Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation

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Severe acute respiratory syndrome (SARS) coronavirus (SCoV) causes a recently emerged human disease associated with pneumonia. The 5′ end two-thirds of the single-stranded positive-sense viral genomic RNA, gene 1, encodes 16 mature proteins. Expression of nsp1, the most N-terminal gene 1 protein, prevented Sendai virus-induced endogenous IFN-β mRNA accumulation without inhibiting dimerization of IFN regulatory factor 3, a protein that is essential for activation of the IFN-β promoter. Furthermore, nsp1 expression promoted degradation of expressed RNA transcripts and host endogenous mRNAs, leading to a strong host protein synthesis inhibition. SCoV replication also promoted degradation of expressed RNA transcripts and host mRNAs, suggesting that nsp1 exerted its mRNA destabilization function in infected cells. In contrast to nsp1-induced mRNA destablization, no degradation of the 28S and 18S rRNAs occurred in either nsp1-expressing cells or SCoV-infected cells. These data suggested that, in infected cells, nsp1 promotes host mRNA degradation and thereby suppresses host gene expression, including proteins involved in host innate immune functions. SCoV nsp1-mediated promotion of host mRNA degradation may play an important role in SCoV pathogenesis.

virus virulence | SARS | mRNA stability | translation inhibition | innate immunity

Severe acute respiratory syndrome (SARS) coronavirus (SCoV) is the etiological agent of a newly emerged disease, SARS, which originated in southern China in 2002 and spread to various areas of the world in the 2003 epidemic (1–4). The SCoV genome is a large single-strand positive-sense RNA and its 5′ end two-thirds constitutes gene 1, which is made up with two large partly overlapping ORFs, ORF1a and ORF1b. Upon infection, the translation of two large precursor gene 1 proteins starts from the incoming viral genomic RNA; one precursor protein is translated from ORF1a, whereas the other precursor protein, which corresponds to the entire length of gene 1, uses a ribosomal frameshift mechanism (5) to extend itself from ORF1a into ORF1b (6). These precursors undergo proteolytic processing via two virally encoded proteinases to generate 16 mature proteins, nsp1 through nsp16. Probably most of the gene 1 proteins are important for viral RNA synthesis (5, 7–11). Some gene 1 proteins are predicted to have biological functions related to RNA synthesis (11), and some have demonstrated functions that appear to be involved in RNA synthesis (7, 8, 10, 12), whereas some gene 1 proteins may have functions other than viral RNA synthesis (13–16).

Host innate immunity functions, including production of IFN-α/β, are the first line of defense against microorganism invasions. Many viruses developed defensive mechanisms to suppress and/or evade host innate immune functions (17). SCoV replication in human 293 cells suppresses IFN-β mRNA accumulation by inhibiting IFN regulatory factor (IRF)-3 phosphorylation, dimerization, nuclear translocation, and association with CBP/p300 (18). Because SCoV is susceptible to the antiviral action of IFNs (19, 20), inhibition of IFN-β transcription appears to be one of SCoV's defenses against host innate immune response. No SCoV gene product(s) is currently known to inhibit IRF-3 function. Some viruses have evolved to generally suppress host mRNA accumulation that would block host responses to viral invasion, and viral proteins that are responsible for host mRNA accumulation inhibition have been identified in a number of viruses (21). For example, NSs protein of Rift Valley fever virus (RVFV), a bunyavirus, inhibits host mRNA transcription, including IFN-β mRNA transcription (22). Herpes simplex virus 1 (HSV-1) virion host shutoff (vhs) protein is known to trigger global mRNA destabilization in infected cells (23). Both NSs and vhs are major viral virulence factors (23, 24), highlighting how effectively viral proteins that stifle host mRNA accumulation advance viral pathogenesis.

We present here that SCoV nsp1 protein inhibited host gene expression, most probably including those involved in host innate immune response, by promoting host mRNA degradation. Our data indicated that SCoV uses nps1 to suppress host innate immune responses in infected cells for advancement of its own replication; SCoV nsp1 may play a major role in SCoV pathogenicity.

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Results

Subcellular Localization of nsp1 in Expressing Cells and in Infected Cells. To study the biological functions of SCoV nsp1 protein, we have constructed a plasmid pCAGGS-nsp1 expressing SCoV nsp1 carrying a myc epitope tag at its C terminus. Western blot analysis using anti-myc antibody demonstrated an accumulation of the ≈20 kDa nsp1 protein in pCAGGS-nsp1-transfected 293 cells (Fig. 1*A*). Confocal microscopic analysis using anti-myc antibody showed that most of the expressed nsp1 was detected in the cytoplasm (Fig. 1*B*). Consistent with a previous report (6), confocal microscopic analysis of nsp1 in SCoV-infected 293 cells, stably expressing the SCoV receptor protein, human angiotensin converting enzyme 2 (25) (293/ACE2 cells), using anti-nsp1 antibody demonstrated that the nsp1 protein was found in the cytoplasm as early as 6 h postinfection (p.i.). Fig. 1*B* shows the cytoplasmic localization of nsp1 at 8 h p.i.

Nsp1 Protein Suppresses IFN-β mRNA Accumulation Without Inhibiting IRF-3 Dimerization. To know whether nsp1 was responsible for inhibition of IFN-β mRNA accumulation in SCoV-infected cells

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Abbreviations: ACE, angiotensin converting enzyme; actD, actinomycin D; CAT, chloramphenicol acetyltransferase; HSV, herpes simplex virus; IRF, IFN regulatory factor; Luc, luciferase; moi, multiplicity of infection; p.i., postinfection; RVFV, Rift Valley fever virus; SCoV, severe acute respiratory syndrome coronavirus; SeV, Sendai virus; vhs, virion host shutoff.

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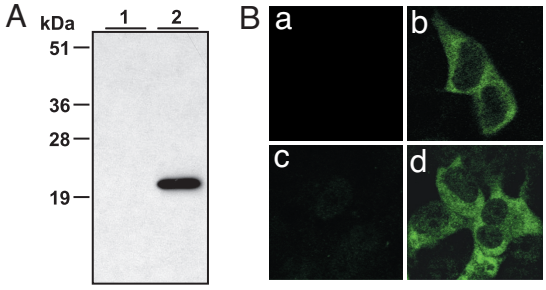

Fig. 1. Subcellular localization of expressed nsp1 and nsp1 in SCoV-infected cells. (*A*) 293 cells were transfected with pCAGGS (lane 1) or pCAGGS-nps1 (lane 2). Total intracellular proteins were extracted at 48 h after transfection, and Western blot analysis was performed by using anti-myc antibody. (*B*) 293T cells were transfected with pCAGGS (*a*) or pCAGGS nsp1 (*b*). 293/ACE2 cells were mock infected (*c*) or infected with SCoV (*d*). At 48 h after transfection or 8 h p.i., subcellular localization of expressed nsp1 protein and SCoV nsp1 protein was examined by using anti-myc antibody (*a* and *b*) and anti-nsp1 antibody (*c* and *d*) as primary antibodies, respectively.

(18), we examined the effect of nsp1 expression on Sendai virus (SeV)-induced IFN-β mRNA accumulation. Cultures of 293 cells were cotransfected with an IFN-β promoter-driven luciferase (Luc) reporter plasmid and pCAGGS-nsp1. As controls, its parental plasmid, pCAGGS, and pCAGGS encoding RVFV NSs (pCAGGS-NSs) were used in place of pCAGGS-nsp1 [RVFV NSs is known to suppress host mRNA transcription (22, 26)]. At 24 h after transfection, the cells were mock infected or infected with SeV, and, at 16 h p.i., cell extracts were prepared to assay for Luc activities. SeV infection substantially increased Luc activities in cells transfected with pCAGGS, whereas SeV-induced Luc activities were very low in cells transfected with pCAGGS-NSs and in those transfected with pCAGGS-nps1 (Fig. 2*A*). Western blot analysis using anti-myc antibody and anti-NSs antibody (27) confirmed expression of nsp1 protein and NSs protein, respectively (data not shown). Western blot analysis of SeV N protein (Fig. 2*B*) and Northern blot analysis of SeV N mRNA (Fig. 7, which is published as supporting information on the PNAS web site) demonstrated that neither nsp1 expression nor NSs expression inhibited SeV replication, eliminating the possibilities that low levels of Luc activities in nsp1-expressing cells and in NSs-expressing cells were because of poor SeV replication. To examine the effect of nsp1 expression on SeV-induced endogenous IFN-β mRNA accumulation, 293 cells were transfected with pCAGGS, pCAGGS-nsp1, or pCAGGS-NSs, and then mock infected or infected with SeV as described earlier. Intracellular RNAs were extracted at 16 h p.i., and amounts of IFN-β mRNAs, which were normalized to 18S rRNA, were determined by using real-time PCR (Fig. 2*C*). SeV infection induced IFN-β mRNA accumulation in cells transfected with pCAGGS, whereas nsp1 expression and NSs expression both strongly inhibited the SeV-induced IFN-β mRNA accumulation, demonstrating that nsp1 protein expression prevented SeV-induced IFN-β mRNA accumulation.

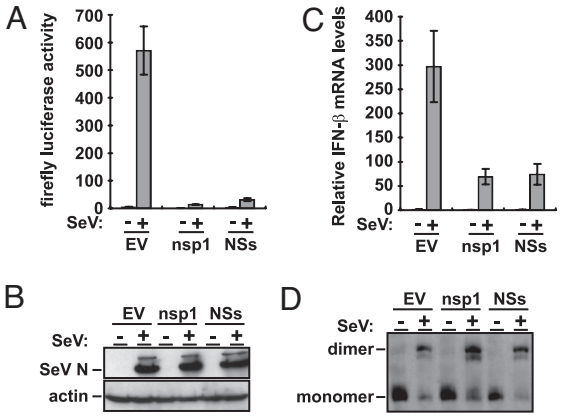

Fig. 2. Effects of nsp1 expression on SeV-induced IFN-β mRNA accumulation. 293 cells were cotransfected with IFN-β-Luc and pCAGGS (EV), IFN-β-Luc and pCAGGS-nsp1 (nsp1), or IFN-β-Luc and pCAGGS-NSs (NSs). At 24 h after transfection, cells were infected with 100 HA units/ml of SeV (+) or mock infected (−). All samples were prepared at 16 h p.i. (*A*) Luc activities were measured. (*B*) Western blot analysis of SeV N protein (SeV N) and actin. (*C*) The relative abundance of IFN-β mRNAs normalized to an endogenous 18S rRNA. (*D*) Western blot analysis of IRF-3 monomers and dimers. *A* and *C* are results of three independent experiments.

IRF-3 phosphorylation, dimerization, nuclear translocation, and association with CBP/p300, all of which are essential for IFN-β mRNA transcription, do not occur in SCoV-infected 293 cells (18). We next examined the effect of nsp1 expression on the SeV-induced IRF-3 homodimerization and found that neither nsp1 nor NSs expression blocked dimerization (Fig. 2*D*) [RVFV NSs does not inhibit SeV-induced IRF-3 dimerization (26)]. These data revealed that the nsp1 expression suppressed SeV-induced INFβ mRNA accumulation without inhibiting IRF-3 dimerization.

Effects of nsp1 Protein Expression on Gene Expression from a Cotransfected Reporter Plasmid. The data shown earlier led us speculate that nsp1 may suppress accumulation of host mRNAs in general. To test this possibility, several different cell lines, including 293 cells, Hec1B cells, Vero cells, and Vero E6 cells, were cotransfected with pCAGGS-nsp1 and a reporter plasmid pCMV-β, in which the β-galactosidase (β-gal) gene was cloned downstream of a CMV promoter. The controls were pCAGGS, pCAGGS-NSs, and pCAGGS-3a, which encodes SCoV 3a accessory protein (28). At 48 h after transfection, cell extracts were prepared. Western blot analysis using anti-myc antibody, anti-NSs antibody (27), and anti-3a antibody (29) confirmed expression of nsp1, NSs, and 3a protein, respectively (data not shown). NSs expression resulted in low β-gal activity most probably because of the NSs-mediated general suppression of mRNA synthesis (22), whereas expression of SCoV 3a protein modestly suppressed β-gal activity. SCoV nsp1 expression suppressed β-gal activity very strongly in all cell lines tested (Fig. 8, which is published as supporting information on the PNAS web site). Cotransfection of pCAGGS-nsp1 and another reporter plasmid, pRL-SV40, in which the Luc gene was cloned downstream of the SV40 promoter, also resulted in suppression of Luc expression (Fig. 8). The viability of 293 cells transfected with pCAGGS-nsp1, pCAGGS-NSs, pCAGGS-3a, and pCAGGS at 48 h after transfection was $\approx$98%, and none of the plasmid-transfected cells showed major morphological change (data not shown), suggesting that reduction of β-gal and Luc activities in cells expressing NSs or nsp1 was real and not an artifact of damage from the exogenous plasmids.

Next, the effect of nsp1 expression on the reporter gene mRNA accumulation was determined. Cultures of 293 cells were cotransfected with pCMV-β and pCAGGS-nsp1. As controls, pCAGGS, pCAGGS-NSs, or pCAGGS-3a was used in place of pCAGGS-nsp1. At 48 h after transfection, intracellular RNAs were extracted. Northern blot analysis using the β-gal specific probe clearly demonstrated that the amounts of β-gal RNA in the cells expressing nsp1 and in those expressing NSs were significantly lower than in those expressing 3a and in those transfected with pCAGGS (Fig. 3*A*). Likewise, when pRL-SV40 was used in place of pCMV-β, the amounts of Luc RNA were clearly lower in the cells expressing nsp1 and in those expressing NSs than in control groups (Fig. 3*B*). In contrast, nsp1 expression did not affect the amounts of 28S and 18S rRNAs (Fig. 3). Expression of nsp1 in Vero E6 cells also resulted in reduction in

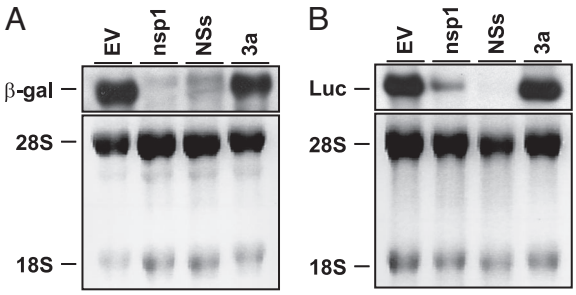

Fig. 3. Effect of nsp1 protein expression on the reporter gene mRNA accumulation. 293 cells were independently cotransfected with pCMV-β and pCAGGS-nsp1 (*A*; nsp1) or pRL-SV40 and pCAGGS-nsp1 (*B*; nsp1). As controls, pCAGGS (EV), pCAGGS-NSs (NSs), or pCAGGS-3a (3a) were used in the place of pCAGGS-nsp1. (*Upper*) At 48 h after transfection, total RNAs were extracted, and Northern blot analysis was performed by using riboprobes specific for β-gal or Luc. (*Lower*) The same RNA samples were separated by agarose gel electrophoresis, and 28S and 18S rRNAs were stained with ethidium bromide.

the abundance of β-gal and Luc RNAs from cotransfected plasmids, although nsp1 expression did not affect the amounts of rRNAs (Fig. 9, which is published as supporting information on the PNAS web site). These data clearly showed that SCoV nsp1 protein suppressed accumulation of reporter gene RNA.

Effect of nsp1 Expression on mRNA Stability and Host Protein Synthesis. To understand the mechanism of nsp1-mediated suppression of the reporter RNA accumulation, we tested whether nsp1 promoted mRNA degradation. 293 cells were transfected with pCMV-β or pRL-SV40. At 16 h after transfection, these cells were transfected independently with one of three *in vitro*-synthesized capped and polyadenylated RNA transcripts: the RNA transcripts encoded nsp1 containing C-terminal myc-His tag (nsp1 RNA transcripts), NSs containing C-terminal myc-His tag (NSs RNA transcripts), and chloramphenicol acetyltransferase (CAT) protein containing C-terminal myc-His tag (CAT RNA transcripts). One hour later, the cells were incubated either in the presence of 4 μg/ml of actinomycin D (actD) to block new RNA synthesis or in the absence of actD. Under the presence of actD, ^{3}H-uridine incorporation to the cells was severely blocked (data not shown), demonstrating that actD treatment blocked new RNA synthesis. Intracellular RNAs and proteins were extracted at 1 h after RNA transfection (Fig. 4 *A* and *B*; 0 h) or 8 h after actD addition (Fig. 4 *A* and *B*; 8 h). Northern blot analysis showed that the amounts of the expressed β-gal and Luc RNAs, and the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin mRNAs in 8-h samples, were clearly lower in nsp1-expressing cells than in those expressing NSs or CAT proteins (Fig. 4*A* and Fig. 10, which is published as supporting information on the PNAS web site). In marked contrast, the amounts of rRNAs were similar in all of the samples. Western blot analysis using anti-myc antibody revealed expression of nsp1, NSs, and CAT proteins from the transfected RNA transcripts; yet, in repeated experiments, the relative amount of nsp1 was less than that of the NSs and CAT proteins (Fig. 4*B*). These data strongly suggested that nsp1 expression promoted degradation of expressed RNAs as well as endogenous mRNAs, without degrading rRNAs.

We tested whether the abundance of expressed nsp1 substantially differed from that of nsp1 accumulation in SCoV-infected cells, and we found that the abundance of the expressed nsp1 in pCAGGS-nsp1-transfected cells at 48 h after transfection and in nsp1 RNA transcripts-transfected cells at 8 h after transfection

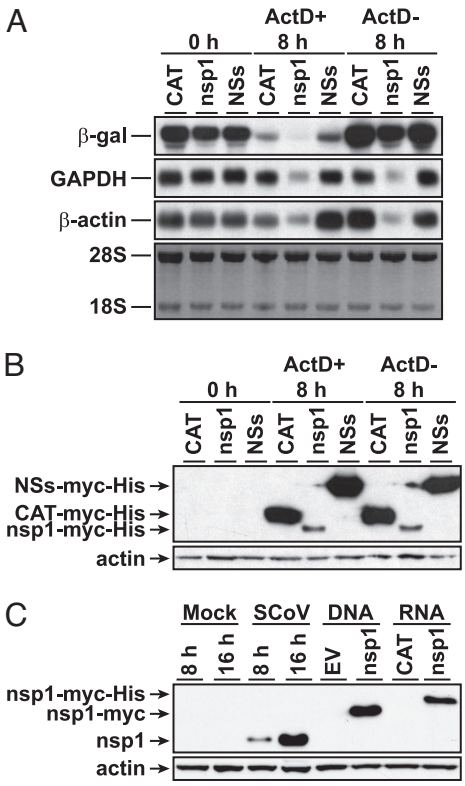

Fig. 4. Effect of nsp1 expression on stabilities of reporter gene RNA and host endogenous mRNAs (*A* and *B*) and accumulation of nsp1 in expressing cells and SCoV-infected cells (*C*). (*A* and *B*) 293 cells were transfected with pCMV-β. At 16 h after transfection, cells were independently transfected with *in vitro*-synthesized CAT RNA transcripts (CAT), nsp1 RNA transcripts (nsp1), or NSs RNA transcripts (NSs). One hour after RNA transfection, cells were incubated with actD (ActD+) or absence of actD (ActD−). Total RNAs were extracted at 0 h (0 h) or 8 h (8 h) after actD addition. (*A*) Abundance of expressed β-gal RNA and endogenous GAPDH and β-actin mRNAs were examined by using Northern blot analysis. (*B*) Total proteins were also extracted at 0 h or 8 h after actD addition, and anti-myc antibody was used to demonstrate expression of CAT, NSs, and nsp1 proteins. (*C*) 293/ACE2 cells were mock-infected (Mock) or infected with SCoV (SCoV) at an moi of 3, and cell extracts were prepared at 8 h and 16 h p.i. 293 cells were transfected with pCAGGS (EV) or pCAGGS-nsp1 (nsp1), and cell extracts were prepared at 48 h after transfection (DNA). 293 cells were transfected with CAT RNA transcripts (CAT) or nsp1 RNA transcripts (nsp1), and cell extracts were prepared at 8 h after transfection (RNA). Western blot analysis was performed to detect nsp1 protein by using anti-nsp1 peptide antibody (29).

were comparable with that of nsp1 in SCoV-infected 293/ACE2 cells at 16 h p.i. (Fig. 4*C*).

Whether nsp1-induced mRNA degradation resulted in host protein synthesis inhibition was investigated. Cultures of 293 cells were transfected with CAT RNA transcripts, nsp1 RNA transcripts, or NSs RNA transcripts, and, 1 h later, actD was added to half of the samples and the other half were untreated. From 8.5–9.5 h after actD addition, the cells were radiolabeled with [^{35}S]methionine, and cell extracts were prepared. Equivalent amounts of cell extracts were applied to SDS/PAGE. In repeated experiments, nsp1 expression, but not NSs or CAT protein expression, strongly suppressed host protein synthesis both in the presence and in the absence of actD (Fig. 5*A*). The nsp1-induced protein synthesis inhibition was stronger in the sample treated with actD than in the untreated sample, indicating that in the absence of new host mRNA synthesis, nsp1 induced efficient degradation of preexisting mRNAs, leading to a strong host protein synthesis inhibition. Colloid Coomassie

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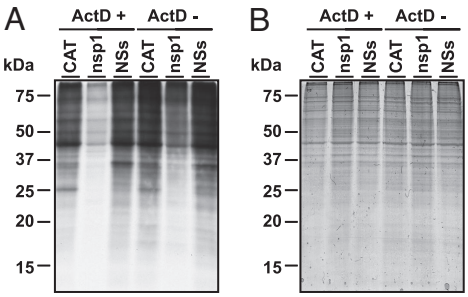

Fig. 5. Effect of nsp1 expression of host protein synthesis. 293 cells were independently transfected with CAT RNA transcripts (CAT), nsp1 RNA transcripts (nsp1), or NSs RNA transcripts (NSs). One hour after RNA transfection, cells were incubated in the presence of actD (ActD+) or absence of actD (ActD−). Cells were labeled with 20 μCi/ml of [^{35}S]methionine from 8.5 to 9.5 h after actD addition. Equivalent amounts of cell extracts were analyzed on a 12.5% SDS/PAGE gel. The gel was exposed to x-ray film (*A*) or stained with colloid Coomassie blue (*B*).

blue staining of the gel confirmed that host protein quantities in these samples were similar (Fig. 5*B*).

Analysis of Reporter Gene RNA and Host mRNA Accumulation in SCoV-Infected Cells. We next investigated whether SCoV replication also induced reduction in the amounts of expressed RNA and endogenous host mRNAs. 293/ACE2 cells were transfected with pCMV-β. At 6 h after transfection, cells were mock infected or infected with SCoV at a multiplicity of infection (moi) of 3. At 6 h and 18 h p.i., intracellular RNAs were extracted, and the amounts of β-gal RNA, GAPDH, and β-actin mRNAs were visualized on Northern blots (Fig. 6*A*). The amounts of β-gal RNA and GAPDH and β-actin mRNAs were clearly reduced in SCoV-infected cells at 18 h p.i. In contrast, SCoV replication had no effect on the amounts of either rRNA.

To know whether the reduction of expressed β-gal RNA and endogenous GAPDH and β-actin mRNAs in SCoV-infected cells was because of degradation of these mRNAs, 293/ACE2 cells were transfected with pCMV-β. At 24 h after transfection, cells were mock infected or infected with SCoV at an moi of 3. After virus adsorption for 1 h, intracellular RNAs were extracted from half of the samples (Fig. 6*B*; 0 h). The remaining cells were incubated in the presence of actD, and intracellular RNAs were extracted at 14 h after actD addition. SCoV replication and nsp1 accumulation were not affected by actD treatment (Fig. 11, which is published as supporting information on the PNAS web site). Northern blot analysis showed that the amounts of β-gal RNA and GAPDH and β-actin mRNAs in the 14-h samples were lower in SCoV-infected cells than in mock-infected cells (Fig. 6*B*), demonstrating that SCoV infection indeed promoted degradation of these mRNAs. These data were consistent with the possibility that nsp1 promoted degradation host mRNAs in SCoV-infected cells.

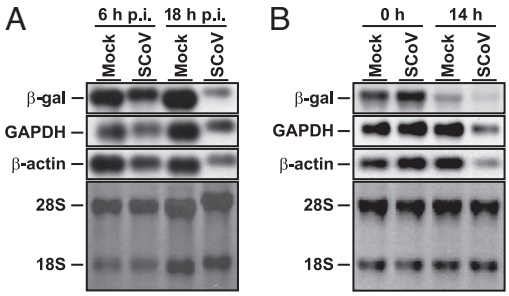

Fig. 6. Effect of SCoV replication on abundance of reporter gene RNA and host endogenous mRNAs. (*A*) 293/ACE2 cells were transfected with pCMV-β. At 6 h after transfection, cells were mock infected (Mock) or infected with SCoV at an moi of 3 (SCoV). At 6 h and 18 h p.i., intracellular RNAs were extracted. (*B*) 293/ACE2 cells were transfected with pCMV-β. At 24 h after transfection, cells were mock infected (Mock) or infected with SCoV at an moi of 3 (SCoV). At 1 h p.i., intracellular RNAs were extracted (0 h) or actD was added to culture. Intracellular RNAs were extracted 14 h after actD addition (14 h). (*A* and *B*) The amounts of β-gal RNA and GAPDH and β-actin mRNAs were determined by using Northern blot analysis. Abundances of 28S and 18S rRNAs in each sample are also shown.

Discussion

The present study explored the biological functions of SCoV nsp1 protein. We first noted that SCoV nsp1 expression inhibited reporter activities of an IFN-β promoter-driven Luc reporter plasmid as well as endogenous IFN-β mRNA accumulation in SeV-infected cells. Because nsp1 expression did not inhibit SeV-induced IRF-3 dimerization, we further investigated the possibility that nsp1 generally kept host mRNA from accumulating. SCoV nsp1 expression indeed blocked reporter gene expression by suppressing accumulation of RNA transcripts from transfected plasmids. The nsp 1 RNA transcripts transfection experiments with subsequent actD treatment strongly suggested that nsp1 promoted degradation of expressed RNA transcripts from plasmids as well as endogenous mRNAs, although our data did not exclude possibilities that nsp1 may also act to reduce mRNA levels at other stages such as transcription or mRNA nucleocytoplasmic transport. We noted efficient accumulation of CAT and NSs proteins from transfected CAT RNA transcripts and NSs RNA transcripts, respectively, whereas nsp1 protein accumulation in nsp1 RNA transcripts-transfected cells was low (Fig. 4*B*); we suspect that the expressed nsp1 probably promoted degradation of its own RNA transcripts. Nevertheless, nsp1-induced promotion of host mRNA degradation strongly inhibited host protein synthesis (Fig. 5). We also observed that SCoV nsp1 expression did not affect the amounts of 28S and 18S rRNAs, indicating that SCoV nsp1 selectively promotes degradation of host mRNAs, because it may leave host translational machineries intact. SCoV infection also resulted in reduction of the amounts of expressed RNA transcripts and endogenous mRNAs, although it did not affect rRNA abundance (Fig. 6), implying that SCoV nsp1 promoted host mRNA degradation in infected cells. Further studies are required to firmly establish nsp1-induced host mRNA degradation promotion in SCoV-infected cells.

The accumulated literature explains how many viruses use different strategies to suppress host gene expression, and, for some RNA viruses, viral proteins that suppress host gene expression have been identified (21, 22, 30–33). To our knowledge, SCoV nsp1 is the first viral protein from any of the RNA viruses that suppresses host gene expression by promoting host mRNA degradation. Another example that a virally encoded protein suppresses host gene expression by promoting host mRNA degradation is seen in HSV-1 vhs protein (23); this viral RNase (34, 35) promotes degradation of both host and viral mRNAs (36) and is considered that vhs-mediated degradation of HSV-1 mRNAs is beneficial for efficient HSV-1 replication (36), which undergoes three regulated viral RNA transcription steps. In contrast, this type of transcriptional control has not been documented in coronaviruses, except that viral RNA replication, but not RNA transcription, occurs very early in infection (37). Also, unlike vhs protein (36), SCoV nsp1 shares no sequence similarities with known RNases (data not shown). If nsp1 induces degradation of SCoV mRNAs, then poor SCoV replication should result. Accordingly, we suspect that the virus has a mechanism(s) that prevents nsp1-induced degradation of SCoV mRNAs in infected cells. If nsp1 does not induce degradation of viral mRNAs in infected cells, then nsp1-induced host mRNA

degradation works in favor of translation of virus-specific proteins; because of reduction of intact host mRNAs, SCoV mRNAs can predominantly use the host translational machinery for virus-specific protein synthesis. Furthermore, nsp1-induced host mRNA degradation most probably inhibited expression of host proteins that are involved in host innate immune responses; as a means of establishing itself in the host, SCoV may use nsp1 to block host innate immune responses. Based on the fact that both RVFV NSs protein and HSV-1 vhs protein are major viral virulence factors (23, 24), SCoV nsp1 likewise may abet SCoV pathogenesis.

In comparison with the heartiness of pCAGGS-nsp1-transfected cells, cells transfected with nsp1 RNA transcripts and those transfected with CAT RNA transcripts showed 33% and 99% cellular viability, respectively, at 48 h after transfection (data not shown). We suspect that rapid and efficient nsp1 accumulation in nsp1 RNA transcript-transfected cells (Fig. 4*C*), resulted in strong host protein synthesis inhibition (Fig. 5), leading to cell death. Although nsp1 expression inhibited host protein synthesis, a reduction in IRF-3 abundance was not noticed in pCAGGS-nsp1-transfected 293 cells (Fig. 2*D*). Perhaps a highly stable IRF-3 (38) masked the putative effect of nsp1-induced IRF-3 synthesis inhibition. We also noted that nsp1 expression did not affect SeV N mRNA abundance (Fig. 7), which could mean that the SeV N mRNA resisted nsp1-induced promotion of mRNA degradation.

SCoV nsp1 expression did not block SeV-induced IRF-3 dimmerization, yet it suppressed IFN-β mRNA accumulation (Fig. 2). We suspect that nsp1 promoted degradation of IFN-β mRNA in SeV-infected cells. Others (18) demonstrated that IRF-3 dimerization is blocked in SCoV-infected 293 cells, so we think that at least two different mechanisms inhibit IFN-β mRNA accumulation in SCoV-infected cells: One suppresses a signaling pathway that activates IRF-3 and the other is the nsp1-mediated promotion of IFN-β mRNA degradation. Dual means of inhibiting IFN-β mRNA accumulation would seem to be crucial for SCoV to establish infection in the host, because SCoV is known to be susceptible to IFN treatment (19, 20).

Materials and Methods

Cell Lines and Viruses. Cultures of 293, 293T, 293/ACE2, Hec1B, Vero, and Vero E6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated FBS. To generate 293/ACE2, ACE2 cDNA was first cloned into a murine leukemia virus expression vector pCX4bsr (39) to yield pCX4bsrACE2 (40). Construct pCX4bsrACE2 was cotransfected with a plasmid expressing amphotropic murine leukemia virus glycoproteins into BOSC23 cells to produce the ACE2-expressing pseudotyped retrovirus (39, 40). Cultures of 293 cells were infected with the ACE2 expressing retrovirus and grown in selection medium containing blasticidin (12 μg/ml) for 3 weeks. The 293/ACE2 cells, stably expressing human ACE2, were selected based on resistance to blasticidin. SCoV Urbani strain grown in Vero E6 cells was used for SCoV infection studies. Sendai virus (SeV) (Cantell stain) was purchased from the Charles River Laboratory (Wilmington, MA).

Plasmid Construction. The RT-PCR product corresponding to SCoV nsp1 ORF was cloned into pCAGGS-MCS, resulting in pCAGGS-nsp1, in which myc-tag was added to the C terminus of the nsp1 ORF. The cDNA encoding the entire wild-type RVFV NSs ORF region (27) was cloned into pCAGGS, yielding pCAGGS-NSs. The construction of pCAGGS-3a, expressing a SCoV accessory protein 3a, was described (28). Three plasmids were generated from pcDNA 3.1 His A myc, each of which encoded the nsp1 ORF, CAT ORF (41), or NSs ORF for use in *in vitro* synthesis of RNA transcripts.

Analysis of Subcellular Localization of nsp1 Protein. Cultures of 293T cells were transfected with pCAGGS or pCAGGS-nsp1 using TransIT-293 reagent (Mirus, Madison, WI). At 48 h after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 30 min and made permeable with PBS containing 0.5% Triton X-100 for 5 min. The cells were incubated with anti-myc antibody as primary antibody (Upstate Biotechnology, Lake Placid, NY) and Alexa Fluor 488-conjugated goat anti-rabbit IgG as secondary antibody (Molecular Probes, Eugene, OR). 293/ACE2 cells were infected with SCoV at an moi of 3. At various times p.i., infected cells were fixed with 4% paraformaldehyde in PBS overnight and made permeable, as described earlier. The samples were incubated with anti-nsp1 antibody as primary antibody (6), followed by the second antibody, Alexa Fluor 488 goat anti-rabbit IgG. The cells were examined by using a Zeiss LSM 510 UV META laser scanning confocal microscope at the University of Texas Medical Branch Infectious Disease and Toxicology Optical Imaging Core (Galveston, TX).

Western Blot Analysis. Western blot analysis was performed as described (28). Anti-myc antibody, anti-actin antibody (Sigma, St. Louis, MO), and anti-SeV N protein (a gift from K. Li, University of Texas Medical Branch, Galveston, TX) were used as first antibodies, and goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, and anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies.

Quantitative Analysis of IFN-β mRNA. Cultures of 293 cells were transfected with pCAGGS, pCAGGS-nsp1, or pCAGGS-NSs using TransIT-293 reagent. At 24 h after transfection, the cells were infected with 100 HA units/ml of SeV or mock infected. At 16 h p.i., total intracellular RNAs were extracted by using an RNA isolation kit (Invitrogen, Carlsbad, CA), and contaminating genomic DNA was digested with DNase I (Promega, Madison, WI). One-step, real-time PCR was used to quantify the expression of IFN-β mRNA and 18S rRNA. Briefly, 80 ng of RNA was transferred to separate tubes for amplifying the target IFN-β mRNA and the endogenous control (18S rRNA) to use with a TaqMan one-step real-time PCR master mix reagent kit. A total of 40 cycles was performed on an ABI PRISM 7000 real-time thermocycler (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The amount of target mRNA was normalized to an endogenous 18S rRNA.

IRF-3 Dimerization Analysis. Cells were lysed in buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, protease inhibitors (Sigma), and phosphatase inhibitors (Sigma) at 16 h p.i., and the samples were incubated on ice for 10 min. After centrifugation (15,000 $\times$ *g* for 15 min at 4°C), the soluble fractions were separated electrophoretically on a 7.5% nondenaturing polyacrylamide gel, with 1% deoxycholate in the cathode buffer [25 mM Tris·HCl (pH 8.4)/192 mM glycine]. IRF-3 monomers and dimers were detected by using Western blot analysis with polyclonal anti-IRF-3 antibody (Upstate Biotechnology) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

Northern Blot Analysis. Northern blot analysis was performed by using total intracellular RNAs as described (41). All digoxigenin-labeled antisense riboprobes (Roche, Indianapolis, IN) were made by using an *in vitro* transcription reaction containing SP6 RNA polymerase or T7 RNA polymerease from pSPT 18 vector (Roche)-based plasmids. The riboprobe genes were generated by using PCR and then cloned into pSPT18. The names, sizes, and PCR templates of each riboprobe are as follows: β-gal probe, 444-nt-long, pCMV-β vector (CLONTECH, Mountain View, CA); *Renilla* Luc probe, 384-nt-long, pRL-SV40 (Promega);

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β-actin probe, 802-nt-long, intracellular RNAs of 293T cells; GAPDH probe, 739-nt-long, intracellular RNAs of 293T cells.

***In Vitro* RNA Synthesis and RNA Transfection.** Capped and polyadenylated CAT RNA transcripts, nsp1 RNA transcripts, and NSs transcripts were synthesized by using mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX). Subconfluent 293 cells were transfected with *in vitro* RNA transcripts using TransIT mRNA (Mirus) according to the manufacturer's instructions.

Radiolabeling of Cells. Cultures of 293 cells were independently transfected with CAT RNA transcripts or nsp1 RNA transcripts. One hour after transfection, cultures were incubated with medium containing 4 μg/ml actD (Sigma). Eight hours later, the cells were incubated in methionine-free medium for 30 min and then incubated in medium containing 20 μCi/ml of [^{35}S]methionine for 1 h. Cell extracts were analyzed on 12.5% SDS/PAGE.

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1. Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H. R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R. A., *et al.* (2003) *N. Engl. J. Med.* **348,** 1967–1976.
2. Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J. A., Lim, W., *et al.* (2003) *N. Engl. J. Med.* **348,** 1953–1966.
3. Poutanen, S. M., Low, D. E., Henry, B., Finkelstein, S., Rose, D., Green, K., Tellier, R., Draker, R., Adachi, D., Ayers, M., *et al.* (2003) *N. Engl. J. Med.* **348,** 1995–2005.
4. Tsang, K. W., Ho, P. L., Ooi, G. C., Yee, W. K., Wang, T., Chan-Yeung, M., Lam, W. K., Seto, W. H., Yam, L. Y., Cheung, T. M., *et al.* (2003) *N. Engl. J. Med.* **348,** 1977–1985.
5. Thiel, V., Ivanov, K. A., Putics, A., Hertzig, T., Schelle, B., Bayer, S., Weissbrich, B., Snijder, E. J., Rabenau, H., Doerr, H. W., *et al.* (2003) *J. Gen. Virol.* **84,** 2305–2315.
6. Prentice, E., McAuliffe, J., Lu, X., Subbarao, K. & Denison, M. R. (2004) *J. Virol.* **78,** 9977–9986.
7. Bhardwaj, K., Guarino, L. & Kao, C. C. (2004) *J. Virol.* **78,** 12218–12224.
8. Fan, K., Wei, P., Feng, Q., Chen, S., Huang, C., Ma, L., Lai, B., Pei, J., Liu, Y., Chen, J., *et al.* (2004) *J. Biol. Chem.* **279,** 1637–1642.
9. Ivanov, K. A., Hertzig, T., Rozanov, M., Bayer, S., Thiel, V., Gorbalenya, A. E. & Ziebuhr, J. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 12694–12699.
10. Ivanov, K. A., Thiel, V., Dobbe, J. C., van der Meer, Y., Snijder, E. J. & Ziebuhr, J. (2004) *J. Virol.* **78,** 5619–5632.
11. Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., Guan, Y., Rozanov, M., Spaan, W. J. & Gorbalenya, A. E. (2003) *J. Mol. Biol.* **331,** 991–1004.
12. Minskaia, E., Hertzig, T., Gorbalenya, A. E., Campanacci, V., Cambillau, C., Canard, B. & Ziebuhr, J. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 5108–5113.
13. Barretto, N., Jukneliene, D., Ratia, K., Chen, Z., Mesecar, A. D. & Baker, S. C. (2005) *J. Virol.* **79,** 15189–15198.
14. Lindner, H. A., Fotouhi-Ardakani, N., Lytvyn, V., Lachance, P., Sulea, T. & Menard, R. (2005) *J. Virol.* **79,** 15199–15208.
15. Sulea, T., Lindner, H. A., Purisima, E. O. & Menard, R. (2005) *J. Virol.* **79,** 4550–4551.
16. Graham, R. L., Sims, A. C., Brockway, S. M., Baric, R. S. & Denison, M. R. (2005) *J. Virol.* **79,** 13399–13411.
17. Hengel, H., Koszinowski, U. H. & Conzelmann, K. K. (2005) *Trends Immunol.* **26,** 396–401.
18. Spiegel, M., Pichlmair, A., Martinez-Sobrido, L., Cros, J., Garcia-Sastre, A., Haller, O. & Weber, F. (2005) *J. Virol.* **79,** 2079–2086.
19. Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H. & Doerr, H. W. (2003) *Lancet* **362,** 293–294.
20. Haagmans, B. L., Kuiken, T., Martina, B. E., Fouchier, R. A., Rimmelzwaan, G. F., van Amerongen, G., van Riel, D., de Jong, T., Itamura, S., Chan, K. H., *et al.* (2004) *Nat. Med.* **10,** 290–293.
21. Weidman, M. K., Sharma, R., Raychaudhuri, S., Kundu, P., Tsai, W. & Dasgupta, A. (2003) *Virus Res.* **95,** 75–85.
22. Le May, N., Dubaele, S., Proietti De Santis, L., Billecocq, A., Bouloy, M. & Egly, J. M. (2004) *Cell* **116,** 541–550.
23. Smiley, J. R. (2004) *J. Virol.* **78,** 1063–1068.
24. Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M. & Haller, O. (2001) *J. Virol.* **75,** 1371–1377.
25. Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K., Greenough, T. C., *et al.* (2003) *Nature* **426,** 450–454.
26. Billecocq, A., Spiegel, M., Vialat, P., Kohl, A., Weber, F., Bouloy, M. & Haller, O. (2004) *J. Virol.* **78,** 9798–9806.
27. Ikegami, T., Peters, C. J. & Makino, S. (2005) *J. Virol.* **79,** 5606–5615.
28. Huang, C., Narayanan, K., Ito, N., Peters, C. J. & Makino, S. (2006) *J. Virol.* **80,** 210–217.
29. Ito, N., Mossel, E. C., Narayanan, K., Popov, V. L., Huang, C., Inoue, T., Peters, C. J. & Makino, S. (2005) *J. Virol.* **79,** 3182–3186.
30. Lo, K., Sheu, G. T. & Lai, M. M. (1998) *Virology* **247,** 178–188.
31. Yuan, H., Yoza, B. K. & Lyles, D. S. (1998) *Virology* **251,** 383–392.
32. von Kobbe, C., van Deursen, J. M., Rodrigues, J. P., Sitterlin, D., Bachi, A., Wu, X., Wilm, M., Carmo-Fonseca, M. & Izaurralde, E. (2000) *Mol. Cell* **6,** 1243–1252.
33. Nemeroff, M. E., Barabino, S. M., Li, Y., Keller, W. & Krug, R. M. (1998) *Mol. Cell* **1,** 991–1000.
34. Everly, D. N., Jr., Feng, P., Mian, I. S. & Read, G. S. (2002) *J. Virol.* **76,** 8560–8571.
35. Taddeo, B., Zhang, W. & Roizman, B. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 2827–2832.
36. Kwong, A. D. & Frenkel, N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1926–1930.
37. An, S., Maeda, A. & Makino, S. (1998) *J. Virol.* **72,** 8517–8524.
38. Prakash, A. & Levy, D. E. (2006) *Biochem. Biophys. Res. Commun.* **342,** 50–56.
39. Akagi, T., Shishido, T., Murata, K. & Hanafusa, H. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7290–7295.
40. Mossel, E. C., Huang, C., Narayanan, K., Makino, S., Tesh, R. B. & Peters, C. J. (2005) *J. Virol.* **79,** 3846–3850.
41. Narayanan, K. & Makino, S. (2001) *J. Virol.* **75,** 9059–9067.