Rift Valley Fever Virus NSs mRNA Is Transcribed from an Incoming Anti-Viral-Sense S RNA Segment

Tetsuro Ikegami,¹ Sungyong Won,¹ C. J. Peters,^{1,2} and Shinji Makino^{1*}

Departments of Microbiology and Immunology¹ and Pathology,² The University of Texas Medical Branch at Galveston, Galveston, Texas

Received 7 April 2005/Accepted 28 June 2005

Analysis of purified Rift Valley fever virus (RVFV) particles demonstrated the presence of three negative-sense RNA genomes, plus three anti-viral-sense RNA segments. The virion-associated anti-viral-sense S segment served as a template for the synthesis of NSs mRNA immediately after infection. NSs protein synthesis also occurred early in infection, suggesting that NSs protein produced early in infection probably has biologically significant roles in virus replication and/or survival in the host. Translation inhibitor treatment of mammalian cells infected with viruses belonging to the *Bunyaviridae* family generally inhibits viral mRNA synthesis. However, RVFV NSs mRNA synthesis, but not N mRNA synthesis, was resistant to puromycin treatment during primary transcription, pointing to the uniqueness of RVFV NSs mRNA synthesis.

Viruses belonging to the *Bunyaviridae* family carry three single-stranded RNAs, designated L, M, and S. Viral RNA synthesis occurs in the cytoplasm, and the host mRNA-derived cap structure is used as a primer for viral mRNA synthesis (16). Host protein synthesis is essential for viral RNA replication (2, 5, 6, 15, 18) and is also important for viral mRNA synthesis in mammalian cells; viral mRNA elongation, but not initiation, is suppressed in the presence of protein synthesis inhibitors (2, 15, 18). How the host protein synthesis machinery facilitates viral mRNA elongation is unknown; it has been suggested that ribosome binding to nascent viral mRNA prevents its annealing to the template RNA and allows mRNA elongation (2).

Rift Valley fever virus (RVFV) (family *Bunyaviridae*, genus *Phlebovirus*) causes severe epidemics among ruminants in the sub-Saharan African and has spread to Egypt, Yemen, and Saudi Arabia. It is also an important human pathogen that causes a syndrome of fever and myalgia, a hemorrhagic syndrome, ocular disease, and encephalitis (1, 13). The anti-viral-sense L segment encodes L protein, a viral RNA polymerase, and the anti-viral-sense M segment encodes two structural glycoproteins, G1 and G2, NSm protein, and a 78-kDa protein. As in other viruses of the genus *Phlebovirus*, RVFV S segment uses an ambisense strategy to express N and NSs proteins, and the viral-sense S and anti-viral-sense S segments serve as templates for N mRNA and NSs mRNA, respectively (16). It has been believed that NSs mRNA is produced only after viral-sense RNA has been copied to anti-viral-sense RNA; therefore, the NSs protein would appear at a later stage in infection than the structural proteins (6, 16).

RVFV NSs protein plays an important role in RVFV pathogenesis and replication. This protein inhibits host mRNA synthesis, including alpha/beta interferon (IFN- α/β) mRNAs (3, 10), hence suppressing host innate immune responses to viral

invasion, and it is a major virus virulence factor (4). We showed that coexpression of RVFV NSs protein with N and L proteins enhances viral RNA accumulation in the RVFV minigenome system (7). Because this effect appears to be independent of the NSs-mediated inhibition of IFN- α/β production, NSs protein most likely augments viral RNA synthesis in the minigenome system (7) and probably during viral infection. The effect of NSs on viral RNA synthesis led us to speculate that RVFV NSs protein might be synthesized early in infection to promote viral RNA synthesis. The present study tested this possibility. Our data indeed support this supposition and showed unexpected properties of NSs mRNA synthesis.

We first examined the possibility of anti-viral-sense S segment incorporation into RVFV particles; if this occurs, the incoming anti-viral-sense S segment may serve as a template for NSs mRNA synthesis immediately after infection. A vaccine strain of RVFV, MP12, which has multiple mutations (19), including the NSs gene, compared with wild-type RVFV, was used as the source of infectious RVFV. MP12 was propagated at 37°C in various mammalian cells, including 293T, Vero E6, and BHK-21 cells, and in mosquito C6/36 cells at 28°C after inoculation at a multiplicity of infection (MOI) of 0.1. At 48 h postinfection (p.i.), culture fluid was collected and clarified by low-speed centrifugation. Then virus particles were partially purified by two subsequent ultracentrifugations on a discontinuous sucrose gradient consisting of 60, 50, 30, and 20% sucrose using a Beckman SW28 rotor (9, 11); the sample was first centrifuged at 28,000 rpm for 3 h, and the virus particles at the interface of 30 and 50% sucrose were further centrifuged at 28,000 rpm for 18 h. The virus particles at the interface of 30 and 50% sucrose were collected, diluted, and then further applied on a continuous sucrose gradient of 20 to 60% sucrose. The samples were centrifuged at 28,000 rpm for 18 h. Subsequently, 10 fractions were collected, and the sucrose density in each fraction was measured. Virus particles in the each fraction were pelleted through a 20% sucrose cushion at 38,000 rpm for 2 h using a Beckman SW 41 rotor (8) and subjected to further analysis. Western blot analysis of the virus sample propagated in Vero E6 cells using anti-RVFV mouse

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1019. Phone: (409) 772-2323. Fax: (409) 772-5065. E-mail: shmakino@utmb.edu.

FIG. 1. Characterization of the virion-associated RNAs. (A) Vero E6 cells were infected with the RVFV MP12 strain at an MOI of 0.1. At 48 h p.i., culture fluid was collected and clarified. Subsequently, a purified virus sample was prepared as described in the text. Ten fractions from a sucrose gradient were collected and examined by Western blot analysis using an anti-RVFV polyclonal antibody. The top panel shows the density of each sucrose fraction. (B) Schematic diagram of the structure of an anti-viral-sense S segment (Svc), anti-viral-sense M segment (Mvc), anti-viral-sense L segment (Lvc), viral-sense S segment (Sv), viral-sense M segment (Mv), and viral-sense L segment (Lv). Riboprobes pN-S(+), pM(+), pL(+), pNS-S(-), pN-S(-), pM(-),

antibody (7) detected the peak viral protein signals, L, G1, G2, and N, at a sucrose density of approximately 1.16 g/cm³ (Fig. 1A, upper panel). The buoyant densities of other virus preparations were from 1.16 to 1.18 g/cm³ (data not shown), similar to previously reported data (16). Northern blot analyses were performed to identify virus-specific RNAs in the purified virion with buoyant densities from 1.16 to 1.18 g/cm³. A total of six digoxigenin-labeled (Roche Applied Science), strand-specific riboprobes were independently synthesized in vitro and grouped into two sets; a mixture of three probes, each of which binds to the viral-sense S, M, or L segments, was designated as probe set A1, while probe set B contained a mixture of three probes, each of which binds to anti-viral-sense S, M, or L segments (Fig. 1B). To confirm the probe specificities, a mixture of the same amounts of in vitro-transcribed, full-length viral-sense S-, M-, and L-segment RNA transcripts, as well as anti-viral-sense transcripts, was separated by agarose gel electrophoresis, and then Northern blot analysis was performed using probe set A1 and probe set B (Fig. 1C, right two panels), which were specifically hybridized to viral-sense RNA transcripts and anti-viral-sense RNA transcripts, respectively, establishing the probe specificities. However, the intensity of each band differed, indicating that the efficiencies of hybridization of each probe to its target RNA transcripts were not the same. A band that migrated between the viral-sense L and M segments probably represented L RNA segment transcripts with a premature termination. Northern blot analysis of virion RNA from purified MP12 that was propagated in 293T cells, Vero E6 cells, BHK-21 cells or C6/36 cells is shown in the two left panels of Fig. 1C. As expected, probe set A1 detected three viral-sense RNA segments in the purified viruses. Unexpectedly, probe set B also demonstrated all three anti-viral-sense RNA segments. Northern blot analysis of intracellular RNA species (see Fig. 2C) showed an accumulation of NS and N mRNAs in infected cells, whereas both were absent in the purified virus sample (Fig. 1C), suggesting that detection of

and pL(-) are complementary to nt 39 to 776 from the 5' end of Svc, nt 1297 to 2102 from the 5' end of Mvc, nt 19 to 756 from the 5' end of Lvc, nt 35 to 781 from the 5' end of Sv, nt 915 to 1652 from the 5' end of Sv, nt 1784 to 2589 from the 5' end of Mv, and nt 5649 to 6386 from the 5' end of Lv, respectively. The viral RNAs and probes are not depicted according to their sizes. (C) Northern blot analysis of virion RNAs of purified MP12 (corresponding to fraction 7 in panel A) that were propagated in 293T, Vero E6 (Vero), BHK-21 (BHK), or C6/36 cells (left two panels), a mixture of in vitro-synthesized, full-length MP12 viral-sense L-segment transcripts (Lv), viral-sense M-segment transcripts (Mv), and viral-sense S-segment transcripts (Sv) (right lanes of the right two panels), and a mixture of anti-viral-sense L-segment transcripts (Lvc), anti-viral-sense M-segment transcripts (Mvc), and anti-viral-sense S-segment transcripts (Svc) (left lanes of right two panels). The same amount of in vitro-transcribed RNA transcripts was applied to the gel electrophoresis in the two panels at right. Probe set A1, a mixture of pN-S(-), pM(-), and pL(-), was used to detect viral-sense RNAs (top two panels), while probe set B, a mixture of pN-S(+), pM(+), and pL(+), was used to detect anti-viral-sense RNAs (bottom two panels). RNAs were denatured and separated on 1.5% denaturing agarose-formaldehyde gels and transferred onto a nylon membrane. Hybridization was performed at 68°C for 18 h in standard hybridization buffer with 50% formamide, and subsequent washing of the membrane with 2× SSC at room temperature and 0.5× SSC at 55°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

anti-viral-sense RNA segments in the purified RVFV was not due to merely contamination of the purified virion sample with intracellular RNAs.

Because an anti-viral-sense S segment was detected in the purified virus, the possibility emerged that the incoming anti-viral-sense S segment serves as a template for NSs mRNA synthesis. As a first step to explore this possibility, we used Northern blot analysis to estimate an approximate molar ratio of viral-sense and anti-viral-sense S segments in the purified virus particles; namely, the amounts of the virion-associated viral-sense S segment and virion-associated anti-viral-sense S segment were independently determined by comparing the former with the known amounts of viral-sense S-segment RNA transcripts and the latter with the known amounts of anti-viral-sense S-segment RNA transcripts. Studies from at least three independent experiments demonstrated that the molar ratio of the anti-viral-sense S segment to viral-sense S segment varies among virus preparations and was in a range from 1:5 to 1:100 (data not shown), demonstrating that the amount of the anti-viral-sense S segment was substantially smaller than that of the viral-sense S segment in the virus particles.

To test the possibility that the incoming anti-viral-sense S segment serves as the template RNA for NSs mRNA synthesis early in infection, we employed an RNase protection assay to examine the synthesis of N and NSs mRNAs. The feasibility of an RNase protection assay was tested first. The ³²P-labeled, 389-nt-long RNA probe (N probe) and the ³²P-labeled, 336-nt-long RNA probe (NSs probe) were independently synthesized by *in vitro* transcription (Fig. 2A); the former contained a 27-nt nonviral sequence at the 5' end and binds to the region from nucleotide (nt) 631 to 990 from the 5' end of the anti-viral-sense S segment and to the 3' region of N mRNA, while the latter contained a 27-nt nonviral sequence at the 5' end and binds to the region from nt 736 to 1036 from the 5' end of the viral-sense S segment and to the 3' region of NSs mRNA (Fig. 2A). Each probe was mixed with intracellular RNAs extracted from MP12-infected Vero E6 cells at 6 h p.i. or purified virion RNAs. An RNase protection assay was performed using an RNase protection assay kit (BD Biosciences) according to the manufacturer's protocol, and the RNase-resistant RNA fragments were separated on a 5% Tris-borate-EDTA-urea gel. As expected, an N probe revealed the virion-associated anti-viral-sense S segment, the intracellular anti-viral-sense S segment, and N mRNA, while an NSs probe detected the virion-associated viral-sense S segment, intracellular viral-sense S segment, and NSs mRNA (Fig. 2B). The synthesis of N and NSs mRNAs early in infection was examined next. To synchronize virus infection, Vero E6 cells were inoculated with MP12 at an MOI of 5, and the cells were kept at 0°C for 1 h. After virus adsorption, cells were washed with chilled medium to remove unadsorbed viruses and then incubated with pre-warmed (37°C) medium to promote synchronized virus penetration. At 0, 20, 40, 60, or 120 min after incubation, total intracellular RNAs were extracted. The RNase protection assay detected signals of both incoming viral-sense and anti-viral-sense S segments in the 0-min sample, and their signal intensities somewhat decreased during 20 min to 60 min incubation (Fig. 2D, left two panels). At 120 min after incubation, both signals increased slightly, indicating that RNA replication was initiated between 60 and 120 min of incubation. Signals of both

N mRNA and NSs mRNA were absent in the 0-min sample, yet accumulation of both mRNAs was evident as early as 20 min of incubation, and their amounts increased consistently thereafter. Intracellular RNAs from mock-infected cells and yeast tRNAs showed no RNA signals. These data demonstrated that the synthesis of N and NSs mRNAs occurred immediately after infection and strongly suggested that N mRNA and NSs mRNA were transcribed from the virion-associated viral-sense S segment and the virion-associated anti-viral-sense S segment, respectively, during primary transcription and prior to viral RNA replication.

To further establish NSs mRNA synthesis during primary transcription, we examined the synthesis of N and NSs mRNAs in the presence of puromycin, a translation inhibitor. It has been reported that bunyavirus genome RNA replication does not occur in the presence of translational inhibitors (2, 5, 6, 15, 18). Accordingly, if NSs mRNA synthesis occurs in the presence of puromycin immediately after infection, then the data would conclusively demonstrate that NSs mRNA synthesis occurred during primary transcription from the incoming anti-viral-sense S segment. To determine the appropriate concentration of puromycin to inhibit viral RNA synthesis, Vero E6 cells were incubated with 0, 0.5, 5.0, 25, 50, or 100 µg/ml of puromycin for 1 h prior to virus adsorption and during virus adsorption (MOI = 5) and incubation of infected cells. Northern blot analysis of intracellular RNAs, which were extracted at 6 h p.i., demonstrated that treatment with 25 to 100 µg/ml of puromycin completely inhibited viral RNA synthesis (Fig. 2C). Accordingly, 100 µg/ml of puromycin was used to examine NSs mRNA synthesis. Vero E6 cells were incubated with puromycin for 1 h, and then virus inoculum containing puromycin was added at an MOI of 5. After 1 h incubation at 0°C and removal of unadsorbed viruses, infected cells were incubated in the presence of puromycin. An RNase protection assay was performed using intracellular RNAs that were extracted at 0, 20, 40, 60, or 120 min incubation. As expected, viral-sense and anti-viral-sense S-segment RNAs did not accumulate (Fig. 2D, right two panels), demonstrating that puromycin treatment inhibited viral RNA replication. Significantly, NSs mRNA synthesis (Fig. 2D, right bottom panel), but not N mRNA synthesis (Fig. 2D, right top panel), occurred in the presence of puromycin. These data conclusively established that NSs mRNA synthesis occurred during primary transcription from the incoming anti-viral-sense S segment. Furthermore, RVFV NSs mRNA synthesis, which was resistant to treatment with the protein synthesis inhibitor puromycin, differed from that in previous reports of mRNA synthesis of other bunyaviruses, in which host translation machineries appeared to be required for viral mRNA transcription (2, 15, 18).

We next examined NSs protein synthesis early in infection. To efficiently detect NSs protein synthesis, we made anti-NSs peptide (N-EESDDDDGFVEVD-C) rabbit polyclonal antibody (ProSci Inc, Poway, CA) and tested its reactivity. Western blot analysis of cell extracts from MP12-infected 293T cells using anti-RVFV antibody demonstrated an accumulation of NSs and N proteins, while anti-NSs antibody detected only NSs protein (Fig. 3A, lane 1); neither antibody detected host protein signals in the mock-infected cell extracts (Fig. 3A, lane 4). To further establish the anti-NSs antibody specificity, 293T cells were independently transfected with a mixture of

FIG. 2. Analysis of NS mRNA and N mRNA synthesis early in infection. (A) Schematic diagram of the structure of the anti-viral-sense S segment (Svc), viral-sense S segment (Sv), NSs mRNA, and N mRNA and binding regions of NSs probe to Sv and NSs mRNA and those of N probe to Svc and N mRNA. (B) An RNase protection assay was used to detect virion Svc and intracellular N mRNA by using an N probe and virion Sv and intracellular NSs mRNA by using an NSs probe. MP12 was propagated in Vero E6 cells and purified as described in the text. Virion RNA extracted from purified MP12 was used for the source of virion RNA, and total RNA from MP12-infected Vero E6 cells at 6 h p.i. was used for intracellular RNA (I.C. RNA). (C) Vero E6 cells were treated with the indicated concentrations of puromycin for 1 h prior to MP12 inoculation at an MOI of 5. After and during 1 h incubation at 0°C, infected cells were incubated at the indicated concentration of puromycin. Total RNA was harvested at 6 h p.i., and analyzed by Northern blotting using probe set A2, a mixture of pNS-S(-), pM(-), and pL(-), to detect viral-sense RNAs (upper panel) and using probe set B, a mixture of pN-S(+), pM(+), and pL(+), to detect anti-viral-sense RNAs (lower panel). Lvc * represents a mixture of anti-viral-sense L segment and L mRNA, and Mvc * represents a mixture of anti-viral-sense M segment and M mRNA. (D) Vero E6 cells were infected with MP12 at an MOI of 5 and incubated at 0°C for 1 h. After removal of unadsorbed viruses, infected cells were incubated with prewarmed medium and kept at 37°C. Addition of prewarmed medium represents 0 min. At indicated times, intracellular RNAs were extracted, and an RNase protection assay using an N probe (upper panel) and an NSs probe (lower panel) was performed. As negative controls, yeast tRNA and intracellular RNAs from mock-infected cells (M) were used. Cells incubated in the absence of puromycin in the samples are shown in the left panel, while cells incubated with 100 μg/ml of puromycin in the samples are at right.

FIG. 3. Accumulation of N and NSs protein early in infection. (A) 293T cells were infected with MP12 at an MOI of 1 (lane 1) or mock-infected (lane 4), and cell extracts were prepared at 8 h p.i. 293T cells were independently transfected with a mixture of pCT7pol (12) and pT7-IRES-NSs (7), which expresses NSs protein (lane 2), or that of pCT7 pol and pT7-IRES-N (7), which expresses N protein (lane 3). After 48 h transfection, cell extracts were prepared and analyzed by Western blot analysis using anti-RVSV antibody (upper panel) or anti-NSs antibody (lower panel). (B) Vero E6 cells were infected with MP12 at an MOI of 5 or mock infected (lane M) in the absence or presence of puromycin, as described for Fig. 2D. At various times p.i., as indicated at the top of the gel, cell extracts were prepared and accumulation of N and NSs proteins examined using anti-RVSV antibody (a-RVSV) and anti-NSs antibody (a-NSs). Antiactin antibody (a-actin) revealed the amounts of actin in each lane (a-actin).

pCT7pol (12), which expresses T7 RNA polymerase, and pT7-IRES-NSs (7) or that of pCT7 pol and pT7-IRES-N (7); pT7-IRES-NSs and pT7-IRES-N express NSs protein and N protein, respectively, by using a cap-independent translation mechanism (7). Forty-eight hours after transfection, cell extracts were prepared and used for Western blot analysis (Fig. 3A, lanes 2 and 3). Anti-RVSV antibody detected both expressed NSs and N proteins, while anti-NSs antibody detected expressed NSs protein but not N protein, demonstrating that anti-NSs antibody specifically recognized NSs protein. Also, anti-NSs antibody was substantially more sensitive than was anti-RVSV antibody in detecting intracellular NSs protein (Fig. 3A). Western blot analysis of intracellular proteins early in RVSV infection demonstrated that anti-RVSV antibody detected the incoming N protein in the 0-min sample (Fig. 3B). In the presence of puromycin treatment, the N protein signals clearly decreased subsequent to incubation at 37°C, implying degradation of the incoming N protein. In the absence of puromycin treatment, the N protein signal in the samples from 20 to 120 min incubation was only slightly lower than that of the 0-min sample, and it clearly increased after 180 min incubation (Fig. 3B). The NSs protein signal was at background level during the first 40 min of incubation, and yet it increased gradually after about 60 to 80 min incubation. Accumulation of NSs protein was quite evident after 180 min incubation and detectable by anti-RVSV antibody. These data demonstrated

that NSs protein synthesis was detectable as early as 60 to 80 min p.i.

To address the possibility that NSs protein synthesis occurs early in infection, we examined whether RVSV carries anti-viral-sense S-segment RNA and used it as a template for NSs mRNA synthesis early in infection. Past studies reported the presence of an anti-viral-sense S-segment RNA in Uukuniemi virus (UUKV) (17), also belonging to the *Phlebovirus* genus. La Crosse virus, belonging to the *Orthobunyavirus* genus, was propagated from C6/36 cells but not from BHK-21 cells and contained an anti-viral-sense S segment in its virions (14). As in these past studies, we used virus particles that were purified by ultracentrifugation and found the presence of not only three viral-sense RNA segments but also all three anti-viral-sense RNA segments in the purified RVSV (Fig. 1C). In the *Bunyaviridae* family, this is the first report that strongly indicates the presence of all three anti-viral-sense RNA segments in the virus particles. We estimated the molar ratio of the viral-sense S segment to the anti-viral-sense S segment in RVSV as ranging between 5:1 and 100:1 in different preparations, while that ratio was reported as 10:1 in UUKV (17). There was a linear correlation between dilution of an MP12-containing sample and the number of plaques (data not shown), suggesting that an infectious particle contains at least all three viral-sense segments. It is unknown how many additional viral RNA segment molecules could be packaged into RVSV particles, yet our data imply that some but not all infectious RVSV particles carry anti-viral-sense segment RNAs. Because synthesis of NSs mRNA occurred immediately after infection in the absence of RNA replication (Fig. 2D), it is difficult to imagine that the anti-viral-sense S segment that was detected in the purified virus particles was a contamination of intracellular RNAs. Testing the presence of anti-viral-sense M and L segments using RVSV that is purified by a different method, e.g., immunoprecipitation, would further confirm the packaging of anti-viral-sense M and L segments into RVSV.

Synthesis of N mRNA and NSs mRNA was detected as early as 20 min p.i. (Fig. 2D), suggesting translation of both N and NSs proteins early in infection. Analysis of intracellular N protein early infection suggested that in the absence of N mRNA synthesis, the incoming N protein underwent substantial degradation (Fig. 3B). In contrast, only a modest reduction in the amounts of N protein occurred after 20 to 120 min incubation in the absence of puromycin. A straightforward interpretation of these data is that N protein synthesis occurred as early as 20 min from newly synthesized N mRNA (Fig. 2D). In contrast, NSs protein accumulation was evident only after 60 to 80 min p.i. (Fig. 3B). Although it is unclear why we were unable to detect NSs protein accumulation during first 40 min p.i., it is conceivable that the amount of NSs mRNA would be substantially lower than that of N mRNA immediately after infection, because the amounts of anti-viral-sense S segment in the virus particles were only 1 to 20% of the viral-sense S segment. Also, the sensitivity of anti-NSs antibody for detecting NSs protein might be lower than that of anti-RVSV antibody for detecting N protein.

Because NSs mRNA synthesis occurred in the absence of viral RNA replication (Fig. 2D, right panel), NSs mRNA that was accumulated early in infection should have been transcribed from the incoming anti-viral-sense S segment. Virions

containing an anti-viral-sense S segment were a minor population in the virus progeny from our cell culture, suggesting that NSs protein synthesis early in infection occurs in only a fraction of cells infected with RVFV. We speculate that the immediate expression of the NSs protein with its inhibition of host mRNA transcription, particularly of the interferon genes, may provide a selective advantage during viral infection. This "head start" may be quite important in particular cells or organs or at a point early in infection at which innate immunity plays a more important role in host resistance. The need for this selective viral advantage may be responsible for the evolutionary retention of the ability of the virions to include subpopulations with diploid genomes. It will be of interest to study the proportions of diploid genomes under different circumstances of passage in vitro and in vivo in interferon-incompetent systems or highly susceptible hosts.

Previous observations that host translation is required for viral mRNA synthesis of viruses of the *Bunyaviridae* family (2, 15, 18) led to the hypothesis that nascent bunyavirus mRNA needs to bind to ribosomes to prevent premature transcription termination; without this binding, the nascent mRNA may interact with its template and stop mRNA elongation (2). Like other bunyaviruses, puromycin treatment inhibited RVFV N mRNA synthesis during primary transcription (Fig. 2D). Unexpectedly, NSs mRNA synthesis occurred in the presence of puromycin. Note that the probes used for the RNase protection assay hybridize only with mature N and NSs mRNAs (Fig. 2A); hence, this assay detected mature mRNAs and not premature transcription products. The simplest explanation for the puromycin-resistant NSs mRNA synthesis is that nascent NSs mRNA forms stable secondary or ternary RNA structures and does not bind to the template RNA, preventing premature transcription termination. Obviously, further studies will be needed to determine the mechanism of puromycin-resistant NSs mRNA synthesis.

We thank R. B. Tesh and S. Higgs for anti-RVFV mouse polyclonal antibody and C6/36 cells, respectively.

This work was supported by grants from NIAID to S.M. and C.J.P. through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research, NIH grant number U54 AI057156.

REFERENCES

1. Balkhy, H. H., and Z. A. Memish. 2003. Rift Valley fever: an uninvited zoonosis in the Arabian peninsula. *Int. J. Antimicrob. Agents* **21**:153–157.
2. Bellocq, C., and D. Kolakofsky. 1987. Translational requirement for La Crosse virus S-mRNA synthesis: a possible mechanism. *J. Virol.* **61**:3960–3967.
3. Billecocq, A., M. Spiegel, P. Vialat, A. Kohl, F. Weber, M. Bouloy, and O. Haller. 2004. NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. *J. Virol.* **78**:9798–9806.
4. Bouloy, M., C. Janzen, P. Vialat, H. Khun, J. Pavlovic, M. Huerre, and O. Haller. 2001. Genetic evidence for an interferon-antagonistic function of Rift Valley fever virus nonstructural protein NSs. *J. Virol.* **75**:1371–1377.
5. Eshita, Y., B. Ericson, V. Romanowski, and D. H. Bishop. 1985. Analyses of the mRNA transcription processes of snowshoe hare bunyavirus S and M RNA species. *J. Virol.* **55**:681–689.
6. Ihara, T., Y. Matsuura, and D. H. Bishop. 1985. Analyses of the mRNA transcription processes of Punta Toro phlebovirus (*Bunyaviridae*). *Virology* **147**:317–325.
7. Ikegami, T., C. J. Peters, and S. Makino. 2005. Rift Valley fever virus nonstructural protein NSs promotes viral RNA replication and transcription in a minigenome system. *J. Virol.* **79**:5606–5615.
8. Ito, N., E. C. Mossel, K. Narayanan, P. V. L., C. Huang, T. Inoue, C. J. Peters, and S. Makino. 2005. Severe acute respiratory syndrome coronavirus 3a protein is a viral structural protein. *J. Virol.* **79**:3182–3186.
9. Kim, K. H., K. Narayanan, and S. Makino. 1997. Assembled coronavirus from complementation of two defective interfering RNAs. *J. Virol.* **71**:3922–3931.
10. Le May, N., S. Dubaele, L. P. De Santis, A. Billecocq, M. Bouloy, and J. M. Egly. 2004. TFIIF transcription factor, a target for the Rift Valley hemorrhagic fever virus. *Cell* **116**:541–550.
11. Narayanan, K., and S. Makino. 2001. Cooperation of an RNA packaging signal and a viral envelope protein in coronavirus RNA packaging. *J. Virol.* **75**:9059–9067.
12. Neumann, G., H. Feldmann, S. Watanabe, I. Lukashevich, and Y. Kawaoka. 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. *J. Virol.* **76**:406–410.
13. Peters, C. J., and J. M. Meegan. 1989. Rift Valley fever. CRC Press, Boca Raton, Fla.
14. Raju, R., and D. Kolakofsky. 1989. The ends of La Crosse virus genome and antigenome RNAs within nucleocapsids are base paired. *J. Virol.* **63**:122–128.
15. Raju, R., and D. Kolakofsky. 1986. Inhibitors of protein synthesis inhibit both La Crosse virus S-mRNA and S genome syntheses in vivo. *Virus Res.* **5**:1–9.
16. Schmaljohn, C., and J. W. Hooper. 2001. *Bunyaviridae*: the viruses and their replication, p. 1581–1602. In D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman, and S. E. Straus (ed.), *Fields virology*, 4th ed. Lippincott, Williams & Wilkins, Philadelphia, PA.
17. Simons, J. F., U. Hellman, and R. F. Pettersson. 1990. Uukuniemi virus S RNA segment: ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *J. Virol.* **64**:247–255.
18. Vialat, P., and M. Bouloy. 1992. Germiston virus transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J. Virol.* **66**:685–693.
19. Vialat, P., R. Muller, T. H. Vu, C. Prehaud, and M. Bouloy. 1997. Mapping of the mutations present in the genome of the Rift Valley fever virus attenuated MP12 strain and their putative role in attenuation. *Virus Res.* **52**:43–50.