vN, and pCAGGS-vG, all of which were 2.2 μ g, and 1.1 μ g of pT7-

IRES-vL using TransIT-LT1 (Mirus, Madison, Wis.). Twenty-four hours later, culture medium was replaced with fresh medium. Five days later, the culture supernatants were passaged into BHK-21 cells.

RT-PCR analysis. Viral RNA was extracted from culture supernatants of Vero E6 cells infected with MP-12 or the recovered viruses by using a high pure viral RNA kit (Roche Applied Science). After DNase I digestion of the samples at 37°C for 1 h, reverse transcription-PCR (RT-PCR) was performed with and without reverse transcription using Ready-To-Go RT-PCR beads (Amersham). Primer pairs to amplify segments were S898F/S1480R, M2681F/M3300R, and L3603F/L4245R, respectively. The letters and numbers refer to the segment, position, and orientation of the primer on the RVFV anti-virus-sense genome.

Plaque assay. After virus adsorption to Vero E6 cells at 37°C for 1 h, inocula were removed, and cells were overlaid with DMEM containing 0.9% agar and 5% FCS. After 2 days of incubation, cells were stained with neutral red for 16 h. Alternatively, we used minimum essential medium containing 0.6% tragacanth gum (MP Biomedicals, Inc.), 2.5% FCS, and 5% tryptose phosphate broth for the overlay. After 3 days, the overlay was removed, fixed, and stained with 0.75% crystal violet, 10% formaldehyde, and 5% ethanol.

Northern (RNA) blotting. RNA was extracted from purified viruses using TRIzol reagent (Invitrogen). Approximately 100 ng of RNA was denatured and separated on 1% denaturing agarose-formaldehyde gels and transferred onto a

TABLE 1. Plasmid combinations for the rescue of RVFV

Plasmid (μg) ^a or parameter	Plasmid combination ^b or value					
	A	B	C	D	E	F
pPro-T7-S(+)(2.2)	+	+	+	+	-	-
pPro-T7-M(+)(2.2)	+	+	+	+	-	-
pPro-T7-L(+)(2.2)	+	+	+	+	-	-
pPro-T7-S(-)(2.2)	-	-	-	-	+	+
pPro-T7-M(-)(2.2)	-	-	-	-	+	+
pPro-T7-L(-)(2.2)	-	-	-	-	+	+
pT7-IRES-vN(2.2)	+	+	+	-	+	-
pT7-IRES-vL(1.1)	+	+	+	-	+	-
pCAGGS-vG(2.2)	+	+	-	-	+	-
pT7-IRES-vNSs(2.2)	-	+	-	-	-	-
Efficiency of virus rescue ^c	3/3	0/3	2/3	1/3	2/3	0/3
Virus titer (PFU/ml) ^d						
Expt 1	1.0×10^5	0	3.0×10^4	4.8×10^5	2.3×10^3	0
Expt 2	5.0×10^4	0	1.3×10^6	0	3.7×10	0
Expt 3	3.0×10^4	0	0	0	0	0

^a Subconfluent monolayers of BHK/T7-9 cells in 60-mm dishes were transfected with the indicated amounts of plasmids.

^b Six different combinations of plasmids (A to F) for virus rescue.

^c Efficiency of rescue in three experiments.

^d Virus titers were measured using culture supernatant from plasmid-transfected BHK/T7-9 cells at 5 days posttransfection.

nylon membrane (Roche Applied Science). Northern blot analysis was performed as described previously (9) with strand-specific RNA probes (8).

Western blot analysis. Western blot analysis was performed as described previously (9). The membranes were incubated with anti-RVFV mouse polyclonal antibody, anti-NSs rabbit polyclonal antibody, or antiactin goat polyclonal antibody (I-19; Santa Cruz Biotechnology) overnight at 4°C and with secondary antibodies for 1 h at room temperature.

RNAse protection assay. MRC-5 cells were independently infected with the rescued viruses at a multiplicity of infection (MOI) of 1.0 at 0°C for 1 h. After the cells were washed with cold medium, cells were incubated for 0, 2, 4, 6, and 8 h. Total RNAs were extracted and mixed with ³²P-labeled multiprobe template hCK-3 (BD RiboQuant RPA kit; BD Biosciences). RNase protection assay was performed according to the manufacturer's instruction. Protected RNAs were analyzed on a 4.75% polyacrylamide gel containing 8 M urea. Undigested probes were used as size markers.

Analysis of viral growth. Vero cells, 293 cells, and MRC-5 cells were infected with viruses at an MOI of 1 or 0.01 at 37°C for 1 h, washed three times with phosphate-buffered saline (PBS), and medium was added. Culture supernatants were harvested at 0, 8, 12, 16, and 20 h postinfection (p.i.), and the virus titers were measured by plaque assay. The points in the growth curves were shown as means \pm standard deviations from three independent experiments.

Analysis of host protein synthesis. Vero cells were infected with viruses at an MOI of 5 at 37°C for 60 min and washed three times with PBS. At 16.5 h p.i., cells were incubated in methionine-free medium for 30 min and then incubated in medium containing 100 $\mu\text{Ci/ml}$ of [³⁵S]methionine for 1 h. Cell extracts were analyzed by 10% polyacrylamide gel electrophoresis.

Analysis of Renilla luciferase expression in infected cells. Vero E6 cells were infected with MP-12 and its mutant virus at 0°C for 60 min and washed three times with cold PBS. Cells were added with warmed medium (0 min) and incubated for various times. Lysates were made and assayed using Renilla luciferase assay system (Promega).

RESULTS

Recovery of RVFV from cDNAs. To recover MP-12 from cDNAs, several plasmids, encoding RVFV MP-12 proteins and viral RNAs, were constructed. The entire N, L, and NSs ORF was placed downstream of the T7 promoter and an encephalomyocarditis virus internal ribosome entry site in each protein expression plasmid (9), while the entire M ORF was cloned in an eukaryotic expression vector, pCAGGS. The entire region of each viral RNA segment was placed between the T7 promoter and hepatitis delta virus ribozyme in each RNA expres-

TABLE 2. Time course of recombinant RVFV production^a

Expt	Virus titer (PFU/ml) at the following time (h) after infection:				
	24	48	72	96	120
4	0	0	2.8×10^2	4.5×10^3	5.3×10^6
5	0	0	2.0×10	1.0×10^3	8.5×10^5

^a Plasmid combination A in Table 1 was used for this study. Virus titers were measured using culture supernatant from plasmid-transfected BHK/T7-9 cells at various time points as indicated.

sion plasmid. To exclude the possibility that recovered viruses represented contamination with MP-12, we modified all three RNA expression plasmids to carry a unique XhoI site, which introduced a silent mutation in the NSs, G1, and L ORF, respectively (Fig. 1A). To recover infectious viruses, BHK/T7-9 cells, which stably express high levels of T7 RNA polymerase under control of the chicken beta-actin promoter (10), were transfected with various combinations of these plasmids (Table 1). At five days posttransfection, the supernatants were transferred into Vero E6 and BHK-21 cells to amplify released viruses. In five repeated experiments, infectious viruses were recovered from BHK/T7-9 cells that were transfected with plasmids expressing all three anti-virus-sense RNA fragments and plasmids expressing all structural proteins (Tables 1 and 2). Low titers of progeny were detected at 3 days posttransfection and steadily increased until 5 days posttransfection (Table 2). The recovery of RVFV was unsuccessful when the plasmid expressing NSs protein (pT7-IRES-vNSs) was included (Table 1). In some experiments viruses were recovered without the plasmid expressing envelope proteins (pCAGGS-vG) and even in the absence of all protein expression plasmids, demonstrating that viral structural protein expression directly from added plasmids was not an absolute requirement for virus recovery. No virus recovery occurred after transfection of plasmids expressing virus-sense RNA segments, yet addition of plasmids expressing L, N, and envelope proteins resulted in virus recovery in two out of three experiments. RT-PCR amplification of the recovered viral RNA and subsequent digestion of the PCR products with XhoI showed that the recovered recombinant MP-12 (rMP-12) carried the introduced XhoI site in each RNA segment (Fig. 1B). The presence of the unique XhoI sites was also demonstrated in each of the three viral RNA segments in sucrose gradient-purified rMP-12 (data not shown), as well as in rMP-12 that was recovered after transfection of plasmids expressing anti-virus-sense RNAs in the absence of all protein expression plasmids (Fig. 1C). These data unambiguously established the validity of this reverse genetics system.

Production of mutants lacking the NSs gene and expressing a foreign gene. We examined whether the NSs protein was essential for RVFV replication cycle by recovering mutant viruses using two pPro-T7-S(+)-derived mutants, one containing an in-frame deletion of 70% of the NSs gene and the other lacking the entire NSs gene; the deletion site in the former mimicked the deletion site of the NSs gene of a naturally occurring RVFV mutant clone 13 (Fig. 2A and B) (15). We have successfully recovered recombinant MP-12 partially lacking NSs ORF (rMP12-C13type) using the former plasmid and completely lacking the NSs ORF (rMP12-NSdel) using the

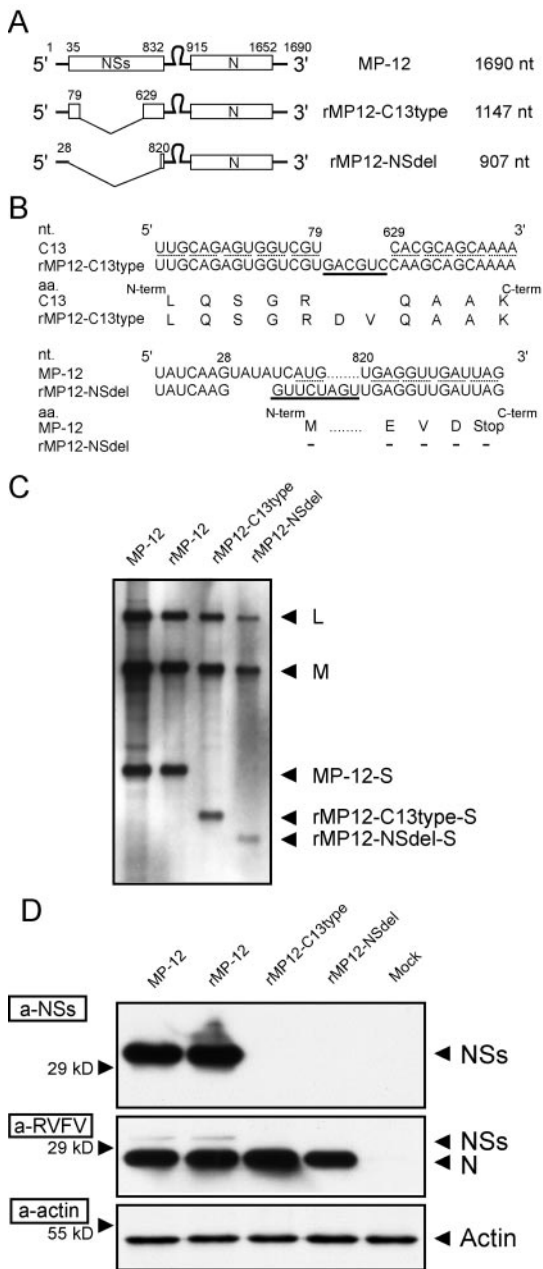


FIG. 2. Production of NSs deletion mutants. (A) Diagrams of NSs deletion mutants. (B) Alignment of nucleotide (nt.) and amino acid (aa.) sequences of clone 13 (C13) and rMP12-C13type or of MP-12 and rMP12-NSdel. The rMP12-NSdel does not contain the first AUG (methionine [M]) and does not produce NSs protein. N-term, N-terminal; C-term, C-terminal. (C) Purified virion RNAs of MP-12, rMP-12, rMP12-C13type, and rMP12-NSdel were analyzed by Northern blotting using virus-sense S-, M-, and L-specific RNA probes (8). (D) Western blotting using anti-NSs (a-NSs), anti-RVSV (a-RVSV), and anti-actin (a-actin) antibodies (8). Vero E6 cells were infected with MP-12 and mutants at an MOI of 1 and were harvested at 6 h p.i.

latter plasmid. Northern blot analysis of the sucrose gradient-purified (8) viruses showed that the L and M segments of MP-12 and all recovered viruses were the same length, while the S segments of rMP12-C13type and rMP12-NSdel were

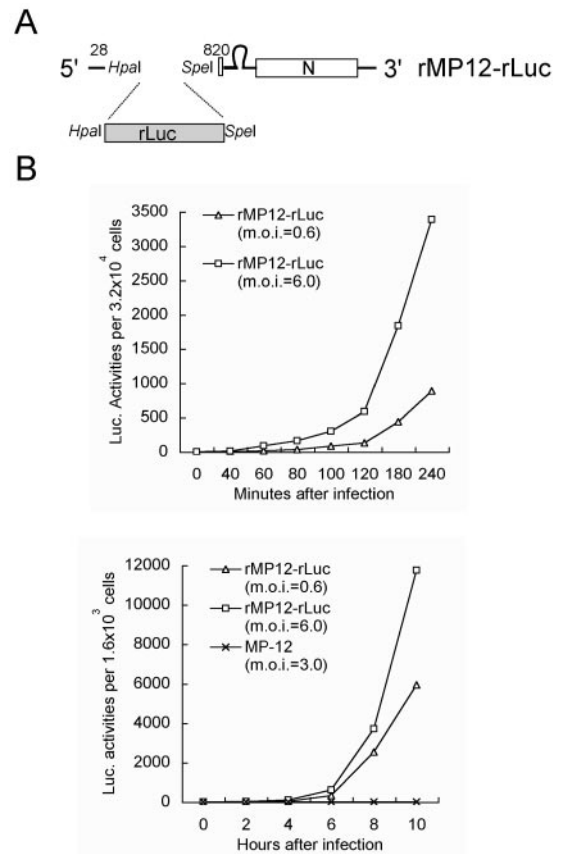


FIG. 3. Production of recombinant MP-12 expressing *Renilla* luciferase. (A) Diagram of the S segment of rMP12-rLuc. *Renilla* luciferase ORF with HpaI and SpeI sites at 5' and 3' ends, respectively, was inserted into the cassette of S segment digested with HpaI and SpeI. (B) Vero E6 cells were infected with rMP12-rLuc or MP-12 at the indicated MOI, and luciferase (Luc.) activity was tested at several time points as shown. Luciferase activities of 3.2×10^4 cells and 1.6×10^3 cells are shown for early (top panel) and late (bottom panel) time points, respectively.

shorter than those of rMP-12 and MP-12 (Fig. 2C) and corresponded to the expected sizes of 1,690 nucleotides (nt) of MP-12 and rMP-12, 1,147 nt of rMP12-C13type, and 907 nt of rMP12-NSdel. Western blot analysis of intracellular viral proteins using anti-NSs and anti-RVSV polyclonal antibodies (8) demonstrated N-protein accumulation in Vero E6 cells infected with each virus, yet NSs protein accumulation occurred only in those infected with MP-12 and rMP-12 (Fig. 2D). We concluded that NSs protein was dispensable for MP-12 replication.

To examine whether replacing the NSs ORF with a foreign gene was compatible with replication of RVSV and expression of the gene, we constructed a pPro-T7-S(+)-derived plasmid containing *Renilla* luciferase (rLuc) ORF instead of the NSs ORF (Fig. 3A). Using this plasmid, we rescued infectious virus, rMP12-rLuc. In Vero E6 cells infected with rMP12-rLuc, luciferase activity was detected as early as 60 min and steadily increased (Fig. 3B), whereas MP-12 did not show any luciferase activity. Those results demonstrated that RVSV can carry a foreign gene in the NSs ORF. rMP12-rLuc retained the inserted *Renilla* luciferase ORF after 10 serial passages at an

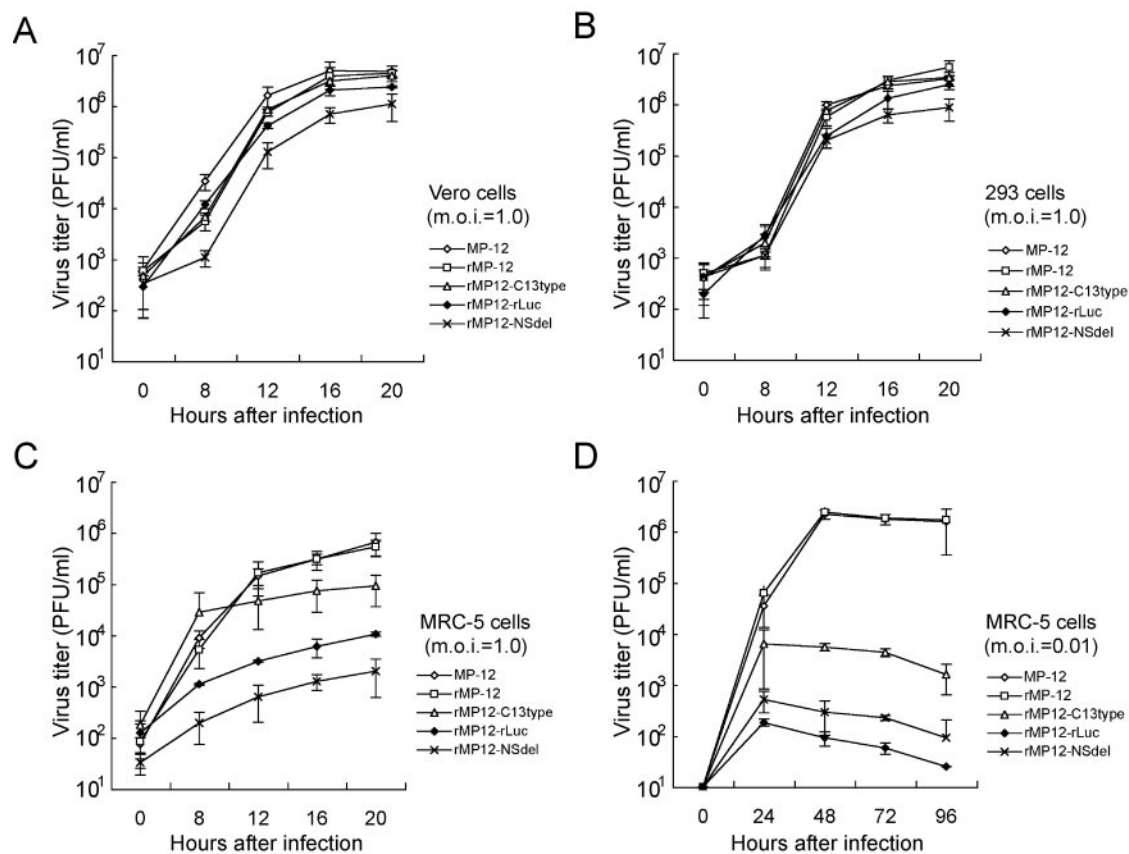


FIG. 4. Growth curves of MP-12 and mutants. Vero cells (A), 293 cells (B), and MRC-5 cells (C and D) were infected with MP-12, rMP-12, rMP12-C13type, rMP12-NSdel, and rMP12-rLuc at an MOI of 1 (A, B, and C) or 0.01 (D), and the culture supernatants were collected at the time points shown. Culture supernatants were collected from three independent wells at each time point, and the virus titers were determined in Vero E6 cells by assay. The values are mean titers \pm standard deviations (error bars) from three independent experiments.

MOI from 0.01 to 0.1 in Vero E6 cells. rMP12-rLuc obtained after 10 passages and unpassaged rMP12-rLuc showed similar luciferase activities (data not shown).

Analysis of growth kinetics of recovered viruses in Vero cells, 293 cells, and MRC-5 cells. Analysis of one-step growth kinetics of MP-12 and all recovered viruses in Vero cells lacking alpha/beta interferon (IFN- α/β) genes (7, 14) and 293 cells showed that the kinetics of infectious rMP-12, rMP12-C13type, and rMP12-rLuc were similar in both cells, yet MP-12 had a slightly higher titer than others in Vero cells (Fig. 4A and B). Titers of rMP12-NSdel in Vero cells were about one-fifth of other viruses throughout infection, and the rMP12-NSdel titers in 293 cells were lower than other virus titers from 12 to 20 h. Deletion of the entire NSs ORF and replacement of the NSs ORF with the rLuc ORF did not apparently inhibit virus replication in these cell lines. In contrast, rMP12-rLuc and rMP12-NSdel replicated to substantially lower titers than rMP-12 or MP-12 throughout infection in MRC-5 cells (Fig. 4C). Also, very limited accumulation of rMP12-C13type occurred after 8 h p.i. in this cell line. After infection of these viruses in MRC-5 cells at a low MOI of 0.01, only MP-12 and rMP-12 replicated efficiently (Fig. 4D); the titers of rMP12-C13type, rMP12-rLuc, and rMP12-NSdel moderately increased during the first 24 h p.i., yet they declined thereafter.

These data suggested that the NSs protein was necessary for efficient viral replication in MRC-5 cells.

Analysis of IFN- β mRNA accumulation in infected MRC-5 cells. Because RRVFV NSs protein inhibits host mRNA transcription, including IFN- α/β mRNAs (1), we suspected that infection of MP-12 mutant viruses lacking the intact NSs ORF triggered IFN- β production in MRC-5 cells, resulting in poor virus replication, yet IFN- β production did not occur in MRC-5 cells that were infected with MP-12 or rMP-12. To test this possibility, accumulation of IFN- β mRNA in infected MRC-5 cells was examined using multiprobe RNase protection assay (Fig. 5). We noted clear substantial increases in the amounts of IFN- β mRNA and tumor necrosis factor alpha (TNF- α) mRNA in rMP12-C13type-infected cells and in rMP12-NSdel-infected cells; both mRNA signals were stronger in the former than in the latter. Mock-infected cells showed neither IFN- β mRNA nor TNF- α mRNA accumulation, while only a minute level of IFN- β mRNA was detected in rMP-12-infected cells. These data supported a notion that infection of MP-12 mutant viruses lacking the NSs ORF triggered IFN- β production in MRC-5 cells, resulting in poor virus replication.

Impaired host protein shutoff in Vero cells infected with rMP12-C13type and rMP12-NSdel. MP-12 and rMP-12 cells

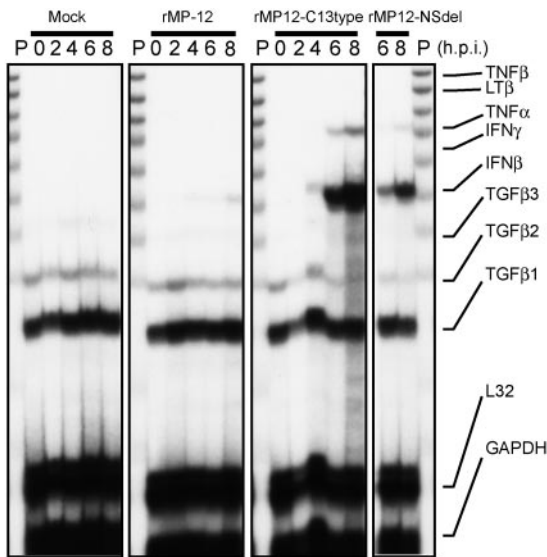


FIG. 5. Accumulation of IFN- β mRNA and TNF- α mRNA in MRC-5 cells infected with NSs deletion mutants. MRC-5 cells were mock infected (Mock) or infected with rMP-12, rMP12-C13type, and rMP12-NSdel at an MOI of 1. Total intracellular RNAs were extracted at the indicated times (hours p.i. [h.p.i.]). RNA samples were hybridized with multiprobe template (hCK-3), and RNase protection assay was performed. P, probes; TGF β 3, transforming growth factor β 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

formed clear plaques with an approximately 1-mm diameter in Vero E6 cells, while rMP12-C13type and rMP12-NSdel made approximately 2-mm-diameter turbid plaques with less defined edges after neutral red staining (Fig. 6A). When plaques were stained with crystal violet, only the former two viruses made clear plaques (Fig. 6A). We also observed that the majority of cells were detached at 2 to 3 days after infection with MP-12 or rMP-12, whereas most of the cells attached to the plates and the severity of CPE was less prominent in the cells infected with rMP12-C13type and rMP12-NSdel (data not shown).

Metabolic radiolabeling analysis of intracellular proteins showed that MP-12 and rMP-12 induced clear shutoff of host protein synthesis, while no obvious decrease occurred in cells infected with rMP12-C13type or rMP12-NSdel (Fig. 6B), demonstrating that expression of NSs was responsible for host protein synthesis shutoff in Vero cells.

DISCUSSION

Control of RVFV after either a natural introduction or bioterrorist attack would require protection of humans and livestock (16). Although there is an inactivated RVFV vaccine that has been widely used in laboratory workers, that immunogen is no longer available (19). An attenuated vaccine, MP-12, is safe and immunogenic but requires further development (6, 13) (P. R. Pittman and C. J. Peters, unpublished observations). One of the critical elements of understanding the safety profile of MP-12 as a human vaccine is to understand the significance of its more than 20 point mutations for attenuation (24). This task and the additional development of an animal vaccine are critically dependent on a reverse genetics system.

We describe an RVFV reverse genetics system, the first for viruses of the *Phlebovirus* genus. Infectious RVFV was consistently rescued by cotransfecting plasmids encoding anti-virus-sense RNA segments and plasmids expressing L, N, and envelope proteins into BHK/T7-9 cells that stably express T7 RNA polymerase (10), while rescue of the infectious viruses did not occur after cotransfection of 293T or BHK-21 cells with pCT7pol plasmid encoding T7 RNA polymerase along with other protein-expressing and RNA-expressing plasmids (data not shown). It is unclear why virus rescue failed using transient T7 polymerase expression. When we cotransfected cells with plasmids bearing RVFV structural protein genes and a plasmid expressing viral minigenome encoding green fluorescent protein (GFP) ORF, production of infectious virus-like particles carrying viral minigenome RNA was more efficient in BHK/T7-9 cells than in 293T or BHK-21 cells transiently expressing T7 polymerase (unpublished data), implying that virus assembly in BHK/T7-9 cells was

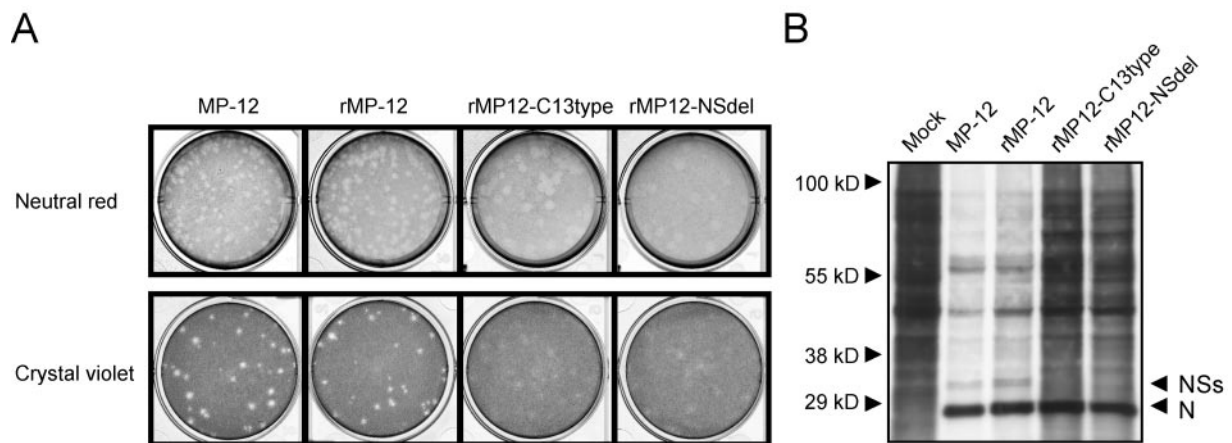


FIG. 6. Responses of host cells infected with MP-12 and mutants. (A) Plaque production in Vero E6 cells by MP-12, rMP-12, rMP12-C13type, and rMP12-NSdel stained with neutral red and crystal violet. (B) Shutoff of host protein synthesis. Vero cells were mock infected (Mock) or infected with MP-12, rMP-12, rMP12-C13type, and rMP12-NSdel at an MOI of 5. Cells were labeled with 100 μ Ci/ml of [35 S]methionine for 1 h at 17 h postinfection. Cell extracts were analyzed on 10% polyacrylamide gel. The positions of synthesized N and NSs proteins are shown by arrowheads to the right of the gel.

more active than in the latter two cells. Not all BHK-derived cell lines constitutively expressing T7 polymerase were suitable for virus rescue, because no infectious viruses were obtained using BHK-21 cells expressing high levels of T7 polymerase induced by an Eastern equine encephalitis virus replicon (data not shown). Although the first reverse genetics system of BUN used vaccinia virus expressing T7 polymerase (4), all recent bunyavirus reverse genetics systems, including this study, have used BHK cells that stably express T7 polymerase without employing any virus vectors (2, 12). The mechanisms for this success are unknown, but the practical implications are obvious.

Transfection of protein expression plasmids inhibits LAC rescue (2) but has no effect on rescue of infectious BUN (12). Cotransfection of protein expression plasmids and RNA expression plasmids resulted in consistent recovery of MP-12 (Tables 1 and 2). Infectious viruses were also recovered in the absence of protein expression plasmids, but virus rescue was not always successful (Table 1). In both BUN (4, 12) and LAC (2) reverse genetics systems, infectious viruses are produced only from cells that express anti-virus-sense RNA transcripts, whereas infectious MP-12 was recovered from cells expressing virus-sense RNA transcripts or anti-virus-sense RNA transcripts (Table 1). These studies indicated that requirements and optimal conditions for virus recovery vary among members of the *Bunyaviridae* family.

Because NSs protein expression promotes RVFV minigenome RNA synthesis in various cell lines, we predicted that NSs protein expression would be important for a successful RVFV reverse genetics system (9). In contrast to this prediction, NSs protein expression suppressed virus recovery (Table 1). Interestingly, NSs protein expression did not increase or decrease minigenome RNA replication in BHK/T7-9 cells (data not shown), so it remains unclear why NSs protein expression should inhibit rescue of infectious viruses.

The RVFV mutant clone 13 carrying an in-frame deletion of about 70% of the NSs gene is viable and fails to inhibit host mRNA transcription (1, 11) and has markedly reduced virulence in interferon-competent mice (3). Nevertheless, it has been unclear whether some portion of the NSs protein and/or its coding region is necessary for virus replication. We demonstrated that rMP12-NSdel and rMP12-rLuc, both of which lacked the NSs ORF, replicated efficiently in Vero and 293 cells (Fig. 4A and B); the former replicated slightly less well than MP-12, yet the latter grew to a level similar to that of MP-12. These data unambiguously demonstrated that NSs protein and its coding region were dispensable for RVFV replication.

In contrast to Vero and 293 cells, both rMP12-C13type and rMP12-NSdel did not replicate efficiently in MRC-5 cells (Fig. 4C and D) and failed to inhibit the accumulation of IFN- β and TNF- α mRNAs (Fig. 5), strongly suggesting that IFN- β was released from infected MRC-5 cells and that it suppressed replication of these mutant viruses. These data were consistent with a previous report that clone 13 does not grow well in MRC-5 cells (15). Efficient replication of the mutant viruses lacking the NSs ORF in Vero cells was most probably due to the absence of the IFN- α/β gene in this cell line (7, 14). Because 293 cells were able to accumulate IFN- β mRNA after Sendai virus infection (data not shown), IFN- β was probably released from 293 cells that were infected with MP-12 mutants

lacking the NSs ORF. Nevertheless, replication of those mutants was not suppressed in 293 cells. We suspect that the induction of IFN- β -induced antiviral responses in 293 cells may not be as efficient as MRC-5 cells, but further studies will be needed to test this possibility.

MP-12 is a highly attenuated virus, and mutations in all three viral RNA segments contribute to its attenuation (16, 21). It has been demonstrated that wild-type (wt) RVFV NSs can decrease general host transcription (11), suppresses IFN- β promoter activation without inhibiting activities of interferon regulatory factor 3, NF- κ B and ATF2/cJun (AP-1) (1) and is a major virus virulence factor (3). There is one amino acid difference between wt RVFV ZH548 NSs protein and MP-12 NSs protein (24), hence possibilities exist that this amino acid substitution alters biological functions of NSs and contributes to the attenuation of MP-12. Like wt RVFV, MP-12 NSs protein was responsible for inhibition of IFN- β mRNA accumulation (Fig. 5) and host protein synthesis shutoff (Fig. 6B) demonstrating that the amino acid substitution in MP-12 NSs did not alter all known biological functions of NSs. The effect of this amino acid substitution in virus virulence remains to be investigated.

It is unclear why the plaques of rMP12-C13type and rMP12-NSdel were larger than those of MP-12 and rMP-12 (Fig. 6A, top panels). MP-12 strain was selected for larger plaques (6), and they are larger than those of wt RVFV strain (20). However, RVFV isolates from nature or laboratory variants forming smaller plaques are usually attenuated in animals (20) (C. J. Peters, unpublished observations). Apparently plaque size has no obligatory correlation with virulence of RVFV. Although LAC and BUN, both of which belong to the genus *Orthobunyavirus*, do not use an ambisense strategy for NSs protein expression and the sizes and amino acid sequences of their NSs proteins differ from those of RVFV (genus *Phlebovirus*), LAC (2) and BUN (5) lacking NSs are also viable.

Taking advantage of the ambisense strategy of the S RNA segment gene expression and the NSs ORF being dispensable for virus replication, we have successfully generated rMP12-rLuc, which expressed enzymatically active luciferase in infected cells. This is the first demonstration of foreign gene expression in any bunyavirus. Consistent with our report that NSs mRNA synthesis occurs early in infection using the virion-associated anti-virus-sense S segment as a template (8), luciferase activity was detectable as early as 60 min p.i. of rMP12-rLuc (Fig. 3B). The rMP12-rLuc virus replicated as efficiently as MP-12 in Vero cells and 293 cells, it stably retained the inserted rLuc ORF after 10 virus passages, and it can be handled in a biosafety level 2 lab. This virus would be useful for rapid screening of RVFV antiviral agents, measurement of neutralizing antibodies, and tracing of viral spread within a susceptible host, such as interferon receptor-deficient mice. We also succeeded in producing another recombinant MP-12, which has GFP ORF instead of NSs ORF (data not shown). Like rMP12-rLuc, the inserted GFP ORF was stable after 10 virus passages in Vero E6 cells and efficient GFP expression was detected throughout virus passages. In addition to the possibility that the RVFV reverse genetics system can be used to study the molecular virology of RVFV, assess current vaccine candidates, produce new vaccines, and incorporate marker genes into animal vaccines, MP-12 may be developed

as an expression vector that can be used for mammalian and insect cells as well as whole animals.

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