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Bunyamwera Bunyavirus Nonstructural Protein NSs Counteracts the Induction of Alpha/Beta Interferon

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Production of alpha/beta interferons (IFN-α/β) in response to viral infection is one of the main defense mechanisms of the innate immune system. Many viruses therefore encode factors that subvert the IFN system to enhance their virulence. *Bunyamwera virus* (BUN) is the prototype of the *Bunyaviridae* family. By using reverse genetics, we previously produced a recombinant virus lacking the nonstructural protein NSs (BUNdelNSs) and showed that NSs is a nonessential gene product that contributes to viral pathogenesis. Here we demonstrate that BUNdelNSs is a strong inducer of IFN-α/β, whereas in cells infected with the wild-type counterpart expressing NSs (wild-type BUN), neither IFN nor IFN mRNA could be detected. IFN induction by BUNdelNSs correlated with activation of NF-κB and was dependent on virally produced double-stranded RNA and on the IFN transcription factor IRF-3. Furthermore, both in cultured cells and in mice lacking a functional IFN-α/β system, BUNdelNSs replicated to wild-type BUN levels, whereas in IFN-competent systems, wild-type BUN grew more efficiently. These results suggest that BUN NSs is an IFN induction antagonist that blocks the transcriptional activation of IFN-α/β in order to increase the virulence of Bunyamwera virus.

Virus infection of mammalian cells prompts the innate immune system to establish a first line of defense against the invading pathogen. As an immediate response to infection, cells synthesize and secrete alpha/beta interferons (IFN-α/β) which prime neighboring cells to express antiviral factors, thereby limiting the extent of virus spread (35).

IFN-α/β are encoded by a single IFN-β gene and a family of closely related IFN-α genes (35). In most cell types of the peripheral tissues, IFN response commences with the production of IFN-β, which then induces the α IFNs in an autocrine and paracrine manner. Consequently, induction of α IFNs is severely impaired in knock-out mice that lack the IFN-β gene (8, 14), emphasizing the central role that is played by IFN-β. Induction of IFN-β occurs primarily at the level of transcriptional initiation and requires several regulatory factors. These factors can be subdivided into those generally activated upon cell stress and those more specific for IFN and other cytokine genes. The first group consists of AP-1 and NF-κB, two universal transcription factors induced upon multiple stress signals (6, 41). The second group consists of IRF-3 and IRF-7, members of the so-called IFN-regulatory factor (IRF) family (25, 41, 43, 46).

Key inducers of the IFN-β promoter are NF-κB and IRF-3 (32). Both factors can be activated by virus infection or by double-stranded RNA (dsRNA), an intermediary of viral RNA synthesis (22, 41, 48). Once secreted, IFN-β then binds to a cell surface receptor common to all IFN-α/β. This results in activation of the signal transducers and transcriptional activators STAT1 and -2, leading to the transcription of genes that contain IFN-stimulated response elements (ISRE) in their promoters. These genes code for a wide array of proteins that are antivirally active (35).

IFN-α/β also have profound immunomodulatory effects and stimulate the adaptive response. Therefore, they establish an important link between innate and adaptive immunity (1). It is becoming increasingly clear that, for a successful infection of the host, viruses must have evolved strategies to subvert the IFN system (17, 21).

The family *Bunyaviridae* contains several members that cause encephalitis or hemorrhagic fevers in humans, e.g., Hantaan, Rift Valley fever, La Crosse, and Crimean-Congo hemorrhagic fever viruses (13). Members of the *Bunyaviridae* are enveloped viruses and have been classified into five genera: *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*. More than 300 named virus isolates, mainly transmitted by arthropods, are contained within the family (12). The prototype of both the family *Bunyaviridae* and the genus *Bunyavirus* is Bunyamwera virus (BUN). All members of the *Bunyaviridae* have a trisegmented single-stranded RNA genome of negative or ambisense polarity, replicate in the cytoplasm, and bud into the Golgi apparatus (12). They encode four common structural proteins: the viral polymerase (L) on the large (L) segment, two glycoproteins (G1 and G2) on the medium (M) segment, and the viral nucleocapsid protein (N) on the smallest (S) segment. Viruses within some genera also encode nonstructural proteins, either on the M segment (termed NSm) or on the S segment (NSs). The functions of these nonstructural proteins are not yet fully understood.

NSs proteins are expressed by members of the *Bunyavirus*,

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Phlebovirus, and *Tospovirus* genera with different coding strategies. For BUN, NSs is a small hydrophobic protein of 101 amino acids expressed from an internal, +1-shifted reading frame within the N gene. We recently succeeded in generating a recombinant BUN that does not express NSs (4). This virus, named BUNdelNSs, contained specific mutations in the S segment that inactivated the NSs start codon without altering the overlapping N reading frame. Compared to the isogenic wild-type virus, BUNdelNSs exhibited a smaller plaque size and grew in several cell types to approximately 10-fold-lower titers. When inoculated by the intracerebral route, BUNdelNSs killed BALB/c mice with a slower time course than wild-type BUN and exhibited a reduced cell-to-cell spread. Interestingly, in vitro experiments with transfected reporter constructs indicated that BUN NSs interferes with IFN induction (4).

Here we analyzed the effects of NSs on the IFN system and examined its role in establishing bunyavirus infection in vitro and in vivo. The results obtained provide evidence for the importance of NSs as a virulence factor that weakens host defense by antagonizing the dsRNA-mediated activation of IFN-β transcription.

MATERIALS AND METHODS

Cells and viruses. Murine BF cells (9), human 293 cells, primary mouse embryo fibroblasts (MEFs) from 129 wild-type and IFNAR$^{0/0}$ mice, and simian Vero cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The wild-type BUN and BUNdelNSs (4) virus stocks used in this study were plaque purified in BHK-21 cells, and working stocks were grown in BHK-21 cells as described before (42).

Plasmids. Plasmid constructs for monitoring IFN-β promoter activation (p-125Luc) and NF-κB activity (p55-A2Luc) and the expression construct for the dominant-negative mutant IRF-3$^{58-427}$ (pEF-HAIRF3$^{58-427}$) were kindly provided by Takashi Fujita, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan (46). The empty vector pEF-BOS was kindly provided by Shigekazu Nagata, Osaka University Medical School, Osaka, Japan. The reporter construct for monitoring ISRE activation [p(9-27)4tkΔ(−39)lucter] was kindly provided by Stephen Goodbourn, St. George's Hospital Medical School, London, United Kingdom (24). The plasmid expressing the vaccinia virus E3L gene, pCMV-E3L, was kindly provided by Robert J. Schneider, New York University School of Medicine (15), and plasmid pAM8-1, expressing T7 RNA polymerase, was kindly provided by Mahito Nakanishi, University of Osaka, Osaka, Japan (49). The expression plasmids pTM1-BUNNSs and pTM1-CTRL contained the appropriate coding sequences under the control of a T7 promoter and the encephalomyocarditis virus internal ribosome entry site (44). The control plasmid pRL-simian virus 40 (Promega) contained the *Renilla* luciferase (REN-Luc) gene under the control of the constitutive simian virus 40 promoter.

Reporter gene assays. Subconfluent monolayers of BF cells were transfected with 1 μg of firefly luciferase (FF-Luc) reporter plasmid DNAs and 0.1 μg of control plasmid pRL-SV40 in 400 μl of Optimem (Gibco-BRL) containing 5 μl of DAC-30 (Eurogentech). After 5 h at 37°C, the liposome-DNA mixture was removed, and cells were infected with 5 PFU of virus per cell. If required, expression constructs were added to the transfection mixture, and cells were incubated for another 24 h before infection. At 16 h postinfection, cells were harvested and lysed in 200 μl of reporter lysis buffer (Promega). An aliquot of 20 μl of lysate was used to measure FF-Luc and REN-Luc activities as described by the manufacturer (Promega). The FF-Luc activities were normalized to the corresponding REN-Luc activities to determine induction.

Treatment with dsRNA. For transfection of cells with dsRNA, 10 μg of poly(I:C) (Sigma) was prepared with 10 μl of DAC-30 liposomes (Eurogentech) in 200 μl of serum-free medium according to the manufacturers' instructions. After 15 min of incubation, the dsRNA-liposome mixture was dropped onto cells without changing the medium.

Assay for IFN. Subconfluent monolayers of BF cells were infected with 5 PFU per cell and incubated for 18 h in DMEM–10% FCS. Supernatants (2 ml) were collected and dialyzed at 4°C two times against 100 mM glycine (pH 2) to inactivate infectious virus and once against phosphate-buffered saline (PBS) for pH neutralization (24 h for each dialysis step). To assay for IFN activity, 1 ml of each of the supernatants was mixed with 1 ml of DMEM–20% FCS and laid onto BF cells that were previously transfected with 1 μg of ISRE-Luc reporter construct p(9-27)4tkd(−39)lucter (24) and 0.1 μg of the control construct pRL-SV40. As a standard, 1,000, 500, 100, and 0 U of human IFN-α A/D (*Bgl*II) (PBL Biomedical Laboratories) per ml, prepared in PBS, was used. The indicator cells were then incubated for 18 h, harvested, and lysed in 200 μl of reporter lysis buffer (Promega).

An aliquot of 20 μl of cell lysate was used to measure FF-Luc induced from p(9-27)4tkd(−39)lucter and REN-Luc constitutively expressed from pRL-SV40) activities as described by the manufacturer (Promega). The ratio of FF-Luc (reflecting IFN response) to REN-Luc (reflecting transfection efficiency) activity was taken as a measure for IFN-α/β in the supernatant. The amount of IFN produced by the virus-infected cells was determined by comparing the normalized luciferase activities with the IFN standard.

IFN-specific RT-PCR. Total RNA was extracted from infected cells with the TriFast reagent (Peqlab). For reverse transcription (RT), 1 μg of total RNA was incubated with 200 U of Superscript II reverse transcriptase (Gibco-BRL) and 100 ng of random hexanucleotides in 20 μl of 1× RT buffer (Gibco-BRL) supplied with 1 mM each of the four deoxynucleotide triphosphates, 20 U of RNasin, and 10 mM dithiothreitol. The resulting cDNA was amplified by 30 cycles of PCR, with each cycle consisting of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C, followed by 10 min at 72°C. Primer sequences for amplifying mouse β-actin were from Shaw-Jackson and Michiels (34), and those for IFN-β were from Marie et al. (26).

Virus growth in tissue culture. Murine 129 wild-type and IFNAR$^{0/0}$ MEFs were grown in six-well dishes to 80% confluency and infected with 0.01 PFU per cell, washed twice with PBS, and overlaid with DMEM–10% FCS. Supernatants were taken at 72 h postinfection and assayed in plaque assays on Vero cells as described before (42).

Pathogenicity studies. IFN-competent mice of inbred strain 129 and transgenic mice with targeted disruptions of the β subunit of the IFN-α/β receptor (IFNAR$^{0/0}$) (28) on an 129 background were obtained from B&K Universal Ltd. (United Kingdom). Five-week-old specific-pathogen-free female mice were inoculated either intraperitoneally or intracerebrally with virus. For intraperitoneal infection, 1,000 PFU of virus was inoculated in 0.1 ml of PBS containing 0.75% (wt/vol) bovine serum albumin (PBSA). For intracerebral infection, 1,000 PFU of virus was inoculated in 0.02 ml of PBSA. The animals were monitored twice daily over an 8-day period. Mice that were moribund or severely paralyzed were killed.

RESULTS

Induction of IFN-α/β by BUNdelNSs. To measure IFN-α/β production of infected cells, we used a bioassay based on transfected reporter plasmids. Mouse fibroblasts were infected with wild-type BUN or BUNdelNSs and incubated overnight, and supernatants were depleted of infectious virus by acidification. To assay for IFN-α/β activity, indicator cells previously transfected with an IFN-responsive (ISRE) reporter plasmid were incubated with the treated supernatants. The amount of firefly luciferase produced from the ISRE construct was linearly dependent on the amount of IFN in the supernatant (data not shown). Therefore, the concentration of IFN could be determined by comparing ISRE activity in indicator cells with a standard curve. Figure 1A shows that supernatants from wild-type BUN-infected cells did not contain detectable amounts (i.e., below 50 U) of IFN-α/β, similar to the situation with mock-infected cells. By contrast, cells infected with BUNdelNSs produced more than 1,000 U of IFN-α/β per ml of medium.

It is conceivable that, in wild-type BUN-infected cells, NSs interferes with the synthesis of IFN by blocking the transcription of the IFN gene, whereas BUNdelNSs is unable to hinder transactivation. Alternatively, the IFN gene could be induced in wild-type BUN- and BUNdelNSs-infected cells to the same extent, but NSs inhibits a posttranscriptional step, e.g., trans-

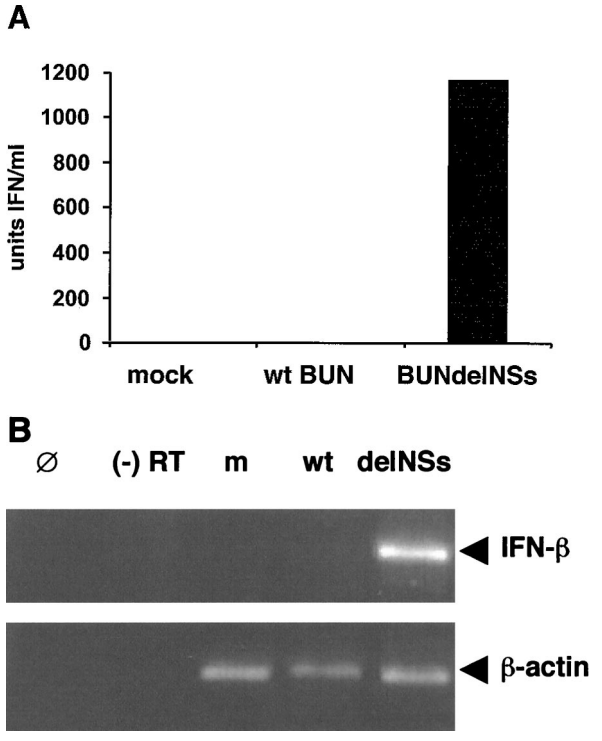

FIG. 1. IFN induction in infected mouse BF cells. (A) IFN-α/β in supernatants. Murine BF cells were either mock infected or infected with 5 PFU of wild-type BUN or BUNdelNSs per cell and incubated for 18 h. Supernatants were collected, and IFN concentration was determined. (B) Detection of IFN-β mRNA. Total RNA was extracted from the cells used in panel A, reverse transcribed with random hexanucleotide primers, and PCR amplified with primers specific for mouse IFN-β (upper panel) or β-actin (lower panel). Cells were either mock infected (lane m) or infected with wild-type BUN (lane wt) or BUNdelNSs (lane delNSs). As controls, water (lane ∅) or total RNA from BUNdelNSs-infected cells without a preceding RT step [lane (−) RT] were used for PCR-amplification.

lation of the IFN mRNA. To distinguish between these possibilities, we analyzed the infected cells for the presence of IFN-β mRNA by RT-PCR. In Fig. 1B (upper panel) it is shown that in cells infected with BUNdelNSs, an RT-PCR signal specific for IFN-β could be detected. By contrast, cells infected with wild-type BUN did not give rise to an RT-PCR signal, similar to mock-infected cells. The signal is due to the presence of IFN-β mRNA and not any residual genomic DNA in the RNA preparation, since PCR without a preceding RT step was negative.

Similarly, the water control did not give rise to a specific signal, indicating the absence of contamination. Figure 1B (lower panel) shows that β-actin mRNA was detected in all RT reactions, but not in the PCR only or the H_2O control, indicating that all preparations contained similar amounts of RNA. Taken together, the results demonstrate that IFN-β mRNA is strongly synthesized and translated in cells infected with BUNdelNSs but not in cells infected with wild-type BUN, suggesting that NSs interferes with transcriptional activation of the IFN-β promoter.

Activation of NF-κB and IRF-3. We investigated the involvement of NF-κB and IRF-3, two key transcription factors, in the induction of IFN-β by BUNdelNSs. For NF-κB, we took advantage of a reporter construct containing the NF-κB binding sequence from the IFN-β promoter, situated upstream of an FF-Luc reporter gene (46). Cells were transfected with the reporter constructs and infected with bunyaviruses. For the reporter assays, we chose an early time point postinfection to exclude the possibility that NF-κB is simply activated by the induced IFN (45). In Fig. 2A it is shown that BUNdelNSs activated the NF-κB reporter by a small but reproducible degree as early as 4 h postinfection, whereas in wild-type BUN- and mock-infected cells, no such activity was detected. These results suggest that the presence of the NSs gene product interferes with the activation of NF-κB.

A different approach was followed to investigate IRF-3 activation. Upon infection with Newcastle disease virus, IRF-3 is essential for the activation of the IFN-β promoter, and blocking its activity results in a loss of IFN-β inducibility (46). We transfected a reporter construct containing FF-Luc under control of an IFN-β promoter together with an expression construct for a dominant-negative IRF-3 mutant, IRF-3^{58-427} (46), and assayed for activation of the IFN-β promoter by bunyavirus infection. In a parallel experiment, the IRF-3^{58-427} construct was replaced with the parental expression vector pEF-

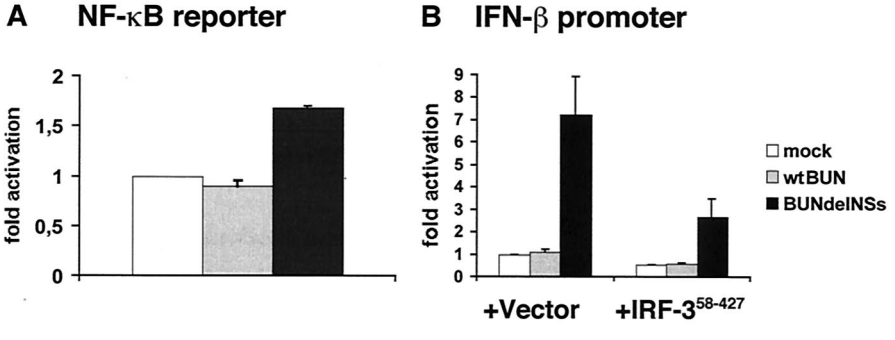

FIG. 2. Involvement of IFN transcription factors NF-κB and IRF-3. (A) NF-κB. Murine BF cells were transfected with the NF-κB reporter plasmid p-125Luc. At 5 h after transfection, the cells were either mock infected or infected with wild-type BUN (wtBUN) or BUNdelNSs. Four hours after infection, firefly luciferase activities in cell lysates were measured. (B) IRF-3. Murine BF cells were transfected with the IFN-β reporter plasmid along with the parental vector pEF-BOS or a plasmid expressing a dominant-negative IRF-3 mutant (IRF3^{58-427}). At 24 h after transfection, the cells were either mock infected or infected with wild-type BUN or BUNdelNSs. Eighteen hours after infection, firefly luciferase activities in the cell lysates were measured. In this and subsequent figures, bars indicate standard error from the results of three experiments.

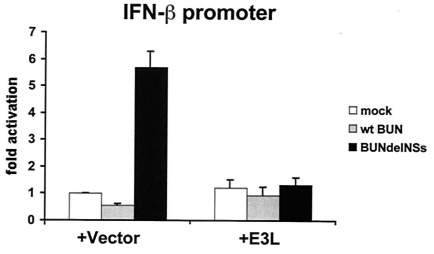

FIG. 3. Effect of dsRNA-binding protein E3L on IFN-β promoter induction by BUNdelNSs. Murine BF cells were transfected with the IFN-β promoter containing reporter plasmid p-125Luc along with 1 μg each of the parental vector pEF-BOS or a plasmid encoding E3L. At 24 h after transfection, the cells were either mock infected or infected with wild-type BUN (wtBUN) or BUNdelNSs. Eighteen hours after infection, firefly luciferase activities in cell lysates were measured.

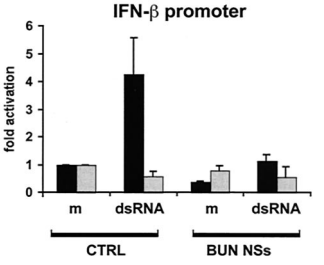

FIG. 4. Effect of NSs on induction of IFN-α/β promoter. Human 293 cells were transfected with the IFN-β promoter containing reporter plasmid p-125Luc (encoding firefly luciferase) along with 0.5 μg each of a T7 polymerase expression plasmid and T7-driven constructs expressing BUN NSs or a control protein (lanes CTRL). At 24 h after transfection, the cells were mock treated (*Renilla* luciferase, bars m) or transfected with dsRNA, and 18 h later, firefly (black bars) and *Renilla* (gray bars) luciferase activities in cell lysates were measured.

BOS. Figure 2B shows that BUNdelNSs was capable of activating the IFN-β promoter of the FF-Luc reporter gene, whereas wild-type BUN was not. Coexpression of the IRF-3$^{58-427}$ mutant, however, resulted in a significant loss of activation by the BUNdelNSs virus. These data demonstrate that IRF-3 is required for IFN-β induction by the mutant virus. Therefore, the induction of IFN-β by BUNdelNSs follows the classical model involving NF-κB and IRF-3.

Involvement of dsRNA. A common activator of NF-κB and IRF-3 is dsRNA, a by-product of viral RNA polymerases (23). We were interested to see whether dsRNA is also involved in IFN induction by BUNdelNSSs. For this purpose, we used E3L, an IFN antagonist encoded by vaccinia virus that binds to dsRNA with high affinity (5). Similar to the experiment with the dominant-negative IRF-3 mutant (see Fig. 2B), we coexpressed E3L in cells transfected with the IFN-β reporter construct. Figure 3 shows that coexpression of E3L did prevent the activation of the IFN-β promoter by BUNdelNSs, whereas a cotransfected control construct had no influence. These data suggest that virally produced dsRNA is the trigger for IFN induction by BUNdelNSs.

Expression of NSs blocks activation of the IFN-β promoter. To determine whether NSs on its own was able to inhibit IFN synthesis, we investigated its influence on the activation of the IFN-β promoter in a virus-free system. Cells were transfected with the IFN-β promoter reporter construct along with plasmids expressing either NSs or the *Renilla* luciferase gene as a control. Subsequently, IFN synthesis was induced by transfection with dsRNA. Figure 4 shows that activation of the IFN-β promoter was observed only in control-transfected cells, but not when NSs was expressed. This indicates that BUN NSs is sufficient to suppress induction of IFN-α/β by dsRNA and that no other viral gene products are needed for its function.

Growth of viruses in IFN-deficient cells. Several bunyaviruses are known to be sensitive to the effects of IFN (16, 31, 39). It was therefore possible that because of the high levels of IFN induced by BUNdelNSs, the mutant virus has a reduced growth rate in IFN-competent cells. We used primary mouse embryo fibroblasts (MEFs) which were derived from transgenic 129 mice either with (wild-type MEFs) or without (IFNAR$^{0/0}$ MEFs) (28) the β subunit of the IFN-α/β receptor as an experimental system. Since these cells have the same genetic background, any observed differences can clearly be ascribed to the presence or absence of the functional IFN system.

As a first step, we investigated the ability of wild-type BUN and BUNdelNSs to form plaques on these cells. Figure 5A shows that on IFNAR$^{0/0}$ MEFs, both wild-type BUN and BUNdelNSs were able to form plaques, with the delNSs mutant having a reduced plaque size. By contrast, on the wild-type MEFs, wild-type BUN but not BUNdelNSs was able to form plaques. To further characterize this effect, cells were infected with wild-type BUN or BUNdelNSs, and virus titers in the supernatants were measured at 72 h postinfection. In Fig. 5B it

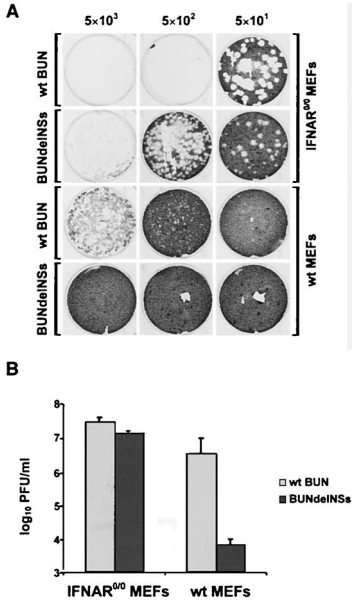

FIG. 5. Growth of viruses in IFN-deficient cells. (A) Comparison of plaques produced by wild-type BUN (wt BUN) and the BUNdelNSs mutant in mouse embryo fibroblasts derived from 129 wild-type mice (wt MEFs) or IFNAR knock-out mice (IFNAR$^{0/0}$ MEFs). Cells were infected with approximately 5×10^3, 5×10^2, or 5×10^1 PFU per dish and fixed and stained 72 h postinfection. (B) Virus titers in wild-type MEFs and IFNAR$^{0/0}$ MEFs. Cells were infected at 0.01 PFU per cell, and the virus concentration in the supernatants was measured 72 h after infection.

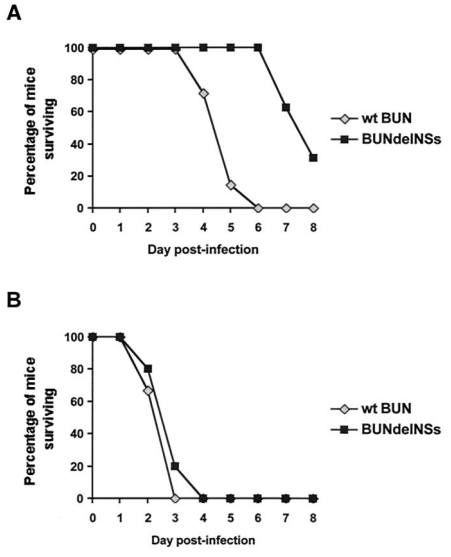

FIG. 6. Virulence of viruses in 129 wild-type (A) and IFNAR$^{0/0}$ (B) mice. Groups of six 5-week-old female mice were inoculated intracerebrally with 1,000 PFU of wild-type BUN (wt BUN) or BUNdelNSs virus. The animals were monitored for 8 days. Mice that were moribund or severely paralyzed were killed and scored dead on that day.

is shown that, on IFNAR$^{0/0}$ MEFs, the difference between wild-type BUN and BUNdelNSs was less than 10-fold. On wild-type MEF cells, however, the difference was significantly more, reaching approximately 1,000-fold. The apparent slight reduction in yield of wild-type BUN on wild-type MEFs probably reflects a very low level of IFN induction upon prolonged incubation. These findings demonstrate that the lack of NSs expression led to severe growth restrictions for BUN in IFN-competent cells but had no significant effect in cells lacking an IFN system.

Virulence of wild-type and delNSs bunyaviruses in mice with a compromised IFN system. We then assessed a possible role of virally induced IFN-α/β in vivo, in order to investigate the function of NSs as a virulence factor. For this purpose, we used the mouse system corresponding to the cells mentioned above, namely, 129 wild-type mice and 129 IFNAR$^{0/0}$ mice. Groups of mice were inoculated intraperitoneally with wild-type BUN or BUNdelNSs and observed for survival. Wild-type mice infected with any of the viruses did not develop symptoms. By contrast, none of the IFNAR$^{0/0}$ mice survived for longer than 96 h following inoculation with either virus. To investigate this further, mice were inoculated intracerebrally and observed for survival. Wild-type 129 mice succumbed to both viruses, but with a clear difference in disease progression. When infected with wild-type BUN, the majority of mice died after 5 days, whereas most of the mice inoculated with BUNdelNSs survived for at least 3 days longer (Fig. 6A). In IFNAR$^{0/0}$ mice, however, this difference was significantly reduced, and both viruses killed their hosts within 3 to 4 days (Fig. 6B).

These data demonstrate that both viruses are equally virulent if the IFN system is absent, strongly indicating that NSs also acts in vivo as an IFN antagonist.

DISCUSSION

In this study, two Bunyamwera viruses which differ genetically by the presence of the NSs reading frame were compared for their ability to interact with the IFN system. The results presented establish that BUN NSs blocks transcriptional activation of IFN-α/β. NSs was found to be of advantage for virus multiplication, but only in cells and animals with a functional IFN-α/β system. Thus, BUN NSs can be designated an IFN antagonist.

Anti-IFN activity has been described for accessory proteins of several other negative-stranded RNA viruses (17, 21). In general, such activities can be distinguished as those interfering with the immediate-early step, induction of IFN (like BUN NSs), and those interfering with the late step, activation of IFN-responsive genes. For simplicity, we propose to designate these factors IFN induction antagonists and IFN response antagonists, respectively. Most factors of negative-stranded RNA viruses characterized so far belong to the second group. For example, the V protein of simian virus 5, a member of the *Paramyxoviridae* family, inhibits the activation of IFN-responsive genes by targeting STAT1 for proteasome-mediated degradation (10). Similarly, the C protein of Sendai virus, another paramyxovirus, inhibits STAT1 activation by hampering phosphorylation (47) and increasing instability (20). Bovine respiratory syncytial virus, also a member of the *Paramyxoviridae*, encodes two nonstructural proteins (NS1 and NS2) that cooperatively mediate resistance to the antiviral actions of IFN (33). These examples illustrate that many negative-stranded RNA viruses have evolved mechanisms to block IFN response in order to survive in the infected cell by hindering activation of antiviral genes.

A different strategy is followed by the IFN induction antagonists. In this case, viruses inhibit the production of IFN-α/β, thus hiding in the host and avoiding a systemic alert that would trigger the innate as well as the adaptive immune response. One of the best-characterized examples is the nonstructural protein NS1 of influenza A virus. Similar to BUN NSs (Fig. 5 and 6), NS1 of influenza A virus was found to be dispensable for growth in IFN-deficient cells and mice (11, 18). The induction of IFN-α/β is prevented by inhibiting the activation of NF-κB (40) and IRF-3 (37), most probably by the ability of NS1 to sequester dsRNA (40). We have found that BUN NSs also prevents activation of NF-κB (Fig. 2A) and IRF-3 (Fig. 2B) and that dsRNA plays a role in induction by the mutant virus (Fig. 3). This could suggest that BUN NSs and influenza A virus NS1 have similar strategies to accomplish IFN inhibition. There is, however, no significant sequence similarity between these two polypeptides. Furthermore, no dsRNA binding could be detected for BUN NSs (F. Weber, unpublished results). Thus, although recombinant BUN and influenza A viruses lacking the NSs or NS1 gene, respectively, both induce IFN by a signal transduction pathway involving dsRNA, NF-κB, and IRF-3, fundamental differences appear to exist in the molecular mechanism of the respective IFN induction antagonists.

The NSs protein of Rift Valley fever virus, a member of the *Phlebovirus* genus in the *Bunyaviridae* family, was also identified as an IFN induction antagonist. It was shown that viruses containing mutations in the NSs gene are strong IFN inducers

and able to grow only in mice with a compromised IFN-α/β system (2). Although the authors could not formally exclude the influence of additional mutations present on the NSs-carrying gene segment, the results strongly imply that NSs is the factor responsible for suppression of IFN induction in wild-type Rift Valley fever virus-infected animals. However, it is not known whether Rift Valley fever virus NSs influences NF-κB and IRF-3, or whether a later step in IFN production is blocked.

It can be speculated that IFN antagonism is a general feature of the NSs proteins of the *Bunyavirus* and *Phlebovirus* genera. Members of the *Tospovirus* genus, which are pathogenic for plants, also encode NSs proteins. Of note, it has been described that dsRNA also plays a role in the defense system of plants against viral pathogens (27). There is, however, a great difference in size, amino acid sequence, and coding strategy between the proteins designated NSs in the different *Bunyaviridae* genera (12). It is therefore conceivable that the NSs proteins evolved independently but in adaptation to a common selection pressure, e.g., the mammalian IFN system. On the other hand, members of both the *Hantavirus* and *Nairovirus* genera do not encode a nonstructural protein in their S segment. To our knowledge, no data on IFN induction are available for any member of the *Nairovirus* genus, but for hantaviruses it was found that infection indeed induces IFN-β (30) and activates IRF-3 and NF-κB (36). Thus, it is possible that bunyaviruses without an NSs gene product may use other strategies to escape the host defense.

In general, negative-stranded viruses, which include many important pathogens, have a relatively small genome with a limited set of genes. Nevertheless, recent advances in reverse genetics technologies (3, 7, 19, 29) revealed that some gene products, often nonstructural proteins, are not essential for virus growth in cell culture but constitute "luxury" functions increasing virulence. Rationally designed virus mutants lacking these genes are promising candidates for live vaccines, since they can be grown to high titers in IFN-deficient cells but are attenuated in the host. This has been exemplified for influenza A and B viruses (38) and may also be a possibility for human-pathogenic members of the *Bunyaviridae* family.

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REFERENCES

1. **Akbar, A. N., J. M. Lord, and M. Salmon.** 2000. IFN-alpha and IFN-beta: a link between immune memory and chronic inflammation. Immunol. Today **21:**337–342.
2. **Bouloy, M., C. Janzen, P. Vialat, H. Khun, J. Pavlovic, M. Huerre, and O. Haller.** 2001. Genetic evidence for an IFN-antagonistic function of Rift Valley fever virus nonstructural protein NSs. J. Virol. **75:**1371–1377.
3. **Bridgen, A., and R. M. Elliott.** 1996. Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. Proc. Natl. Acad. Sci. USA **93:**15400–15404.
4. **Bridgen, A., F. Weber, J. K. Fazakerley, and R. M. Elliott.** 2001. Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. Proc. Natl. Acad. Sci. USA **98:**664–669.
5. **Chang, H. W., J. C. Watson, and B. L. Jacobs.** 1992. The E3L gene of vaccinia virus encodes an inhibitor of the IFN induced, double-stranded RNA-dependent protein kinase. Proc. Natl. Acad. Sci. USA **89:**4825–4829.
6. **Chu, W. M., D. Ostertag, Z. W. Li, L. Chang, Y. Chen, Y. Hu, B. Williams, J. Perrault, and M. Karin.** 1999. JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity **11:**721–731.
7. **Conzelmann, K. K.** 1998. Nonsegmented negative-strand RNA viruses: genetics and manipulation of viral genomes. Annu. Rev. Genet. **32:**123–162.
8. **Deonarain, R., A. Alcami, M. Alexiou, M. J. Dallman, D. R. Gewert, and A. C. Porter.** 2000. Impaired antiviral response and alpha/beta IFN induction in mice lacking beta IFN. J. Virol. **74:**3404–3409.
9. **Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall.** 1999. Sendai virus and simian virus 5 block activation of IFN-responsive genes: importance for virus pathogenesis. J. Virol. **73:**3125–3133.
10. **Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall.** 1999. The V protein of simian virus 5 inhibits IFN signaling by targeting STAT1 for proteasome-mediated degradation. J. Virol. **73:**9928–9933.
11. **Egorov, A., S. Brandt, S. Sereinig, J. Romanova, B. Ferko, D. Katinger, A. Grassauer, G. Alexandrova, H. Katinger, and T. Muster.** 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. J. Virol. **72:**6437–6441.
12. **Elliott, R. M.** 1996. The Bunyaviridae. Plenum Press, New York, N.Y.
13. **Elliott, R. M.** 1997. Emerging viruses: the Bunyaviridae. Mol. Med. **3:**572–577.
14. **Erlandsson, L., R. Blumenthal, M. L. Eloranta, H. Engel, G. Alm, S. Weiss, and T. Leanderson.** 1998. IFN-beta is required for IFN-alpha production in mouse fibroblasts. Curr. Biol. **8:**223–226.
15. **Feigenblum, D., R. Walker, and R. J. Schneider.** 1998. Adenovirus induction of an IFN-regulatory factor during entry into the late phase of infection. J. Virol. **72:**9257–9266.
16. **Frese, M., G. Kochs, H. Feldmann, C. Hertkorn, and O. Haller.** 1996. Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein. J. Virol. **70:**915–923.
17. **Garcia-Sastre, A.** 2001. Inhibition of IFN-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology **279:**375–384.
18. **Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster.** 1998. Influenza A virus lacking the NS1 gene replicates in IFN-deficient systems. Virology **252:**324–330.
19. **Garcia-Sastre, A., and P. Palese.** 1993. Genetic manipulation of negative-strand RNA virus genomes. Annu. Rev. Microbiol. **47:**765–790.
20. **Garcin, D., J. Curran, and D. Kolakofsky.** 2000. Sendai virus C proteins must interact directly with cellular components to interfere with IFN action. J. Virol. **74:**8823–8830.
21. **Goodbourn, S., L. Didcock, and R. E. Randall.** 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. **81:**2341–2364.
22. **Iordanov, M. S., J. Wong, J. C. Bell, and B. E. Magun.** 2001. Activation of NF-κB by double-stranded RNA (dsRNA) in the absence of protein kinase R and RNase L demonstrates the existence of two separate dsRNA-triggered antiviral programs. Mol. Cell. Biol. **21:**61–72.
23. **Jacobs, B. L., J. O. Langland, and T. Brandt.** 1998. Characterization of viral double-stranded RNA-binding proteins. Methods **15:**225–232.
24. **King, P., and S. Goodbourn.** 1998. STAT1 is inactivated by a caspase. J. Biol. Chem. **273:**8699–8704.
25. **Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott.** 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol. Cell. Biol. **18:**2986–2996.
26. **Marie, I., J. E. Durbin, and D. E. Levy.** 1998. Differential viral induction of distinct IFN-alpha genes by positive feedback through IFN regulatory factor-7. EMBO J. **17:**6660–6669.
27. **Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo, and H. Vaucheret.** 2000. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:**533–542.
28. **Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet.** 1994. Functional role of type I and type II interferons in antiviral defense. Science **264:**1918–1921.
29. **Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. USA **96:**9345–9350.
30. **Pensiero, M. N., J. B. Sharefkin, C. W. Dieffenbach, and J. Hay.** 1992. Hantaan virus infection of human endothelial cells. J. Virol. **66:**5929–5936.
31. **Peters, C. J., J. A. Reynolds, T. W. Slone, D. E. Jones, and E. L. Stephen.** 1986. Prophylaxis of Rift Valley fever with antiviral drugs, immune serum, an IFN inducer, and a macrophage activator. Antiviral Res. **6:**285–297.
32. **Schafer, S. L., R. Lin, P. A. Moore, J. Hiscott, and P. M. Pitha.** 1998. Regulation of IFN-α/β gene expression by IFN regulatory factor-3. J. Biol. Chem. **273:**2714–2720.

33. **Schlender, J., B. Bossert, U. Buchholz, and K. K. Conzelmann.** 2000. Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize alpha/beta IFN induced antiviral response. J. Virol. **74:**8234–8242.
34. **Shaw-Jackson, C., and T. Michiels.** 1999. Absence of internal ribosome entry site-mediated tissue specificity in the translation of a bicistronic transgene. J. Virol. **73:**2729–2738.
35. **Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annu. Rev. Biochem. **67:**227–264.
36. **Sundstrom, J. B., L. K. McMullan, C. F. Spiropoulou, W. C. Hooper, A. A. Ansari, C. J. Peters, and P. E. Rollin.** 2001. Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. J. Virol. **75:**6070–6085.
37. **Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre.** 2000. Activation of IFN regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J. Virol. **74:**7989–7996.
38. **Talon, J., M. Salvatore, R. E. O'Neill, Y. Nakaya, H. Zheng, T. Muster, A. Garcia-Sastre, and P. Palese.** 2000. Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. Proc. Natl. Acad. Sci. USA **97:**4309–4314.
39. **Temonen, M., H. Lankinen, O. Vapalahti, T. Ronni, I. Julkunen, and A. Vaheri.** 1995. Effect of IFN-alpha and cell differentiation on Puumala virus infection in human monocyte/macrophages. Virology **206:**8–15.
40. **Wang, X., M. Li, H. Zheng, T. Muster, P. Palese, A. A. Beg, and A. Garcia-Sastre.** 2000. Influenza A virus NS1 protein prevents activation of NF-κB and induction of alpha/beta IFN. J. Virol. **74:**11566–11573.
41. **Wathelet, M. G., C. H. Lin, B. S. Parekh, L. V. Ronco, P. M. Howley, and T. Maniatis.** 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Mol. Cell **1:**507–518.
42. **Watret, G. E., C. R. Pringle, and R. M. Elliott.** 1985. Synthesis of bunyavirus-specific proteins in a continuous cell line (XTC-2) derived from Xenopus laevis. J. Gen. Virol. **66:**473–482.
43. **Weaver, B. K., K. P. Kumar, and N. C. Reich.** 1998. IFN regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. Mol. Cell. Biol. **18:**1359–1368.
44. **Weber, F., E. F. Dunn, A. Bridgen, and R. M. Elliott.** 2001. The Bunyamwera virus nonstructural protein NSs inhibits viral RNA synthesis in a minireplicon system. Virology **281:**67–74.
45. **Yang, C. H., A. Murti, S. R. Pfeffer, L. Basu, J. G. Kim, and L. M. Pfeffer.** 2000. IFNalpha/beta promotes cell survival by activating NF-κB. Proc. Natl. Acad. Sci. USA **97:**13631–13636.
46. **Yoneyama, M., W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita.** 1998. Direct triggering of the IFN-α/β system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J. **17:**1087–1095.
47. **Young, D. F., L. Didcock, S. Goodbourn, and R. E. Randall.** 2000. Paramyxoviridae use distinct virus-specific mechanisms to circumvent the IFN response. Virology **269:**383–390.
48. **Zamanian-Daryoush, M., T. H. Mogensen, J. A. DiDonato, and B. R. Williams.** 2000. NF-κB activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF-κB-inducing kinase and IκB kinase. Mol. Cell. Biol. **20:**1278–1290.
49. **Zhang, J., O. Yamada, T. Ito, M. Akiyama, Y. Hashimoto, H. Yoshida, R. Makino, A. Masago, H. Uemura, and H. Araki.** 1999. A single nucleotide insertion in the 5′-untranslated region of hepatitis C virus leads to enhanced cap-independent translation. Virology **261:**263–270.