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Infectious Bronchitis Coronavirus Limits Interferon Production by Inducing a Host Shutoff That Requires Accessory Protein 5b

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ABSTRACT

During infection of their host cells, viruses often inhibit the production of host proteins, a process that is referred to as host shutoff. By doing this, viruses limit the production of antiviral proteins and increase production capacity for viral proteins. Coronaviruses from the genera *Alphacoronavirus* and *Betacoronavirus*, such as severe acute respiratory syndrome coronavirus (SARS-CoV), establish host shutoff via their nonstructural protein 1 (nsp1). The *Gammacoronavirus* and *Deltacoronavirus* genomes, however, do not encode nsp1, and it has been suggested that these viruses do not induce host shutoff. Here, we show that the *Gammacoronavirus* infectious bronchitis virus (IBV) does induce host shutoff, and we find that its accessory protein 5b is indispensable for this function. Importantly, we found that 5b-null viruses, unlike wild-type viruses, induce production of high concentrations of type I interferon protein *in vitro*, indicating that host shutoff by IBV plays an important role in antagonizing the host's innate immune response. Altogether, we demonstrate that 5b is a functional equivalent of nsp1, thereby answering the longstanding question of whether lack of nsp1 in gammacoronaviruses is compensated for by another viral protein. As such, our study is a significant step forward in the understanding of coronavirus biology and closes a gap in the understanding of some IBV virulence strategies.

IMPORTANCE

Many viruses inhibit protein synthesis by their host cell to enhance virus replication and to antagonize antiviral defense mechanisms. This process is referred to as host shutoff. We studied gene expression and protein synthesis in chicken cells infected with the important poultry pathogen infectious bronchitis virus (IBV). We show that IBV inhibits synthesis of host proteins, including that of type I interferon, a key component of the antiviral response. The IBV-induced host shutoff, however, does not require degradation of host RNA. Furthermore, we demonstrate that accessory protein 5b of IBV plays a crucial role in the onset of host shutoff. Our findings suggest that inhibition of host protein synthesis is a common feature of coronaviruses and primarily serves to inhibit the antiviral response of the host.

Viruses are dependent on the host cell machinery for translation of their proteins. To maximize the production of viral proteins and limit the production of antiviral proteins, viruses have evolved strategies to interfere with the host cell machinery at various levels (1). Betacoronaviruses, such as mouse hepatitis coronavirus (MHV), severe acute respiratory syndrome coronavirus (SARS-CoV), and several bat coronaviruses, limit host translation using virus-encoded nsp1 (2–4), which induces degradation of host, but not viral, RNA (3, 5, 6). Alternatively, nsp1 of transmissible gastroenteritis coronavirus (TGEV) (genus *Alphacoronavirus*) inhibits host translation through an unknown mechanism that does not seem to involve degradation of host mRNA (4, 7).

In addition to inhibiting host translation, nsp1 also counteracts the innate immune response (reviewed in reference 8). Observations on recombinant SARS-CoV expressing a truncated nsp1 indicate that inhibition of translation by nsp1 limits the production of cytokines, as the mutant virus induced considerably higher production of interferon (IFN) in HEK 293 cells than the parental virus (9). In addition, the virus was attenuated in IFN-competent cells (10). In line with this, MHV-nsp1 mutant viruses were severely attenuated in IFN-competent, but not in type I IFN receptor-deficient (IFNAR$^{-/-}$), mice (11, 12). Unlike the SARS-CoV nsp1 mutant virus, the MHV-nsp1 mutant did not elicit more production of interferon alpha (IFN-α) but was significantly more sensitive to treatment with IFN-α in macrophages than the parental virus (11). Experimental data suggest that nsp1 proteins from alpha- and betacoronaviruses inhibit reporter gene expression driven by an interferon beta gene (*Ifn*β) promoter, as well as from an IFN-inducible promoter (4, 10, 12–14).

Although nsp1 proteins of alpha- and betacoronaviruses exhibit remarkably similar biological functions, they are different in

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size and lack significant similarity in protein sequence (15, 16). Interestingly, of the four coronavirus genera, only alpha- and betacoronaviruses encode nsp1 (17–20). This observation has prompted the question of whether gamma- and deltacoronaviruses inhibit translation of host mRNA, and if so, which viral protein is involved. Wang et al. found that the *Gammacoronavirus* infectious bronchitis virus (IBV) does not reduce translation of host proteins (21), although the same group reported earlier that the spike protein of IBV inhibits host translation through interaction with eIF3f (22). Because of these conflicting reports, it has remained unclear whether IBV uses a host shutoff mechanism to enhance virus replication. In this study, we show that IBV inhibits synthesis of host proteins, including that of type I interferons, and we present evidence that accessory protein 5b is, at least partly, responsible for the IBV-induced host shutoff. Similar to *Alphacoronavirus* TGEV, inhibition of protein synthesis by IBV does not involve degradation of host mRNA. Taken together, our results suggest that *Gammacoronavirus* accessory protein 5b acts as the functional equivalent of *Alpha-* and *Betacoronavirus* nsp1. As such, this study closes a gap in the understanding of *Gammacoronavirus* virulence strategies and shows that evolutionarily distant coronaviruses use similar strategies to manipulate host cells.

MATERIALS AND METHODS

Cells. Chicken embryonic kidneys were aseptically removed from 17- to 19-day-old chicken embryo's (Charles River SPAFAS). A cell suspension was obtained by trypsinization for 30 min at 37°C and filtered through a 100-μm mesh. The resulting chicken embryo kidney (CEK) cells were seeded at 4×10^5 cells/cm^2 in 199 medium (Invitrogen) supplemented with 0.5% fetal bovine serum (FBS) (SAFC) and 1% PenStrep (Gibco, Invitrogen). DF-1, Vero, and CEC-32 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen) supplemented with 10% FBS and 1% PenStrep. All the cells were incubated in a humidified incubator at 37°C and 5% CO_2.

Viruses. IBV-M41, IBV-QX, and IBV-Italy-O2 and Rift Valley fever virus clone 13 (RVFV Cl13) were obtained from Merck Animal Health, Boxmeer, The Netherlands. Sindbis virus (SinV) was a kind gift from G. P. Pijlman (Laboratory of Virology, Wageningen University, Wageningen, The Netherlands). IBV Beaudette, strain Beau-R and the generation of the ScAUG3a, ScAUG3b, ScAUG5a, ScAUG5b, ScAUG3ab, and ScAUG5ab Beau-R-null viruses were published previously (23–25). In these mutant IBVs, the start codons of the indicated accessory genes were mutated to stop codons. All the IBVs were amplified and titrated on the cells in which the experiment was carried out. SinV was amplified on BHK cells and titrated on CEK cells. RVFV Cl13 was amplified and titrated on Vero cells.

cDNA synthesis, RNA isolation and gene expression analysis. Total RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacturer's instructions, including on-column DNase treatment (Qiagen). Approximately 8×10^5 CEK cells were lysed in RLT buffer (Qiagen) at various time points after infection. The RLT cell lysis buffer was spiked with 1 ng/sample of luciferase mRNA (Promega) immediately prior to RNA isolation as an external reference gene for normalization during the gene expression analysis. An external reference gene was used for normalization because none of the endogenous genes tested were suitable as housekeeping genes during viral infections. Prior to cDNA synthesis, a second DNase treatment was performed using amplification grade DNase I (Invitrogen), and subsequently, 0.5 to 1.0 μg RNA was used for cDNA synthesis using SuperScript III (Invitrogen) and random-hexamer primers. cDNA samples were diluted 1:50 in nuclease-free water before real-time quantitative PCR (RT-qPCR) analysis on a Rotor-Gene 6000 (Corbett Research), using Brilliant SYBR green quantitative PCR (Stratagene) and the primers listed in Table 1 (26–31). Cycle thresholds and amplification efficiencies were calculated with Rotor-Gene software (version 1.7) using the comparative-quantitation method. The relative expression ratio of the target gene was calculated using the average reaction efficiency for each primer set and the cycle threshold (C_t) deviation of sample versus control at the 0-h time point, as previously described (32). Because the expression of various housekeeping genes was unstable during virus infections at time points later than 24 h (data not shown), gene expression ratios were normalized using an external reference (luciferase) gene.

Chicken type I IFN bioassay. Bioactive chicken type I interferon (chIFN) was measured using a bioassay based on the CEC-32 quail reporter cell line expressing luciferase under the control of the chicken *mx* promoter (33) (kindly provided by Peter Staeheli). Briefly, CEC-32 cells were incubated with serial dilutions of chIFN-containing samples for 6 h, after which luciferase activity was quantified and IFN concentrations were calculated using a chIFN standard. To avoid the influence of virus on the assay, samples were heat inactivated at 56°C for 30 min, which did not influence the bioactivity of type I chIFN.

Luciferase expression assay. Before seeding at 100,000 cells/well in 96-well plates, CEK cells were electroporated using the Amaxa Nucleofector II (solution V; program W001), applying 2 μg pGL3-Firefly luciferase reporter plasmid (pGL3-FFluc) per 4 million cells. Vero and DF-1 cells at 80 to 90% confluence in 96-well plates were transfected with 100 ng pGL3-FFluc per well using FuGene HD (Promega) at a 1:3 ratio of DNA to FuGene according to the manufacturers' specifications. At 24 h posttransfection, the cells were infected with IBV M41 (CEK) or Beau-R (DF-1 and Vero), and 22 h later, luciferase activity was quantified using the Bright-Glo luciferase assay (Promega) and a Filtermax F5 luminometer (Molecular Devices).

Transfection of accessory proteins and cytotoxicity assay. Plasmids encoding Flag-tagged accessory proteins were constructed as follows. Accessory genes 3a, 3b, 5a, and 5b were PCR amplified with *Taq* polymerase (Invitrogen) using template cDNA from IBV-infected samples. The primers used for cloning are listed in Table 1. The PCR products were ligated into pFLAG-CMV-2 (Sigma-Aldrich) at the EcoRI site, after which the sequences were verified. Vero and DF-1 cells at 80 to 90% confluence were transfected using FuGene HD (Promega) at a 1:3 ratio of DNA to FuGene in 96-well plates according to the manufacturers' specifications using 10 ng pRL-SV40 *Renilla* luciferase plasmid and 90 ng pFLAG-Beau-R 3a/3b/5a/5b-GFP or pEGFP-MHV-nsp1 per well. At 18 h posttransfection, luciferase activity was quantified using the *Renilla* luciferase assay (Promega) and a Filtermax F5 luminometer (Molecular Devices). In parallel wells, the cytotoxicity of accessory proteins was quantified using the CellTiter 96 cell proliferation assay (Promega). At 18 h posttransfection, 20 μl AQueous One solution was added per well and incubated at 37°C for 4 h, after which the absorbance at 485 nm was measured using a FilterMax F5 luminometer. The absorbance value for 0% cell viability was established by incubating nontransfected cells for 15 min in 2% Triton X-100 (Bio-Rad) in medium prior to addition of the Aqueous One solution.

To visualize expression of Beau-R accessory proteins, the above-mentioned transfection method was used to transfect Vero cells at 60% confluence, cultured on 8-well Lab-Tek number 1.0 borosilicate cover glasses (Sigma-Aldrich). At 18 h posttransfection, the cells were fixed with 3.7% paraformaldehyde and permeabilized using 0.1% Triton X-100 in phosphate-buffered saline (PBS). Flag-tagged accessory proteins were detected using anti-Flag M2 antibody (Sigma-Aldrich) and visualized using Alexa 488-labeled goat anti-mouse antibody (Invitrogen). The antibodies were diluted 1:1,000 in PBS supplemented with 5% FBS. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were imaged using a Zeiss Primo Vert microscope and Axiovision software. Image overlays were obtained in ImageJ.

Host mRNA stability assay. The stability of host mRNAs was quantified by comparing the fold change in gene expression between infected and noninfected cells after treatment with 10 μg/ml actinomycin D (ActD) (Sigma-Aldrich). To this end, CEK cells were infected with Beau-R at a multiplicity of infection (MOI) of 10 or mock treated, and 5 h later,

TABLE 1 Primers used in this study

Gene or product	Orientation[a]	Sequence (5′–3′)[b]	Accession no.	Reference
*Ifn*β	FW	GCTCTCACCACCACCTTCTC	NM_001024836	6
	RV	GCTTGCTTCTTGTCCTTGCT		
*Ifn*α	FW	ATCCTGCTGCTCACGCTCCTTCT	XM_004937096	1
	RV	GGTGTTGCTGGTGTCCAGGATG		
Irf3	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
	RV	TGCATGTGGTATTGCTCGAT		
Tlr3	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	1
	RV	GGCGTCATAATCAAACACTCC		
Mda5	FW	TGGAGCTGGGCATCTTTCAG	GU570144	6
	RV	GTTCCCACGACTCTCAATAACAGT		
Mx	FW	TTGTCTGGTGTTGCTCTTCCT	GQ390353	6
	RV	GCTGTATTTCTGTGTTGCGGTA		
Oas	FW	CACGGCCTCTTCTACGACA	NM_205041	2
	RV	TGGGCCATACGGTGTAGACT		
Il8	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	2
	RV	GGAGCAGGAGGAATTACCAGTT		
Pkr	FW	CCTCTGCTGGCCTTACTGTCA	NM_204487	3
	RV	AAGAGAGGCAGAAGGAATAATTTGCC		
Adar	FW	TGTTTGTGATGGCTGTTGAG	AF403114	6
	RV	AGATGTGAAGTCCGTGTTG		
Mhc-I	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	2
	RV	GCCACTCCACGCAGGT		
Isg20	FW	TCTGGAAAGGTGGTGGTT	EU602349	
	RV	AAGGGGATTTTGGATGTGT		
Caspase 3	FW	GTTAGAAACGCAAACCTGA	NM_204725	
	RV	TGAAGATACGAAACCAAACCA		
Rpl17	FW	TGGATTCTCTGGTGATTGAG	XM_004949013	
	RV	CTTCTTCCTCTGGCTTGG		
Gapdh	FW	CATCACAGCCACACAGAAG	NM_204305	
	RV	GGTCAGGTCAACAACAGAGA		
Eef1α1	FW	CTGATTGTGCTGTCCTGATT	NM_204157	
	RV	TTCGTATCTCTTCTGGCTGT		
RVFV	FW	AAAGGAACAATGGACTCTGGTCA	AF134508	4
	RV	CACTTCTTACTACCATGTCCTCCAAT		
SinV	FW	CCCAGGAACCCGCAAGTATG	GM893992	5
	RV	CGTGAGGAAGATTGCGGTTC		
IBV-N	FW	GAAGAAAACCAGTCCCAGA	AY851295	6
	RV	TTACCAGCAACCCACAC		
Luciferase	FW	TGTTGGGCGCGTTATTTATC	X65316	6
	RV	AGGCTGCGAAATGTTCATACT		
3a	FW	GCCGC*GAATTC*GATGATCCAAAGTCCCACG		
	RV	TATCGAT*GAATTC*GC**TTA**GTCTAGACTGTGCCAAAGG		
3b	FW	GCCGC*GAATTC*AATGTTAAACTTAGAAGTAATTATTGAAACTG		
	RV	TATCGAT*GAATTC*GC**TTA**TTCAATAAATTCATCATCACC		
5a	FW	GCCGC*GAATTC*AATGAAATGGCTGACTAGTTTTG		
	RV	TATCGAT*GAATTC*GC**TCA**TGCCAGCGATTGGGTGG		
5b	FW	GCCGC*GAATTC*AATGAATAATAGTAAAGATAATCCTTTTCG		
	RV	TATCGAT*GAATTC*GC**CTA**GTTTAATGACTGGCGCTG		

[a] FW, forward; RV, reverse.
[b] EcoRI sites are italicized, start codons are underlined, and stop codons are in boldface.

ActD was added to all the cells and incubation was continued for an additional 6 h. Before ($t = 0$ h) and after ($t = 6$ h) ActD treatment, samples were taken for RNA isolation, cDNA synthesis, and RT-qPCR. mRNA stability was defined as the fold change in gene expression after ActD treatment. All fold changes were calculated relative to 0 h and normalized to an external reference (luciferase) gene, which was added as mRNA to the RLT lysis buffer.

Radioactive labeling. Approximately 2×10^5 Vero cells were seeded in 6-well clusters and infected 24 h later with either Beau-R or ScAUG3ab, ScAUG5ab, ScAUG5a, or ScAUG5b Beau-R-null virus at an MOI of 20. Cell lysates were collected at 6, 12, and 24 h postinfection (hpi). At the indicated time points, the cells were starved in methionine- and cysteine-deficient medium for 30 min and incubated with ^{35}S-*trans*-label (Amersham) for 15 min. The cells were washed three times in phosphate-buffered saline, trypsinized, spun down, and lysed in TEN-L buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% NP-40, and protease inhibitor cocktail [Roche]). The lysates were cleared for 15 min at 20,000 $\times$ *g*, and the supernatants were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent fluorography as previously described (2). Sample quantities loaded on the SDS-PAGE gel were adjusted so that each slot contained equal scintillation counts. For quantification of protein translation levels, radioactive signal from the gel was imaged using a Storm 860 PhosphorImager (Molecular Dynamics). The signal intensity in the images was quantified using ImageJ software.

Statistics. Statistical analyses were performed in GraphPad Prism 6.0. Significant differences were determined using an unpaired *t* test or a one-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test or a two-way ANOVA followed by a Dunnet multiple-comparison test.

RESULTS

IBV inhibits production of type I IFN. To characterize the production of type I IFN by chicken cells in response to virus infection, primary CEK cells were infected with IBV, RVFV Cl13, or SinV. Subsequently, *Ifn*β mRNA was quantified, as it is the primary IFN gene transcribed in response to viral infection of CEK cells and a proxy for innate immune activation (28). Infection of CEK cells with IBV, RVFV Cl13, and SinV induced production of *Ifn*β mRNA as early as 6 hpi (Fig. 1A). However, IFN protein was detected only in the supernatant of cells infected with RVFV Cl13 and Sindbis virus (Fig. 1B). The supernatant of IBV-infected cells contained almost no IFN, although the virus replicates well in these cells (Fig. 1C), indicating that IBV inhibits the production of type I IFN protein. To assess whether inhibition of IFN production is a common feature of IBV, we investigated IFN production by CEK cells upon infection with five serotypes of IBV. We found that at 24 hpi, all the serotypes induced production of *Ifn*β mRNA (Fig. 1D), but no IFN protein was detected in the cell culture supernatant (Fig. 1E). At 48 hpi, cytopathic effect (CPE) was extensive (data not shown), and low concentrations of IFN (30 to 180 U/ml) were detected in the supernatants of cells infected with IBV strains M41, It02, QX, and 4/91. A concentration of 1,000 U/ml was detected in the supernatant of cells infected with strain Beau-R, which showed even more extensive CPE. Titration of cell supernatants showed that the titer of the cell culture-adapted Beau-R strain was higher than those of the other IBV strains (Fig. 1F), which could explain why Beau-R induced higher production of IFN. Notably, *Ifn*β mRNA levels were not higher in Beau-R-infected cells, suggesting that Beau-R allows more production of IFN protein than the other serotypes studied. Taken together, we conclude that IBV efficiently inhibits production of IFN protein by primary chicken kidney cells during the first 24 h of infection.

IBV inhibits translation of host proteins. The observation that IBV-infected cells synthesize *Ifn*β mRNA but do not produce IFN protein suggests that IBV infection might inhibit synthesis of host proteins. To test this hypothesis, a ^{35}S-labeling experiment was performed, which showed that in IBV-infected Vero cells, synthesis of host proteins is severely reduced at both 12 and 24 hpi (Fig. 2A). In addition, we found that IBV infection reduced luciferase expression from a constitutively active promoter (Fig. 2B) in three different cell types, i.e., Vero, CEK, and DF-1 cells. Taken together, these results indicate that IBV inhibits the synthesis of host proteins. The betacoronaviruses SARS-CoV and MHV also inhibit synthesis of host proteins, and in cells infected with these viruses, degradation of host mRNA was observed (5, 6). To investigate whether the mechanism by which IBV inhibits host protein synthesis is comparable to those of SARS and MHV, we investigated the stability of host mRNA in IBV-infected cells. To this end, we compared mRNA levels before and after inhibition of *de novo* mRNA transcription with ActD in IBV-infected and noninfected (mock-infected) cells (Fig. 2C). Using RT-qPCR, we quantified mRNA and calculated the percentage of mRNA that remained after 6 h of treatment with ActD. The percentage of mRNA that remains after ActD treatment is a function of the stability of that specific mRNA and the duration of the treatment. We quantified mRNA levels of housekeeping genes (*Gapdh*, *Eef1*α*1*, and *Rpl17*), as well as genes involved in the innate antiviral response. Upon ActD treatment, mRNA levels for most genes decreased between 30 and 90% in both IBV-infected and noninfected cells. For several immune-related genes involved in antiviral responses (*Irf3*, *Mda5*, *Tlr3*, *Isg20*, and *Ifn*β), mRNA levels were less reduced in IBV-infected cells. In conclusion, no evidence was found to support the hypothesis that IBV infection decreases the stability of host mRNAs. As such, degradation of host mRNA is most probably not the mechanism by which inhibition of host protein synthesis in IBV-infected cells occurs.

Accessory protein 5b inhibits synthesis of host proteins. Unlike alpha- and betacoronaviruses, the genome of gammacoronaviruses does not encode an nsp1 homologue; consequently, another viral protein must be responsible for inhibition of host protein synthesis. Plausible candidate proteins that could fulfill this function are the genus-specific accessory proteins. Therefore, we investigated the inhibitory potential of the four IBV accessory proteins on protein synthesis. Plasmids that encoded individual IBV accessory proteins were constructed with an N-terminal Flag tag (Fig. 3A). These plasmids were transfected into Vero and DF-1 cells, together with a plasmid that encodes *Renilla* luciferase (Fig. 3B and C). It was observed that cotransfection of the plasmid encoding accessory protein 3a moderately reduced luciferase activity in DF-1 cells (Fig. 3B), whereas cotransfection of accessory protein 3b slightly increased luciferase activity in both DF-1 and Vero cells (Fig. 3B and C). Importantly, the only plasmids that significantly reduced luciferase expression in both DF-1 and Vero cells were those encoding accessory protein 5b of IBV and nsp1 of MHV. The reduction in expression of *Renilla* luciferase was not due to cytotoxic effects of either the transfection procedure or the plasmids (Fig. 3B and C, right *y* axes). Taken together, these overexpression studies suggest that accessory protein 5b plays the most prominent role in the reduction of host protein production.

To investigate whether accessory protein 5b is involved in inhibition of host protein production during IBV infection, cells

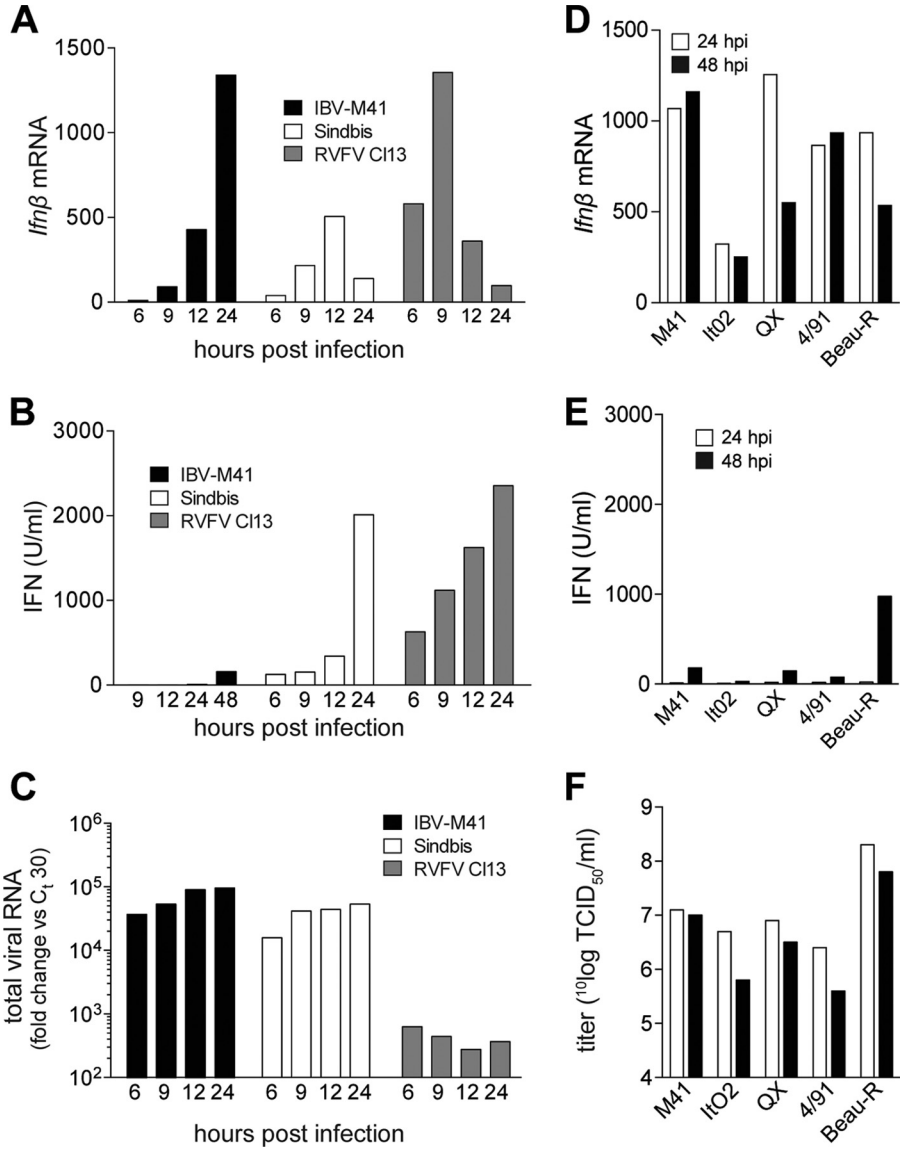

FIG 1 IBV induces transcription of *Ifn*β but limits production of interferon protein. CEK cells were infected with IBV-M41 (MOI, 5), Sindbis virus (MOI, 1), or RVFV Cl13 (MOI, 5). At the indicated time points, *Ifn*β mRNA (A), extracellular IFN protein (B), and total viral RNA (C) in the supernatant were quantified. (D to F) CEK cells were infected with the indicated strains of IBV (MOI, 1) and *Ifn*β mRNA (D) and IFN protein (E), and virus titers were determined at 24 and 48 hpi (F). The values represent the results of one experiment, which was performed twice with comparable results.

were infected with Beau-R or Beau-R accessory-gene-null mutant virus (ScAUG), and *de novo* protein synthesis was measured using ^{35}S pulse-labeling at 6, 12, and 24 hpi (Fig. 4A). Figure 4A shows three regions that contain mostly host proteins, labeled H1, H2, and H3. Quantification of the ^{35}S signal in these regions was used to measure *de novo* synthesis of host proteins in virus- and mock-infected cells at 12 and 24 hpi (Fig. 4B and C). The results of the quantification indicated that all the viruses except ScAUG5b and ScAUG5ab decreased translation of host proteins at both 12 and 24 hpi. Next, we investigated whether increased synthesis of host proteins in ScAUG5b- and ScAUG5ab-infected cells corresponds to a decrease in synthesis of viral proteins. To do this, we quantified the ^{35}S signal of the spike (S), nucleocapsid (N), and membrane (M) proteins of IBV and found synthesis of these proteins to be comparable in all the viruses (Fig. 4D). Overall, these results indicate that accessory protein 5b is required for inhibition of host protein synthesis by IBV.

Accessory protein 5b inhibits production of IFN. Subsequently, we explored whether 5b could be responsible for the lack of IFN production by IBV-infected cells observed in Fig. 1. To investigate the role of 5b, we quantified the production of type I IFN by DF-1 cells infected with Beau-R or accessory-gene-null mutant virus (Fig. 5A). We found that infection with ScAUG5b, as well as ScAUG5ab, resulted in significantly higher production of IFN by DF-1 cells than infection with Beau-R. In fact, ScAUG5b-infected cells produced 55 and 30 times more IFN at 36 and 48 hpi, respectively. To verify the relevance of this finding, the experiment was repeated in primary (immunocompetent) CEK cells, where we found that both ScAUG5b- and ScAUG5ab-infected cells produced up to 15 times more IFN than Beau-R-infected cells

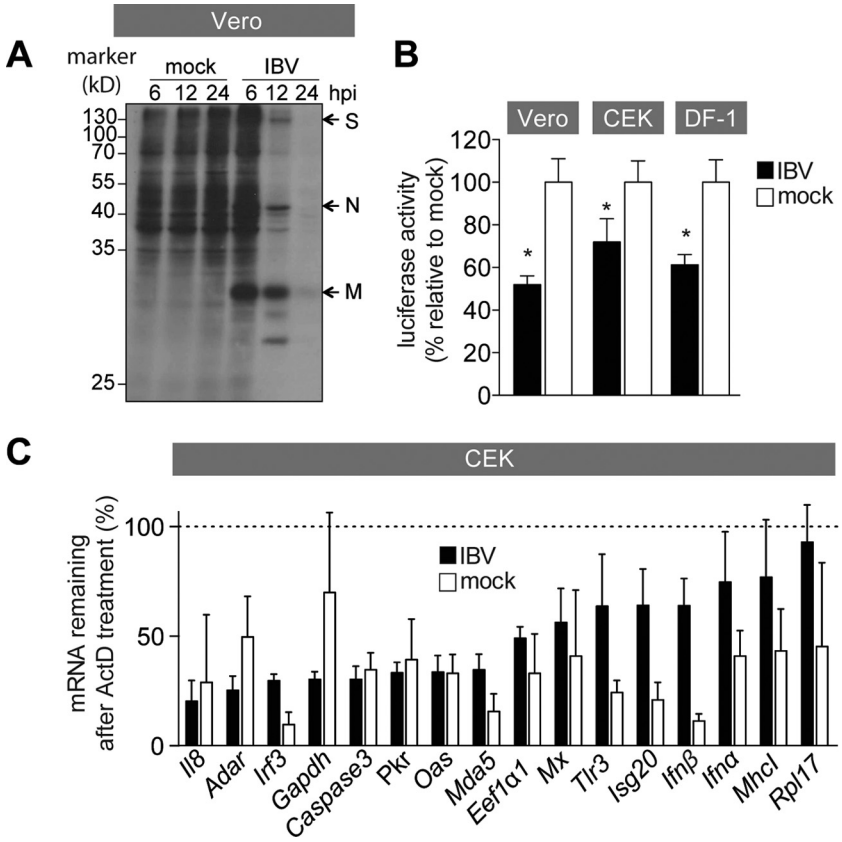

FIG 2 IBV induces host shutoff without degradation of host mRNA. (A) Vero cells were infected with Beau-R (MOI, 20), and at 6, 12, and 18 hpi, newly synthesized proteins were radioactively labeled for 1 h with [^{35}S]methionine. The cells were subsequently lysed, and proteins were separated using SDS-PAGE. after which ^{35}S was visualized using a phosphorimager. Virus proteins are indicated by S, N, and M. (B) Cells were electroporated (CEK) or transfected (DF-1 and Vero) with pGL3-SV40-Firefly luciferase plasmid and 24 h later infected with IBV-M41 (MOI, 10). At 22 hpi, luciferase activity was quantified. The bars represent mean luciferase activities of triplicate measurements from two experiments. The error bars indicate standard deviations, and the asterisks indicate statistically significant differences ($P < 0.0001$) compared to mock-infected cells, as determined using an unpaired Student's *t* test. (C) CEK cells were infected with Beau-R (MOI, 10), and at 5 hpi, transcription was inhibited using ActD (10 μg/ml) for an additional 6 h, after which mRNA was quantified using RT-qPCR. The bars indicate the mean percentages of mRNA remaining after ActD treatment from triplicate wells of a representative example of two biological replicates. The error bars indicate standard deviations.

(Fig. 5B). It was also found that levels of *Ifn*β mRNA were significantly higher in cells infected with any of the accessory-gene-null viruses (Fig. 5C), which is consistent with findings from a previous study (28). Despite the overall increase in *Ifn*β mRNA transcription observed in cells infected with any of the accessory-gene-null viruses, only in 5b mutant virus-infected cells were increased IFN protein levels detected. This observation is consistent with the hypothesis that 5b inhibits the translation of *Ifn*β mRNA. At 36 hpi, when IFN levels were significantly higher for the accessory-gene-null viruses than for Beau-R, the titers of all accessory-gene-null viruses were moderately lower than that of Beau-R (Fig. 5D). These growth characteristics are generally in line with previous observations (24). Taken together, our results indicate that accessory protein 5b plays a major role in the inhibition of general host protein synthesis, thereby inhibiting production of IFN by IBV-infected cells.

DISCUSSION

The type I IFN response has been shown to be important for clearance of coronavirus infection *in vivo*, and coronaviruses have evolved multiple mechanisms to delay and antagonize it (reviewed in reference 34). One of the strategies of alpha- and betacoronaviruses is to inhibit production of host proteins, including type I IFN, via the viral nsp1 protein (2–4, 7). Gamma- and deltacoronaviruses lack nsp1, and it is therefore unclear whether and how these viruses antagonize host translation. Previously, we demonstrated that the *Gammacoronavirus* IBV elicits remarkable induction of transcription of *Ifn*β mRNA in avian cells but that this is delayed with respect to the peak of viral replication. Here, we further show that IBV-induced *Ifn*β transcription does not lead to production of significant levels of IFN protein until well after the onset of transcription. In fact, we found that IBV inhibits IFN production by blocking host translation, better known as host shutoff, and we show that accessory protein 5b is required for this function.

Various alpha- and betacoronaviruses have been shown to induce host shutoff (2, 3, 7), and the only study on a gammacoronavirus (IBV) reported absence of host shutoff in IBV-infected cells (21). Our observation that IBV infection induces transcription of *Ifn*β, but not production of IFN protein, prompted us to reevaluate whether IBV induces host shutoff. Using the same cell line and the same IBV strain that Wang et al. (21) used, we found

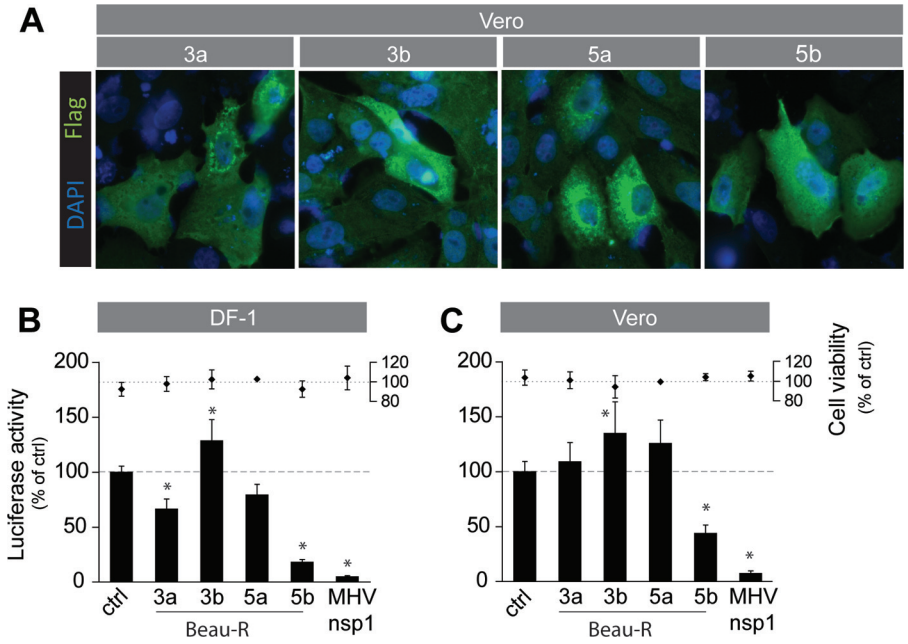

FIG 3 Accessory protein 5b inhibits luciferase activity in DF-1 and Vero cells Vero cells were transfected with plasmids expressing Flag-tagged accessory proteins, and 22 h later, the proteins were detected using a Flag-specific antibody. (A) Detection of IBV accessory proteins. (B and C) DF-1 (B) and Vero (C) cells were seeded in 96-well plates and transfected with 10 ng *Renilla* luciferase-expressing plasmid plus 90 ng of plasmid expressing the indicated accessory protein of Beau-R or MHV nsp1, or empty plasmid (ctrl). At 18 h posttransfection, luciferase activity was quantified, and it is plotted on the left *y* axis. In parallel wells, the cytotoxicity of each construct was investigated using the cell titer 96 cytotoxicity assay. The results are plotted on the right *y* axis. All the values represent the means of quadruplicate measurements from two independent experiments. The error bars indicate standard deviations, and the asterisks indicate significant differences ($P < 0.001$) compared to the control as assessed by one-way ANOVA, followed by a Bonferroni *post hoc* test.

that IBV infection severely reduced synthesis of host proteins. The apparent discrepancy between our results and those of Wang et al. was probably caused by the application of a higher MOI in our case (20 instead of 2).

During beta- but not alphacoronavirus infection, host shutoff is accompanied by degradation of host mRNAs (5, 6). To investigate whether IBV infection induces degradation of mRNA, we inhibited transcription in IBV-infected and noninfected cells with actinomycin D and quantified the decrease in host mRNA levels. In contrast to infection with MHV and SARS-CoV (5, 6), we did not observe a decrease in the stability of host mRNAs. In fact, a subset of mRNAs, including *Irf3*, *Mda5*, *Tlr3*, *Isg20*, and *Ifn*β, appeared to show increased stability in IBV-infected cells. The reason for the increased stability of these innate-immune mRNAs is unclear, but it could be the result of transcription induced in response to IBV infection. An alternative explanation is that during IBV infection a subset of mRNAs are recruited to structures such as stress granules (SG). SG are temporary repositories of mRNAs, and they are formed in response to stress-induced translational arrest (35, 36). SG have been shown to prevent degradation of mRNAs by cellular ribonucleases, and increased phosphorylation of eIF2α, which frequently accompanies virus-induced host shutoff, is one of the triggers for SG formation (37). Many viruses have been shown to modulate the formation of SG, but in most cases it is unclear whether SG formation is beneficial to the host or to the virus (reviewed in reference 38). Stress granules have been observed in TGEV- and MHV-infected cells (2, 39), and in the case of TGEV, formation of SG coincided with decreased viral RNA synthesis, suggesting that SG are detrimental to virus replication. The observation that IBV does not decrease mRNA stability may suggest that IBV induces host shutoff via a mechanism similar to that of alphacoronaviruses, which also do not induce degradation of host mRNA (4, 7).

For both alpha- and betacoronaviruses, the viral nsp1 protein was shown to be essential for establishment of host shutoff. The genomes of gamma- and deltacoronaviruses do not encode nsp1, so it is unclear which IBV protein could play a role in IBV-induced host shutoff. We speculated that one of the accessory proteins might be involved in the IBV-induced host shutoff. In a previous study, we showed that absence of IBV accessory protein 3b increases production of type I interferon protein late (≥36 hpi) during infection (28). In addition to this, it was shown that accessory protein 3a confers resistance to IFN through an unknown mechanism (40). To investigate the influence of individual IBV accessory proteins on gene expression, we overexpressed each of the four accessory proteins and found that not 3a or 3b but 5b alone decreased expression from a constitutive promoter in both a chicken and a mammalian cell line. Inhibition by IBV 5b was less pronounced than inhibition by MHV-nsp1; similar differences in inhibition efficiency were reported for nsp1 proteins from various betacoronaviruses (4). Next, we investigated the effect of 5b in the context of a virus infection using mutant viruses that do not express one or more accessory proteins. Using radioactive labeling of *de novo* protein synthesis, we found that both 5a/5b-null (ScAUG5ab) and 5b-null (ScAUG5b) viruses were less efficient at inhibiting host translation than the parental virus. The difference in inhibition efficiency between the 5b-null virus and the parental virus was comparable to the difference previously

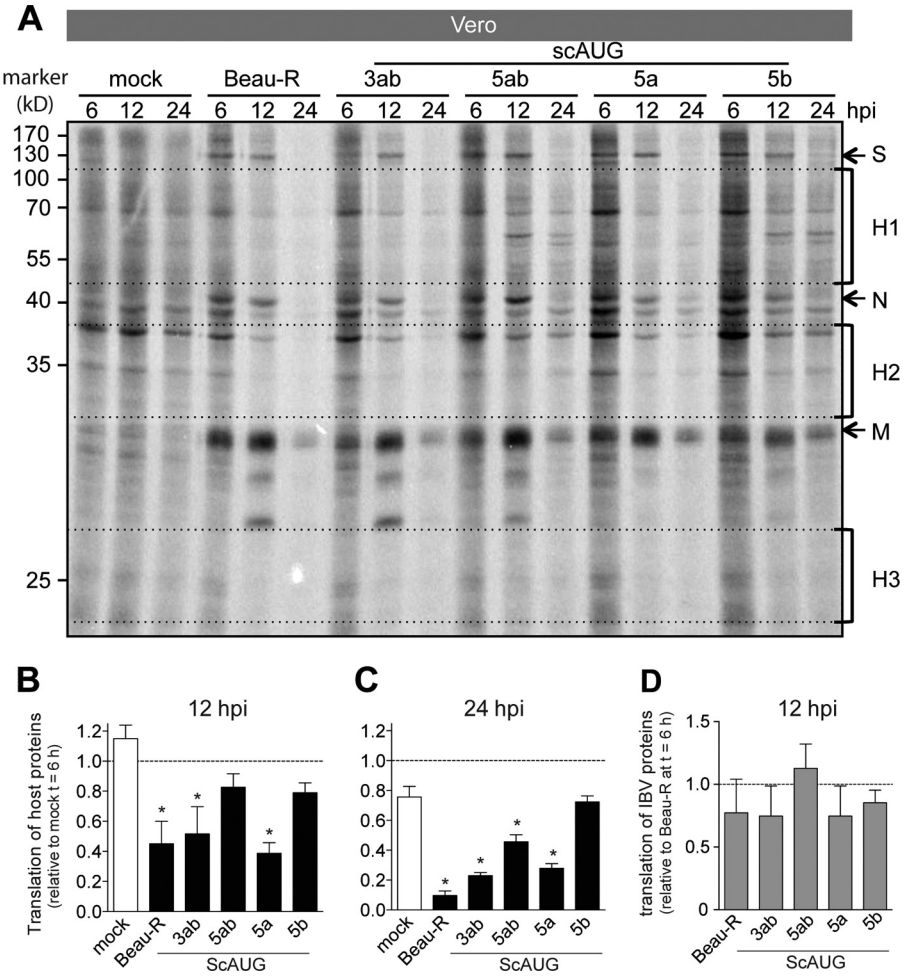

FIG 4 Accessory protein 5b is required for induction of host shutoff. (A) Vero cells were mock treated or infected with the indicated mutant viruses not expressing one or two accessory proteins (MOI, 20). At the indicated time points after infection, *de novo*-synthesized proteins were labeled with [^{35}S]methionine for 1 h. Subsequently, the cells were lysed, proteins were separated using SDS-PAGE, and the ^{35}S-labeled proteins were visualized using a phosphorimager. Areas containing host proteins are labeled H1 to H3. Viral spike (S), nucleocapsid (N), and membrane (M) proteins are also indicated. (B and C) Host protein synthesis at 12 hpi (B) and 24 hpi (C) was approximated by quantification of ^{35}S signal intensity in the areas H1 to H3 indicated in panel A and expressed as a ratio relative to mock-infected cells at 6 hpi. (D) Synthesis of IBV proteins was approximated by quantification of the ^{35}S signal intensities of IBV S, N, and M proteins at 12 hpi relative to Beau-R-infected cells at 6 hpi. The bars indicate the means of the three values determined for either virus or host proteins at the indicated time points, and the error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.001$) compared to mock infection, as assessed by one-way ANOVA followed by a Bonferroni *post hoc* test.

found between wild-type SARS-CoV and SARS-CoV nsp1 mutant virus (9).

We then examined whether inhibition of host protein synthesis by 5b is responsible for the block in IFN production observed in IBV-infected chicken cells. We found that both 5a/5b- and 5b-null viruses induced up to 90 times higher production of type I IFN in chicken cells than the parental virus. The extent of the difference in IFN production is comparable to previous observations of SARS-CoV nsp1 mutant virus (9). The functional resemblance between IBV-5b and nsp1 prompted us to compare the two proteins. Amino acid sequence alignment of 5b and nsp1 proteins from various viruses yielded no significant similarity (<20% [data not shown]). Additionally, phylogenetic analysis showed that nsp1 proteins from both alpha- and betacoronaviruses do not cluster with any of the four gammacoronavirus accessory proteins (data not shown). Although 5b and nsp1 do not show sequence similarities, they both evolved to fulfill the same biological function, i.e., inhibition of host translation. The *Coronavirinae* split 2 million years ago (41) to yield the ancestor of all alpha- and betacoronaviruses and the ancestor of both gamma- and deltacoronaviruses. The question that remains to be answered is whether the common ancestor of all coronaviruses encoded nsp1 or 5b. The lack of sequence homology between 5b and nsp1, their different locations in the genome, and the evolutionary history of the viral genomes carrying them suggest that their functional homology is the result of convergent evolution. Interestingly, nsp1 is the first protein to be synthesized in alpha- and betacoronavirus-infected cells, because it is translated directly from genomic viral RNA. In contrast, 5b can be translated only from subgenomic RNAs that are produced only later during infection. This may indicate that inhibition of host protein synthesis during the initial stages of infection may not be required for the *Gammacoronavirus* IBV.

The finding that 5b and nsp1 are functionally equivalent an-

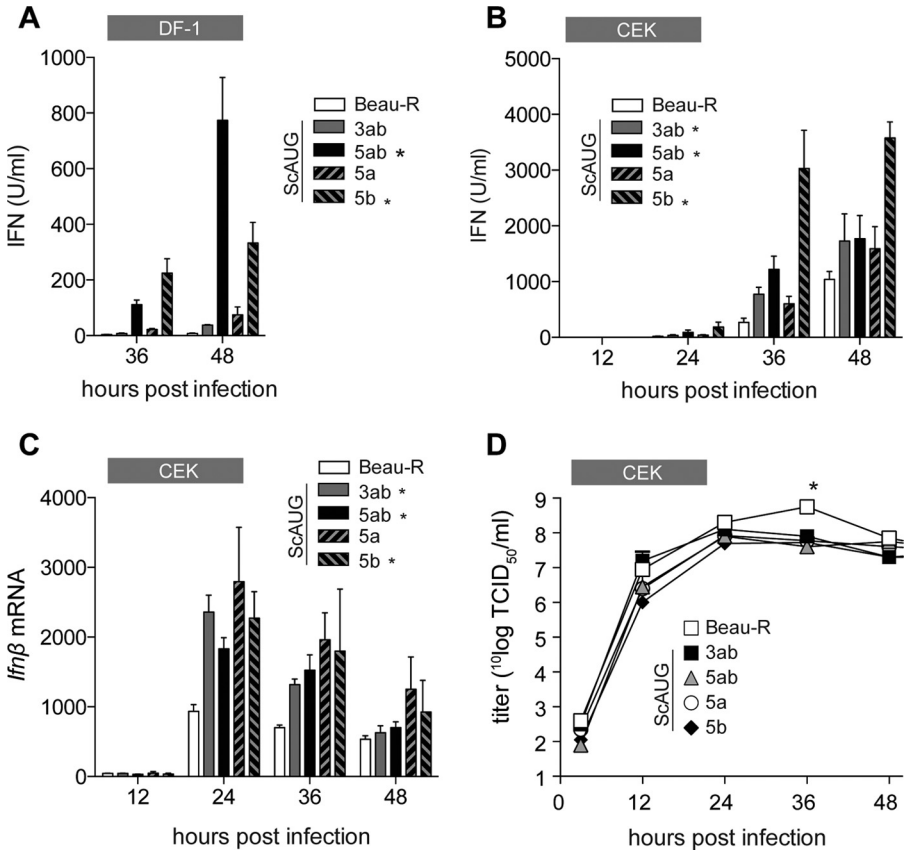

FIG 5 Host shutoff induced by accessory protein 5b limits production of IFN. DF-1 (A) and CEK (B to D) cells were infected (MOI, 0.1) with Beau-R or Beau-R mutant virus that did not express the indicated accessory proteins. IFN protein in the supernatant (A and B), *Ifn*β mRNA (C), and virus in the supernatant (D) were quantified. The values represent the means of the results of a representative experiment performed in triplicate, and the error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) compared to the parental Beau-R virus as assessed by two-way ANOVA followed by a Dunnet multiple-comparison test.

swers the longstanding question of whether lack of nsp1 in gammacoronaviruses is compensated for by another viral protein. As such, our study is a significant step forward in the understanding of coronavirus biology. Although the mechanism by which 5b inhibits host translation remains to be investigated, our *in vitro* results indicate that it may be an important virulence factor of gammacoronaviruses and a potential target for the rational design of live-attenuated virus vaccines against this important pathogen.

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