Transactivation of programmed ribosomal frameshifting by a viral protein

Yanhua Li\textsuperscript{a,1}, Emmely E. Treffers\textsuperscript{c,d}, Sawsan Naphthine\textsuperscript{e}, Ali Tas\textsuperscript{1}, Longchao Zhu\textsuperscript{b,1}, Zhi Sun\textsuperscript{1}, Susanne Bell\textsuperscript{a}, Brian L. Mark\textsuperscript{b}, Peter A. van Veelen\textsuperscript{d}, Martijn J. van Hemert\textsuperscript{c}, Andrew E. Firth\textsuperscript{1}, Ian Brierley\textsuperscript{e,2}, Eric J. Snijder\textsuperscript{c,2}, and Ying Fang\textsuperscript{b,1,2}

\textsuperscript{a}Department of Veterinary and Biomedical Sciences and Department of Biology/Microbiology, South Dakota State University, Brookings, SD 57007; \textsuperscript{b}Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506; \textsuperscript{c}Molecular Virology Laboratory, Department of Medical Microbiology, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands; \textsuperscript{d}Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands; \textsuperscript{e}Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom; and \textsuperscript{f}Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada R3T 2N2

Programmed –1 ribosomal frameshifting (−1 PRF) is a widely used translational mechanism facilitating the expression of two polypeptides from a single mRNA. Commonly, the ribosome interacts with an mRNA secondary structure that promotes −1 frameshifting on a homopolymeric slippery sequence. Recently, we described an unusual −2 frameshifting (−2 PRF) signal directing efficient expression of a transframe protein (nonstructural protein 2TF (nsp2TF)) of porcine reproductive and respiratory syndrome virus (PRRSV) from an alternative reading frame overlapping the viral replicase gene. Unusually, this arterivirus PRF signal lacks an obvious stimulatory RNA secondary structure, but as confirmed here, can also direct the occurrence of −1 PRF, yielding a third, truncated nsp2 variant named “nsp2N.” Remarkably, we now show that both −2 and −1 PRF are transactivated by a protein factor, specifically a PRRSV replicase subunit (nsp1β). Embedded in nsp1β’s papain-like autoproteinase domain, we identified a highly conserved, putative RNA-binding motif that is critical for PRF transactivation. The minimal RNA sequence required for PRF was mapped within a 34-nt region that includes the slippery sequence and a downstream conserved CCCANCUCU motif. Interaction of nsp1β with the PRF signal was demonstrated in pull-down assays. These studies demonstrate for the first time, to our knowledge, that a protein can function as a transactivator of ribosomal frameshifting. The newly identified frameshifting determinants provide potential antiviral targets for arterivirus disease control and prevention. Moreover, protein-induced transactivation of frameshifting may be a widely used mechanism, potentially including previously undiscovered viral strategies to regulate viral gene expression and/or modulate host cell translation upon infection.

Significance

Ribosomes synthesize proteins by translating mRNAs into linear chains of amino acids through the decoding of consecutive nucleotide triplets (codons). Specific mRNA signals, however, can stimulate ribosomes to shift into an alternative triplet reading frame (ribosomal frameshifting) resulting in translation of a different protein. Typically, such signals are regions of intramolecular nucleotide base-pairing in the mRNA which form structures that stall ribosome progress. Here we show that the frameshifting signal used to express the nsp2TF and nsp2N proteins of porcine reproductive and respiratory syndrome virus, an important swine pathogen, requires the action of a transacting viral protein rather than a structured RNA. This novel mechanism of gene expression may also be used by other viruses or in cellular gene expression.


Conflict of interest statement: The authors have filed a patent application that relates to some aspects of this work.

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1Present address: Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506-5705.

2To whom correspondence may be addressed. E-mail: yfang@vet.k-state.edu, ib103@mole.bio.cam.ac.uk, or ejsnijder@lumc.nl.

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subunit is involved in diverse steps of the arterivirus replicative cycle, including replicase polyprotein processing (16), the formation of replication structures (17, 18), and innate immune evasion (19–22). At the PRRSV −2 PRF signal, a proportion of ribosomes back up 2 nt, to generate a transframe fusion protein (nsp2TF) comprising the N-terminal two-thirds of nsp2 and the ribosomes back up 2 nt, to generate a transframe fusion protein the end of ORF1a. The TF ORF overlaps the central ORF1a region in the −2 reading frame and is accessed via −2 PRF (13). A −1 frameshift at the same site generates the nsp2N product (see details under the section “Alternative −2 and −1 PRF at the Same PRRSV Slippery Sequence”). The vertical red line indicates the location of the RG_GUU_UUU shift site (R = A or G, in different arteriviruses). Domains in nsp2/nsp2TF: C, Cys-rich domain HVR, hypervariable region; PLP2, papain-like protease; TM/TM′, putative transmembrane domains. (B) Sequence of the SD01-08 RNA in the region of the −2/−1 PRF signal, with the slippery sequence (red) and C-rich motif (blue) highlighted. The −1 reading frame stop codon is underlined and codons for each of the reading frames are indicated. (C) Features of the canonical −1 PRF signal present in the PRRSV ORF1a/ORF1b overlap region. The stimulatory RNA pseudoknot is composed of two stems connected by single-stranded loops.

In this report, we identify PRRSV replicase subunit nsp1β as a transactivator of efficient −2 and −1 PRF at the same slippery sequence and provide evidence that its frameshift-stimulatory activity requires interaction with the viral mRNA. In support of this, a highly conserved putative RNA-binding motif (GKYLOQRLO), integrated into the structure of nsp1β’s papain-like autoproteinase domain, was found to be critical for the stimulation of frameshifting and for interacting with the RNA sequence of the PRRSV PRF signal. The minimal RNA sequence required to direct efficient PRF was mapped within a 34-nt region of the PRRSV nsp2-coding sequence that includes the shift site and the conserved CCCANCUCC motif. Our findings reveal an unusual noncanonical translation mechanism in which a viral protein functions as a transactivator of efficient −2 and −1 PRF. This study advances our understanding of noncanonical translation, suggests that viruses may use additional strategies to modulate viral and potentially host cell translation during infection, and has practical implications in biotechnology and the design of antiviral strategies.

Results

Alternative −2 and −1 PRF at the Same PRRSV Slippery Sequence. Previously (13), we demonstrated expression of the PRRSV TF ORF (Fig. 1A) using a rabbit antiserum raised against the epitope on the C terminus of the polyepitope it encodes. Sub-
sequently, the frameshift product was immunopurified from infected cells and mass spectrometry (MS) was used to identify both the site (RG_GUU_UUU) and direction (−2, rather than +1) of ribosomal frameshifting. In both PRRSV-infected cells and an ORF1a expression system, and using distantly related type 1 and type 2 PRRSV isolates, the same studies revealed an additional nsp2-related product (nsp2N) with a size consistent with −1 PRF occurring at the same site (estimated efficiency ∼7%) (13). However, a stop codon is present in the −1 frame immediately downstream of the RG_GUU_UUU slippery sequence (Fig. 1A) and consequently, if nsp2N were derived from −1 frameshifting, it would lack a unique C-terminal sequence that could be discriminated from a product derived through the internal proteolytic cleavage of full-length nsp2. In an attempt to confirm the occurrence of −1 PRF by immunoprecipitation and mass spectrometric analysis (MS), we sought to extend the potential −1 frameshift product with a unique C-terminal signature. In a full-length cDNA clone of the previously used PRRSV isolate SD01-08 (a type 1 virus) (30), the −1 frame stop codon (UGA) was replaced by a tryptophan codon (UGG), extending the −1 frame by an additional 87 codons (Fig. S1, SD01-08-M1). However, this point mutation unavoidably also introduced amino acid substitutions in the overlapping 0 and −2 frames encoding nsp2 and nsp2TF (Glu→Gly and Lys→Glu, respectively), and perhaps as a consequence, the resulting recombinant virus was severely crippled [titers reduced to 10^3 fluorescent-focus units (FFU)/mL], preventing us from immunopurifying sufficient nsp2N for reliable MS analysis. We, therefore, reverted to a type 2 PRRSV isolate (SD95-21) (31) and introduced the same A-to-G mutation, which in this case extended the −1 ORF by 23 additional codons to generate mutant SD95-21-M1 (Fig. S1). Fortunately, despite carrying Asp→Gly and Thr→Ala mutations in the nsp2 and nsp2TF products, respectively, this recombinant virus replicated to much higher titers and Thr thereupon able to direct both nsp2-Coding Region.

Previously we demonstrated that translation extended the nsp2N product and introduced the same A-to-G mutation, which in this case therefore, reverted to a type 2 PRRSV isolate (SD95-21) (31) immunopurifying sufficient nsp2N for reliable MS analysis. We, therefore, reverted to a type 2 PRRSV isolate (SD95-21) (31) and introduced the same A-to-G mutation, which in this case extended the −1 ORF by 23 additional codons to generate mutant SD95-21-M1(Fig. S1). Fortunately, despite carrying Asp→Gly and Thr→Ala mutations in the nsp2 and nsp2TF products, respectively, this recombinant virus replicated to much higher titers (10^5.2 FFU/mL) and the C-terminally extended nsp2N product (nsp2N)* could be immunopurified from infected MARC-145 cells. A gel slice containing the nsp2N* band was analyzed by liquid chromatography tandem MS (LC-MS/MS) and a QVFWPR tryptic peptide that spanned the frameshift site and is compatible with −1 PRF at the RG_GUU_UUU sequence was identified (Fig. S2). To verify correct identification of this peptide, a synthetic dem mass spectrum of this synthetic peptide was found to be identical to that of the peptide derived from the nsp2N*-containing gel slice (Fig. S2D), confirming that nsp2N is indeed translated via −1 PRF at the RG_GUU_UUU slippery sequence, which is, therefore, able to direct both −1 and −2 PRF.

**PRRSV nsp1β Is Required for Efficient −1 and −2 Frameshifting in the nsp2-Coding Region.** Previously we demonstrated that translation of the complete PRRSV ORF1a sequence is sufficient to allow efficient −2 PRF (13). To define the minimal sequence requirements for −2/−1 PRF in PRRSV isolate SD01-08, we focused our attention on the N-terminal half of ORF1a (the nsp1α-nsp3 region) and generated a panel of truncated ORF1a constructs (Fig. 2A) for expression in the recombinant vaccinia virus–T7 RNA polymerase system (32). Following radiolabeling of proteins synthesized in transfected RK-13 cells, expression of nsp2, nsp2TF, and nsp2N was analyzed by immunoprecipitation using monoclonal antibody (mAb) α-EU-nsp2 and rabbit antisera α-EU-TF, recognizing all three nsp2-related products and the unique C-terminal epitope of nsp2TF, respectively (see Fig. S1B for a summary of antibody nomenclature and epitopes recognized). As shown in Fig. 2B, constructs lacking the nsp1α- and/or nsp3-coding region still efficiently expressed nsp2TF and nsp2N. In contrast, constructs lacking the nsp1β-coding region expressed nsp2 but only trace amounts of nsp2TF or nsp2N were detected. This indicates that nsp1β, or the RNA sequence encoding nsp1β, is required for efficient −2/−1 PRF at the RG_GUU_UUU slippery sequence in the nsp2-coding region, located some 2.5 kb downstream of the nsp1β-coding region.

Extending this further, using the same expression system, nsp2 and nsp1β were expressed from separate, cotransfected plasmids (pLns2p and pLns1β) rather than as a self-cleaving nsp1β-2 polyprotein (pLns1β-2). Again, both nsp2TF and nsp2N were
produced (Fig. 2C), indicating that nsp1β can stimulate −2/−1 PRF in the nsp2-coding region in *trans*. To establish whether this effect was mediated by the nsp1β protein or the nsp1β-coding RNA sequence, a drastically altered version of the nsp1β-2 expression vector was produced in which almost every codon of the nsp1β-coding sequence was mutated simultaneously, while avoiding rare codons (mutant pLns1β[cc-2]; Fig. S3). This pLns1β[cc-2] construct expresses an unaltered nsp1β protein, but the nucleotide sequence encoding it is changed to such an extent that we would expect to have disrupted any primary sequence or RNA secondary structure elements that might be involved in −2 PRF (for example, an element having a long-range interaction with the PRF region in the nsp2-coding sequence). Immunoprecipitation analysis revealed that nsp2TF and nsp2N were expressed with equal efficiency in cells transfected with pLns1β[cc-2] and wild-type (WT) pLns1β-2 (Fig. 2C), indicating that PRF stimulation involves the nsp1β protein rather than an RNA signal in the nsp1β-coding sequence.

**Minimal RNA Sequence Requirements for −2/−1 PRF.** We next set out to define the minimal RNA sequences in the nsp2-coding region that are required for efficient −2/−1 PRF. To this end, we prepared a reporter gene construct in which PRRSV RNA sequences from the PRF-inducing region were placed between two luciferase genes [pDluc (33, 34); Fig. 3A]. Whereas the ORF1a frame of the PRRSV insert was placed in-frame with the upstream (*Renilla*) luciferase gene, the downstream (firefly) luciferase was in the −2 frame and thus its expression depended on the occurrence of −2 frameshifting. Also, −1 PRF could be monitored, because the native stop codon in the −1 frame was retained and −1 PRF would, therefore, yield a polypeptide slightly shorter than the product resulting from translation termination in the zero reading frame. As controls, an in-frame control (IFC) construct was also prepared in which the two luciferase genes were aligned in the same frame by inserting two nucleotides (CU) immediately downstream of the slippery sequence. A previously described PRF knockout construct (KO2; Fig. S1) (13) containing point mutations within both slippery sequence and downstream C-rich region was also included in the analysis.

Initially, a 79-nt region spanning 5 nt upstream of the slippery sequence to 66 nt downstream (including the conserved CCCANCUCC motif) was cloned between the two luciferase genes (construct pDluc-WT). Frameshifting efficiencies were determined by comparing the ratio of enzymatic activities of firefly and *Renilla* luciferase such that the ORF1a frame of all pDluc translation constructs containing a WT or mutant nsp1β cassette was removed (Fig. 3C). The ORF1a frameshift efficiency was calculated by comparing the ratio of firefly and *Renilla* luciferase activities, using the IFC mutant (Fig. 3A). As shown in Fig. 3B, in comparison with the IFC control, the WT PRRSV −2 PRF efficiency was ~38%, and this high level of −2 PRF was only observed in cells cotransfected with the nsp1β-expressing plasmid; in the absence of the transactivator, only low levels of −2 PRF (<5%) were observed. As expected, frameshifting was not observed in cells transfected with pDluc-KO2. Western blot analysis of transfected cell lysates revealed that both efficient −2 PRF and efficient −1 PRF could be observed with pDluc-WT provided that an nsp1β expression plasmid was cotransfected (Fig. 3C). These data indicated that the 79-nt PRRSV sequence included in pDluc-WT contains all cis-acting sequences required for efficient −2/−1 PRF, and that, as documented above, both types of frameshifting depend on the presence of nsp1β. In the absence of this transactivator, only low levels of PRF were observed.

To further investigate the key RNA sequences required for PRF, in-frame deletions were introduced into pDluc-WT, starting from the 3′ end of the PRRSV insert. As shown in Fig. 3C, an initial deletion that reduced the PRRSV sequence downstream of the shift site to 45 nt (pDluc-45) led to a small reduction in −2 PRF (about twofold), albeit with a concurrent increase in −1 PRF. Subsequent deletions had no further effect until part of the conserved CCCANCUCC motif was removed (Fig. 3C; compare pDluc-21 and pDluc-15). In pDluc-15, which lacked the second half (CUCCC) of the conserved motif, the capacity for transactivation of PRF by nsp1β was lost. These data provided further support for a role of the C-rich motif in PRF, and allowed us to define the functional PRRSV −2/−1 PRF cassette as a 34-nt region containing the slippery sequence and the 3′ C-rich motif.

**Identification of a Conserved nsp1β Motif That Is Critical for PRF TransActivation.** The nsp1–nsp1β region has previously been implicated in a variety of processes in the arterivirus replicative
and His residues of the papain-like proteinase domain (PLP1) that constitutes the C-terminal two-thirds of nsp1 of both type 1 and type 2 PRRSV, forms one of three RNA interaction domains. This sequence, GKYLQRRLQ in a previously identified conserved sequence motif as a potential transcriptional control (36, 37), and innate immune evasion (31, 38). An analysis of nsp1β sequence conservation (Fig. 4A), together with the published crystal structure of nsp1β from a type 2 PRRSV isolate (PRRSV XH-GD) (39) are shown above the alignment and are color matched to the nsp1β structure in B. Conserved basic residues in PLP1β helix αβ are boxed in orange. #, residues mutated in mutant 1βKO (see details in Results, Identification of a Conserved nsp1β Motif That Is Critical for PRF Trans-Activation); *, PLP1β active site residues. PRRSV sequences are numbered (black) from the nsp1/nsp1β cleavage site, whereas all other sequences are numbered (gray) starting from the N terminus of the pp1a polyprotein. The names of specific isolates used are indicated. GenBank accession nos. of sequences used are as follows: EU624117 (PRRSV XH-GD), DQ489311 (PRRSV SD01-08), KC469618 (PRRSV SD95-21), NC_001639 (LDV P), NC_003092 (SHFV LVR), HQ845737 (SHFV krc1), HQ845738 (SHFV krc2), JX473847 (SHFV krtg1), and NC_002532 (EAV Bucyrus). (**B**) Cartoon representation of the crystal structure of the nsp1β dimer from a type 2 PRRSV isolate (PRRSV XH-GD; PDB ID code 3MTV) (39). For both monomers, the N-terminal domain is colored purple, whereas the PLP1β domain and the C-terminal extension (leading up to the nsp1β/nsp2 site cleaved by PLP1β) are colored green and red, respectively. Helix αβ of PLP1β, containing the conserved GKYLQRRLQ motif, is colored orange with basic residues represented as sticks. (**C**) Electrostatic surface representation of the nsp1β dimer showing the positively charged (blue) patches on helix αβ of PLP1β (boxed in orange) created by the basic residues of the GKYLQRRLQ motif. Both patches reside on the same side of the structure, potentially allowing for RNA to bind across the entire dimer surface.

Fig. 4. PRRSV nsp1β sequence and structure. (**A**) Amino acid sequence alignment of the PLP1β domains from selected arterivirus nsp1β proteins. Secondary structure elements (based on the published crystal structure from type 2 PRRSV isolate XH-GD) (39) are shown above the alignment and are color matched to the nsp1β structure in B. Conserved basic residues in PLP1β helix αβ are boxed in orange. #, residues mutated in mutant 1βKO (see details in Results, Identification of a Conserved nsp1β Motif That Is Critical for PRF Trans-Activation); *, PLP1β active site residues. PRRSV sequences are numbered (black) from the nsp1/nsp1β cleavage site, whereas all other sequences are numbered (gray) starting from the N terminus of the pp1a polyprotein. The names of specific isolates used are indicated. GenBank accession nos. of sequences used are as follows: EU624117 (PRRSV XH-GD), DQ489311 (PRRSV SD01-08), KC469618 (PRRSV SD95-21), NC_001639 (LDV P), NC_003092 (SHFV LVR), HQ845737 (SHFV krc1), HQ845738 (SHFV krc2), JX473847 (SHFV krtg1), and NC_002532 (EAV Bucyrus). (**B**) Cartoon representation of the crystal structure of the nsp1β dimer from a type 2 PRRSV isolate (PRRSV XH-GD; PDB ID code 3MTV) (39). For both monomers, the N-terminal domain is colored purple, whereas the PLP1β domain and the C-terminal extension (leading up to the nsp1β/nsp2 site cleaved by PLP1β) are colored green and red, respectively. Helix αβ of PLP1β, containing the conserved GKYLQRRLQ motif, is colored orange with basic residues represented as sticks. (**C**) Electrostatic surface representation of the nsp1β dimer showing the positively charged (blue) patches on helix αβ of PLP1β (boxed in orange) created by the basic residues of the GKYLQRRLQ motif. Both patches reside on the same side of the structure, potentially allowing for RNA to bind across the entire dimer surface.

In a recent study (31), the GKYLQRRLQ motif was targeted by site-directed mutagenesis and the Lys and the first Arg of the motif were replaced with Ala (mutant 1βKO, Fig. S1). For both PRRSV genotypes, the replication of the 1βKO mutant in MARC-145 cells was found to be seriously crippled. The fact that we had observed similar defects in mutants in which the −2/−1 PRF signal had been inactivated, or in which the expression of a functional nsp2TF was prevented (13), prompted us to investigate whether this KR→AA double mutation affected nsp2TF/nsp2N expression. Strikingly, upon expression of nsp1β-nsp2 from either PRRSV genotype carrying these nsp1β mutations, neither nsp2TF nor nsp2N could be detected (Fig. 5). These data indicate that the GKYLQRRLQ motif plays a key role in PRF activation.

To investigate nsp1β transactivation of PRF in the context of PRRSV infection, we analyzed nsp2 expression using the 1βKO mutant of both PRRSV genotypes. As controls, we included the corresponding KO2 mutants, which carry mutations within the slippery sequence and C-rich region that eliminate frameshifting (Fig. S1) (13). Using reverse genetics, KO2 and 1βKO mutant viruses were recovered from full-length infectious clones of the two PRRSV genotypes. Both mutants replicated poorly in MARC-145 cells, but for the type 2 PRRSV isolate (SD95-21), they produced titers (105.1 and 105.3 FFU/mL for KO2 and 1βKO, respectively) that sufficed for the subsequent experiments of infection, metabolic labeling, and radioimmunoprecipitation.
and 1 KO mutant carried a double Aia substitution of basic residues in the highly conserved GKYLQRRLQ motif of nsp1β (see also Fig. 4 and Fig. S1). Expression products were immunoprecipitated with mAbs recognizing the common N-terminal domain of the nsp2-related products. Following cotransfection of vectors expressing RNA target and nsp1β, we used constructs pFLAG-nsp1β-WT and pFLAG-nsp1β-KO, producing WT and mutant (K130A/R134A) nsp1β, respectively, each fused to a N-terminal triple FLAG tag. The empty vectors pFLAG and pEGFP were included as negative controls.

Following cotransfection of vectors expressing RNA target and nsp1β into 293T cells, cell lysates were prepared. Western blot and qRT-PCR analysis (Fig. 7B) were first used to determine the expression levels of nsp1β bait and target RNA, respectively, and confirmed the presence of similar amounts of both molecules in all cotransfection samples. Subsequently, we immunoprecipitated FLAG-nsp1β using an anti-FLAG mAb and analyzed these samples for co-immunoprecipitation of target RNA using the same qRT-PCR method, while verifying successful immunoprecipitation of nsp1β with a specific mAb (Fig. 7C). A strong and specific RNA immunoprecipitation signal analysis. As expected (Fig. 6A), the expression of nsp2, nsp2TF, and nsp2N was detected in SD95-21-WT-infected cells, whereas only nsp2 was recovered from cells infected with either SD95-21-KO2 or SD95-21-1KO, whereas their nsp1β was expressed at a level similar to that observed with the WT virus.

Unfortunately, the 1KO mutant of the PRRSV type 1 isolate (SD01-08) yielded very low titers in MARC-145 cells (10^2 FFU/mL). Considering the number of viral functions and properties potentially affected by nsp1β mutations (Discussion), we therefore performed a so-called first-cycle analysis of the phenotypes of SD01-08 WT, KO2, and 1KO. The three viruses were launched by transfecting in vitro-transcribed full-length RNA into BHK-21 cells, which support replication of transfected PRRSV RNA but cannot be infected by the progeny virus released from the transfected cells, due to the lack of the appropriate receptor(s) on their surface (40). Moreover, BHK-21 cells have a defect in IFN production (41), thus minimizing the (potential) impact of host innate responses on the comparison of viral replication phenotypes. Following metabolic labeling of protein synthesis in transfected cells, a radioimmunoprecipitation analysis revealed that SD01-08-1KO produced large amounts of nsp2, whereas the production of nsp2TF was greatly reduced and nsp2N was not detected (Fig. 6B). As previously established, SD01-08-KO2 produced only nsp2, whereas SD01-08-WT produced all three nsp2 variants. Equal expression of nsp1β in WT-, KO2-, and 1KO-transfected cells was confirmed by immunoprecipitation with an nsp1β-specific mAb. We also investigated whether the mutations in 1KO affected the activity of the PLP1 protease or the (potential) involvement of nsp1β in the control of viral subgenomic mRNA synthesis. Although the total amount of nsp1β and viral RNA was somewhat reduced in 1KO-transfected cells, cleavage of the site between nsp1β and nsp2 and subgenomic mRNA production (Fig. S4 B and C) were not affected by the mutations in the GKYLQRRLQ motif nor did they affect nsp1β stability (Fig. S4D). Finally, we included a double transfection of BHK-21 cells with KO2 and 1KO full-length RNA (Fig. 6B) and demonstrated complementation between the two PRF-negative mutants leading to reactivation of nsp2TF/nsp2N expression. As expected, the WT nsp1β expressed by mutant KO2 was able to transactivate −2/−1 PRF on the WT PRF signal in the 1KO genome, again confirming that the GKYLQRRLQ motif plays a critical role in the PRF stimulatory activity of nsp1β in PRRSV-infected cells.

PRRSV nsp1β interacts with the RNA signals that direct −2/−1 PRF. To test the hypothesis that nsp1β, and specifically in the GKYLQRRLQ motif, interacts with the PRRSV RNA sequences that direct −2/−1 PRF, we developed an RNA-binding protein immunoprecipitation assay. To produce a RNA target, we engineered plasmid pR79WT-EGFP yielding an RNA in which a 79-nt PRRSV SD01-08 RNA sequence (Fig. 3A) containing the shift site and conserved CCCAUCCUC motif was fused to the EGFP ORF (Fig. 7A). The latter served as a target for quantitative RT-PCR (qRT-PCR) amplification, which we included plasmids pR79KO2-EGFP and pR79CC2-EGFP, containing combinations of point mutations in the shift site and/or CCCAUCCUC motif that were previously demonstrated to completely inactivate PRF (Fig. S1). To express the nsp1β bait, we used constructs pFLAG-nsp1β-WT and pFLAG-nsp1β-KO, producing WT and mutant (K130A/R134A) nsp1β, respectively, each fused to an N-terminal triple FLAG tag. The empty vectors pFLAG and pEGFP were included as negative controls.

Following cotransfection of vectors expressing RNA target and nsp1β into 293T cells, cell lysates were prepared. Western blot and qRT-PCR analysis (Fig. 7B) were first used to determine the expression levels of nsp1β bait and target RNA, respectively, and confirmed the presence of similar amounts of both molecules in all cotransfection samples. Subsequently, we immunoprecipitated FLAG-nsp1β using an anti-FLAG mAb and analyzed these samples for co-immunoprecipitation of target RNA using the same qRT-PCR method, while verifying successful immunoprecipitation of nsp1β with a specific mAb (Fig. 7C). A strong and specific RNA immunoprecipitation signal
was detected only in samples from cells cotransfected with pFLAG-nsp1 and pR79WT-EGFP. In contrast, when mutant nsp1KO carrying the K130A/R134A double mutation in the GKYLQRRLQ motif was used, only very low levels of target RNA were pulled down, suggesting that the K130A/R134A mutations impaired the interaction of nsp1β with PRRSV RNA. Only background signal was detected when using a negative control mouse IgG; Western blot analysis was used to monitor the use of equal amounts of cell lysate (β-tubulin control; Bottom). Lane numbers are explained in C. (C) Following FLAG-nsp1β immunoprecipitation, the amount of coprecipitating target RNA was determined by qRT-PCR (A). Western blot analysis using a mAb α-Eu-nsp1β was used to monitor the amount of immunoprecipitated nsp1β. A legend explaining the cotransfected plasmids for each lane number is given on the right.

Discussion

In this paper, we report the remarkable discovery that efficient ribosomal frameshifting in the expression of the PRRSV nsp2TF and nsp2N proteins requires the viral nsp1 protein as a trans-activator. Protein-stimulated PRF is unprecedented. It has been reported that cellular annexin A2 may interact with the −1 PRF signal of the coronavirus infectious bronchitis virus, but its role appears to be to down-regulate frameshifting through destabilization of the stimulatory pseudoknot (43), and no specific frameshift-stimulatory protein factors have been identified to date. Although down-regulation of eukaryotic translation release factor levels can lead to a low-level stimulation of PRF (44, 45), this is a poorly characterized phenomenon, likely to be a rather nonspecific effect brought about by changes in translation rates (46). It is known that −1 PRF at the HIV type 1 slippery sequence can be promoted by replacing the natural stimulatory RNA with a combination of the iron-responsive element (IRE) RNA and its cognate binding partner (the IRE-binding element AUCUCUCUCUCUUCUCUC).
Rather than the modes, perhaps a reflection of differences in mRNA tension PRRSV structure affected the relative utilization of GU_UUU_UUA slippery sequence (29), it was noted that the RNA secondary structure-stimulated does not appear to be present (55, 56). In a recent study on unusual single-tRNA slippage events seen in prokaryotic systems "−associated with single mismatches in the anticodon ′ of the slippery sequence was mRNA tension. although it remains to be determined whether this is linked to protein (or complex), that can influence frameshift magnitude, although it remains to be determined whether this is linked to mRNA tension.

Analysis of the published structure of nsp1β from a type 2 PRRSV isolate provides insights into the mechanism of how the protein may interact with viral RNA. The PLP1β domain of nsp1β adopts a papain-like fold consisting of three α-helices that pack against a β-sheet of four antiparallel strands (39) (Fig. 4B). One of the helices (helix α4 in the overall nsp1β structure) contains a conserved GKYLQRRLQ motif that we now show plays a critical role in the transactivation of frameshifting. The crystal structure of nsp1β suggests that the protein exists as a homodimer (39) and, interestingly, helix α4 of both nsp1β monomers resides on the same side of the dimer, which may generate a continuous, positively charged surface that could bind a long single- or double-stranded RNA molecule (Fig. 4C). The involvement of an α-helix in RNA binding is consistent with the observation that nucleoproteins of many RNA viruses encapsidate the viral genome using domains of α-helical structure (57, 58). Thus, it is plausible that the GKYLQRRLQ motif of helix α4 directly binds viral RNA, although we cannot exclude the possibility that this helix may be a binding site for a cellular protein that in turn could bind to the PRF signal in the viral RNA.

Except for equine arteritis virus (EAV), the −2 PRF mechanism seems to be conserved in all currently known arteriviruses as judged by the presence of a TF ORF overlapping ORF1a and a conserved slippery sequence and downstream C-rich region (13). In PRRSV and lactate dehydrogenase-elevating virus (LDV) −1 PRF can occur, but in contrast the RG_GUC_UCU shift site in some of the recently identified simian hemorrhagic fever virus (SHFV)-like viruses (59) would preclude −1 PRF while still allowing −2 PRF. It is expected that such PRF events in LDV and SHFV would also be controlled by nsp1β, and indeed the transactivating motif in nsp1β was found to be largely conserved in these viruses (Fig. 4A). Possibly, the nsp1β component of the frameshift mechanism, which is encoded several kilobases upstream of the PRF site, evolved secondarily, for example to enhance the efficiency of nsp2TF/nsp2N expression because in the absence of nsp1β low levels of PRF could still be observed (Fig. 2A). Amino acid sequence comparisons reveal that the GKYLQRRLQ motif-containing helix is highly conserved in the PLP1β domains of PRRSV, SHFV, and LDV, but the motif is lacking in EAV. For the latter virus, the three helices of the PLP1β domain are reduced to two α-helices (57). The insertion of 3 aa in the EAV equivalent of the α helix compared with the other arteriviruses. The nsp2-encoding region of EAV lacks an equivalent of the (overlapping) TF ORF and produces a substantially smaller nsp2. Assuming the TF ORF was lost at some point during the evolution of the EAV lineage, changes in this helix may have been tolerated when it was no longer required to stimulate PRF in trans. Although an alternative evolutionary scenario (i.e., a common ancestor of PRRSV, SHFV, and LDV independently acquiring a TF ORF) cannot be excluded, loss of the requirement to transactivate PRF may also explain a second remarkable difference between the nsp1 region of EAV and other arteriviruses: the inactivation of the proteolytic activity of the PLP1α protease, resulting in the synthesis of a single nsp1 protein rather than nsp1α and nsp1β (35, 60). In particular, the N-terminal zinc finger of nsp1 (EAV) or nsp1α (PRRSV) has been implicated in the control of viral subgenomic mRNA synthesis (37, 61–63), a function that may not be compatible with a role in PRF transactivation, thus requiring the internal cleavage of nsp1 by PLP1α in arteriviruses that use nsp1β-mediated transactivation of TF ORF expression.

The capacity of nsp1β to stimulate both −1 and −2 PRF suggests that protein transactivation could be used more widely in the induction of programmed frameshifting events in diverse systems. With regards to arteriviruses, it is possible that nsp1β might also modulate translation of host cell mRNAs containing appropriate signals. A cursory search of porcine mRNAs re-
vealed hundreds of −1 and/or −2 frameshift-compatible shift sites followed by C-rich motifs at an appropriate spacing, although no site that is exactly identical to the PRRSV minimal PRF cassette (−8 nt shift site plus the downstream 21 nt). Whether and to what extent frameshifting occurs at such sites remains to be investigated. Although the occurrence of nsp1β-responsive frameshift signals in host mRNAs would presumably be sporadic, the overall effect may perturb cellular gene expression, thus adding an extra dimension to virus–host interactions.

When screening PRRSV nonstructural proteins for their capacity to suppress type I IFN expression, both nsp1β and nsp2 were found to possess such activities (20, 22, 31, 38, 49). In reporter gene-based assays, nsp1β had the strongest potential to inhibit IFN-β promoter activity and could also inhibit downstream IFN-induced signaling pathways for expression of IFN-stimulated genes (ISGs), including ISG15 (31, 38, 49, 64, 65). On the other hand, the PLP2 activity of nsp2 is able to disrupt innate immune signaling by removing ubiquitin (Ub) and Ub-like modifiers from host cell substrates, exhibiting a general deubiquitinating (DUB) activity toward cellular Ub conjugates and also cleaving the Ub homolog ISG15 (19–22). As documented here, nsp1β transactivates both nsp2TF and nsp2N expression, resulting in the synthesis of three nsp2-related proteins (nsp2, nsp2TF, and nsp2N) that have the N-terminal PLP2-DUB domain in common. Thus, it remains to be established to which extent nsp1β directly modulates the innate immune response or does so by stimulating the expression of nsp2TF and nsp2N. Furthermore, nsp1β may affect the immune response through modulation of host cell mRNA translation. The identification of viral/host elements responsible for innate immune evasion is fundamental for the development of modified live virus vaccines. As illustrated by our reverse genetics studies, mutagenesis of key regulatory sequences and the involvement of the C-terminal epitope (CFLKVGVKSAGDLV) of nsp2TF of type 2 PRRSV was generated by GenScript.

3. Antibodies. Antibodies recognizing PRRSV proteins (see also Fig. 51B for the nomenclature used in this paper), including mAb 22-28 (α-EU-nsp1), mAb 123-128 (α-Na-nsp1), mAb 36-19 (α-EU-PLP2), mAb 58-46 (α-EU-nsp2), mAb 140-68 (α-Na-nsp2), mAb 148-83 (α-Na-nsp2), and a rabbit antisera recognizing the C-terminal epitope (CFLKVGVKSAGDLV) of nsp2TF of type 2 PRRSV was generated by GenScript. For detection of FLAG-tagged proteins, an anti-FLAG mAb was obtained from Sigma Life Science. Anti-β-tubulin and anti-dsRNA (J2-0601) mAbs were obtained from Landa Biotech and English and Scientific Consulting, respectively.

DNA Constructs and Reverse Genetics. Except for the KO2 (Fig. 51) and pLns1p1cc-2 (Fig. 53) mutants, for which synthetic DNA was used, all other constructs were made by standard PCR-based mutagenesis and recombinant DNA techniques. Procedures for the construction of plasmids are provided in SI Materials and Methods. Methods for in vitro transcription, virus rescue from full-length cDNA clones, and virus titration were described previously (13, 30, 31).

MS. Nsp2N was immunoprecipitated from SD95-21-M1–infected MARC-145 cell lysate using mAb α-Na-PLP2 and samples were separated on a 6% (wt/vol) SDS/PAGE gel, which was fixed and stained with Coomassie Brilliant Blue G-250 (Bio-Rad). The band expected to contain nsp2N* (based on predicted protein size) was excised. Trypsin digestion and LC-MS/MS analysis were performed as described previously (67). MS spectra were searched against a custom-made protein database containing the nsp2N sequence. For positive control, a synthetic version of the identified frameshift peptide was made and analyzed by LC-MS/MS.

Immunoads. Different regions of PRRSV ORF1a were transiently expressed in RK-13 or HEK-293T cells using truncated derivatives of expression plasmid pL1a and the recombinant vaccinia virus–T7 polymerase expression system (66). Expression products were 35S labeled, immunoprecipitated, and analyzed by SDS/PAGE and autoradiography as described previously (13). Alternatively, nsp1β- and nsp2N-related products were detected by consecutive immunoprecipitation of (unlabeled) proteins and Western blot analysis, using a combination of PRRSV nsp-specific mAbs as described previously (13, 31). WT and mutant SD01-08 viruses were launched by transfecting in vitro-transcribed full-length RNA into BKH-21 cells, and radioimmunoprecipitation was conducted to detect the expression of nsp1β- and nsp2-related products (see SI Materials and Methods for detailed procedures).

Dual Luciferase Assay. Using FuGENE HD transfection reagent (Roche Molecular Biochemicals), HEK-293T cells were cotransfected with 0.2 μg dual luciferase plasmid containing the PRRSV PRF sequence and 50 ng pFLAG-nsp1β. At 24 h posttransfection, cells were harvested and luciferase expression was measured using the Dual Luciferase Stop & Glo Reporter Assay System (Promega) and a luminometer (Berthold). Frameshifting efficiencies were calculated from the ratio of firefly to Renilla luciferase activities, using the IFC control construct as the standard.

Analysis of Protein Sequences and Structure. Sequence alignment of the PLP1 domain of PRRSV, LDV, and SHFV nsp1β and EAV nsp1 was performed using the MUSCLE algorithm in Geneious 6 (Biomatters Ltd, Auckland, NZ). Potential RNA-binding residues in nsp1β were identified using the program BindN (68). Images of the crystal structure of the PRRSV nsp1β dimer [Protein Data Bank (PDB) ID code 3MTV] (39) were created using PyMOL (69).

Assays for Detecting Interactions Between nsp1β and Viral RNA. Immunoprecipitation assays to detect RNA-binding proteins were performed using a Magna RIP kit (Millipore) and a RiboTrap kit (Medical & Biological Laboratories) following the manufacturer's instructions. The amount of target mRNA bound to nsp1β was determined by qRT-PCR, and the presence of nsp1β in RNA–protein complexes verified by Western blot. Detailed experimental procedures are presented in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

DNA Constructs. Plasmids for expression of full-length or partial porcine reproductive and respiratory syndrome virus (PRRSV) ORF1a were constructed by RT-PCR amplification of corresponding regions from genomic RNA (nucleotides 191–7702 of the SD95-21 genome; nucleotides 222–736 of the SD01-08 genome). The PCR product was digested with Ncol and NotI restriction enzymes and ligated into a pL1a backbone digested with the same enzymes. The design of pL1a was described previously (1). For the KO2 (Fig. S1) and nsp1βec-2 (Fig. S3) constructs, which were cloned with NotI, all mutations were introduced by using the Quick-Change site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions.

Plasmid pLspn1βec-2 was constructed by extensively mutating the SD01-08 PRRSV nsp1β-coding region with synonymous replacements (Fig. S3), while keeping the encoded amino acid sequence intact and avoiding rare codons. The modified sequence (nsp1βec) was produced as a synthetic gene and fused back with the nsp2-coding region to generate the nsp1βec-2 construct. To construct plasmids for the dual-luciferase assay, a 79-nt oligonucleotide (nucleotides 3506–3584 of SD01-08 genome) containing the wild-type (WT) sequence or mutations (Fig. S3, IFC and KO2) at the ribosomal frameshifting region was synthesized and cloned into the dual luciferase vector pDluc as described previously (2, 3).

The plasmid expressing FLAG-tagged nsp1β (pFLAG-nsp1β) was generated by PCR amplification of the nsp1β-coding region (nucleotides 762–1376 of the SD01-08 genome) and cloned into the plasmid vector pXFLAG-CMV-24 (Sigma). Plasmids expressing the EGFP-tagged PRF sequence (pR79WT-EGFP, pR79KO2-EGFP, and pR79CC2-EGFP) were generated by mutating the PRF region (nucleotides 3506–3584 of SD01-08) into the plasmid vector pEGFP-N1 (Clontech).

In Vitro Transcription and RNA-Protein Immunoprecipitation Analysis of SD01-08 WT and Mutants. Full-length SD01-08 WT, KO2, or 1KO RNA was transcribed from 1 µg linearized plasmid DNA using the mMESSAGE mMACHINE T7 kit (Ambion) following the manufacturer’s instructions. BHK-21 cells (4 × 10^6) were electroporated with 8 µg in vitro-transcribed RNA using program T-020 of the Amaxa Nucleofector and kit T (Lonza). Newly synthesized proteins were labeled from 16.5 to 18.5 h posttransfection for 15 min in cysteine- and methionine-free medium containing 200 µCi/mL [35S]-methionine and [35S]-cysteine (Perkin-Elmer). After removal of the label medium, cells were either lysed immediately or chased for 1 h in the presence of an excess of unlabeled methionine and cysteine. Immunoprecipitation was performed as described previously (4) with mAb α-EU-nsp1β. Precipitated proteins were separated on a 12% (wt/vol) SDS/PAGE gel and phosphorimaging was performed as described above.

Immunofluorescence Microscopy of Transfected BHK-21 Cells. Following electroporation with in vitro-transcribed SD01-08 WT, KO2, or 1KO RNA, 0.15 × 10^6 BHK-21 cells were seeded on glass coverslips. At 18 h posttransfection, cells were fixed in 3% (wt/vol) paraformaldehyde in PBS. Cells were double labeled with a mAb recognizing dsRNA and Hoechst 33342 to stain nuclear DNA.

RNA Isolation, Denaturing Formaldehyde Gel Electrophoreses, and in Gel Hybridization. BHK cells (0.75 × 10^6) were electroporated with in vitro-transcribed RNA of SD01-08 WT, KO2, and 1KO. At 18 h posttransfection, cells were lysed in 20 mM Tris-HCl (pH 7.4), 100 mM LiCl, 2 mM EDTA, 5 mM DTT, 5% (wt/vol) lithium dodecyl sulfate, and 100 µg/mL proteinase K. Total RNA was extracted and separated on a denaturing formaldehyde gel. Positive-stranded viral RNA was visualized by gel drying and hybridization with a 32P-labeled oligonucleotide probe (PRRSV-hyb1 5’-TCGCCCTAATTGAATAGGTG-3’) complementary to the 3’ end of the viral genome and therefore recognizes all viral mRNAs; 18S ribosomal RNA was used as a loading control and was detected with probe 5’-ATGCCCCGGCGCGTCCTCTC-3’. Hybridized gels were analyzed by phosphorimaging as described above. Correction for loading variations was performed using the amount of 18S RNA in the same lane. The sum of the signal for all viral mRNAs in each lane was used to calculate the relative abundance of each individual mRNA.

RNA-Binding Protein Immunoprecipitation and RNA Pull-Down Assay. RNA-binding protein immunoprecipitation was performed using a Magna RIP kit (Millipore) according to the manufacturer’s instructions. Briefly, HEK-293T cells seeded in 10-cm Petri dishes cotransfected with plasmids expressing the RNA bait (R79WT-EGFP, R79KO2-EGFP, and R79CC2-EGFP, or pEGFP, 8 µg) and the nsp1β bait (nsp1β-WT, nsp1β-KO, or pFLAG; 2 µg). At 24 h posttransfection, cell lysates were prepared for communoprecipitation. FLAG-tagged nsp1β was immunoprecipitated using an α-FLAG mAb, and coprecipitating target RNA was quantified by quantitative RT-PCR using a TaqMan Gene Expression Assay kit (Life Technologies) targeting the EGFP RNA sequence. The expression of nsp1β in all cotransfected samples was determined by Western blot analysis using an nsp1β-specific mAb. A RiboTrap kit (Medical & Biological Laboratories) was used to further confirm the interaction between nsp1β and the 79-nucleotide RNA sequence from the PRRSV PRF region. The R79WT, R79KO2, or R79CC2 RNA were labeled with 5-bromo-U and in vitro synthesized using the MEGAAscript T7 Kit (Life Technologies). The 5-bromo-U-labeled RNA transcripts were bound to magnetic beads conjugated with anti-B-U mAb. Subsequently, these magnetic beads were incubated with lysates of HEK-293T cell expressing FLAG-tagged 1KO or WT of nsp1β. The amount of nsp1β pulled down with the RNA bait was determined by Western blot analysis using an nsp1β-specific mAb.


Fig. S1. Overview of mutants and antibodies used in this study. (A) List of WT and mutant sequences of the PRRSV PRF region (GGUUUUU shift site and conserved CCCANCUCC motif indicated with orange and cyan boxes, respectively). Mutated nucleotides are highlighted in magenta. Coordinates of starting nucleotides refer to PRRSV sequences DQ489311 (type 1 PRRSV) and KC469618 (type 2 PRRSV). CC2, disrupted CCCANCUCC motif; IFC, in-frame control; KO2, knockout mutant 2 (premature −2 frame termination codon and disrupted frameshift cassette); M1, mutated −1 frame termination codon to C-terminally extend nsp2N; 1βKO, nsp1β knockout mutant (double mutation introduced into the nsp1β GKYLQRRLQ motif). (B) Sizes of nsp2-related polypeptides described in this study and location of epitopes recognized by the PRRSV-specific antibodies in the nsp1β-2 region. Nsp2N* refers to the C-terminally extended version of nsp2N that is produced by mutant M1, due to removal of the stop codon (A). The origin and original name of each antibody are provided in Materials and Methods. Sizes (in number of amino acids) for nsp2-related products are shown for GenBank sequences DQ489311 (SD01-08, PRRSV type 1) and KC469618 (SD95-21, PRRSV type 2).
Fig. S2. Mass spectrometric (MS) analysis of nsp2N* (a C-terminally extended version of nsp2N) purified from cells infected with mutant SD95-21-M1. (A) PRRSV-infected or mock-infected MARC-145 cell lysates were immunoprecipitated with nsp2-specific α-NA-PLP2. Immunoprecipitated proteins were separated by SDS/PAGE and stained with Coomassie Blue. The positions of nsp2, nsp2TF, and nsp2N are indicated. (B) Peptide sequence of the nsp2N*−1 frameshift-specific peptide. The fragment ions that were identified in the liquid chromatography tandem MS analysis of the gel slice are indicated. (C) Complete amino acid sequence of nsp2N* comprising nsp2N and a 23-amino acid C-terminal extension (highlighted in gray). Peptides identified by MS are depicted in red. The peptide spanning the −1 frameshift site is underlined in green. (D) Fragmentation spectrum of the −1 frameshift-specific peptide QVFWPR. (E) Nucleotide sequence and −1 PRF-directed translation of nsp2N* at the frameshift site.

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Fig. S3. Nucleotide sequences of the WT and synonymously mutated nsp1β-coding region. For each block, the top, middle, and bottom lines give the WT nsp1β-coding sequence (black), the synonymously mutated sequence (mutations present in pLnsp1βcc-nsp2 given in red), and the (unchanged) translation into amino acids (blue), respectively.

Fig. S4. First-cycle analysis of RNA synthesis and nsp1β stability of WT and mutant (KO2 or 1βKO) SD01-08 virus in BHK-21 cells. (A) Immunofluorescence microscopy analysis of transfection rate in BHK-21 cells electroporated with in vitro-transcribed full-length PRRSV RNA. Transfected cells were double labeled with a mAb specific for viral dsRNA (green) and Hoechst 33342 (blue) for staining of DNA in cell nuclei. (B and C) Gel hybridization analysis and quantification of PRRSV-specific mRNA accumulation in cells transfected with mutants KO2 and 1βKO or a WT control. (B) Total intracellular RNA was isolated at 18 h posttransfection and resolved by denaturing formaldehyde agarose gel electrophoresis. PRRSV-specific mRNAs were detected by hybridization of the dried gel with a 32P-labeled probe complementary to the 3′-end of the viral genome and subsequent phosphorimaging. The positions of the PRRSV genome (RNA1) and the six subgenomic mRNAs (RNA2 to RNA7) are indicated. (C) The volume of the bands corresponding to each of the viral mRNAs was quantified by phosphorimaging and adjusted for the control 18S ribosomal RNA band in the same lane. The sum of the signals for all viral mRNA bands in each lane was used to calculate the relative abundance of each individual mRNA. (D) Pulse–chase analysis of nsp1β expression. BHK-21 cells were transfected with RNA transcribed from WT or mutants of PRRSV full-length cDNA clones. At 16.5 h posttransfection and resolved by denaturing formaldehyde agarose gel electrophoresis. PRRSV-specific mRNAs were detected by hybridization of the dried gel with a 32P-labeled probe complementary to the 3′-end of the viral genome and subsequent phosphorimaging. The positions of the PRRSV genome (RNA1) and the six subgenomic mRNAs (RNA2 to RNA7) are indicated. (C) The volume of the bands corresponding to each of the viral mRNAs was quantified by phosphorimaging and adjusted for the control 18S ribosomal RNA band in the same lane. The sum of the signals for all viral mRNA bands in each lane was used to calculate the relative abundance of each individual mRNA. (D) Pulse–chase analysis of nsp1β expression. BHK-21 cells were transfected with RNA transcribed from WT or mutants of PRRSV full-length cDNA clones. At 16.5 h posttransfection, protein synthesis was labeled for 15 min and chased for 1 h. Cells were lysed and, following immunoprecipitation with mAb α-EU-nsp1β, the production and turnover of nsp1β were analyzed using SDS/PAGE and autoradiography.