Tula and Puumala Hantavirus NSs ORFs Are Functional and the Products Inhibit Activation of the Interferon-Beta Promoter

Krisi M. Jääskeläinen,1 Pasi Kaukinen,1 Ekaterina S. Minskaya,2 Angelina Plyusnina,1 Olli Vapalahti,1 Richard M. Elliott,2 Friedemann Weber,3 Antti Vaheri,1 and Alexander Plyusnin1*

1Department of Virology, Haartman Institute, FIN-00014 University of Helsinki, Finland
2Division of Virology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G11 5JR, United Kingdom
3Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Freiburg, Germany

The S RNA genome segment of hantaviruses carried by Arvicolinae and Sigmodontinae rodents encodes the nucleocapsid (N) protein and has an overlapping (+1) open reading frame (ORF) for a putative nonstructural protein (NSs). The aim of this study was to determine whether the ORF is functional. A protein corresponding to the predicted size of Tula virus (TULV) NSs was detected using coupled in vitro transcription and translation from a cloned S segment cDNA, and a protein corresponding to the predicted size of Puumala virus (PUUV) NSs was detected in infected cells by Western blotting with an anti-peptide serum. The activities of the interferon beta (IFN-β) promoter, and nuclear factor kappa B (NF-κB)- and interferon regulatory factor-3 (IRF-3) responsive promoters, were inhibited in COS-7 cells transiently expressing TULV or PUUV NSs. Also IFN-β mRNA levels in IFN-competent MRC5 cells either infected with TULV or transiently expressing NSs were decreased. These data demonstrate that Tula and Puumala hantaviruses have a functional NSs ORF. The findings may explain why the NSs ORF has been preserved in the genome of most hantaviruses during their long evolution and why hantavirus-infected cells secrete relatively low levels of IFNs. J. Med. Virol. 79:1527–1536, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: hantavirus; TULV; PUUV; NSs ORF; innate immunity; IFN

INTRODUCTION

Hantaviruses (family Bunyaviridae, genus Hantavirus) are rodent-borne viruses present throughout the world; the infection is asymptomatic in rodents but in humans some hantaviruses cause hemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome [Nichol et al., 2005]. Like other bunyaviruses, hantaviruses have a segmented single-stranded RNA genome of negative polarity. The small (S) segment encodes the nucleocapsid protein (N), the medium (M) segment encodes the glycoprotein precursor that is further cleaved to Gn and Gc glycoproteins, and the large (L) segment encodes the RNA polymerase that acts also as a helicase and transcriptase [Nichol et al., 2005]. The S segment of hantaviruses carried by Arvicolinae and Sigmodontinae rodents (voles and lemmings of the northern hemisphere and New World mice and rats) has, in addition to the nucleocapsid protein (N) open reading frame (ORF), an overlapping (+1) ORF for a putative nonstructural protein (NSs; Fig. 1a), which is not found in the S segments of hantaviruses associated with Murinae rodents (Old World mice and rats).
This ORF is in the same relative location in the S segment, and is of similar size, as the NSs ORF in orthobunyaviruses that are also in the family Bunyaviridae [Nichol et al., 2005]. The part of the hantavirus S segment having the double coding capacity is more conserved than the surrounding sequence, suggesting that the NSs ORF may be functional [Plyusnin et al., 1994; Bowen et al., 1995]. The corresponding protein has, however, not yet been detected in hantavirus-infected cells and thus the question whether the NSs ORF is active remains open.

In three of the other four genera in the Bunyaviridae family, the viruses encode a nonstructural protein in their S segment. The NSs protein of Rift Valley fever virus (RVFV, genus Phlebovirus) is a general inhibitor of transcription; it prevents the formation of mature transcription factor TFIIH complex by binding to the p44 subunit and forming nuclear filamentous structures [Le May et al., 2004]. The NSs protein from the plant virus Tomato spotted wilt virus (genus Tospovirus) suppresses post-transcriptional gene silencing [Takeda et al., 2002]. The silencing of the NSs ORF converts the prototype virus in the family, Bunyamwera (BUNV, genus Orthobunyavirus), from an interferon (IFN) noninducer to an inducer [Bridgen et al., 2001]. Similarly, an attenuated RVFV strain with a defective NSs gene is highly virulent in IFNAR−/− mice [Bouloy et al., 2001]. In mice and cultured cells lacking an active IFN-α/β system, the genetically engineered BUNV with the silenced NSs ORF grows to titers similar to those of the wild-type (wt) virus whereas in IFN-competent systems wt virus grows better [Weber et al., 2002].
reporter gene assay system, BUNV NSs antagonizes IFN induction. BUNV NSs suppresses activation of a nuclear factor kappa B (NF-κB)-dependent promoter [Weber et al., 2002] and inhibits signaling downstream of interferon regulatory factor 3 (IRF-3) [Kohl et al., 2003]. Further work showed that BUNV NSs functions by blocking one step in the phosphorylation of the C terminal domain of RNA polymerase II and thus causes generalized inhibition of transcription [Thomas et al., 2004]. Recent reviews of other viral proteins that act as IFN-antagonists are given by García-Sastre [2004], Weber et al. [2004], and Haller et al. [2006], and describe the mechanism of inhibition concerning host cell gene expression, IFN synthesis, IFN signaling, or IFN action.

Data on hantaviruses and IFNs/IFN-regulated proteins are limited. They, nevertheless, clearly show that IFN-mediated responses play an important role in a host cell defense [Tamura et al., 1987; Pensiiero et al., 1992; Temon et al., 1995; Geimonen et al., 2002; Kraus et al., 2004; Khaiboullina et al., 2005; Prescott et al., 2005]. Not surprisingly, hantaviruses grow best in Vero E6 cells, which are IFN-deficient [Diaz et al., 1988]. In this study we aimed to determine whether the hantaviral NSs ORF is functional and, in particular, whether the NSs protein inhibits the IFN-β promoter. Toward this goal, the expression of Tula virus (TULV) NSs ORF was first examined in a S segment cDNA clone with the coupled in vitro transcription and translation system, and the expression of Puumala virus (PUUV) NSs ORF in infected cells with anti-NSs ORF antibodies. Further, an inhibitory activity of transiently expressed TULV and PUUV NSs proteins on the IFN-β promoter and also NF-κB- and IRF-3-responsive promoters was analyzed in a reporter-based system. Finally, the effect(s) of overexpressed TULV NSs protein on the activity of the IFN-β gene were studied in infected, IFN-competent MRC5 cells.

**MATERIALS AND METHODS**

**Cells, Viruses, Plasmids, and Antibodies**

COS-7 and Vero E6 cells were grown in modified Eagle’s medium and MRC5 cells in Dulbecco’s modified Eagle’s medium, both supplemented with 10% foetal calf serum, 2 mM l-glutamine, penicillin and streptomycin, in 5% CO2 at 37°C. TULV, strain Tula/Moravia/Ma5302V/94 [Vapalahti et al., 1996], was used at an MOI 0.1-0.8. PUUV, strain Gis18-20, was grown in Vero E6 cells. A recombinant vaccinia virus vTF7-3, which synthesizes bacteriophage T7 RNA polymerase [Fuerst et al., 1986], was a gift from Bernard Moss (NIH, Bethesda, MD).

The following plasmids with a firefly luciferase reporter gene were included in transfections [Yoneyama et al., 1996]: p125-luc containing the IFN-β promoter; p55A2-luc containing a shorter promoter, which consists of repeated PRD II elements of the IFN-β promoter that can be activated by NF-κB; and p55C1B-luc containing repeated PRD I elements of the IFN-β promoter that respond to IRF-3. The controls were plasmid pRL-SV40 (Promega, Madison, WI) containing Renilla luciferase gene for monitoring transfection efficiency, and plasmid pcDNA3 (Invitrogen, Carlsbad, CA) containing amino acids 121–435 of mouse adaptin gene. The following pcDNA3-based plasmids were used: TULwtNSs construct contains the NSs ORF from wt strain TUL/Moravia/5302Ma/94; it codes for a protein of 90 aa residues [Plyusnin et al., 1995] (Fig. 1b). TULvNSs and TULvS constructs contain the NSs- and N-ORFs from the cell culture-adapted isolate Tula/Moravia/Ma5302V/94 [Vapalahti et al., 1996]; both carry a stop codon as the 15th triplet of the NSs ORF and encode a protein of 67–68 aa residues depending on the initiating Met24 or Met25 (Fig. 1b). PUU NSs construct contained the NSs ORF from strain Sotkamo [Vapalahti et al., 1992] (Fig. 1b). Translation of full-sized NSs was blocked in TULwtNSs-stop, TULvNSs-stop, and TULvS-stop constructs by introduction of two stop codons after methionine codons 24 and 25 and in PUU NSs-stop construct by introduction of one stop codon after methionine codon 25 (Fig. 1b). PUU S construct contains the N-ORF from PUUV strain Sotkamo.

pTMPUUUS contains nucleotides 43–1,344 of the PUUV S segment cDNA cloned between Ncol and PstI restriction sites in plasmid pTM1 [Fuerst et al., 1986] under control of bacteriophage T7 promoter and encephalomyocarditis virus internal ribosome entry site sequences. The sequence encoding PUUV ORF2, bases 83–353, was amplified by PCR from pTMPUUUS using primers that incorporated Ncol (5’) and PstI (3’) restriction sites. The amplified DNA was then digested with Ncol and PstI and ligated into similarly digested plasmid pTM1 to give pTMPUUORF2.

PUUV ORF2 rabbit polyclonal peptide antibodies 46–50 were prepared as follows. Peptides representing PUUV Sotkamo NSs aa 9–24, 28–43, 38–56, 42–60, and 74–90 were synthesized, rabbits were immunized and sera were collected at 3 months after immunization. Rabbit polyclonal antibody to PUUV N has been described earlier [Vapalahti et al., 1995]. Rabbit polyclonal antibody to myxovirus-resistance protein A (MxA) [Halminen et al., 1997] was a kind gift from Dr. Ilkka Julkunen.

**Transfection Experiments**

Transfection of COS-7 cells used FuGENE6 reagent (Roche, Basel, Switzerland) and of MRC5 cells Lipofectamine 2000 reagent (Invitrogen), following the manufacturer’s recommendations.

Transient expression of the PUUV ORF2 protein was performed in Vero E6 cells as follows. Cells were infected with vTF7-3 (MOI 5 pfu/cell) for 1 hr, washed with Opti-MEM, and then transfected with 1 μg of plasmid DNA using Lipofectamine (Invitrogen) as transfectant according to the manufacturer’s instructions. Cell extracts were processed at 24 hr post-transfection.

**UV Inactivation of Virus**

TULV was irradiated at 254 nm in an open 3 cm-diameter Petri dish 10 cm from 30 W UV lamp for 30 min [Li et al., 2004].
Poly(I:C) Induction of the IFN-β Promoter

COS-7 cells were transfected with 1 μg p125-luc, p55A2-luc or p55C1B-luc DNA, 1 ng plasmid pRL-SV40 and 1 μg pcDNA3 plasmid containing the appropriate viral gene or mouse adaptin gene. After 24 hr the IFN-β promoter was induced by transfecting cells with 10 μg of synthetic double-stranded RNA (dsRNA), poly(I:C) (Sigma-Aldrich, St. Louis, MO). Eighteen hours later cell extracts were prepared and luciferase activities measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions. Luciferase activities were measured from triplicate samples with a DCR-1 luminometer (Dige Diagnostics, Inc., Gaithersburg, MD).

Coupled In Vitro Transcription and Translation and Fluorography

Coupled in vitro transcription and translation of pcDNA3 plasmids used in the above transfection experiments was carried out with TnT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer’s instructions. The proteins were labeled with Redivue[^1]S-methionine (Amersham Biosciences, Piscataway, NJ). The samples were analyzed by SDS–PAGE on 18% polyacrylamide gels and the proteins in the gel were fixed with 50% methanol, 10% acetic acid in PAGE on 18% polyacrylamide gels and the proteins in PAGE were washed with trypsin-EDTA and suspended to PBS. Cells were washed three times with PBS and 20% and 5% of cells were put onto immunofluorescence-quality glass chamber at 37 °C until 70 °C and then the membranes were washed with 0.5% Tween-20 in TEN. Antibody reactions were incubated at RT for 1 hr and then the membranes were washed with 0.5% Tween-20 in TEN. Antibody reactions were incubated at RT for 1 hr and then the membranes were washed with 0.5% Tween-20 in TEN. The immunoblots were developed with enhanced chemiluminescence reaction.

Immunofluorescence Assay

MRC5 cells grown on 6-well plates were infected with TULV. After 5, 9, and 15 days infection cells were washed with trypsin-EDTA and suspended to PBS. Cells were washed three times with PBS and 20% and 5% of cells were put onto immunofluorescence-quality glass slides. Cells were fixed with cold acetone at room temperature (RT) for 7 min and stored at −70 °C until analyzed. Antibody reactions were done in a moist chamber at 37 °C for 30 min. Rabbit polyclonal antibody PUU-N(2/3)-GST was used at a dilution of 1:50 and anti-rabbit IgG-FITC conjugated antibody at a dilution of 1:40 in PBS. Between antibody incubations, the slides were washed three times with PBS. Samples were examined with an Olympus BX-50 microscope at a magnification of 20.

IFN Biological Assay

MRC5 cells grown on 6-well plates were infected with TULV, and at 1, 4, and 7 d.p.i. the culture medium was collected and dialyzed against acidic glycine buffer (pH 2), and then twice against PBS. IFN-α/β levels were monitored by measuring vesicular stomatitis virus plaque reduction in Hep2 cells [Cantell et al., 1991]. The detection limit for the assay was <1 IU/ml. An international IFN-α control was utilized as an internal laboratory standard.

Western Blot

Extracts of MRC5 cells infected with TULV or Vero E6 cells infected with PUUV or transfected with PUUV ORF2 expressing plasmids were analyzed by SDS–PAGE and immunoblotted according to standard protocols. Proteins were transferred to a nitrocellulose membrane and blocking was carried out overnight (o/n) in 5% milk, 0.05% Tween-20 in TEN buffer. Antibody reactions were performed with 1:400 dilution of rabbit polyclonal to PUUV N, 1:10 dilution of rabbit anti-peptide sera against ORF2 protein, or 1:1,000 dilution of rabbit polyclonal to MxA, and 1:1,000 dilution of swine anti-rabbit-conjugated to horseradish peroxidase, in 1% milk, 0.05% Tween-20 in TEN. Antibody reactions were incubated at RT for 1 hr and then the membranes were washed with 0.5% Tween-20 in TEN. The immunoblots were developed with enhanced chemiluminescence reaction.

RT-PCR Analysis

Total RNA was isolated from samples with 1 ml of TriPure Isolation Reagent (Roche) mainly according to the manufacturer’s instructions. Isopropanol precipitation was carried out o/n at −70 °C and the precipitate washed with 99% EtOH. The RNA was reprecipitated with 75% EtOH and 0.02 M Na-acetate pH 5.3 o/n at −70 °C. RNA was dissolved in 20 μl H2O. Reverse transcriptase (RT) PCR was performed with SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) using random hexamer primers. When producing cDNA for interferon stimulated gene 56 (ISG56) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification, RNA was denatured by incubation for 5 min at RT with 3 mM CH3HgOH. On other occasions thermal denaturation (65 °C for 5 min) was used. Amplification PCR was done with AmpliTaq® DNA Polymerase (Applied Biosystems, Foster City, CA). In one reaction, 2.5 μl of cDNA was amplified with 0.03 U/μl of the polymerase, 4 mM MgCl2, 250 μM dNTPs and 1 μM of primers. Five microliters of cDNA was amplified with ISG56 primers. Primers for amplification were the following: GAPDH, forward 5'-CGGAGTCAACGGATTTGGTCG-3', and reverse 5'-AGCCTTCTCCATGGTGTTGGAAG-3' (modified from Collins et al., 2004); and TULV S segment, forward 5'-CCTGTAAGAAAAATGGTCTCCTGT-3', and reverse 5'-GGGTCTCAGATATGACTGATAG-3'. IFN-β, MxA and ISG56 primers have been described earlier [King et al., 2004; Spiegel et al., 2005; Weber et al., 2006].

Real-Time PCR

Relative quantification real-time PCR was performed with LightCycler FastStart DNA Master SYBR Green I (Roche) according to the manufacturer’s instructions. PCR for IFN-β was carried out with 4 mM MgCl2 and 0.4 μM primers, and for porphobilinogen deaminase with 2 mM MgCl2 and 0.9 μM primers. cDNA was diluted 1:3. IFN-β primers were purchased from SuperAssay Bioscience Corporation (Frederick, MD). Porphobilinogen deaminase primers were as published by Mensink

et al. [1998]. Samples were analyzed in the LightCycler 2.0 instrument. Gene expression changes were calculated by comparing the changes in the mean Ct values in IFN-β expression with normalized Ct values of porphobilinogen deaminase (method 2⁻^Ct).

**Statistics**

P values were calculated with F-test and t-test. P values less than 0.05 were considered significant.

**RESULTS**

**Analysis of expression of TULV and PUUV NSs ORFs**

The overall organization of the S segments of TULV and PUUV is shown in Figure 1a. Analysis of TULV NSs ORF-containing plasmids with a coupled in vitro transcription and translation system showed that TULwtNSs construct, containing the NSs sequence from wt TULV (Fig. 1b), expressed a protein of approximately 13 kDa (Fig. 1c, lane 2). TULvNSs construct containing the NSs from cell culture-adapted TULV (Fig. 1b), expressed a slightly shorter protein (Fig. 1c, lane 3; although electrophoresis resulted in bands on a slant, the difference in size of wtNSs and vNSs is still evident) while both TULwtNSs-stop and TULvNSs-stop constructs with silenced NSs ORFs failed to produce any protein (Fig. 1c, lanes 6 and 7). A lower level of NSs protein expression was observed from the TULvS plasmid, containing both N and NSs ORFs of cell culture-adapted TULV (Fig. 1c, lane 4). This construct expressed mostly N protein (seen as the full-length molecule of approximately 48 kDa and truncated molecules of 40 and 28 kDa, probably initiated from Met74 and Met181). These shorter forms of the N protein are sometimes seen in transfected cells (unpublished observations). Silencing of the NSs ORF in this construct by introduction of a stop codon into the NSs ORF was efficient, but did not affect the level of the N protein expression (Fig. 1c, lane 5). These data illustrated that the NSs ORFs compete in the context of the viral S segment.

Our attempts to detect the product of TULV NSs ORF in infected Vero E6 cells using rabbit antibodies raised against peptides from the known TULV NSs sequence were unsuccessful. Of five anti-PUUV NSs (ORF2) antibodies, however, two appeared efficient in the detection of PUUV ORF2 in Western blot (data not shown). Antibody 49, that reacted more strongly, was used to detect the NSs protein in transfected and also in infected cells (Fig. 2a,b). Similarly to what was observed with TULV-based constructs, the level of expression of the NSs ORF from the PUUORF2 construct was higher than from the PUUS construct (Fig. 2a) confirming that the genetic background of the S segment can influence the level of NSs protein expression. Most notably, the ORF2 protein was seen in infected Vero E6 cells at 2, 4, and 6 days p.i. (Fig. 2b). The very weak band in the control lane in Figure 2b most probably arose by leakage from the neighboring well containing 2 d.p.i. sample.

Attempts to detect the NSs protein in lung tissues of TULV-infected M. arvalis and PUUV-infected C. glareolus using the anti-peptide antibodies were unsuccessful (data not shown) suggesting that the level of the NSs protein expression in a natural rodent host is low.

**Effect of the NSs ORF on the Activity of Poly(I:C)-Induced IFN-β Promoter**

COS-7 cells were transfected with the plasmids containing the luciferase reporter gene under control of the IFN-β promoter, NF-κB-responsive promoter or IRF-3-responsive promoter, and also with test plasmids expressing various TULV and PUUV ORFs. The promoters were induced with poly(I:C). The activity of all three promoters was inhibited by the product of TULwtNSs ORF (NSs from wt TULV; Fig. 3a–c). The level of inhibition varied slightly, but was always significant. The results demonstrate inhibition of approximately 30% (P = 0.004) for the IFN-β promoter, 20% (P = 0.016) for the NF-κB-responsive promoter, and 35% (P = 0.011) for the IRF-3-responsive promoter.

**Fig. 2. Detection of PUUV NSs (ORF2) protein.** a: Western blot analysis with anti-ORF2 antibody 49. Vero E6 cells were infected with vaccinia virus vTF7-3 (VV) or mock-infected (m), and then transfected with PUUORF2 (ORF2) or PUUS (S) constructs. Cell extracts were separated by 20% SDS–PAGE and subjected to Western blot analysis with anti-ORF2 peptide antibody 49. The protein (NSs) produced from the ORF2 construct is shown on the top. The NSs protein produced from the PUUS construct is shown on the bottom. b: Western blot analysis of PUUV infected cells. Vero E6 cells were infected with PUUV or mock-infected (m), and cell extracts prepared on days 2, 4, or 6 post-infection were separated by 20% SDS–PAGE and analyzed by Western blot with anti-ORF2 antibody 49. Arrows indicate the location of the NSs protein.
(Fig. 3a–c). With the TULwtNSs-stop mutant the inhibitory effect was totally abolished (data not shown). Analysis of plasmids with a coupled in vitro transcription and translation system showed that TULwtNSs expressed a protein of approximately 13 kDa while TULwtNSs-stop construct, with the silenced NSs ORF, failed to produce any protein (Fig. 1c). Transiently expressed TULwtNSs inhibited the IFN-β promoter and NF-κB- and IRF-3-responsive promoters. Also, the NSs protein of PUUV (ORF2) showed statistically significant inhibition of approximately 30% (P = 0.001), 45% (P = 0.006), and 30% (P = 0.015) for the three promoters (Fig. 3a–c). Again, an introduction of a stop codon in the NSs ORF (Fig. 1b) diminished the inhibitory effect, totally (for the IFN-β and IRF3-responsive promoters) or to a lesser extent for the NF-κB-responsive promoter (data not shown). In the PUU NSs-stop construct, L26 was converted to a stop codon (Fig. 1b). It might be that this construct produces a small protein, comprising aa 1–25, that could have an inhibitory effect on the NF-κB-responsive promoter. By comparison, the level of inhibition by the NSs proteins of BUNV and RVFV is in the range of 75% [Weber et al., 2002] and 95% [Billecocq et al., 2004]. This indicates that hantavirus NSs is less active in inhibiting IFN induction, possibly because hantavirus glycoproteins also have IFN antagonist effect [Alff et al., 2006; Spiropoulou et al., 2007].

These data indicate that the NSs ORFs in TULV and PUUV can be expressed and that the products, designated as NSs proteins, modulate the IFN-β promoter.

**Effect of Hantavirus Replication on the Expression of IFN-β Related Genes**

MRC5 cells were discovered to be IFN-competent [Young et al., 2003] and here it is shown that they can be infected with hantaviruses (Fig. 4). Although the percentage of infected MRC5 cells is usually not very high, this cell line seems to be a valuable tool for studying innate immune responses during hantaviral infection and has, in our opinion, several important advantages compared to HUVEC where hantaviruses are concerned (see Discussion). TULV, a nonpathogenic virus that does not require to be handled under P3 biosafety conditions, was mostly used to study the effect of hantavirus replication on the expression of IFN-β-related genes. Confluent MRC5 cells were either infected for 24 hr with TULV or treated with UV-inactivated virus. Mock-infected cells and poly(I:C) transfected cells were used as negative and positive controls. RT-PCR amplification of GAPDH mRNA was used to confirm that the samples contained similar amounts of cDNA (and therefore RNA). RT-PCR amplification of the TULV S segment confirmed that only infected samples contained the viral gene.

Although the level of the IFN-β protein in infected cells was below the limit of detection, it was observed that two IFN-β-inducible genes, MxA and ISG56, were expressed in TULV-treated MRC5 cells. The MxA protein was seen in the immunoblots 4 d.p.i and 7 d.p.i. (Fig. 5) and ISG56 mRNA was detected already 1 d.p.i. by RT-PCR (data not shown). ISG56 mRNA expression was also observed 1 day after infection of MRC5 cells with PUUV (data not shown). With real-time PCR the level of IFN-β mRNA in TULV-infected MRC5 cells was also examined 5 days p.i. and the level was 20% lower than in cells treated with inactivated TULV (Fig. 6). Thus, TULV replication caused some but not total inhibition of the IFN-β gene expression. These results correlate with our observations in Vero E6 cells where TULV infection reduced the expression of the IFN-β reporter constructs by 25–40% (data not shown).

**Effect of Transiently Expressed NSs Protein on Activation of IFN-β Gene**

MRC5 cells, grown to 70% confluence, were infected with TULV and at 3, 7, or 13 days p.i. were co-transfected with pcDNA3-based constructs TULwtNSs and TULwtNSs-stop. Two days later, RNA was extracted and reverse-transcribed. cDNA was amplified
with GAPDH and TULV primers to ensure that the amount of cDNA and infection rate were similar in all samples, and the expression levels of IFN-β and MxA mRNAs was determined. A similar level of MxA mRNA was apparent in all samples (data not shown). At 5 days p.i. no difference in the IFN-β mRNA level between cells transiently expressing TULwtNSs and TULwtNSs-stop was seen. However, at 9 days p.i. and 15 days p.i. a difference was evident (Fig. 7a), suggesting that TULwtNSs was able to inhibit IFN-β expression. The fact that on day 15 p.i. the level of IFN-β mRNA went down, whereas the virus RNA level stayed constant, provides another hint to a viral IFN-β antagonistic effect. Although at day 5 p.i. very few cells showed positive immunofluorescence when examined for the presence of hantaviral N protein, at days 9 and 15 p.i., approximately 10–15% of cells were IFA-positive (Fig. 4). In another experiment, samples were analyzed by real-time PCR and it was observed that at 5 days p.i., IFN-β expression in cells over-expressing the NSs protein (i.e., cells infected with TULV and co-transfected with the TULwtNSs construct) was reduced to 70% compared to cells transfected with the TULwtNSs-stop construct (Fig. 7b).

From these results it was concluded that infection of MRC5 cells with TULV activates the IFN-β promoter and that the NSs protein of the virus can, to some extent, counteract this activation.

**DISCUSSION**

To the best of our knowledge, this is the first time that the NSs ORF of a hantavirus has been shown to produce a functional protein. Not only was a protein of appropriate size detected following coupled in vitro transcription and translation of an S segment cDNA, but a correctly sized protein could be detected in infected cells with antibodies raised against corresponding peptides. As for the protein’s function(s), the NSs proteins of TULV and PUUV inhibited the expression of the IFN-β gene, and more specifically, dsRNA-induced activation of the IFN-β promoter. This effect was observed in COS-7 cells and, more importantly, in the IFN-competent MRC5 cells. The inhibition of IFN-β gene expression, however, appeared to be rather weak. This might be due to particular experimental settings of this study: cell cultures, recombinant constructs, etc. Another explanation for the observed effect is that the hantaviral NSs protein is a genuinely weak IFN antagonist, in contrast to, for example, the NSs proteins of BUNV and RVFV. Hantaviruses are the only bunyaviruses causing persistent asymptomatic infection in their natural rodent hosts [Plyusnin and Morzunov, 2001; Schmaljohn and Hooper, 2001]. This
might be the main reason why they grow slowly and reach low titers in cultured cells. A low rate of replication and consequently a limited dsRNA production during infection leads to a modest induction of IFN and downstream-activated components of an innate immune response(s), and can therefore enable hantaviruses, TULV including, to manage without a strong IFN antagonist. The mechanism(s) of anti-IFN activity of hantaviral NSs protein remain unknown. Since host protein synthesis is not severely affected in TULV- and PUUV-infected cells (our unpublished observations), the hantaviral NSs proteins are apparently not strong inhibitors of cellular transcription. NSs proteins of BUNV, La Crosse virus (family Bunyaviridae, genus Orthobunyavirus) and RVFV shut down host transcription and protein synthesis by inhibiting cellular RNA polymerase II [Bridgen et al., 2001; Billecocq et al., 2004; Le May et al., 2004; Thomas et al., 2004; Blakqori and Weber, 2005]. The NSs protein of BUNV inhibits phosphorylation of serine residues within the C-terminal domain of the polymerase [Thomas et al., 2004], while the NSs protein of RVFV prevents the formation of mature transcription factor TFIHH complex by binding to the p44 subunit [Le May et al., 2004]. NSs proteins of TULV and PUUV failed to interact with the p44 protein in the yeast-two-hybrid assay (M. Bouloy and N. Le May, personal communication) suggesting that hantaviruses inhibit cellular transcriptions by other means.

IFNs as well as IFN-regulated proteins seem to play a role during hantavirus infection, but the details of this interplay remain unknown largely due the fact that hantaviruses grow poorly in cultured cells which possess an active type I IFN system. IFN-β treatment before Hantaan virus (HTNV) infection enhances survival of mice and reduces plaque formation in Vero E6 cells [Tamura et al., 1987]. In monocytes/macrophages, IFN-α pretreatment causes a dose-dependent reduction in PUUV production; furthermore, with application of anti-IFN-α antibodies, the yield increases. PUUV also induces, besides very low levels of IFN-α, MxA protein [Temomon et al., 1995], which is a hallmark of type I IFN action [Haller and Kochs, 2002]. Since the 1990s it has been known that HTNV infection in human endothelial cells from saphenous veins induces IFN-β, resulting in a late decrease in virus yield [Pensiero et al., 1992]. Until recently cells of this type were not used to study details of interplay between hantaviruses and IFN-competent cells. In their pioneering work with human umbilical vein endothelial cells (HUVEC) Geimonen et al. [2002] demonstrated a significant difference in the temporal regulation of IFN-stimulated genes by nonpathogenic Prospect Hill virus compared to pathogenic HTNV and New York virus. In the studies that followed [Kraus et al., 2004; Khailoullina et al., 2005; Prescott et al., 2005] several aspects of an innate immunity response to hantaviruses have been studied with HUVEC. Despite some controversy, for example, on the issue as to whether hantavirus replication in these cells is required for induction of innate immunity response genes, these studies established HUVEC as a useful, albeit not perfect, tool. In this paper it is shown that MRC5 is a promising candidate of an IFN-competent hantavirus-sensitive cell culture type, which is easy to achieve and maintain.

Hantavirus glycoproteins have recently been shown to inhibit the IFN response. The cytoplasmic tail of Gn protein from New York virus has been found to decrease

Fig. 7. a: Levels of IFN-β mRNA in cells infected with TULV and co-transfected with the NSs-protein expressing construct. MRC5 cells were infected with TULV and co-transfected with TULwtNSs or TULwtNSs-stop plasmid. After RT, the PCR was done with IFN-β, TULV, or GAPDH gene specific primers. b: Effects of transiently expressed TULV NSs protein (from the TULwtNSs construct) on the expression of the IFN-β gene. MRC5 cells were infected for 3 days with TULV and then transfected with TULwtNSs or TULwtNSs-stop constructs. Two days post transfection total RNA was extracted and real-time PCR was done with IFN-β gene specific primers. Results were normalized to PBGD gene expression with 2^(-ΔΔCt) method.
the activities of retinoic acid-inducible gene I (RIG-I) and TANK binding kinase 1 (TBK-1), which leads to inhibition of the IFN response [Alff et al., 2006]. Further, it has been shown that nonpathogenic Prospect Hill virus induces a great IFN-β response in HMVEC-L cells early whereas the response is low after infection with pathogenic Andes virus. Glycoproteins from both viruses reduce IFN-induced signal transducers and activators of transcription 1 and 2 (STAT-1/2) activity [Spiropoulou et al., 2007]. Many viruses have several proteins that can block the IFN response [Haller et al., 2006] and thus these studies do not rule out the function of hantavirus NSs protein as IFN antagonist.

Studies with genetically engineered hantaviruses containing silenced NSs ORF are pending, but the lack of reverse genetics system has so far hampered generation of a knock-out virus.

There are other questions concerning the hantaviral NSs protein that remain unanswered. Of them, two seem to be most important: (1) how and when is the NSs protein expressed during the hantaviral replication; and (2) why do some hantaviruses lack the NSs-ORF? To address the first question, one needs better tools to detect naturally or/and transiently expressed NSs protein. The available anti-peptide antibodies are of limited use and alternative possibilities are being explored. The relatively low yields of hantaviruses in cell cultures make the NSs protein detection quite difficult. Similarly, the hantaviral L protein is not seen in infected cells by IFP with highly specific polyclonal antibodies and can only be visualized on immunoblots [Kukkonen et al., 2004]. There is also the question of how expression of the NSs ORF is regulated in infected cells. The genetic background of the S segment may not be favorable for the production of the NSs protein. When COS-7 cells were transfected with plasmids containing the entire TULV or PUUV N-ORFs none of the three promoters, IFN-β, NF-κB, or IRF-3, was inhibited, suggesting that expressing the NSs ORF in the TULV/ PUUV N gene context under a cellular promoter was not sufficient to detect IFN suppression in our reporter system. This result was in agreement with the data observed with the coupled in vitro transcription and translation system: the NSs band expressed from the vS construct was weaker than the band expressed from the NSs constructs (Fig. 1c). Similarly, the PUUV NSs band was weaker in cells transfected with the N-ORF construct than with the NSs-ORF construct (Fig. 2a). These data showed the dominance of the N protein expression from the hantaviral S segment. The reasons for this dominance remain unknown. Both TULV and PUUV NSs ORF start codons are surrounded by a more favorable “Kozak sequence” (CCC GCC ATG A) than TULV and PUUV N ORF start codons (GCT GGT ATG A and TCT GGA ATG A, respectively) [Kozak, 1984, 1990, 1991]. This difference cannot explain the different expression levels of the N protein and the NSs protein from the constructs carrying two overlapping ORFs. Therefore, other factors may be involved in the low-level NSs protein expression.

The NSs ORF has been preserved in the genome of most hantaviruses during their long evolution [Plyussnin, 2002]. It is a widely accepted view that genetic drift would inevitably destroy any ORF, unless its product performs an important function and stabilizing selective pressure works against appearance of stop-mutations [Kimura, 1983]. This is especially true for viral genomes tightly packed with genetic information. Why hantaviruses carried by Murinae rodents lack the NSs ORF remains unknown. One possibility is that functions of the NSs protein in these hantaviruses are delegated to another viral protein, for example, to the N protein that has to perform additional tasks besides encapsidation of the viral RNA [Kaukinen et al., 2005]. This hypothesis is currently under evaluation.

The poor induction of type I IFNs in human cells infected with TULV and PUUV [Temonen et al., 1995; Kraus et al., 2004] could be explained by an inhibitory activity of the NSs protein. Several other studies are not contradictory to the idea that hantaviruses encode type I IFN-antagonist(s) [Geimonen et al., 2002; Prescott et al., 2005]. Further efforts are needed to clarify the role of the NSs protein in a course of hantaviral infection.

ACKNOWLEDGMENTS

The authors are grateful to Mitsutoshi Yoneyama and Takashi Fujita for providing luciferase reporter plasmids, to Rick Randall and Dan Young for providing MRC5 cells, to Bernard Moss for providing recombinant vaccinia virus, to Ilkka Julkunen for a friendly advice and for providing MxA antibody, to Hilikka Lankinen for an advice with NSs peptides, and to Brian Hjelle and Ake Lundkvist for a helpful discussion. Hanna Värttinen is thanked for help with the IFN measurements. Leena Kostamovaara and Tytti Manni are acknowledged for excellent technical assistance. This work was supported by the University of Helsinki (the Young Scientist’s grant for KMJ), The Academy of Finland, Sigrid Juselius Foundation and EU (grant QLK2-CT-2002-01358).

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