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 glycoproteins (G1 and G2) on the medium (M) segment, important link between innate and adaptive immunity (1). It is an 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: Bunavirus, 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 Bunavirus 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,
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 BUN-delNSs, 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, BUN-delNSs exhibited a smaller plaque size and grew in several cell types to approximately 10-fold-lower titers. When inoculated by the intracerebral route, BUN-delNSs 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 embryoblast fibroblasts (MEFs) from 129 wild-type and IFNAR knockout 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 BUN-delNSs (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 (p55-2A-Luc) and the expression construct for the dominant-negative mutant IRF-3 (pEF-HAIRF358-472) 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 [p9-(2-7)]48kD (–39)Luc 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 FastTrak 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 IFN-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 knockout 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 knockout (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 BUN-delNSs.** 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 BUN-delNSs 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 BUN-delNSs 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 BUN-delNSs is unable to hinder transactivation. Alternatively, the IFN gene could be induced in wild-type BUN- and BUN-delNSs-infected cells to the same extent, but NSs inhibits a posttranscriptional step, e.g., trans-
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 H2O 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, IRF3 58-427 (46), and assayed for activation of the IFN-β promoter by bunyavirus infection. In a parallel experiment, the IRF3 58-427 construct was replaced with the parental expression vector pEF-
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-358-427 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 BUNdelNSs. 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-deficient 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-deficient 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
is shown that, on IFNAR<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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-xB (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-xB (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-xB, 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-deicient 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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